(19) DANMARK

(10) **DK/EP 3287471 T3**

Patent- og
Varemærkestyrelsen

(12) Oversættelse af
europæisk patentskrift

(51) Int.Cl.: ***C 08 B 37/00 (2006.01)***

(45) Oversættelsen bekendtgjort den: **2021-01-11**

(80) Dato for Den Europæiske Patentmyndigheds
bekendtgørelse om meddelelse af patentet: **2020-12-16**

(86) Europæisk ansøgning nr.: **17189957.8**

(86) Europæisk indleveringsdag: **2010-11-16**

(87) Den europæiske ansøgnings publiceringsdag: **2018-02-28**

(30) Prioritet: **2009-12-11 EP 09178876**

(62) Stamansøgningsnr: **10776375.7**

(84) Designerede stater: **AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO RS SE SI SK SM TR**

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(54) Benævnelse: **Polysaccharid, der er egnet til modulering af immunreaktion**

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DESCRIPTION

[0001] The present invention relates to an edible product or pharmaceutical composition for use in the treatment of cold or flu, said edible product or pharmaceutical composition being obtained by method that comprises incorporating a polysaccharide that can be used to modulate immune response. The invention also relates to a method for preparation of an edible product or pharmaceutical composition comprising the polysaccharide.

[0002] Many adults in the western world suffer two to five colds a year, and infants and preschool children even have an average of four to eight colds a year. The upper respiratory tract infections, like common colds and flu, are together with gastrointestinal infections the most important reasons of absenteeism at work or school. In a lifetime of 75 years, we suffer on average from over 200 episodes of common cold. This means that if each cold lasts for five to seven days we spend around three years of our life coughing and sneezing with colds. The need and interest of a consumer in 'self prevention' and 'self treatment' of these acute infections are therefore high.

[0003] A recent interest in the field of functional food ingredients is the use of immunomodulators for enhancing host defence responses, for instance, to provide more protection against the common cold. An important part of the host defence response is the innate immune system. The innate arm of the immune system is a rapidly activated first line of defence against pathogens. It involves amongst others phagocytic and natural killer (NK) cells. Phagocytic cells such as neutrophils, monocytes and macrophages can generate reactive oxygen species (ROS) to kill pathogens such as fungi, bacteria and virus-infected cells. NK cells can kill target cells that have lost or express insufficient amounts of MHC class I, a frequent event in tumor- or virus-infected cells.

[0004] Some edible products or food products have been reported to have immunostimulating properties. For example, US 2005/0002962 A1 discloses a melanin preparation of botanicals such as Echinacea, American ginseng, black walnut, green tea, Parthenium integrifolium, Korean ginseng, alfalfa sprouts, ginger, goldenseal, red clover, dandelion, black cohosh, licorice, chamomile, milk thistle, alfalfa, horsetail, astragalus, gotu kola, feverfew, valerian, hawthorn, rosemary, saw palmetto, ephedra, pau d'arco, ginkgo, garlic, St. John's wort, Agaricus bisporus (common mushroom), Agaricus bisporus brown strain (portabella mushroom), Lentinus edodes (shiitake mushroom) or Boletus edulis (porcini mushroom) as an immune stimulatory composition.

[0005] WO 2009/071425 A1 and WO 2009/080447 A1 disclose the immunostimulating effects of fractions including substantial amounts of oligosaccharides and/or low molecular weight from plants of the Asclepiadoideae subfamily (such as hoodia) and the Alliaceae family (such as onion and garlic from the *Allium* genus), respectively.

[0006] Westereng et al. (Mol. Nutr. Food Res. 2009, vol. 53, p. 780-789) describe release and

characterization of single side chains of pectic material extracted from white cabbage (*Brassica oleracea* var. Capitata, Bartolo cultivar) and their complement-fixing activity (which plays a role in the human immune system). Westereng et al. focus on the side chains as the elements of the pectic material being active in complement fixing, and the authors indicate that structural elements containing multiple side chains are necessary for efficient complement-fixing activity. The pectic material contained two fractions, having a molecular weight of 630 and 40 kDa, respectively, and was active in complement fixing. The average ratio between galacturonyl acid and rhamnosyl residues of the polysaccharides was 5.7. The pectic material was degraded by beta-elimination into a charged fraction and two neutral fractions, having molecular weights of 90, 17, and 8 kDa, respectively. The neutral fractions consist of side chains released by beta-elimination, and the fraction of 17 kDa showed complement fixing activity, while the 8 kDa fraction did not show complement fixing activity; the 90 kDa fraction was not tested. Apparently, based on molecular weight, the large neutral fraction contains molecules having at least two side chains, while the small neutral fraction contains only single side chains.

[0007] Sakurai et al (Immunology, 1999, vol. 97, p. 540-547) disclose a pectic polysaccharide fraction (called bupleuran 2IIc) that was prepared from a medicinal herb (*Bupleurum falcatum* L.) and administered orally to mice. The polysaccharide comprises a galacturonic region and a rhamnogalacturonan core rich in neutral sugar chains, and it is suggested that the side chains of the RG core are important for the mitogenic activity of the polysaccharide.

[0008] WO 2001/76609 A1 discloses the use of polygalacturonic acids as ingredients in food. The polygalacturonic acids are prepared by endopolygalacturonidase treatment of plants contain pectin. The polygalacturonic acids are useful as viscosifier in food products. The pectins are known to have a useful physiological effect.

[0009] US 6,794,495 discloses a composition of extensin and pectin, enhancing the immune system. Extensin is hydroxyproline-rich glycoprotein abundant in the cell walls of dicotyledons.

[0010] Lau et al. (Carbohydrate Research, 1985, vol. 137, p. 111-125) disclose an RG-I polysaccharide, M_W about 200 kDa, alternating sequence of Rha and GalA about 400 residues. Nature of side chains is still in question, although they contain L-arabinosyl and D-galactosyl residues.

[0011] US 2003/0159178 A1 discloses a rhamongalacturonan-I polysaccharide having a ratio of GalA/Rha -1.3. The polysaccharides can be derived from genetically modified potato, wherein the modification of the plant has resulted into reduced amount of arabinose or galactose in the side chains of the RG-I.

[0012] EP 1 550 448 A1 discloses a histamine release inhibitor comprising a pectin.

[0013] Hansen et al. (Journal of AOAC International, 2001, vol. 84, p. 1851-1854) discloses a method for analysis of pectin.

[0014] Nergard et al. (Carbohydrate Research, 2005, vol. 340, p. 115-130) disclose polysaccharides from the plant *Vernonia kotschyana,* and their effect on complement fixing.

[0015] Zhao et al. (Food Research International, 2006, vol. 39, p. 917-923) disclose polysaccharides involved in immunological acitivity, although it is not specified which part of the polysaccharide is involved in modulation of immune response.

[0016] Nakamura et al. (Biosci. Biotechnol. Biochem., 2001, vol. 65, p. 2249-2258) disclose the structure of polysaccharides derived from soybean.

[0017] Oechslin et al. (Carbohydrate Polymers, 2003, vol. 51, p. 301-310) disclose the structure of RG-I isolated from apple cellulosic residue.

SUMMARY OF THE INVENTION

[0018] In spite of the disclosure of the prior art, there is a desire to increase the natural defence of the human body against intruders which may cause a cold or the flu. The consumer is especially interested to achieve this goal with naturally occurring compounds or ingredients, especially as part of a common food product or diet, as the consumer generally does not want to rely on medicaments only. In order to achieve this, the consumer may consume one or more ingredients or edible products that modulate the immune response of the consumer and provide the required natural defence. Hence there is a constant need for new or alternative edible products, especially food products having such immunostimulating properties. It is an objective of the present invention to provide such edible products, especially food products.

[0019] In order to be a useful ingredient for an edible product or a food product, the properties of the ingredient should be such that the ingredient can be used in an edible product or a food product without negative influence on the properties of the edible product or food product. These properties may be related to the organoleptic properties of the ingredient, like taste, flavour, smell, colour. For example the ingredient should not have a bad taste or smell, otherwise consumers will not like the edible product and will not consume it. On the other hand, the property may also be related to some technical aspects of the ingredient when being used as an ingredient in an edible product. For the food manufacturer it is important that the ingredient does not negatively influence technical properties of the edible product or food product, such as texture, viscosity, homogeneity, emulsification, and other relevant properties.

[0020] These requirements are met by a polysaccharide obtained from plants of the species *Camellia sinensis,* wherein the backbone of the polysaccharide comprises alternating rhamnogalacturonan-I cores and alpha(1,4)-linked polygalacturonic acid or alpha(1,4)-linked oligogalacturonic acid cores, wherein the molar ratio of galacturonyl acid residues to rhamnosyl residues in the backbone of the polysaccharide ranges from 2.5:1 to 1:1, and that has a molecular weight of at least 70 kDa. Hence the amount of alpha(1,4)-linked polygalacturonic acid and/or alpha(1,4)-linked oligogalacturonic acid cores in this polysaccharide is very low.

The immuno-stimulation, immuno-suppression or immuno-modulation effects in the prior art are based on various molecular structures, including small alkaloid type molecules and glucan type polysaccharides or very heterologous combinations of these. Their effects differ in magnitude. The present invention employs well characterized isolated polysaccharide materials with well characterized activities.

[0021] The advantage of the polysaccharide according to the invention is that it is not only suitable to modulate immune response, and therewith increase the natural defence of a consumer against the cold or flu, or a similar condition, but it is also useful as an ingredient in edible products, more specifically food products. Compared to standard pectins, the thickening effect of the polysaccharide according to the invention is only very limited. This way the ingredient may be used in various food products, especially liquid food products such as beverages and soups, without negatively affecting the properties such as viscosity and texture of those food products. Additionally the use of the polysaccharide is not limited to food products, it may also be used as a medicament.

[0022] Accordingly in a first aspect the present invention provides a method for the preparation of an edible product or a pharmaceutical composition, said method comprising incorporating 0.5-10% by weight of a polysaccharide having a backbone comprising alternating rhamnogalacturonan-I cores and alpha(1,4)-linked polygalacturonic acid or alpha(1,4)-linked oligogalacturonic acid cores or having a backbone consisting of a rhamnogalacturonan-I core;
wherein the polysaccharide has a molecular weight of at least 70 kD; and
wherein the polysaccharide is obtained from plants of the species *Camellia sinensis* and wherein the molar ratio of galacturonyl acid residues to rhamnosyl residues in the backbone of the polysaccharide ranges from 2.5:1 to 1:1.

