

IMMUNOLOGICAL EXTRACT AND METHOD OF PRODUCTION

Technical Field of Invention

The present invention relates to methods of preparing an immunological extract from mixtures comprising bird's nest raw materials, and optionally other materials, and the extracts obtainable from the methods.

Background of Invention

Edible bird's nest (EBN) is the nest made from the saliva of swiftlets naturally found in the South-east Asian region. The abandoned nests are harvested from the wild or from specially built housing for swiftlets. It has been reported that EBN exhibited various bioactivities and nutritional value that include potential for mitogenic response, epidermal growth factor (EGF)-like activity, anti-influenza virus, haemagglutination-inhibitory activity, lectin-binding activity, improvement of bone strength and dermal thickness, and hormone content. Processing of EBN can be different depending on the application. Ongoing investigations have been carried out to elucidate the biological and medical functions of the edible bird's nest.

Currently, EBN is used in the form of a soup or other drinks by boiling the EBN in water and consuming. The molecules of EBN in such a scenario are large biomacromolecules that are difficult for the body to digest and absorb. As a result, the bioavailability of the beneficial components of EBN prepared in such a manner is low, and the beneficial effects of EBN is not maximised.

However, consuming whole EBN may lead to immunoglobulin E (IgE) mediated anaphylaxis (Goh et al., 2001, J. Allergy Clin. Immun., 107(6), 1082–1088) and EBN is thought to be the most common cause of food-induced anaphylaxis which could be life-threatening among children.

Another problem with crude EBN is the presence of undesirable compounds either due to natural causes or added intentionally during processing. Adulteration of EBN commonly takes place decreasing the quality of the EBN. Adulterants used include pig skin, agar, red

seaweed and karaya gum. In order to camouflage adulterants and waste matters, bleaches are often added.

Of particular concern is the presence of nitrite salts which is derived mainly from the faeces
5 of the swiftlets. Nitrites can also be added to white bird's nest during processing to turn it into red bird's nest which is commercially more valuable. Ingestion of excessive nitrites had been linked to cancer (Bryan et al. Food Chem. Toxicol. 2012, 50 (10), 3646-3654).

Viruses, bacteria and fungi could contaminate EBN in the wild or in the factory during
10 processing. Concerns with regards to avian flu in wild birds can lead to restriction of imports of whole EBN itself.

Therefore, there is a need to improve the processing of EBN to improve the overall quality and beneficial properties to the consumer. By extracting and isolating desirable
15 compounds from EBN, harmful effects are avoided or minimised while maximising the therapeutic benefits of EBN.

Further, bioactive molecules like opsonins, complement proteins, lectins, ficolins, collectins, and pentraxins are extracted mainly from animal parts or genetically modified
20 microorganisms cloned in bioreactors. There is a lack of a standard for consistency in purity and yields and safety aspects of the extracted products. The process described herein solves the issues of safety and sustainability as edible bird's nest (EBN), or EBN mixtures containing hasma or coral seaweed, are very rich and abundant sources for such bioactive factors.

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The listing or discussion of an apparently prior-published document in this specification should not necessarily be taken as an acknowledgement that the document is part of the state of the art or is common general knowledge.

30 Any document referred to herein is hereby incorporated by reference in its entirety.

Summary of Invention

In a first aspect of the invention, there is provided a method of preparing an extract from edible bird's nest, the method comprising: preparing an edible bird's nest (EBN) mixture; and contacting the mixture with an extraction solution to bind a molecule in the mixture, wherein the extraction solution comprises at least one binding moiety selected from the group comprising an opsonin binding moiety, a complement protein binding moiety, a lectin binding moiety, a ficolin binding moiety, a collectin binding moiety, and a pentraxin binding moiety.

The term "contacting" refers to the components of the EBN mixture and extraction solution interacting with each other, preferably the interaction should lead to the formation of a bond, which is reversible, between the binding moiety and the target molecule.

The term "binding moiety" refers to any molecule and/or functional group that selectively targets and binds to the molecule of interest. For example, an opsonin binding moiety is any molecule that binds to an opsonin molecule. Examples of suitable binding moiety may include antibodies, antibody fragments, antibody mimetics, cells with receptors, and molecules that mimic the binding function of the receptor. For example, a "lectin binding moiety" could be a cell expressing a lectin binding receptor on the cell surface, and so forth. In another example, a "lectin binding moiety" includes an antibody which binds lectin, or the lectin (antigen) binding site of an antibody, antibody fragment, or antibody mimetic, and so forth. The binding moiety should preferably bind to the molecule with a K_D in the micromolar or lower region. For example, the binding moiety could bind to the molecule with a K_D of less than 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} , 10^{-8} , 10^{-9} , 10^{-10} , 10^{-11} , 10^{-12} , or less. The lower the K_D value the stronger the binding affinity. The binding moiety of the extraction solution is preferably capable of binding to its target with an affinity that is at least two-fold, 10-fold, 50-fold, 100-fold or greater than its affinity for binding to another non-target molecule.

The binding moiety to be used may be naturally occurring, semi-synthetic or synthetic, for example tagged binding moiety that can facilitate the separation of the binding moiety from the EBN mixture may be used. Additionally, the binding moiety used to extract the target molecule could be bound to a solid support. The solid support could be made of a ferromagnetic material or conventional inert support material. The binding moiety may be

commercially available and can be used as such. If modifications of the binding moiety are desired, there are many methods as commonly known in the literature to modify to obtain the desired characteristics.