[0023] In a second aspect the present invention provides an edible product or pharmaceutical composition for use in the treatment of cold or flu, said edible product or pharmaceutical composition being obtained by a method comprising incorporating 0.5-10% by weight of a polysaccharide having a backbone comprising alternating rhamnogalacturonan-I cores and alpha(1,4)-linked polygalacturonic acid or alpha(1,4)-linked oligogalacturonic acid cores or having a backbone consisting of a rhamnogalacturonan-I core;
wherein the polysaccharide has a molecular weight of at least 70 kD; and
wherein the polysaccharide:

- is obtained from plants of the species *Camellia sinensis* and wherein the molar ratio of galacturonyl acid residues to rhamnosyl residues in the backbone of the polysaccharide ranges from 2.5:1 to 1:1; or
- is obtained from plants of the species *Daucus carota* and wherein the molar ratio of galacturonyl acid residues to rhamnosyl residues in the backbone of the polysaccharide ranges from 2.5:1 to 1:1; or
- is obtained from the species *Glycine max* and wherein the molar ratio of galacturonyl acid residues to rhamnosyl residues in the backbone of the polysaccharide ranges from 1.5:1 to 1:1; or

- is obtained from the species *Malus domestica* and wherein the molar ratio of galacturonyl acid residues to rhamnosyl residues in the backbone of the polysaccharide ranges from 1.5:1 to 1:1; or
- is obtained from the species *Beta vulgaris L.* and wherein the molar ratio of galacturonyl acid residues to rhamnosyl residues in the backbone of the polysaccharide ranges from 1.5:1 to 1:1;

wherein the molar ratio of galacturonyl acid residues to rhamnosyl residues in the backbone of the polysaccharide is determined using the NMR method described in the Examples.

DETAILED DESCRIPTION

[0024] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art.

[0025] All percentages, unless otherwise stated, refer to the percentage by weight.

[0026] In case a range is given in the context of the present invention, the indicated range includes the mentioned endpoints.

[0027] Abbreviations: kDa - kilo Dalton; Gal - D-Galactose; GalA- D-Galacturonic acid; Rha - L-Rhamnose; Ara - L-Arabinose; Fuc - L-Fucose; Glc - D-Glucose; GlcA - D-Glucuronic acid. The L- and D- forms of these monomers as indicated here also apply to the monomers as indicated in the rest of this specificiation (which may not be abbreviated but written in full).

Immune Response

[0028] By the term 'modulating immune response' as used herein, is meant that the activity or capacity of the immune system to defend the body is modulated. This may relate to immuno-stimulation or immuno-suppression. The primary task of the immune system is to protect against pathogens such as fungi, bacteria, viruses, protozoa and parasites. In this context, modulating immune response preferably means stimulation of the immune response. Suitably, stimulation of the immune response contributes to an enhanced natural defence of the human body. On the other hand, the immune system sometimes mounts an immune response against harmless substances, like house mite, dust or pollen, resulting in allergy. In addition, many physiological disorders, like hypercholesterolemia and obesity, result in a low-grade inflammatory status. Immune modulation in the context of abnormal immune responses, like allergy or inflammation, means dampening or counteracting the hypersensitivity immune response. The present invention not only relates to the primary task of the immune system, but also to this second 'abnormal' immune response.

[0029] Several assays can be used to identify components that could modify immunity. The

present inventors chose the use of phagocytic and natural killer (NK) cells to aid the identification of immunostimulating compounds as these cells are part of the innate immune system, which is a rapidly activated non-specific first line of defence against pathogens.

[0030] Phagocytic cells such as neutrophils, monocytes and macrophages can generate reactive oxygen species (ROS) to kill pathogens such as fungi and bacteria. The effect of ingredients on phagocytosis activity can be measured *ex vivo* with fresh blood of healthy human volunteers after incubation with FITC-labelled *E. coli* bacteria. The percentage of phagocytosing cells in the granulocyte population can be determined by flow cytometry. The results are typically normalized to the effect of lipo-polysaccharide (LPS), which is a well known potent immunostimulating reference compound. Suitably, a normalized percentage phagocytosing granulocytes of more than 40% is regarded as a significant immune stimulating effect.

[0031] NK cells can kill target cells that have lost or express insufficient amounts of MHC class I, a frequent event in tumor- or virus-infected cells. The effect of ingredients on NK cell activity can *ex vivo* be measured with peripheral blood mononuclear cells (PBMC) isolated from fresh blood of healthy human volunteers. After pre-incubation of the PBMCs with the ingredient, pre-labelled K562 target cells are usually added and after subsequent incubation, propidium iodide can be added for detection of dead cells. The percentage of dead target cells can be determined with flow cytometry. The results are typically normalized to the effect of interleukin-2 (IL-2), which is a well known potent NK cell stimulating reference compound. Suitably, a normalized % NK cell activity of more than 17% is regarded as a significant immune stimulating effect.

Pectin and Rhamnogalacturonan Polysaccharides

[0032] Pectin is a complex mixture of colloidal polysaccharides found in the primary cell walls of both monocotyledons (monocots) and dicotyledons (dicots). It is characterized by the presence of rhamnosyl, galacturonyl acid, arabinosyl, and galactosyl residues as the main components, and at least occasionally xylosyl, mannosyl, glucosyl and apiosyl residues. Traditionally, pectin is known for its gelling and viscosifying properties utilized in industrial and household preparations of jellies, jam, and marmalade and it is widely used for its thickening and viscosifying properties. Pectin is generally regarded to be poly-D-galacturonic acid (homogalacturonan, HG), wherein the galacturonyl acid moieties are linked via alpha(1-4) linkages. The carboxyl group of a galacturonyl acid residue may be esterified with methanol, to create high methoxy and low methoxy pectins. The degree of methyl esterification influences the gelling behaviour of pectin. Moreover the galacturonic acid may be acetylated, in addition to the methyl esters. In that case one of the hydroxyl groups 2-OH and 3-OH positions are substituted by esterification to yield the acetates. Acetylation generally prevents gel-formation but increases the stabilising and emulsifying effects of pectin. Pectic polysaccharide is a heterogenous group of polysaccharides including various amounts of various components sometimes present or absent such as (i) homogalacturonan (HG), (ii) xylogalacturonan (XGA),

(iii) rhamnogalacturonan-I backbone, encompassing arabinan and arabinogalactan I and II side-chains (RG-I), and (iv) rhamnogalacturonan-II (RG-II) (Ralet et al., Carbohydrate Research, vol. 344, 2009, p. 1798-1807). Pectic polysaccharide composition and fine structure vary widely depending on the plant source and the extraction conditions applied. The poly-GalA core can have a length of about 100 consecutive D-GalA residues. The RG-I core containing the side chains is usually called the 'ramified region' or 'hairy region', while the poly-GalA core (between 2 RG-I cores) is not typically substituted with oligosaccharides.

[0033] Rhamnogalacturonans are a group of closely related cell wall pectic polysaccharides that contain a backbone of the repeating disaccharide:
[→4)-D-GalA-alpha(1→2)-L-Rha-alpha(1→]
(which can also be represented as: [-4)-D-GalA-alpha(1-2)- L-Rha(p)-alpha(1-])

[0034] Rhamnogalacturonan-I (RG-I) is referred to as regions with 30-40 repeats of GalA and rhamnose (Rha) pairs [Westereng 2009 *ibid*.], with varying numbers of Rha residues. The GalA residues are linked to the Rha residues via the 1 and 4 positions, while the Rha residue is linked to the GalA residue via the anomeric and 2-OH positions. In general about 20-80% of the Rha residues is branched at the 4-OH position (depending on the plant source and the method of isolation), with neutral and acidic side chains (or 4-OH position). These side chains consist mainly of Ara and Gal residues linked in various manners, constituting polymers known as arabinogalactan I (AG-I) and/or AG-II. AG-I is composed of a beta (1,4)-linked D-Gal backbone with substitutions at 3-OH of alpha-L-arabinosyl groups; the Gal backbone can have interspacing alpha(1,5)-L-Ara units. AG-II consists of highly ramified galactan with predominantly interior beta(1,3)-linked D-Galp with substitutions of short (1,6)-linked chains exteriorly. The latter has further attachments of (1,3)- and/or alpha(1,5)-linked L-Ara. The oligosaccharide side chains may be linear or branched, and some of these side chains may be terminated with alpha-L-fucosides, beta-D-glucuronides, and 4-O-methyl beta-D-glucuronyl residues.

Polysaccharide from Camellia sinensis

[0035] In one embodiment of the invention, the edible product or pharmaceutical composition for use in the treatment of cold or flu comprises a polysaccharide obtained from plants of the species *Camellia sinensis.*

[0036] The species *Camellia sinensis,* commonly known as the tea plant, belongs to the Theaceae family. Preferably the polysaccharide is obtained from the leaves of this plant.

[0037] The molar ratio of galacturonyl acid residues to rhamnosyl residues refers to the total number of residues of galacturonyl acid present in the polysaccharide according to the invention. Consequently this includes both the GalA residues present in the RG-I core linked to Rha residues, as well as the GalA residues present in the alpha(1,4)-linked polygalacturonic acid or alpha(1,4)-linked oligogalacturonic acid cores, hence linked to other GalA residues.

Polygalacturonic acid is considered to be a (part of a) polymer of at least 10 galacturonyl acid residues linked to each other; oligogalacturonic acid is considered to be a (part of a) molecule of 2 to 10 galacturonyl acid residues linked to each other.

[0038] For example if the molar ratio of galacturonyl acid residues to rhamnosyl residues is 2:1, then the polysaccharide contains per rhamnosyl residue (which is present in the RG-I core) one corresponding GalA residue (which is also present in the RG-I core linked to a Rha residue), as well as one GalA residues in the alpha(1,4)-linked polygalacturonic acid or alpha(1,4)-linked oligogalacturonic acid core.

[0039] If, for example, the molar ratio of galacturonyl acid residues to rhamnosyl residues is 1:1, then the polysaccharide contains per rhamnosyl residue (present in the RG-I core) one corresponding alpha(1,2)-linked GalA residue (also present in the RG-I core linked to a Rha residue). In that case the polysaccharide does not contain GalA residues from the alpha(1,4)-linked polygalacturonic acid or alpha(1,4)-linked oligogalacturonic acid core.

[0040] Preferably the molar ratio of galacturonyl acid residues to rhamnosyl residues in the backbone of the polysaccharide ranges from 1.2:1 to 1:1. In case the molar ratio is 1:1 or close to 1:1, the polysaccharide consists entirely or nearly entirely of an RG-I core, without or a very low proportion of alpha(1,4)-linked polygalacturonic acid or alpha(1,4)-linked oligogalacturonic acid cores.