5 Figure 9 shows how cells with the desired receptor may be produced. In Figure 9 panel (a), all mRNA is extracted from cells that normally express the receptor of interest and reverse-transcribed into double-stranded cDNA. The entire population of cDNAs is inserted into plasmid expression vectors in between a strong promoter and a terminator of transcription. The plasmids are transfected into bacterial cells that do not normally express
10 the receptor of interest. The resulting cDNA library is divided into pools, each containing about 1000 different cDNAs. Panel (b) of Figure 9 further shows that the plasmids in each pool are transfected into a population of cultured cells (e.g., COS cells) that lack the receptor of interest. Only transfected cells that contain the cDNA encoding the desired receptor synthesise it; other transfected cells produce irrelevant proteins. To detect the
15 few cells producing the desired receptor, a radiolabeled ligand specific for the receptor is added to the culture dishes containing the transfected cells; the cells are fixed and subjected to autoradiography. Positive cells synthesizing the specific receptor will be covered with many grains. Alternatively, transfected cells can be treated with a fluorescent-labeled ligand and passed through a fluorescence-activated cell sorter. Cells
20 expressing the receptor will bind the fluorescent label and be separated from those that do not. Plasmid cDNA pools giving rise to a positive signal are maintained in bacteria and subdivided into smaller pools, each of which is rescreened by transfection into cultured cells. After several cycles of screening and subdividing positive cDNA pools, a pure cDNA clone encoding the desired receptor is obtained. Detailed methods may be found in A.
25 Aruffo and B. Seed, *Molecular Cloning of a CD28 cDNA by a high-efficiency COS cell expression system*, Proc. Nat'l. Acad. Sci. USA, 1987, 84, 8573; and A. D'Andrea, H. F. Lodish, and G. Wong, *Expression Cloning of the murine erythropoietin receptor*, Cell, 1989, Vol. 57, 277, which are both incorporated herein by reference. The pure cDNA clone may then be used to produce cells with the desired receptor of interest.

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The cDNA refers to complementary DNA and refers to nucleic acid molecules having a nucleotide sequence complementary to a desired coding polynucleotide, for example RNA,

in particular mRNA. The term “complementary” refer to sequences of polynucleotides which is capable of forming Watson and Crick base pairing with nother specified throughout the entirety of the complementary region. Complementary bases are generally, A and T (A and U), or C and G. The desired coding polynucleotide includes
5 sequences having at least 50%, 60%, 70%, 80%, 90%, 95%, or 99% identity to a polynucleotide sequence which encodes the desired polypeptide.

In an example, the binding moiety may be a protein produced by the expression of the cDNA in an expression system. Common examples of expression systems include cell-based
10 systems and cell-free systems. Cell-based systems include those derived from bacteria, yeast, insect cells, mammalian cells and filamentous fungi. Non-limiting examples of bacteria expression systems include *Escherichia coli* (*E. coli*), and *Pseudomonas fluorescens* (*P. fluorescens*). Non-limiting examples of eukaryotic systems include yeasts like *Saccharomyces cerevisiae* (*S. cerevisiae*) and *Pichia pastoris*, filamentous fungi like
15 *Aspergillus*, *Trichoderma*, and *Myceliphthora thermophile*, insect cells infected with and without baculovirus like Sf9 and Sf21 from *Spodoptera frugiperda* cells, and mammalian cells like Chinese Hamster ovary (CHO) and Human embryonic kidney (HEK) cells. Cell-free production of proteins may also be performed in-vitro using purified RNA polymerase, ribosomes, tRNA and ribonucleotides which may be obtained synthetically, from cells and/or
20 from a cell-based expression system. The different expression systems each have its own advantages and the choice of the expression system depends in part on the nature of the protein and the intended use. For example, if post-translation modification of the protein is required a eukaryotic system is generally a better choice. It may also be possible for multiple cells to be used in the expression system to produce the protein. Often the
25 expression systems includes an affinity tag attached to the protein to facilitate the purification of the expressed protein. These affinity tags bind specifically with specific partner ligands, for example immobilised on a solid support, enabling separation of the affinity tag and protein construct. The expressed protein may be isolated with the affinity tag or by cleavage of the affinity tag to release the desired protein. Non-limiting examples
30 includes a His tag and a Strep-tag. The His-tag binds strongly to divalent metal ions like nickel and cobalt, while the Strep-tag binds specifically to an engineered streptavidin. Other purification methods may also be utilised as required.

If antibodies are used, the antibodies may exist in a variety of forms including, for example, polyclonal antibodies, monoclonal antibodies, humanised antibodies and human antibodies. Examples of antibody fragments may include Fab, Fab', F(ab')₂, Fv, linear antibodies, single chain (scFv) antibodies, single-domain antibodies (sdAb). Methods of
5 producing these binding moieties may be made by any suitable method. One method of obtaining antibodies is to immunise suitable host animals with an immunogen and to follow standard procedures for polyclonal or monoclonal antibody production. The immunogen will facilitate presentation of the immunogen on the cell surface. Immunisation of a suitable host can be carried out in a number of ways. Nucleic acid
10 sequences encoding a polypeptide or immunogenic fragments thereof, can be provided to the host in a delivery vehicle that is taken up by immune cells of the host. The cells will in turn express the receptor on the cell surface generating an immunogenic response in the host. Alternatively, nucleic acid sequences encoding the polypeptide, or immunogenic surfaces thereof, can be expressed in cells in vitro, followed by isolation of the polypeptide
15 and administration of the polypeptide to a suitable host in which antibodies are raised. Alternatively, antibodies against the polypeptide may be derived from an antibody phage display library. A bacteriophage is capable of infecting and reproducing within bacteria, which can be engineered, when combined with human antibody genes, to display human antibody proteins. Phage display is the process by which the phase is made to "display" the
20 human antibody proteins on its surface. Genes from the human antibody gene libraries are inserted into a population of phage. Each phage carries the genes for a different antibody and thus displays a different antibody on its surface.