[0041] The length of a RG-I core preferably ranges from 10 to 60 disaccharide units, wherein a disaccharide unit contains a rhamnosyl and a galacturonyl acid residue (as explained before). Preferably the length of the RG-I core is between 20 and 50, more preferably between 20 and 40 disaccharide units.

[0042] All polymers according to the invention modulate immune response, preferably stimulate immune response. Preferably with increasing molecular weight, the modulation of the immune response increases as well per weight amount of polysaccharide. The molecular weight of the polysaccharide according to the invention is at least 70 kDa. Preferably the polysaccharide according to the invention has a molecular weight between 70 and 2,000 kDa. The polysaccharide according to the invention preferably has a molecular weight between 70 and 110 kDa, more preferably between 110 and 2,000 kDa. Suitably the polysaccharide according tot the invention has a molecular weight between 110 and 1,000 kDa, preferably between 110 and 500 kDa, or between 500 and 1,000 kDa.

[0043] Preferably the polysaccharide according to the invention comprises is a polysaccharide wherein said rhamnogalacturonan-I core comprises one or more side chains, wherein the one or more side chains comprise a backbone of at least one or more alpha(1,5)-linked arabinosyl residues and wherein the one or more side chains are substituted at the 4-OH position of the rhamnosyl residues. Said preferred side chain comprising alpha(1,5)-linked arabinosyl residues may be substantially linear or branched. In case that side chain is primarily linear, the side chain primarily comprises alpha(1,5)-linked arabinosyl residues, which form the backbone

of the side chain. In case said side chain is a branched side chain, then one or more alpha-arabinosyl residues are linked to the 2-OH and/or 3-OH to the of alpha(1,5)-linked arabinans.

[0044] The preferred molecular weight of the preferred side chain comprising an alpha(1,5)-linked arabinosyl residue can be expressed as a relative number: the molar ratio between the number of arabinosyl residues and the number of rhamnosyl residues. If the polysaccharide of the invention comprises a side chain comprising an alpha(1,5)-linked arabinosyl residue, then preferably the molar ratio of arabinosyl residues to rhamnosyl residues is between 50:1 and 1:2, more preferably between 40:1 and 1:2, more preferably between 30:1 and 1:2, more preferably between 20:1 and 1:2, more preferably between 20:1 and 1:1, and more preferably between 20:1 and 2:1. Alternatively, preferably the ratio is between 10:1 and 1:2, preferably between 6:1 and 1:2, preferably between 4:1 and 1:2.

[0045] If side chains comprising arabinosyl monomers are present, the length of the side chains (expressed as number of monomer units) preferably is between 1 and 100 monomer units, more preferably between 1 and 50 units, even more preferably between 1 and 30 units.

[0046] Another preferred polysaccharide according to the invention is a polysaccharide wherein said rhamnogalacturonan-I core comprises one or more side chains, wherein the one or more side chains comprise a backbone of at least one or more beta(1,4)-linked galactosyl residues and wherein said one or more side chains are substituted at the 4-OH position of the rhamnosyl residues. If the preferred polysaccharide according to the invention comprises a side chain comprising one or more beta(1,4)-linked galactan residues, then the side chain is mostly a linear unsubstituted chain. Preferably other galactans like beta(1,3)-linked galactan and/or beta(1,6)-linked galactan are absent, or at least substantially absent, which means that preferably less than 10 mol% of galactan residues are present in side chains are beta(1,3)-linked or beta(1,6)-linked galactan residues, preferably less than 5 mole%, preferably less than 2 mole%, preferably less than 1 mole%.

[0047] The preferred molecular weight of the preferred side chain comprising a beta(1,4)-linked galactan residue can be expressed as a relative number: the molar ratio between the number of galactosyl residues and the number of rhamnosyl residues. If the polysaccharide of the invention comprises a side chain comprising a beta(1,4)-linked galactan residue, then preferably the the molar ratio of galactosyl residues to rhamnosyl residues is between 80:1 and 1:1, more preferably between 60:1 and 1:1, more preferably between 50:1 and 1:1, more preferably between 30:1 and 1:1, more preferably between 20:1 and 1:1, more preferably between 20:1 and 2:1, and more preferably between 20:1 and 3:1. Alternatively, preferably the ratio is between 30:1 and 1:2, preferably between 25:1 and 1:1, preferably between 20:1 and 1:1, preferably between 10:1 and 2:1, preferably between 6:1 and 2:1.

[0048] If side chains comprising galactosyl monomers are present, the length of the side chains (expressed as number of monomer units) preferably is between 1 and 100 monomer units, more preferably between 1 and 50 units, even more preferably between 1 and 30 units.

[0049] If the polysaccharide according to the invention comprises side chains which are substituted at the 4-OH position of rhamnosyl residues of the rhamnogalacturonan-I core, then preferably at most 5% of the side chains are arabinogalactan side chains, more preferably at most 1% of the side chains are arabinogalactan side chains. This should be understood to mean that preferably the polysaccharide according to the invention is substantially free from arabinogalactan side chains, more preferably free from arabinogalactan side chains. Arabinogalactan side chains are side chains which comprise both arabinosyl and galactosyl residues. Arabinogalactan I (AGI) and AGII have been described above.

[0050] Preferably the RG-I core of the polysaccharide of the invention comprises side chains, such that at least 20 mole% of the rhamnosyl residues is substituted at the 4-OH position, preferably at least 30 mole%, preferably at least 40 mole%, preferably at least 45 mole%, and preferably at most 90 mole%, preferably at most 80 mole%.

[0051] Preferably, in general the polysaccharide according to the invention has a small, even if any, effect on viscosity or thickening of liquid compositions when dissolved, as compared to standard pectins. With increasing molecular weight, the thickening effect preferably increases per unit weight, however this thickening effect preferably is still small. Preferably the effect as a thickener depends on the degree of branching of possible side chains and/or the average length of side chains of the RG-I core: with increasing branching and/or increasing average length of side chains, the thickening effect becomes less.

[0052] The polysaccharide according to the invention comprises residues of the monomers rhamnose, galacturonic acid, and if the polymer comprises one or more side chains, then the polysaccharide may contain residues of arabinose and/or galactose as well. In addition the polysaccharide may contain minor amounts of residues of the monomers fucose, glucose, glucuronic acid, xylose, and/or uronic acid. These monomers may for instance terminate side chains, if these are present. In a preferred embodiment the invention is directed to polysaccharides comprising xylose.

[0053] The methods for determining the structures of the polysaccharides of the present invention are known to the skilled person. These methods include analysis using ^{1}H and ^{13}C NMR.

[0054] A suitable method for intake of the polysaccharides or the said preparation according to the invention may be oral intake of the edible product or pharmaceutical composition. Alternatively the polysaccharide or the said preparation may be taken in as ingredients in a pharmaceutical composition which is common in the field. Differences may exist between immuno-modulating effects between different animal species including humans. The immunological effects in the present invention have been established using human immune cells and are therefore most pertinent to immunomodulation in humans and related mammals.

[0055] The physiological immune response of a consumer that consumes the polysaccharide of the invention, may be determined by *ex vivo* analysis of the activity of phagocytic and natural

killer (NK) cells of that consumer. The immune modulating response upon ingestion of the polysaccharides according to the invention generally occurs within a few hours, e.g. 2 or 3 hours. The effect may last for about 24 hours or longer. Suitably, upon continued consumption of products containing the polysaccharide according to the invention, for example once or twice a day at consecutive days, the immune response can be stimulated and prolonged, and the natural defence of the consumer against the flu or cold can be enforced.

[0056] The daily dose of a polysaccharide according to the invention required to obtain the preferred immune response modulating effect, preferably is between 1 and 10,000 milligram per day. More preferred the amount of polysaccharide dosed is between 5 and 10,000 milligram per day, preferably between 10 and 10,000 milligram per day, even more preferred between 10 and 5,000 milligram per day. More preferred the amount dosed is between 10 and 1,000 milligram per day and most preferred between 10 and 500 milligram per day. This amount may be dosed as a single dose per day, or as 2, 3, 4, 5, 6, 7, 8, 9, or 10 doses per day. Preferably the suitable amount of polysaccharides according to the invention are delivered by 1 or 2 doses per day.

Edible product or pharmaceutical composition

[0057] Preferably the concentration of the polysaccharide in the edible product or pharmaceutical composition for use in the treatment of cold or flu is between 1% and 10% by weight, more preferred between 2% and 10% by weight, more preferred between 3% and and 10% by weight, more preferred between between 4% and 10% by weight, and most preferred between 5% and 9% by weight. The polysaccharides according to the invention may be present in the edible product or pharmaceutical composition in its native form, meaning as a constituent of a vegetable material which is used in the edible product or pharmaceutical composition. Nevertheless preferably the edible product or pharmaceutical composition is enriched with the polysaccharide according to the invention. This means that the polysaccharide is possibly not only present in its native form as a constituent of a vegetable material, but that in addition also the polysaccharide is added as an ingredient to the edible product or pharmaceutical composition. The polysaccharide may be added in pure form or as part of an extract enriched in the polysaccharide or in any other suitable form. Hence preferably, the edible product or pharmaceutical composition according to the invention preferably comprises from 0.0001 to 25% by weight of the polysaccharide, wherein at least a part of the polysaccharides is added to the edible product or pharmaceutical composition in an enriched form. Preferably the concentration of the polysaccharide in the composition according to the invention is between 0.5% and 10% by weight, preferably between 1% and 10% by weight, more preferred between 2% and 10% by weight, more preferred between 3% and and 10% by weight, more preferred between between 4% and 10% by weight, and most preferred between 5% and 9% by weight, wherein at least a part of the polysaccharides is added to the edible product or pharmaceutical composition in an enriched form. The polysaccharides according to the invention may be added to the edible product or pharmaceutical composition in a specific salt form.

[0058] The edible product according to the present invention may take any physical form. In particular, it may be a food product, a beverage, a dietary food product, or a clinical food product. It may also be a dietary supplement, in the form of a beverage, a tablet, a capsule, or any other suitable form for a dietary supplement. Preferred edible products for incorporation of the polysaccharide according to the invention are in the form of a liquid, such as a soup or a beverage, a spread, a dressing, a dessert or a bread. If the preferred edible product is a soup, this may be a liquid soup, or a dried soup to which hot water can be added by the consumer. The edible product may be in liquid or spreadable form, it may be a spoonable solid or soft-solid product, or it may be a food supplement. Preferably the edible product is a liquid product. The edible product may suitably take the form of e.g. a soup, a beverage, a spread, a dressing, a dessert, a bread. More preferably, the edible product is a beverage, a dessert or a spread. More preferably, the edible product is a beverage or a spread, especially a spread in the form of an oil-in-water emulsion or a water-in-oil emulsion. The term 'spread' as used herein encompasses spreadable products such as margarine, light margarine, spreadable cheese based products, processed cheese, dairy spreads, and dairy-alternative spreads. Spreads as used herein (oil-in-water or water-in-oil emulsions) may have a concentration of oil and/or fat of between about 5% and 85% by weight, preferably between 10% and 80% by weight, more preferred between 20% and 70% by weight. Preferably the oil and/or fat are from vegetable origin (such as but not limited to sunflower oil, palm oil, rapeseed oil); oils and/or fats of non-vegetable origin may be included in the composition as well (such as but not limited to dairy fats, fish oil).

[0059] Most preferably, the product is a beverage. Such a beverage typically contains at least 60% by weight water and 0 to 20% by weight of dispersed oil or fat. Preferably, such beverage contains at least 70% by weight water and 0 to 10% by weight of dispersed oil or fat.

[0060] A dressing in the context of the present invention generally is an oil-in-water emulsion, which may contain between 0.1 and 85% of oil and/or fat. Mayonnaise is an example of a dressing within the context of the present invention. Dressings as used herein (oil-in-water emulsion) may have a concentration of oil and/or fat of between about 50.1 and 85% by weight, preferably between 5% and 80% by weight, more preferred between 10% and 70% by weight. Preferably the oil and/or fat are from vegetable origin (such as but not limited to sunflower oil, palm oil, rapeseed oil); oils and/or fats of non-vegetable origin may be included in the composition as well (such as but not limited to dairy fats, fish oil).

[0061] A pharmaceutical composition in the context of the present invention encompasses, but is not limited to, prescription drugs, non-prescription drugs, over the counter medicines, dietary supplements, dietary foods, clinical foods, edible products, tablets, capsules, pills, and food products such as beverages or any other suitable food product, and any other composition which is commonly known to the skilled person. Alternatively the medicament may be an injectable substance or an inhalable substance, such as a nasal spray. In case of a pharmaceutical composition, the compostion may contain more than 25% by weight of the polysaccharide according to the invention, preferably more than 30% by weight, preferably

more than 40% by weight, or preferably more than 50% by weight, or preferably even more than 75% by weight.

[0062] Edible products suitable for this invention can be any food product, including beverages, dietary food products and clinical food products. The concentration of the polysaccharide according to the invention should be such that modulation of the immune response occurs after consumption of the food product at a regular amount. A regular amount is the amount that an average consumer consumes of such a food product at a specific consumption moment.

[0063] The concentration of polysaccharides required in the edible product depends on the specific edible product and how much of such a product is usually consumed. Preferably the polysaccharides according to the invention are incorporated in edible products which are usually consumed at a predefined amount. For example a cereal bar is usually packed per single bar, and also consumed per single bar. Usually the weight of such a bar is between 40 and 80 gram. Similarly, dairy mini-drinks are consumed from small bottles, having a volume of about 100 milliliter.

[0064] By incorporating the polysaccharides in such food products, the daily intake of the polysaccharides can in principle be controlled. Preferably the polysaccharides are delivered to the consumer in 1 or 2 doses per day. The skilled person is able to calculate the required concentration of the polysaccharide in a unit amount of the edible product, preferably food product.

[0065] A unit amount of a food product is a quantity of a food product which is usually consumed as a single serving. The unit amount or serving size of such food products depends on the specific product. A few non-limiting examples of typical serving sizes are:

milk, yoghurt: 200 mL

natural cheese: 43 gram

processed cheese: 57 gram

fruit juice: 177 mL

soft drink: 200 mL

bread: 1 slice, 35 gram

coffee: 125 mL

tea: 150 mL

cereal bar, candy bar: 50 gram

chocolate: 30 gram

ice cream: 100 mL

spread: 15 gram

soup: 250 mL

cocoa beverage: 200 mL

[0066] A unit amount of a food product in the context of the present invention may be packed and sold as a single portion. For example, ice cream may be packed as individual units, therewith making such an individual portion a unit amount in the context of the present invention. The actual weight or volume of such an individually packed product may be higher or lower than indicated above for a standard serving size. For example probiotic dairy drinks are consumed from small bottles, individually packed, having a volume of about 100 mL.

Polysaccharides from Daucus carota

[0067] In one embodiment of the invention, the edible product or pharmaceutical composition for use in the treatment of cold or flu comprises polysaccharides that are obtained from plants of the species *Daucus carota.* Most preferred the polysaccharide is obtained from the root of the species *Daucus carota* subsp. *sativus.*

[0068] The species *Daucus carota* includes wild carrot, of which the root is edible. Preferably the polysaccharide is obtained from the species *Daucus carota* subsp. *sativus,* which is called the commonly cultivated carrot. The carrot is a well known root vegetable, usually orange, red, purple, white or yellow in colour.

[0069] When the edible product or pharmaceutical composition according to the invention comprises polysaccharides which are obtained from the species *Daucus carota* subsp. *sativus,* more preferably from the root of the species *Daucus carota* subsp. *sativus* (from carrot), then the molar ratio of galacturonyl acid residues to rhamnosyl residues in the backbone of the polysaccharide ranges from 1.5:1 to 1:1, preferably from 1.2:1 to 1:1.

[0070] Preferably the polysaccharide has a molecular weight of at least 110 kDa, most preferably between 110 and 2,000 kDa.

[0071] Preferably the polysaccharide according to the invention that is obtained from *Daucus carota* subsp. *sativus,* contains side chains connected to the 4-OH position of the rhamnosyl residues, wherein the side chains comprise arabinosyl residues, and wherein the molar ratio of arabinosyl residues to rhamnosyl residues of the polysaccharide is between 50:1 and 5:1, more preferably between 30:1 and 5:1, most preferably between 20:1 and 5:1.

[0072] Preferably the polysaccharide according to the invention that is obtained from *Daucus carota* subsp. *sativus,* contains side chains connected to the 4-OH position of the rhamnosyl residues, wherein the side chains comprises galactosyl residues, and wherein the molar ratio of galactosyl residues to rhamnosyl residues of the polysaccharide is between 50:1 and 5:1, more preferably between 30:1 and 5:1, most preferably between 20:1 and 5:1.

[0073] Preferably the number of side chains is such that at least 40 mole% of the rhamnosyl residues of the rhamnogalacturonan-I core is substituted, preferably at least 50 mole%, and preferably at most 90 mole%, more preferably at most 80 mole%.

[0074] In another preferred embodiment, when the edible product or pharmaceutical composition according to the invention comprises polysaccharides which are obtained from the root of the species *Daucus carota* subsp. *sativus* (from carrot), then the molar ratio of galacturonyl acid residues to rhamnosyl residues in the backbone of the polysaccharide may range from 25:1 to 1:1, preferably from 20:1 to 1:1, more preferably from 20:1 to 10:1. In that case, preferably the polysaccharide has a molecular weight between 70 and 110 kDa.

[0075] Moreover, the said polysaccharide obtained from *Daucus carota* subsp. *sativus,* contains side chains connected to the 4-OH position of the rhamnosyl residues, wherein the side chains comprise arabinosyl residues, and wherein the molar ratio of arabinosyl residues to rhamnosyl residues of the polysaccharide is between 20:1 and 2:1, more preferably between 15:1 and 3:1, most preferred between 14:1 and 7:1. Moreover, the said polysaccharide obtained from *Daucus carota* subsp. *sativus,* preferably contains side chains connected to the 4-OH position of the rhamnosyl residues, wherein the side chains comprises galactosyl residues, and wherein the molar ratio of galactosyl residues to rhamnosyl residues of the polysaccharide is between 20:1 and 2:1, more preferably between 15:1 and 3:1, most preferred between 10:1 and 3:1.

[0076] Preferably the number of side chains is such that at least 50 mole% of the rhamnosyl residues of the rhamnogalacturonan-I core is substituted, preferably at least 60 mole%, and preferably at most 90 mole%, more preferably at most 80 mole%.

Polysaccharides from Glycine max

[0077] In another embodiment, the edible product or pharmaceutical composition for use in the treatment of cold or flu comprises a polysaccharide that is obtained from one or more plants belonging to the species *Glycine max,* commonly known as soya.

[0078] When the edible product or pharmaceutical composition according to the invention comprises polysaccharides which are obtained from the species *Glycine max,* more preferably from soyabean, then the molar ratio of galacturonyl acid residues to rhamnosyl residues in the backbone of the polysaccharide ranges from 1.5:1 to 1:1, preferably from 1.2:1 to 1:1.

[0079] Preferably the polysaccharide has a molecular weight of between 70 and 2000 kDa, more preferably between 70 and 110 kDa, more preferably at least 110 kDa, most preferably between 110 and 2,000 kDa.

[0080] Preferably the polysaccharide according to the invention that is obtained from *Glycine max,* contains side chains connected to the 4-OH position of the rhamnosyl residues, wherein the side chains comprises arabinosyl residues, and wherein the molar ratio of arabinosyl residues to rhamnosyl residues of the polysaccharide is between 50:1 and 1:2, more preferred between 40:1 and 1:1, and more preferred between 30:1 and 2:1, more preferred between 25:1 and 3:1.

[0081] Preferably the polysaccharide according to the invention that is obtained from *Glycine max,* contains side chains connected to the 4-OH position of the rhamnosyl residues, wherein the side chains comprises galactosyl residues, and wherein the molar ratio of galactosyl residues to rhamnosyl residues of the polysaccharide is between 70:1 and 1:2, more preferred between 60:1 and 1:1, and more preferred between 50:1 and 2:1, more preferred between 40:1 and 5:1.

[0082] Preferably the number of side chains is such that at least 40 mole% of the rhamnosyl residues of the rhamnogalacturonan-I core is substituted, preferably at least 50 mole%, and preferably at most 90 mole%, preferably at most 80 mole%.

Polysaccharides from Malus domestica

[0083] In another embodiment, the edible product or pharmaceutical composition for use in the treatment of cold or flu comprises a polysaccharide according to the invention, said polysaccharide being obtained from one or more plants belonging to the species *Malus domestica,* commonly known as the apple. Most preferably the polysaccharide is obtained from the fruit of the species *Malus domestica,* which is the commonly known apple.

[0084] When the edible product or pharmaceutical composition according to the invention comprises polysaccharides which are obtained from the species *Malus domestica,* more preferably from the apple, then the molar ratio of galacturonyl acid residues to rhamnosyl residues in the backbone of the polysaccharide ranges from 1.5:1 to 1:1, preferably from 1.2:1 to 1:1.