Antibodies made by any method known in the art may be further purified from the host.
25 Antibody purification methods may include salt precipitation, ion exchange chromatography, gel filtration chromatography, and affinity chromatography, for example with protein A, protein G, hydroxyapatite and anti-immunoglobulin.

Antibodies may be conveniently produced from hybridoma cells engineered to express the
30 antibody. Methods of making hybridomas are well known in the art. The hybridoma cells can be cultured in a suitable medium and spent medium can be used as an antibody source. Polynucleotides encoding the antibody of interest can in turn be obtained from the

hybridoma that produces the antibody, and then the antibody may be produced synthetically or recombinantly from these DNA sequences. For the production of large amounts of antibody, it is generally more convenient to obtain an ascites fluid. The method of raising ascites generally comprises injecting hybridoma cells into an immunologically
5 naïve histocompatible or immunotolerant mammal, especially a mouse. The mammal may be primed for ascites production by prior administration of a suitable composition (e.g. Pristane).

The term “antibody mimetic” or “antibody mimic” means a molecule which specifically
10 binds an antigen, but is not structurally related to antibodies. Typically, antibody mimetics specifically binding to a target are produced by screening libraries of mutagenized molecular scaffolds. Examples of molecular scaffolds include, without limitation, a fibronectin III (FN3) domain. The molecular scaffold is typically a smaller molecule than an antibody (e.g. about 50-200 residues). Examples of antibody mimetics include, without
15 limitation, affibodies, affilins, affitins, anticalins, avimers, DARPs, Kunitz domain derived peptides, knottins, and monobodies. A monobody comprise a fibronectin type III domain (FN3) as a molecular scaffold. Monobodies are produced from combinatorial libraries in which portions of the FN3 scaffold are diversified using highly tailored mixtures of amino acids by utilising phage display and yeast surface display techniques.

20 The term “lectin” in the context of the invention is a carbohydrate-binding protein and excludes ficolins and collectins. The term “opsonin” refers to any compound or molecules that enhances phagocytosis excluding complement proteins, lectins, ficolins, collectins, and pentraxins.

25 Preferably, the mixture further comprises seaweed and/or hasma. Hasma (hashima) is made from the dried fatty tissue near the fallopian tubes of true frogs. Seaweed or macroalgae refers to several species of macroscopic, multicellular, marine algae, and only includes those which are edible. Common edible seaweed that may be used include red algae, brown algae and green algae.

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Preferably, the opsonin binding moiety has a molecular weight of about 10 kDa to about 750 kDa. In an example, the opsonin binding moiety comprises SEQ ID No. 1.

Preferably, the complement protein binding moiety has a molecular weight of about 20 kDa to about 7000 kDa. Examples of the complement protein binding moiety include a molecular weight of about 50 kDa to about 7000 kDa, and a molecular weight of about 20 kDa to about 1000 kDa. In an example, the complement protein binding moiety comprises SEQ ID No. 2.

Preferably, the lectin binding moiety has a molecular weight of about 20 kDa to about 1000 kDa. In an example, the lectin binding moiety comprises SEQ ID No. 3.

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Preferably, the ficolin binding moiety has a molecular weight of about 15 kDa to about 900 kDa. In an example, the ficolin binding moiety comprises SEQ ID No. 4.

Preferably, the collectin binding moiety has a molecular weight of about 15 kDa to about 900 kDa. In an example, the collectin binding moiety comprises SEQ ID No. 5.

Preferably, the pentraxin binding moiety has a molecular weight of about 20 kDa to about 1000 kDa. In an example, the pentraxin binding moiety comprises SEQ ID No. 6.

Preferably, the binding moiety is a receptor protein. In an example, the receptor protein is anchored to a cell or may be isolated in purified form. The molecular weights of the binding moieties above refer to the receptor protein molecular weight.

Preferably, the at least one binding moiety comprises any one selected from:

- i) the lectin binding moiety and the opsonin binding moiety;
- ii) the lectin binding moiety, the complement protein binding moiety, the opsonin binding moiety, the ficolin binding moiety, the collectin binding moiety, and the pentraxin binding moiety;
- iii) the lectin binding moiety, the complement protein binding moiety, and the opsonin binding moiety;
- iv) the opsonin binding moiety, and the pentraxin binding moiety;

- v) the ficolin binding moiety, the collectin binding moiety, and the pentraxin binding moiety;
- vi) the complement protein binding moiety, the ficolin binding moiety, and the collectin binding moiety; and
- 5 vii) the lectin binding moiety, the opsonin binding moiety, and the collectin binding moiety.

More preferably, the at least one binding moiety comprises any one selected from:

- i) 50% to 90% of the lectin binding moiety and 10% to 50% of the opsonin binding
10 moiety;
- ii) 10% to 30% of the lectin binding moiety, 10% to 30% of the complement protein binding moiety, 10% to 30% of the opsonin binding moiety, 5% to 15% of the ficolin binding moiety, 5% to 15% of the collectin binding moiety, and 10% to 30% of the pentraxin binding moiety;
- 15 iii) 5% to 45% of the lectin binding moiety, 35% to 65% of the complement protein binding moiety, and 5% to 45% of the opsonin binding moiety;
- iv) 10% to 90% of the opsonin binding moiety, and 10% to 90% of the pentraxin binding moiety;
- v) 30% to 50% of the ficolin binding moiety, 10% to 50% of the collectin binding
20 moiety, and 10% to 50% of the pentraxin binding moiety;
- vi) 55% to 85% of the complement protein binding moiety, 5% to 15% of the ficolin binding moiety, and 10% to 30% of the collectin binding moiety; and
- vii) 5% to 15% of the lectin binding moiety, 10% to 30% of the opsonin binding moiety, and 55% to 85% of the collectin binding moiety. The percentage provided for each binding
25 moiety is the percentage weight of each binding moiety relative to the total weight of the binding moieties present. Therefore, the binding moiety may be dissolved in a solvent like water, or buffer, but the percentage is for the binding moiety/moieties present.

Preferably, the at least one binding moiety comprises any one selected from:

- 30 i) 70 % lectin of the binding moiety and 30 % opsonin of the binding moiety;

- ii) 20 % lectin of the binding moiety, 20 % complement protein of the binding moiety, 20 % opsonin of the binding moiety, 20 % pentraxin of the binding moiety, 10 % ficolin of the binding moiety, and 10% collectin of the binding moiety;
- iii) 50% complement protein of the binding moiety, 25 % lectin of the binding moiety and 25 % opsonin of the binding moiety;
- iv) 50% opsonin of the binding moiety and 50% pentraxin of the binding moiety;
- v) 40% ficolin of the binding moiety, 30% collectin of the binding moiety and 30% pentraxin of the binding moiety;
- vi) 10% ficolin of the binding moiety, 20% collectin of the binding moiety and 70% complement protein of the binding moiety; and
- vii) 70% collectin of the binding moiety, 20% opsonin of the binding moiety and 10% lectin of the binding moiety.

Preferably, preparing the EBN mixture comprises washing the mixture, and filtering the washed mixture.

More preferably, the washing step comprises exposing the mixture to a first enzyme solution, and soaking the mixture and the first enzyme solution in water. For example, the exposing step is done at ambient temperatures for about 5 minutes, and the soaking step for a further 5 minutes. Ambient or room temperature refers to a temperature in the range of 20 to 30 °C. Even more preferably, the first enzyme solution comprises a nitrite reductase. In an embodiment, the water is obtained from a reverse osmosis process. More preferably, the washing step comprises washing the mixture in oxygenated water for about 10 minutes followed by a drying period for about 12 hours at 70°C.

Preferably, preparing the EBN mixture comprises dipping the mixture in oil prior to the contacting step, in particular after the mixture has been washed. The oil should be a food oil, i.e. an oil that is edible like a vegetable oil. The presence of the oil may facilitate the contacting step by ensuring that the mixture is surrounded by the oil to increase the affinity of the binding moiety and the target molecules. It is believed that the presence of the oil makes the mixture more lipid permissible and compatible with the target molecules which are often associated with the cell membrane, a predominantly lipid structure.

Preferably, preparing the EBN mixture comprises sterilising the washed EBN or EBN mixture prior to the contacting step, optionally at 121°C for at least 10 minutes, for example about 10 to 20 minutes.

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Preferably, the contacting step is carried out in the presence of ascorbic acid and gold nanoparticles. The ascorbic acid provide anti-oxidant properties, while the gold nanoparticles provide a stable non-reactive environment. Individually or in combination, the ascorbic acid and gold nanoparticles maintain and preserves the integrity of the extract mixture with minimised or no change to the target molecules.

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Preferably, the contacting step is carried out at between 4°C to 37°C for at least 20 minutes. For example, the contacting step is carried out at between 25°C to 37°C for about 20 to 120 minutes.

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Preferably, the method further comprises hydrolysing the bounded molecules with an acidic solution.

Preferably, isolating the molecule comprises separating the at least one binding moiety and bounded molecules from the mixture; releasing the bounded molecules; and obtaining the released molecules by dialysis.

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Preferably, the method further comprises treating the dialysed molecule with a second enzyme solution comprising a vegetable protease and/or a fruit protease.

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Preferably, the method comprises at least one of the following conditions:

(a) a concentration of the second enzyme solution is from about 10 µg/ml to 100 µg/ml; and

(b) treating of the isolated molecule with the second enzyme solution is carried out at 45°C for 60 minutes at pH 6.5 to 9.0, and denaturing the second enzyme solution, i.e. only the enzymes in the second enzyme solution but not the target molecules.

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Preferably, the method further comprises drying the molecules. The drying should be done after the molecules have been isolated, and is typically by freeze drying to preserve the quality and properties of the molecules.

- 5 In a second aspect of the invention, there is a bird's nest extract obtainable from the methods of the first aspect. In an embodiment, the bird's nest extract may further comprise an extract from seaweed and/or hasma.

- 10 In a third aspect of the invention, there is a bird's nest extract comprising a plurality of molecules selected from at least two groups comprising: opsonins, complement proteins, lectins, ficolins, collectins, and pentraxins. In an embodiment, the bird's nest extract may further comprise an extract from seaweed and/or hasma.