[0085] Preferably the polysaccharide has a molecular weight of between 70 and 2,000 kDa, preferably between 70 and 110 kDa, preferably between 110 and 2,000 kDa. Preferably the polysaccharide according to the invention that is obtained from *Malus domestica,* contains side chains connected to the 4-OH position of the rhamnosyl residues, wherein the side chains comprises arabinosyl residues, and wherein the molar ratio of arabinosyl residues to rhamnosyl residues of the polysaccharide is between 10:1 and 1:2, preferably between 5:1 and 1:2.

[0086] Preferably the polysaccharide according to the invention that is obtained from *Malus domestica,* contains side chains connected to the 4-OH position of the rhamnosyl residues, wherein the side chains comprises galactosyl residues, and wherein the molar ratio of galactosyl residues to rhamnosyl residues of the polysaccharide is between 10:1 and 1:2, preferably between 5:1 and 1:1.

[0087] Preferably the number of side chains is such that at least 50 mole% of the rhamnosyl residues of the rhamnogalacturonan-I core is substituted, preferably at least 60 mole%, and preferably at most 90 mole%, preferably at most 80 mole%.

***Polysaccharides from Beta vulgaris* L.**

[0088] In another embodiment, the edible product or pharmaceutical composition for use in the treatment of cold or flu comprises a polysaccharide according to the invention, said polysaccharide being obtained from one or more plants belonging to the species *Beta vulgaris* L.. Most preferably the polysaccharide is obtained from the root of the species *Beta vulgaris* L., which is the commonly known sugar beet.

[0089] When the edible product or pharmaceutical composition according to the invention comprises polysaccharides which are obtained from the species Beta vulgaris L., more preferably from the root of Beta vulgaris L. (sugar beet), then the molar ratio of galacturonyl acid residues to rhamnosyl residues in the backbone of the polysaccharide ranges from 1.5:1 to 1:1, preferably from 1.2:1 to 1:1.

[0090] Preferably the polysaccharide has a molecular weight of at least 110 kDa, more preferred between 110 and 2,000 kDa.

[0091] Preferably the polysaccharide according to the invention that is obtained from Beta vulgaris L., contains side chains connected to the 4-OH position of the rhamnosyl residues, wherein the side chains comprises arabinosyl residues, and wherein the molar ratio of arabinosyl residues to rhamnosyl residues of the polysaccharide is between 50:1 and 1:2, preferably between 40:1 and 1:1, more preferred between 35:1 and 5:1.

[0092] Preferably the polysaccharide according to the invention that is obtained from Beta vulgaris L., contains side chains connected to the 4-OH position of the rhamnosyl residues, wherein the side chains comprises galactosyl residues, and wherein the molar ratio of galactosyl residues to rhamnosyl residues of the polysaccharide is between 10:1 and 1:2, preferably between 5:1 and 1:1.

[0093] Preferably the number of side chains is such that at least 40 mole% of the rhamnosyl residues of the rhamnogalacturonan-I core is substituted, preferably at least 50 mole%, and preferably at most 90 mole%, preferably at most 80 mole%.

[0094] Preferred food products according to the invention may be dried and contain less than 40% water by weight of the composition, preferably less than 25%, more preferably from 1 to 15%. Alternatively, the food may be substantially aqueous and contain at least 40% water by weight of the composition, preferably at least 50%, more preferably from 65 to 99.9%.

[0095] The food preferably comprises nutrients including carbohydrate (including sugars and/or starches), protein, fat, vitamins, minerals, phytonutrients (including terpenes, phenolic compounds, organosulfides or a mixture thereof) or mixtures thereof. The source of the protein material is not limited, and may for example be from dairy origin (such as casein, lactoglobulin), or vegetable origin (such as soya, pea, etcetera). The food may be low calorie (e.g. have an energy content of less than 100 kCal per 100 gram of the composition) or may have a high calorie content (e.g. have an energy content of more than 100 kCal per 100 g of the composition, preferably between 150 and 1000 kCal). The food may also contain salt, flavours, colours, preservatives, antioxidants, non-nutritive sweetener or mixtures thereof. Suitable vitamins and minerals include but are not limited to vitamin A, vitamin D, vitamin E, vitamin C, thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12 (cyanocobalamin), biotin, pantothenic acid, calcium, phosphorous, potassium, iron, zinc, copper, iodine, selenium, sodium, magnesium, manganese, molybdenum, vitamin K, chromium and mixtures thereof. The preferred ingredients to deliver vitamins and minerals include but are not limited to potassium phosphate, calcium phosphate, magnesium oxide, magnesium phosphate ascorbic acid, sodium ascorbate, vitamin E acetate, niacinamide, ferric orthophosphate, calcium pantothenate, zinc oxide, zinc gluconate, vitamin A palmitate, pyridoxine hydrochloride, riboflavin, thiamin mononitrate, biotin, folic acid, chromium chloride, potassium iodide, sodium molybdate, sodium selenate, phytonadone (vitamin K), cholecalciferol (vitamin D3), manganese sulfate and mixtures thereof. Preferably, the product according to the invention contains at least 10% or more of the recommended daily amount ('RDA') of the vitamins and minerals.

[0096] The products according to the invention may further include meat, fish, meat and fish extracts, fruit, dried fruit, fruit concentrates, fruit extracts, fruit juices, tea (e.g. green tea), vegetables, vegetable extracts and concentrates, nuts, nut extracts, chocolate, bread, vinegar, salt, pepper, cocoa powder, herbs (e.g. parsley), herb extracts, spices (e.g. cinnamon), spice extracts, emulsifiers, acidity regulators (e.g. phosphoric, malic, citric, tartaric acids and salts thereof), flavonoids, preservatives (e.g. lactic acid, EDTA, tocopherols, sodium benzoate), colours (e.g. beta carotene, lycopene, caramel, carmine red), fibers (e.g. soya), leavening agents (e.g., sodium bicarbonate), pectin, citric acid, yeast, salt, glycerin, and mixtures thereof.

[0097] The present invention further provides a method for preparation of an edible product or pharmaceutical composition said method comprising incorporating 0.5-10% by weight of a polysaccharide having a backbone comprising alternating rhamnogalacturonan-I cores and alpha(1,4)-linked polygalacturonic acid or alpha(1,4)-linked oligogalacturonic acid cores or having a backbone consisting of a rhamnogalacturonan-I core;
wherein the polysaccharide has a molecular weight of at least 70 kD; and

wherein the polysaccharide is obtained from plants of the species *Camellia sinensis* and wherein the molar ratio of galacturonyl acid residues to rhamnosyl residues in the backbone of the polysaccharide ranges from 2.5:1 to 1:1.

[0098] The polysaccharide according to the invention may be incorporated into an edible product or pharmaceutical composition by bringing the polysaccharide in purified form into contact with other ingredients of the edible product or pharmaceutical composition. Additionally, or alternatively, a vegetable material that contains the polysaccharides according to the invention may be brought into contact with other ingredients of the edible product or pharmaceutical composition. These steps may be performed in any stage of the production process. For example, the polysaccharides may be mixed into a ready or nearly ready made edible product or pharmaceutical composition or it may be brought into contact with ingredients, such that the ingredients are subsequently mixed and the edible product or pharmaceutical composition is prepared.

DESCRIPTION OF FIGURES

[0099]

Figure 1: Schematic representation of a preferred polysaccharide according to the invention:

1. 1: galacturonyl acid residue in RG-I core
2. 2: rhamnosyl residue in RG-I core
3. 3: galactosyl residue in side chain, connected to 4-OH position of the rhamnosyl residue
4. 4: arabinosyl residue in side chain, connected to 4-OH position of the rhamnosyl residue

Figure 2: Immune modulating effect of polysaccharides, obtained from the fruit of the species *Malus domestica* (apple); whole blood cell assay.
Concentration of extract in microgram per milliliter (x-axis) versus percentage phagocytosis (y-axis).

Figure 3: Immune modulating effect of polysaccharides, obtained from the bean of the species *Glycine max* (soyabean); HL60 cell assay.
Concentration of extract in microgram per milliliter (x-axis) versus percentage phagocytosis (y-axis).

EXAMPLES

[0100] The following non-limiting examples illustrate the present invention.

Methods

Separation of polysaccharides by molecular weight

[0101] Polysaccharides obtained by boiling water extraction are separated by gel filtration chromatography on a column of Superdex 200 (1 × 30 cm; GE Healthcare). The polysaccharide samples are dissolved in 0.1 M ammonium bicarbonate and aliquots of 5-15 mg were run on the column in the same buffer. Eluting components are detected by absorption at 214 nm.

[0102] The polysaccharides are pooled into three fractions: M_W > 110 kDa, 70-110 kDa and 40-70 kDa. The pooling limits are determined by comparing to elution positions of Dextran standards 40 kDa, 70 kDa and 110 kDa (ex GE Healthcare).

Substitution and composition analysis of polysaccharides

[0103] The composition of polysaccharide fractions as obtained from the extraction process are determined using NMR analysis. Prior to NMR analysis, dry polysaccharide samples (1-10 mg) are dissolved in deuterium oxide (D_2O) and dried in a vacuum centrifuge. Samples are then dissolved in 600 microliter of D_2O. Spectra are collected on a Varian Unity 500 NMR spectrometer at 296 K and referenced to internal acetone standard (2.225 ppm).

[0104] Referring to table 1, the polysaccharide substitution level and the molar composition of the building units are analysed by using the NMR data as follows:

- Rhamnogalacturonan substitution level
  The ratio of 4-substituted rhamnosyl units and nonsubstituted units is estimated by integrating the splitted rhamnose -CH_3 signals. The -CH_3 protons in 4-substituted rhamnosyl units resonate around 1.32 ppm, while those of the nonsubstituted rhamnosyl units at 1.25 ppm.
- Molar ratio of galactans and arabinans
  The molar amount of galactans and arabinans is analysed by integrating the observed β1,4-galactan H-1 signal (4.64 ppm) and the arabinan H-1 signals (-5Ara H-1, 5.09 ppm; -3,5Ara H-1 5.12 ppm; terminal Araα1-3 5.15 ppm; terminal Araα1-2 5.18 ppm; -2,3,5Ara H-1 5.26 ppm. These integration values are compared to the integrated rhamnose -CH_3 signals.
- Polygalacturonic acid/RG-I ratio
  The ratio of polygalacturonic acid to RG-I is analysed as follows: The total amount of galacturonic acid H-4 signals is integrated between 4.42 and 4.47 ppm, and the amount of rhamnose is obtained by integration of the -CH_3 signals (1.25-1.32 ppm). The H-4 signal of the RG-I specific GalAα1-2 unit is located in the same 4.42-4.47 signal, and its

portion has to be deducted from the total H-4 signal. This value is the same as rhamnose amount, as RG-I is a 1:1 polymer of Rha and GalA. The remaining H-4 signal represents the share of polygalacturonic acid type GalAα1-4 H-4 signal.

In vitro assays

[0105] Two assays are used to determine the immunomodulating response of the polysaccharides *in vitro,* both based on phagocytosis activity. These assays are:

Whole blood cell assay

[0106] Phagocytosis activity in whole blood is evaluated using the Phagotest® kit of Orpegen Pharma (Heidelberg, Germany) using an adjusted protocol. Fresh blood is obtained from healthy human volunteers in sodium heparin vacutainers (BD Biosciences). 30 microliter of whole blood and 5 microliter of the ingredient are incubated in duplicates for 30 minutes in a polypropylene 96-well plate at 37°C in a water bath. Control incubations consisted of PBS (=basal phagocytosis activity) or 100 ng/mL *E. coli*-lipopolysaccharide (LPS) (= positive control sample) in triplicate measurements. After the incubation step, 10 microliter of FITC-labeled *E. coli* (white blood cell to *E. coli* ratio of 25:1) is added. This incubation at 37°C is stopped after 6.5 minutes by adding 50 microliter of ice-cold quencher solution. The cells are washed three times by adding 230 microliter of ice-cold wash-buffer and centrifugation for 3 min at 300g (4°C). The erythrocytes are lysed using 290 microliter of lysis buffer. After incubation in the dark for 20 minutes at room temperature, the cells are centrifuged for 5 min at 300g (4°C). Cells are resuspended in 150 microliter of wash-buffer and stained with propidium iodide. Analysis is performed by flow cytometry (Coulter FC500 MPL flow cytometer, Beckman Coulter Nederland BV, Mijdrecht). Within the leucocytes, granulocytes are gated according to the FSC/SSC profile. The percentage of phagocytosing cells in the granulocyte population is determined. The results are normalized to the dynamic range between basal and LPS-stimulated phagocytosis and expressed as a relative percentage phagocytosis activity. A normalized percentage of more than 40% is considered to be positive.

HL60 cell assay

[0107] The human promyelocytic leukaemia cell line HL60 is used to determine the phagocytosis-enhancing capacity of the ingredients. This cell line can be differentiated towards the monocyte lineage with vitamin D3 and the cells subsequently obtain phagocytic capacity. 48 hours before the start of the assay, the HL60 cells are differentiated along the monocytic lineage by the addition of 1α,25-dihydroxyvitamin D3 (VitD3) to the medium. Upon

differentiation, 200 microliter of HL60 cells ($8x10^5$ cells/ml) are transferred to 96-wells flat-bottom plates in triplicates. Fluorescent labeled microspheres (ratio beads: cells, 19:1) are added and incubated for 24 hrs at 37°C. Control incubations consist of differentiated HL60 cells in PBS (= basal phagocytosis level) or 100 ng/mL E. coli-lipopolysaccharide (LPS) (= positive control sample) in triplicate measurements. After the incubation period, the cells are transferred to a 96-wells V-bottom plate, washed three times and fixed with formaldehyde. For analysis, the cells are transferred to a 96-wells clear-bottom plate and fluorescence intensity is analyzed using a Flex Station II fluorometer. The data are normalized using the positive control and expressed as a relative percentage phagocytosis activity. A normalized percentage of more than 40% is considered to be positive.

Example 1: Isolation and characterisation of polysaccharides

[0108] Various plant materials were used to extract polysaccharides. Polysaccharides were obtained from the following raw materials:

- leaves of the species *Camellia sinensis* (tea); the composition of polysaccharides from several samples was determined;
- roots of the species *Daucus carota* subsp. *sativus* (carrot);
- fruit of the species *Malus domestica* (apple);
- root of the species *Beta vulgaris* L. (sugar beet);
- bean of the species *Glycine max* (soyabean), source of the polysaccharides is water-soluble soya polysaccharides ex Fuji Oil (Japan).

[0109] The procedure to obtain the materials was as follows. 25 g of methanol insoluble plant material was washed 2 times with 200 ml of 85% ethanol (VWR Prolabo) in water for 2.5 hours at 80°C and 1 time with 200 ml of 85% ethanol in water for 1.5 hours at 80 °C. After decanting the ethanol, the pellet was dried overnight in a fuming cabinet. The polysaccharides were extracted by adding 200 ml of demineralised (MilliQ) water and boiling for 3 hours at atmospheric pressure. After centrifugation at 2,000g for 20 min at room temperature (RT), the pellet was re-suspended in 200 ml of demineralised (MilliQ) water and boiled again for 3 hours. The supernatants of the first and second extraction were collected, freeze dried and stored at room temperature.

[0110] From the polysaccharide enriched freeze dried extracts a 2% (w/w) suspension in demineralised water was made and autoclaved at a temperature of about 121 °C during about 15 minutes. The suspension was centrifuged at 2,000g for 30 minutes at room temperature. The supernatants were further purified by filtering through a 0.2 micrometer filter, divided in small portions and stored at -20°C.

[0111] Subsequently, about 650 mg of extract was dissolved in 30 ml of 20 mM Tris-HCl, pH

7.5, and allowed to dissolve with frequent mixing for 1 hour. Insoluble matter was removed by centrifugation and the clear supernatant was subjected to anion-exchange chromatography on a column of DEAE-sepharose (50 x 150 mm, ca. 290 ml) equilibrated with 20 mM Tris-HCl, pH 7.5, at a flow rate of 10 ml/min. After injection, the column was run isocratically with the equilibration buffer for 30 min, followed by a gradient of 0-1 M NaCl over 30 min, and 1 M NaCl for additional 40 min. Absorbance at 214 nm was recorded and fractions of 25 ml collected. The acid fraction was concentrated and desalted by ultrafiltration over a 10 kDa membrane prior to GPC. The yield was not measured at this point.

[0112] The acid fractions obtained above were combined (from multiple runs) and then subjected to gel-filtration chromatography on a column of Superdex 200 (5 cm diameter, 95 cm length). Superloop was used in the injection due to large sample volumes. The column was eluted at a flow rate of 5 ml/min with 100 mM ammonium bicarbonate and the absorbance at 214 nm was recorded. Fractions of 25 ml were collected.

[0113] Polysaccharides from *apple* were obtained in the following way. Fresh apples were grated and treated with an experimental pectolytic enzyme preparation, Rapidase C600 (0.02% w/w, 4 h at 45°C; enzyme obtained from Gist Brocades, Delft, the Netherlands). The soluble part was recovered by centrifugation, and subjected to ultrafiltration on a 60 kDa molecular weight cut-off membrane, and then lyophilized. This fraction was further saponified to remove labile methyl and acetyl esters: a sample of 50.4 mg of apple RG was dissolved in 5 ml of 0.2 M sodium carbonate, pH 10, and allowed to react for 18 h at room temperature. The reaction mixture was then dialyzed for 24 h with three solution changes against 50 mM ammonium bicarbonate in MWCO 6000-8000 dialysis tubing. Finally, the sample was subjected to SPE in C-18 silica.

[0114] The following compositions of polysaccharides obtained from various sources were determined.

Table 1 Substitution and composition analysis of polysaccharides obtained from various sources; polysaccharide fractions separated on Superdex 200.

Material and M_W fraction	**Rha 4-OH subst. (1)**	**β1-4Gal (2)**	**Ara (3)**	**α1-4GalA (4)**
Tea #7				
> 110 kDa	45%	3	5	__(5)
70-110 kDa	45%	2	3	__(5)
40-70 kDa (comparative as M_W <70)	65%	3	3	__(5)
Tea #15				
> 110 kDa	50%	6.5	0.5	__(5)
70-110 kDa	70%	8	1.5	__(5)

Tea #15				
40-70 kDa (comparative as M_W <70kDa)	60%	9	0.8	--(5)
Tea #218				
> 110 kDa	35%	17	19	--(5)
70-110 kDa (comparative as GalA:Rha >2.5)	70%	8	20	46
Tea #244				
> 110 kDa	37.50%	7.5	11	--(5)
70-110 kDa (comparative as GalA:Rha >2.5)	50%	3	--	
Apple				
> 110 kDa	80%	3	3	--(5)
70-110 kDa	60%	1.5	1	1.2
40-70 kDa (comparative as M_W <70kDa)	80%	2	0.7	1.5
Sugar beet				
> 110 kDa	60%	1.7	29	--(5)
70-110 kDa	60%	1.9	20	--(5)
40-70 kDa (comparative as M_W <70kDa)	45%	2.1	11	--(5)
Material and M_W fraction	Rha **4-OH** subst. (1)	**β1-4Gal** (2)	**Ara** (3)	**α1-4GalA** (4)
Carrot				
> 110 kDa	55%	15	17	--(5)
70-110 kDa (comparative as GalA:Rha>2.5)	70%	5	10	16
40-70 kDa (comparative as M_W <70kDa)	40%	1.5	6	16
Soya				
> 110 kDa	55%	44	23	--

Soya				
70-110 kDa	50%	26	13	--
40-70 kDa (comparative as M_W <70kDa)	40%	6	4	3

Legend
(1) *Rha 4-OH subst.:* molar fraction of the Rha moieties in the RG-I core which is substituted at the C-4 position with a side chain; as measured from the Rha CH_3 signal shift;
(2) *β1-4Gal:* molar ratio of beta(1,4)-linked Gal as compared to Rha (mol/mol); relates to the length of side chains containing beta(1,4)-linked galactan residues;
(3) *Ara:* molar ratio of alpha(1,5)-linked arabinan as compared to Rha (mol/mol); relates to the length of side chains containing alpha(1,5)-linked arabinosyl residues;
(4) *α1-4GalA:* alpha-1,4-galacturonyl acid residues vs. rhamnosyl residues (mol/mol); i.e. this number indicates the molar ratio between GalA residues in the alpha(1,4)-linked polygalacturonic acid or alpha(1,4)-linked oligogalacturonic acid cores in the polysaccharide and Rha residues in the RG-I core of the polysaccharide;
(5) Below measurable level; i.e. the amount of alpha(1,4)-linked GalA residues present in the polysaccharide originating from the alpha(1,4)-linked polygalacturonic acid or alpha(1,4)-linked oligogalacturonic acid cores is so low that it is not detectable. If this is the case, then the ratio GalA:Rha is 1.

Example 2: Immunomodulating effect of polysaccharides from various sources

[0115] The immuno-modulating effect of various samples have been determined at various concentrations, using the two assays as described above. The results are given in the following table.