Preferably, the plurality of molecules is any one selected from:

- 15 i) lectins and opsonins;
 ii) lectins, complement proteins, opsonins, ficolins, collectins, and pentraxins;
 iii) lectins, complement proteins, and opsonins;
 iv) opsonins and pentraxins;
 v) ficolins, collectins, and pentraxins;
 20 vi) complement proteins, ficolins, and collectins; and
 vii) lectins, opsonins, and collectins.

More preferably, the plurality of molecules is any one selected from:

- i) 50% to 90% of lectins, and 10% to 50% of opsonins;
 25 ii) 10% to 30% of lectins, 10% to 30% of complement proteins, 10% to 30% of opsonins, 5% to 15% of ficolins, 5% to 15% of collectins, and 10% to 30% of pentraxins;
 iii) 5% to 45% of lectins, 35% to 65% of complement proteins, and 5% to 45% of opsonins;
 iv) 10% to 90% of opsonins, and 10% to 90% of pentraxins;
 30 v) 30% to 50% of ficolins, 10% to 50% of collectins, and 10% to 50% of pentraxins;
 vi) 55% to 85% of complement proteins, 5% to 15% of ficolins, and 10% to 30% of collectins; and

vii) 5% to 15% of lectins, 10% to 30% of opsonins, and 55% to 85% of collectins.

The percentage provided for each type of molecule is the percentage weight of each type of molecule relative to the total weight of the types of molecules present.

5 More preferably, the plurality of molecules is any one selected from:

- i) 70% of lectins, and 30% of opsonins;
- ii) 20% of lectins, 20% of complement proteins, 20% of opsonins, 10% of ficolins, 10% of collectins, and 20% of pentraxins;
- iii) 25% of lectins, 50% of complement proteins, and 25% of opsonin;
- 10 iv) 50% of opsonins, and 50% of pentraxins;
- v) 40% of ficolins, 30% of collectins, and 30% of pentraxins; and
- vi) 70% of complement proteins, 10% of ficolins, and 20% of collectins;
- vii) 10% of lectins, 20% of opsonins, and 70% of collectins.

15 Preferably, the bird's nest extract are hydrolysed by treatment with an acid solution and/or an enzymatic solution. This breaks the extracted molecules down into smaller molecules which are more readily absorbed by the body. In an example, the hydrolysed product comprises peptides and/or free amino acids with molecular weights below 1000 Daltons, 750 Daltons, 500 Daltons, or 300 Daltons.

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Preferably, the bird's nest extract further comprises maltodextrin.

A composition may be provided comprising the bird's nest extract according to the second and third aspects; and a pharmaceutically-acceptable carrier, excipient or diluent. As used
25 herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible.

Preferred pharmaceutically acceptable carriers comprise aqueous carriers or diluents.
30 Examples of suitable aqueous carriers that may be employed in the pharmaceutical compositions of the invention include water, buffered water and saline. Examples of other carriers include ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol,

and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants. In many cases, it will be preferable
5 to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, or sodium chloride in the composition.

Preferably, the composition or formulation is a unit dosage containing a daily dose or unit, daily sub-dose or an appropriate fraction thereof, of the active ingredient. The
10 compositions of the present invention may normally be administered orally or by any parenteral route, in the form of a pharmaceutical composition comprising the immunological concentrate, optionally in the form of a non-toxic organic, or inorganic, acid, or base, addition salt, in a pharmaceutically acceptable dosage form. Depending upon the condition, disorder and patient to be treated, as well as the route of administration,
15 the compositions may be administered at varying doses.

In human therapy, the immunological concentrate/extract or compositions of the invention can be administered alone but will generally be administered in admixture with a suitable pharmaceutical excipient diluent or carrier selected with regard to the intended
20 route of administration and standard pharmaceutical practice. They may be administered orally (via tablets and capsules) or parenterally, for example, intravenously, intra-arterially, intraperitoneal, intrathecal, intraventricular, intrastemally, intracranially, intra-muscularly or subcutaneously, or they may be administered by infusion techniques. They are best used in the form of a sterile aqueous solution which may contain other substances, for example,
25 enough salts or glucose to make the solution isotonic with blood. The aqueous solutions should be suitably buffered (preferably to a pH of from 3 to 9), if necessary. The preparation of suitable parenteral formulations under sterile conditions is readily accomplished by standard pharmaceutical techniques well-known to those skilled in the art.

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Compositions or formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers,

bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. These compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents.

5 Prevention of presence of microorganisms may be ensured both by sterilization procedures, supra, and by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form
10 may be brought about by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin. Therapeutic compositions typically must be sterile and stable under the conditions of manufacture and storage. The composition can be formulated as a solution, microemulsion, liposome, or other ordered structure suitable to high drug concentration.

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The formulations may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilised) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be
20 prepared from sterile powders, granules and tablets of the kind previously described.

For oral and parenteral administration to human patients, the daily dosage level of the compounds of the invention will usually be from 1mg/kg to 30 mg/kg. Thus, for example, the tablets or capsules of the compound of the invention may contain a dose of active
25 compound for administration singly or two or more at a time, as appropriate. The physician in any event will determine the actual dosage which will be most suitable for any individual patient and it will vary with the age, weight and response of the particular patient. The above dosages are exemplary of the average case. There can, of course, be individual instances where higher or lower dosage ranges are merited and such are within the scope
30 of this invention.

Alternatively, the compositions of the invention can be administered in the form of a suppository or pessary, or they may be applied topically in the form of a lotion, solution, cream, ointment or dusting powder. The compositions of the invention, particularly the bird's nest extracts, may also be transdermal administered, for example, by the use of a skin patch. They may also be administered by the ocular route, particularly for treating diseases of the eye. For application topically to the skin, the compounds of the invention can be formulated as a suitable ointment containing the active compound suspended or dissolved in, for example, a mixture with one or more of the following: mineral oil, liquid petrolatum, white petrolatum, propylene glycol, polyoxyethylene polyoxypropylene compound, emulsifying wax and water. Alternatively, they can be formulated as a suitable lotion or cream, suspended or dissolved in, for example, a mixture of one or more of the following: mineral oil, sorbitan monostearate, a polyethylene glycol, liquid paraffin, polysorbate 60, cetyl esters wax, cetearyl alcohol, 2-octyldodecanol, benzyl alcohol and water.

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Generally, in humans, oral or topical administration of the compositions of the invention is the preferred route, being the most convenient. In circumstances where the recipient suffers from a swallowing disorder or from impairment of drug absorption after oral administration, the drug may be administered parenterally, e.g. sublingually, buccally, transmucosal or transdermal means.

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In an embodiment, the bird's nest extract according to the second and third aspects may be consumed as a nutraceutical or health supplement. For example, a nutraceutical for use in modulating an immune system of a subject, the nutraceutical comprising the bird's nest extract according to the second or third aspects of the invention. The extract may be consumed as a powder, or combined with other food or drink.

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In a fourth aspect of the invention, the bird's nest extract of the second and third aspects may be for use in medicine, or use in the manufacture of a medicament.

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Preferably, the bird's nest extract may be for use to inhibit dengue virus replication, or for use in modulating an immune system of a subject, or for use in inducing interferon

regulatory factor 3 (IRF3) phosphorylation. The subject may be any animal, preferably a mammal, more preferably a human being. More preferably, modulating the immune system is by inducing production of inflammatory cytokines, or induction of the NF- κ B pathway and/or MAPK pathway. The inhibition of the dengue virus replication is preferably
5 to prevent at least 50% of the dengue virus from replicating, at least 60% of the dengue virus from replicating, at least 70% of the dengue virus from replicating, at least 80% of the dengue virus from replicating, at least 90% of the dengue virus from replicating, at least 95% of the dengue virus from replicating, or essentially 100% of the dengue virus from replicating.

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Alternatively, the bird's nest extract may be used in a method of inhibiting dengue virus replication, or modulating an immune system of a subject, or inducing interferon regulatory factor 3 (IRF3) phosphorylation, the method comprises administering the bird's nest extract of the second and third aspects to the subject, particularly in a biologically
15 effective amount.

The methods described allows for the preparation of a bird's nest extract, and optionally containing seaweed and/or hasma, that is enriched in compounds compared to natural bird's nest. Further, the process hydrolyses the extracted products to supply the
20 compounds in high purity and high bioavailability after consumption. The compounds extracted may be varied for different purposes by varying the extraction solution. The products isolated in this process is amenable to be delivered to the user in a variety of ways and methods to allow for effective and quick delivery of the bioactive molecules to the sites of action. The bird's nest extract may provide immune boosting effects and anti-viral
25 properties, and may be consumed as a nutraceutical or health product. The products are a low cost source of active nutraceutical ingredients (ANI) but possess the high efficacies of active pharmaceutical ingredients (APIs).

In the Figures:

30 Figure 1 shows a flow chart of a method of preparing a concentrate from bird's nest.

Figure 2 shows the effects of a 10% mixture of a bird's nest extract (E1) on the inhibition of dengue virus (DV1) replication in macrophages. RT-PCT of DV1 negative strand (DV1neg) mRNA assayed 72 hours after DV1 infection. GAPDH served as the loading control.

- 5 Figure 3 shows the effects of a 10% mixture of a bird's nest extract (E2) on macrophages. The figure shows that E2 can elicit the production of IFN- β and other inflammatory cytokines in macrophages. Panels A-E shows the qPCR results of macrophages after treatment with E2, with Poly(I:C) and LPS of 2 strains (0111:B4 and 055:B5) as positive controls. Boxes highlight the induction of various cytokine genes expression by E2.

10

Figure 4 shows the western blot analysis of EBN-induced macrophages. Panel A shows the absence of pIRF3 band in 10% mixture of a negative control (SF) lane. Panel B shows the presence of pIRF3 band in 10% mixture of a bird's nest extract (E3) lane.

- 15 Figure 5 shows the western bolt analysis of a 50% mixture of an edible bird's nest extract (E4) and a 50% mixture of a negative control (SF) induced macrophages on the NF κ B pathway and MAPK pathway in Panels A and B respectively.