[0116] Polysaccharides were obtained from the following raw materials:

- leaves of the species *Camellia sinensis* (tea); the immuno modulating effect of a pooled mix of samples of tea #7, tea #15, tea #218, tea #244 (see table 1) is indicated in the table below;
- roots of the species *Daucus carota* subsp. *sativus* (carrot);
- fruit of the species *Malus domestica* (apple);
- root of the species *Beta vulgaris* L. (sugar beet);
- bean of the species *Glycine max* (soyabean).

Table 2 *In vitro* immune modulating activity of various samples, tested using whole blood cell assay, and/or HL60 cell assay. Concentration of extract (microgram per milliliter) obtained according to method above, versus phagocytosis activity of assay.

	whole blood cell assay		HL60 cell assay	
Material and M_W fraction	3 microgram/ml	30 microgram/ml	0.3 microgram/ml	3 microgram/ml
Tea				
> 110 kDa	+	++	+	++
70-110 kDa	-	++	-	+
Apple				
> 110 kDa	++	++	++	++
70-110 kDa	-	++	-	+
40-70 kDa (comparative as M_W <70kDa)				
Sugar beet				
> 110 kDa	+	++		
Carrot				
> 110 kDa	++	++	-	++

Legend:
++ very positive effect (% phagocytosis >80%)
+ positive effect (% phagocytosis 40%-80%)
- no effect
blank: not tested

[0117] Figures 2 and 3 indicate the measured phagocytosis percentage, for some of the polysaccharide samples:

Figure 2: Apple (as in table 1 and 2), whole blood cell assay.

Figure 3: Soyabean; HL60 cell assay; this sample not in table 2, soyabean polysaccharides: Rhamnogalacturonan (Soy Bean) ex Megazyme International Ireland Ltd. (Wicklow, Ireland).

[0118] Two of the samples obtained from the tea plant (*Camellia sinensis*) were also tested in the two tests, at lower concentrations this time. These samples were tea #7 and tea #15. Also the samples from carrot and apple were tested. The structural features of these materials have already been given in table 1. The results of these tests are the following:

Table 3 *In vitro* immune modulating activity of two samples obtained from the plant *Camellia sinensis,* and from carrot and apple, tested using whole blood cell assay, and/or HL60 cell assay. Concentration of extract (microgram per milliliter) obtained according to method above, versus phagocytosis activity of assay.

	whole blood cell assay		HL60 cell assay	
Material and M_W fraction	0.03 microgram/ml	0.3 microgram/ml	0.003 microgram/ml	0.03 microgram/ml
Tea #7				
> 110 kDa	++	++		
70-110 kDa	++	++		
40-70 kDa	-	++		
Tea #15				
> 110 kDa	++	++	++	++
70-110 kDa	++	++	-	-
40-70 kDa	-	++	-	-
Carrot				
> 110 kDa	-	-		
70-110 kDa	-	-		
40-70 kDa	-	-		
Apple				
> 110 kDa	-	-		
70-110 kDa	-	-		
40-70 kDa	-	-		

Legend:
++ very positive effect (% phagocytosis >80%)
+ positive effect (% phagocytosis 40%-80%)
- no effect
blank: not tested

[0119] This example shows that the extracts obtained from the plant *Camellia sinensis* showed very high immunostimulating activity *in vitro.* Already at concentrations as low as 0.003 microgram per millileter, already a very high immunostimulating activity was measured in the

whole blood cell assay, as compared to various other polysaccharides obtained from other vegetable materials. Polysaccharides obtained from apple or from carrot did not show immunostimulating effect in the whole blood cell assay at concentrations of 0.03 and 0.3 microgram per millilter.

Example 3: Properties of polysaccharide

[0120] During the isolation of polysaccharides from carrot, it was observed that these polysaccharides did not lead to thickening of fluids in which it was dissolved. This is in contrast to normal pectins, wherein similar concentrations of polymer lead to thickening of the fluids.

REFERENCES CITED IN THE DESCRIPTION

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Patentkrav

1. Fremgangsmåde til fremstilling af et spiseligt produkt eller en farmaceutisk sammensætning, hvilken fremgangsmåde omfatter at inkorporere 0,5-10 vægt-% af et polysaccharid, som har et backbone omfattende vekslende rhamnogalacturonan-I-kerner og alpha(1,4)-bundet polygalacturonsyre- eller alpha(1,4)-bundet oligogalacturonsyre-kerner, eller som har et backbone bestående af en rhamnogalacturonan-I-kerne;
hvor polysaccharidet har en molekylvægt på mindst 70 kD; og
hvor polysaccharidet opnås ud fra planter af arten *Camellia sinensis*, og hvor molforholdet mellem galacturonylsyrerester og rhamnosylrester i polysaccharidets backbone ligger i området fra 2,5:1 til 1:1;
hvor molforholdet mellem galacturonylsyrerester og rhamnosylrester i polysaccharidets backbone bestemmes under anvendelse af NMR-fremgangsmåden beskrevet i eksemplerne.

2. Fremgangsmåde ifølge krav 1, hvor molforholdet mellem galacturonylsyrerester og rhamnosylrester i polysaccharidets backbone ligger i området fra 2:1 til 1:1, fortrinsvis fra 1,5:1 til 1:1.

3. Fremgangsmåde ifølge krav 1 eller 2, hvor polysaccharidet har en molekylvægt mellem 70 og 2.000 kDa, fortrinsvis mellem 110 og 2.000 kDa.

4. Fremgangsmåde ifølge et hvilket som helst af kravene 1 til 3, hvor fremgangsmåden omfatter at inkorporere mindst 1 vægt-% af polysaccharidet.

5. Fremgangsmåde ifølge et hvilket som helst af kravene 1 til 4,
hvor rhamnogalacturonan-I-kernen omfatter en eller flere sidekæder,
hvor den ene eller de flere sidekæder omfatter et backbone af mindst en eller flere alpha(1,5)-bundne arabinosylrester; hvor den ene eller de flere sidekæder er substituerede ved 4-OH-positionen af rhamnosylresterne; og hvor molforholdet mellem arabinosylrester og rhamnosylrester er mellem 50:1 og 1:2.

6. Fremgangsmåde ifølge et hvilket som helst af kravene 1 til 5, hvor rhamnogalacturonan-I-kernen omfatter en eller flere sidekæder,
hvor den ene eller de flere sidekæder omfatter et backbone af mindst en eller flere beta(1,4)-bundne galactosylrester; hvor den ene eller de flere sidekæder er substituerede ved 4-OH-positionen af rhamnosylresterne; og hvor molforholdet mellem galactosylresterne og rhamnosylresterne er mellem 80:1 og 1:1.

7. Fremgangsmåde ifølge et hvilket som helst af kravene 1 til 6, hvor polysaccharidet opnås ved en proces, der omfatter følgende trin:
• at bringe det vegetabilske materiale i kontakt med en alkohol ved en temperatur på 60-80 °C i et tidsrum på mellem 15 minutter og 5 timer;
• at separere den faste rest fra væsken;
• at ekstrahere den faste rest med mindst en tredobbelt mængde vand (v/v) ved en temperatur på 60-100 °C i mindst 1 time til opnåelse af ekstraheret polysaccharid.

8. Fremgangsmåde ifølge krav 7, hvor det ekstraherede polysaccharid udsættes for yderligere behandling, hvilken yderligere behandling omfatter mindst et af følgende trin:
• dialyse af det ekstraherede polysaccharid til fjernelse af molekyler med en molekylvægt under 10 kDa;
• separation af det ekstraherede polysaccharid i en sur fraktion og ikke-sure forbindelser under anvendelse af ionbytning.

9. Spiseligt produkt eller farmaceutisk sammensætning til anvendelse ved behandling af forkølelse eller influenza, hvor det spiselige produkt eller den farmaceutiske sammensætning kan opnås ved en fremgangsmåde, der omfatter at inkorporere 0,5-10 vægt-% af et polysaccharid, som har et backbone omfattende vekslende rhamnogalacturonan-I-kerner og alpha(1,4)-bundet polygalacturonsyre- eller alpha(1,4)-bundet oligogalacturonsyre-kerner, eller som har et backbone bestående af en rhamnogalacturonan-I-kerne;
hvor polysaccharidet har en molekylvægt på mindst 70 kD; og
hvor polysaccharidet:

• kan opnås ud fra planter af arten *Camellia sinensis*, og hvor molforholdet mellem galacturonylsyrerester og rhamnosylrester i polysaccharidets backbone ligger i området fra 2,5:1 til 1:1; eller
• kan opnås ud fra planter af arten *Daucus carota*, og hvor molforholdet mellem galacturonylsyrerester og rhamnosylrester i polysaccharidets backbone ligger i området fra 2,5:1 til 1:1; eller
• kan opnås ud fra arten *Glycine max*, og hvor molforholdet mellem galacturonylsyrerester og rhamnosylrester i polysaccharidets backbone ligger i området fra 1,5:1 til 1:1; eller
• kan opnås ud fra arten *Malus domestica*, og hvor molforholdet mellem galacturonylsyrerester og rhamnosylrester i polysaccharidets backbone ligger i området fra 1,5:1 til 1:1; eller
• kan opnås ud fra arten *Beta vulgaris L.*, og hvor molforholdet mellem galacturonylsyrerester og rhamnosylrester i polysaccharidets backbone ligger i området fra 1,5:1 til 1:1;
hvor molforholdet mellem galacturonylsyrerester og rhamnosylrester i polysaccharidets backbone bestemmes under anvendelse af NMR-fremgangsmåden beskrevet i eksemplerne.

10. Spiseligt produkt eller farmaceutisk sammensætning ifølge krav 9, hvor polysaccharidet indgives i en daglig dosis på mellem 10 og 1.000 milligram om dagen.

11. Spiseligt produkt eller farmaceutisk sammensætning ifølge krav 9 eller 10, hvor molforholdet mellem galacturonylsyrerester og rhamnosylrester i polysaccharidets backbone ligger i området fra 2:1 til 1:1, fortrinsvis fra 1,5:1 til 1:1.

12. Spiseligt produkt eller farmaceutisk sammensætning ifølge et hvilket som helst af kravene 9 til 11, hvor polysaccharidet har en molekylvægt mellem 70 og 2.000 kDa, fortrinsvis mellem 110 og 2.000 kDa.

13. Spiseligt produkt eller farmaceutisk sammensætning ifølge et hvilket som helst af kravene 9 til 12, hvor det spiselige produkt eller den farmaceutiske

sammensætning kan opnås ved en fremgangsmåde, der omfatter at inkorporere mindst 1 vægt-% af polysaccharidet.