- Figure 6 shows the relative expression of cytokines in 10% mixture of edible bird's nest extract (E5) induced B lymphocytes at a time point of 4 hours. Panel A shows IFN β , Panel B shows IL-10, Panel C shows TNF α , Panel D shows IL-6 and Panel E shows IL-12.
- 20

- Figure 7 shows the dose-dependent effects on B cells for cytokine production. The relative expression of cytokines in 10% and 50% mixture of edible bird's nest extract (E6) induced B lymphocytes harvested overnight. Panel A shows IFN β , Panel B shows IL-10, Panel C shows TNF α , Panel D shows IL-6 and Panel E shows IL-12.
- 25

- Figure 8 shows the qPCR results of B lymphocytes after treatment with edible bird's nest extract (E7). Actin was used as the endogenous control where all gene expressions were normalised to. Expression of target genes were compared to Media with Poly(I:C), and LPS (0111:B4) as positive controls.
- 30

Figure 9 shows the identification and isolation of a cDNA encoding a desired cell-surface receptor by plasmid expression cloning.

Detailed Description

- 5 Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art to which this invention belongs.

The terms “about”, “approximately”, “substantially” must be read with reference to the context of the application as a whole, and have regard to the meaning a particular technical
10 term qualified by such a word usually has in the field concerned. For example, it may be understood that a certain parameter, function, effect, or result can be performed or obtained within a certain tolerance, and the skilled person in the relevant technical field knows how to obtain the tolerance of such term.

- 15 In the following description, numerous specific details are set forth in order to provide a thorough understanding of various illustrative embodiments of the invention. It will be understood, however, to one skilled in the art, that embodiments of the invention may be practiced without some or all of these specific details. It is understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not
20 intended to limit the scope of the invention. In the drawings, like reference numerals refer to same or similar functionalities or features throughout the several views.

The human immune system protects the body from foreign organisms, and comprise an innate immune system and an adaptive immune system. The innate immune system
25 provides immediate defence against infection, while the adaptive immune system provides a long-lasting immunity against specific foreign organisms. Parkin and Cohen (Lancet 2001, 357, 1777-1789) provided an overview of the immune system and the main components in the human body.

- 30 Major functions of the innate immune system includes recruiting immune cells to infection sites through the production of chemical factors including cytokines and to promote

removal of the foreign organisms; activation of the complement cascade; and activation of the adaptive immune system.

An opsonin is any molecule that enhances phagocytosis, and includes antibodies, complement proteins, and circulating proteins (for example pentraxins, collectins and ficolins). An opsonin typically marks an antigen for an immune response or mark dead cells for recycling. Most, if not all, cell membranes maintain a non-zero transmembrane potential and makes it difficult for two cells to come together. Opsonins generally work by binding to their target cells and enhance phagocytosis, i.e. the opsonin serve as a linker.

The complement system is a part of the immune system that enhances the ability of antibodies and phagocytic cells to clear microbes and damaged cells from organisms. The complement system comprises a number of complement proteins which circulate in the blood as inactive precursors and are activated by biochemical pathways. Pentraxins, collectins, and ficolins are soluble innate immune pattern-recognition proteins which identify non-self or altered-self molecular patterns on the surfaces of dying cells and promotes the programmed cell death (e.g. apoptosis) and the clearance of dying cells and cellular material by macrophages and other phagocytic cells. As defined above, the term “opsonin” in the context of the invention excludes complement proteins, lectins, ficolins, collectins and pentraxins.

20

Lectins are carbohydrate-binding proteins and perform recognition on the cellular and molecular level. Within the animal lectins, C-type lectins are the most abundant and are grouped into three major families: selectins, collectins and endocytic lectins. Collectins are believed to be involved in the pattern recognition of respiratory viruses and pathogenic bacteria. Examples include the collagenous lectins such as mannose binding proteins (MBP), pulmonary surfactant SP-A and SP-D and conglutinin. MBP is an example of a protective collectin that is able to bind oligomannose residues of bacterial and fungal cell surface oligosaccharides. MBP is also able to active the classical and the alternative complement pathways. Another endogenous collectin is a mannose receptor and is expressed on macrophage and dendritic cell surfaces, and are able to recognise and bind bacteria. As defined above, the term “lectin” in the context of the invention excludes ficolins and collectins, i.e. lectins other than ficolins and collectins.

25

30

Figure 1 shows the method to extract and isolate the bioactive molecules of interest from edible bird's nest (EBN):

- (a) cleaning EBN to remove contaminants;
- 5 (b) grounding the cleaned EBN and sifting through a mesh;
- (c) placing the EBN powder in water to provide an EBN mixture; hasma and/or seaweed may optionally be cleaned and added into the EBN mixture as well;
- (d) sterilising the EBN or EBN mixture;
- (e) dipping the EBN or EBN mixture into oil;
- 10 (f) treating the EBN mixture with an extraction solution comprising at least one binding moiety, the binding moiety being selected from the group comprising an opsonin binding moiety, a complement protein binding moiety, a lectin binding moiety, a ficolin binding moiety, a collectin binding moiety, and a pentraxin binding moiety;
- (g) hydrolysing the bounded molecules partially with an acidic solution;
- 15 (h) separating the binding moiety and bioactive molecules from the EBN mixture;
- (i) releasing the bounded molecules by addition of peptides of large molecular weight;
- (j) obtaining the released bioactive molecules via dialysis;
- (k) treating the isolated fraction from dialysis with a second enzymatic solution to further break down the bioactive molecules;
- 20 (l) denaturing and removing the enzymes of the second enzymatic solution;
- (m) drying the isolated fraction to obtain a solid product.

Certain steps described may be omitted, or performed in a different order. For example, steps (a)-(e), individually or in combination, may be considered as steps in preparing the EBN mixture for treating (or contacting) with the extraction solution. Hydrolysis of the target molecules is beneficial to maximise their biological effects. The time and temperature of the process may be varied to determine optimal parameters depending on the enzyme, binding moiety, and bioactive molecules being extracted.