14. Spiseligt produkt eller farmaceutisk sammensætning ifølge et hvilket som helst af kravene 9 til 13, hvor polysaccharidet kan opnås ud fra roden af arten *Daucus carota* subsp. *sativus* (gulerod), hvor molforholdet mellem galacturonylsyrerester og rhamnosylrester i polysaccharidets backbone ligger i området fra 1,5:1 til 1:1.

DRAWINGS

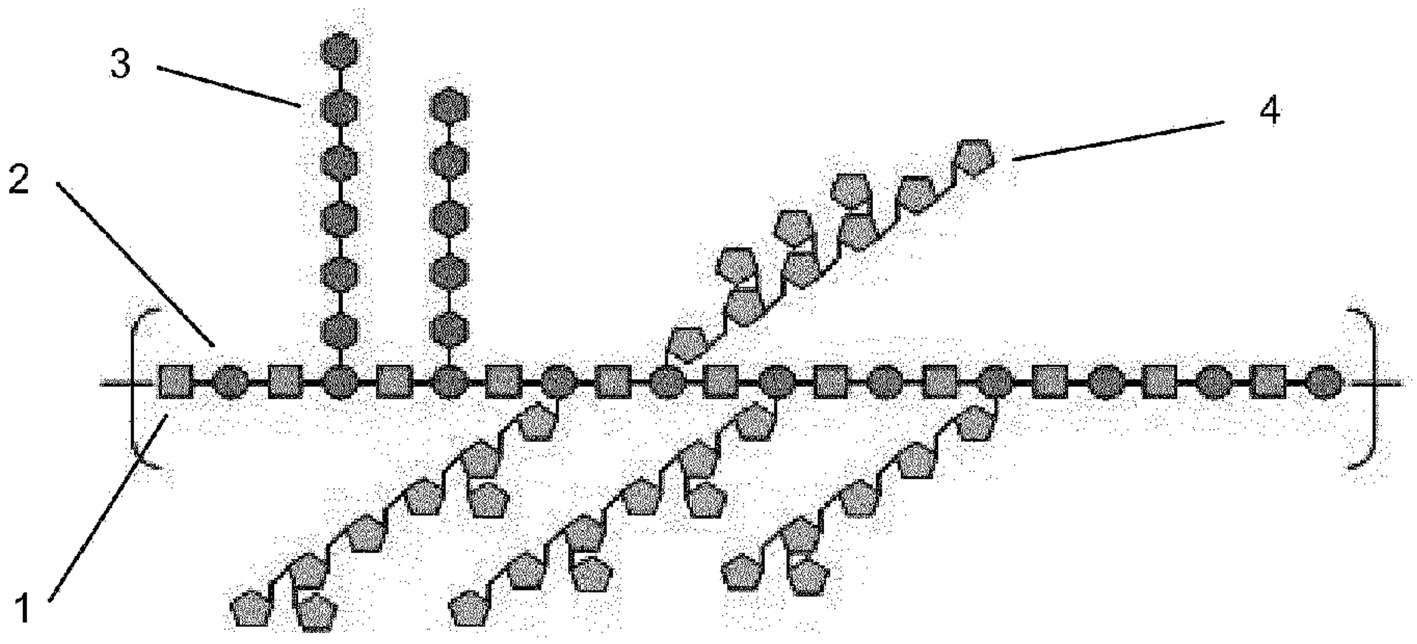

Figure 1

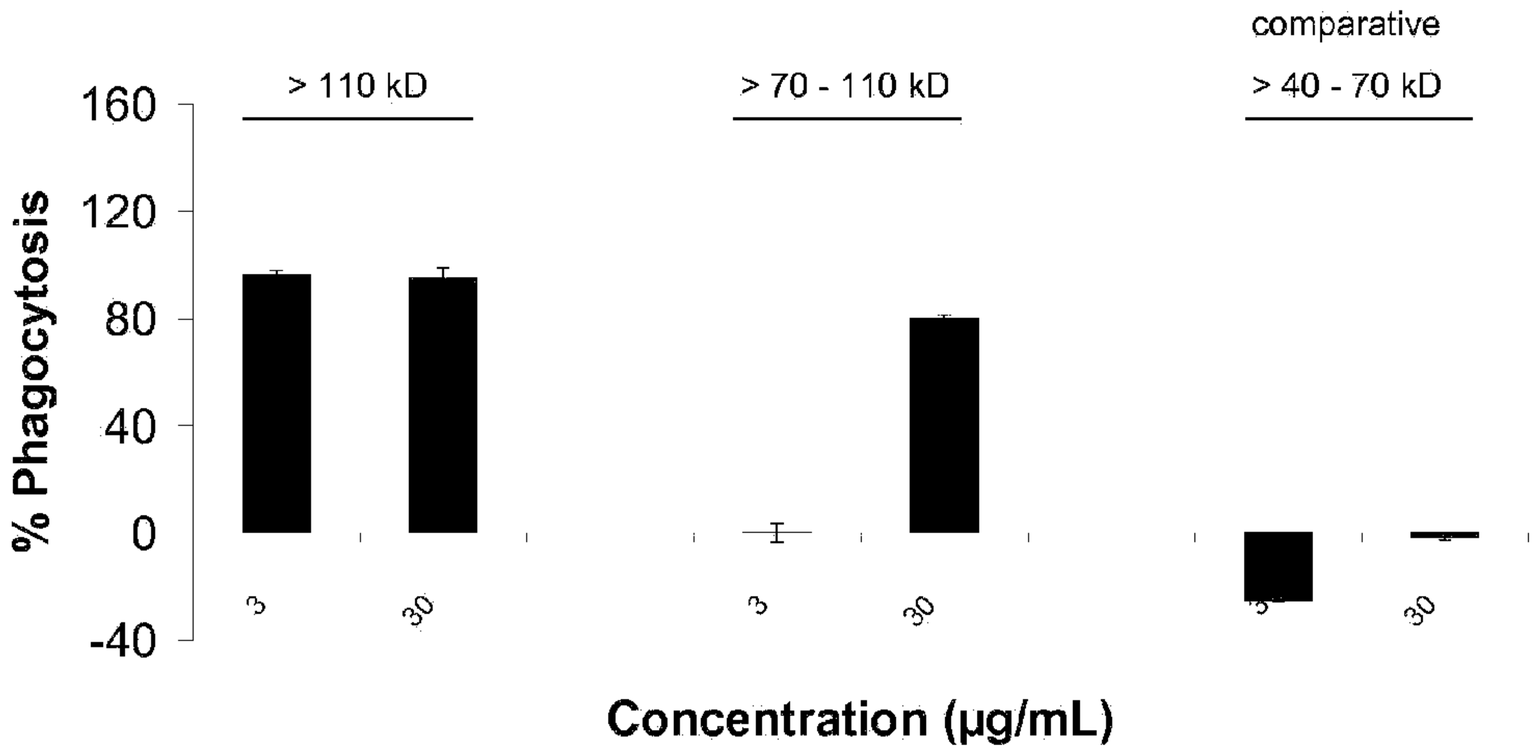
comparative
> 110 kD
> 70 - 110 kD
> 40 - 70 kD
160
120
80
40
0
-40
% Phagocytosis
3
30
3
30
3
30
Concentration (µg/mL)

Figure 2

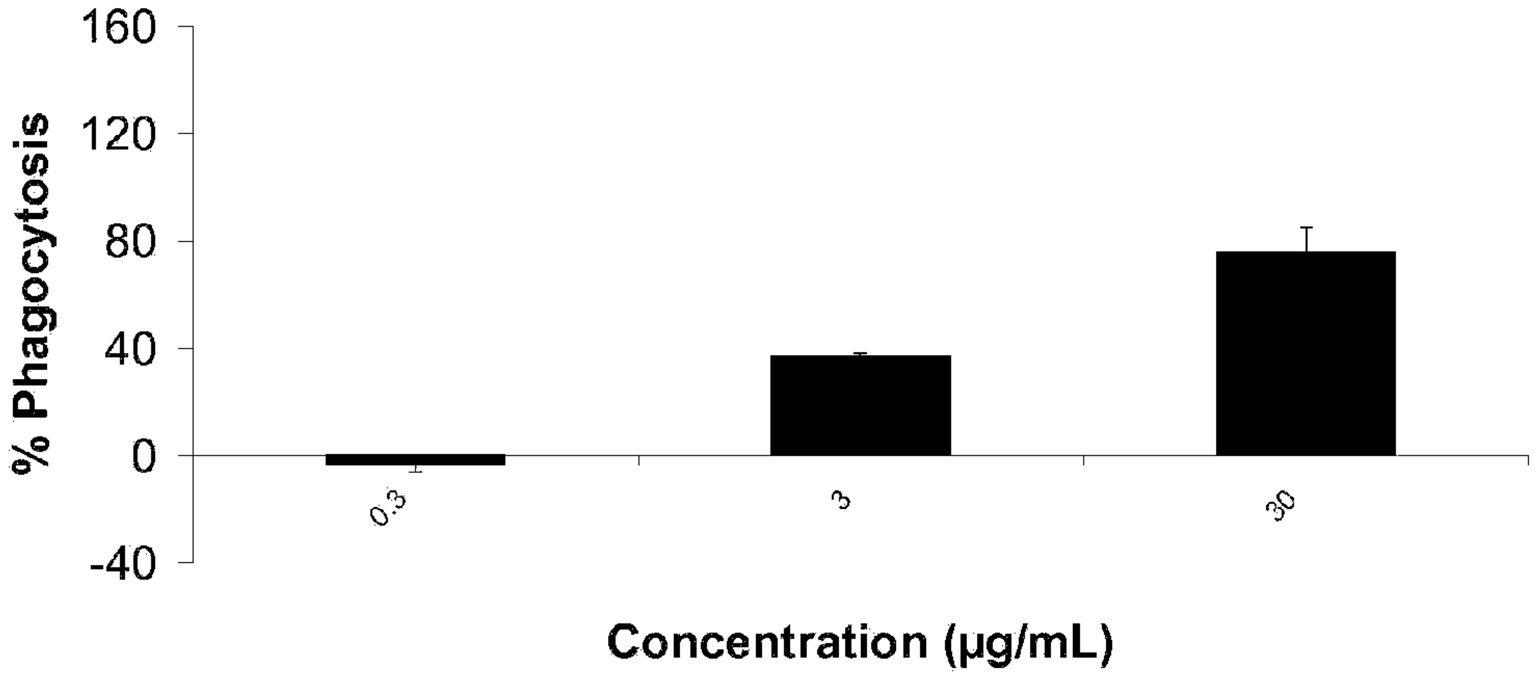

Figure 3