30 The crude EBN (1 piece is approximately 10 to 50 g) is cleaned by soaking in water to remove nitrites, mites and other contaminants. The other possible contaminants that are removed may include heavy metals, bleach and other minute debris, including stains.

An effective method to remove the nitrites is to use a solution containing nitrite reductase enzymes from fruits, plants and soil. Additionally, the solution may contain another enzyme to inactivate any accompanying bacteria that produce the nitrite. To remove mites,
5 a solution containing special fruit proteinases are used. Such examples include any such protease from papaya (papain), kiwifruit (actinidin), pineapple (bromelain), fig (ficin) etc. These proteases may be used in any suitable concentration that will allow for the inactivation of the bacteria.

10 The EBN mixture was treated sequentially with each enzymatic solution for at least 5 minutes from room temperature to 40 °C. Nanobubbling of the resultant suspension of EBN in the enzymatic solution will cause the degraded cellular debris to float to the surface of the water where it can be easily removed. The enzymatic solution is subsequently removed from the solid EBN. The solid EBN can be further washed to remove any residual
15 enzymes and contaminants. The cleaned EBN is dried to remove excess water, preferably at 70 °C for 12h.

The cleaned EBN is grounded and sifted through a mesh. The size of the mesh should be sufficient to remove any large impurities left, preferably in a size of 200 to 700 µm. Most
20 preferably the mesh size is 600 µm.

The EBN powder is placed in water, preferably distilled or deionised water, at 5 °C for 5 hours. A suitable concentration is 25 g of EBN in 1000 mL of water. The mixture may be further sterilised at 121 °C for 10 to 20 minutes if desired. Hasma and/or seaweed may also
25 be cleaned as above and soaked in the mixture with the EBN. Alternatively, the hasma and/or seaweed may be prepared separately and added to the EBN mixture. Subsequently, the EBN mixture is dipped into oil to enhance the binding in the subsequent treatment with the binding moiety through enhanced interaction and affinity of the binding moiety and the bioactive molecules.

30

The EBN mixture is treated with an aqueous solution containing at least one binding moiety in a temperature range from 4 to 37 °C for at least 20 minutes. With a temperature of 25

to 37 °C, 20 to 120 minutes suffice, but could be kept longer overnight at a lower temperature. With a temperature of 4 °C, the mixture of antibody and EBN is kept for at least 9 hours. Generally, the lower the temperature the longer the time required for the binding moiety solution to completely bind to the targeted compounds. The binding moiety is selected from the group comprising an opsonin binding moiety, a complement protein binding moiety, a lectin binding moiety, a ficolin binding moiety, a collectin binding moiety, and a pentraxin binding moiety. In an embodiment, the contacting or mixing step of the EBN mixture and binding moiety is carried out in the presence of ascorbic acid and/or gold nanoparticles. The ascorbic acid provides anti-oxidant properties and prevents or minimises the degradation of the bioactive molecules, for example from reactive oxygen species. The gold nanoparticles provide a stable and non-reactive environment which enhances the binding of the target molecules and binding moiety. The at least one binding moiety present in the extraction solution will bind to the targeted molecule and allow the bounded molecule to be extracted out of the EBN mixture.

15

Examples of binding moieties

An example of an opsonin binding moiety protein is GP-340, a putative opsonin receptor for lung surfactant protein D. The protein has the following sequence (SEQ ID No. 1) with a molecular weight of 260.79 kDa:

```

20      1 mgistvilem cllwgqvlst ggwiprttdy aslipsevp1 dqtvaegsp1f psestlesta
      61 aegspisles tlestvaege lipsestles tvaegsdsgl alrlvngdgr cqgrveilyr
      121 gswgtvcdds wtdndanvvc rqlgcgwams apgnawfgqg sgpialdvdr csghesylws
      181 cphngwlshn cghgedagvi csaaqpqstl rpeswpvris ppvptegses slalrlvngg
      241 drcrgrvevl yrsgwtvcd dywdtdanv vcrqlgcgwa msapgnagfg qsggpivldd
25      301 vrcsghesyl wscphngwlt hncghsedag vicsapqsrp tpspdtwpts hastagpess
      361 lalrlvnggd rcqgrvevly rgswgtvcdd swdtsdanvv crqlgcgwat sapgnarfqq
      421 gsgpivlddv rcsqyesylw scphngwlsh ncghsedagv icsaahswst pspdtlptit
      481 lpastvgsses slalrlvngg drcqgrvevl yrsgwtvcd dswdtdanv vcrqlgcgwa
      541 mlapgnarfqq qsggpivldd vrcsgnesyl wscphngwls hncghsedag vicsgppessl
30      601 alrlvnggdr cqgrvevlyr gswgtvcdds wtdndanvvc rqlgcgwams apgnarfqqg
      661 sgpiavddvdr csghesylws cpnngwlshn cghhedagvi csaaqsrstp rpdltlstitl
      721 ppstvgsses ltlrlvngsd rcqgrvevly rgswgtvcdd swdtdanvv crqlgcgwam
      781 sapgnarfqq gsgpivlddv rcsghesylw scphngwlsh ncghhedagv icsvsgsrpt
      841 pspdtwptsh astagsessl alrlvnggdr cqgrvevlyr gswgtvcdds wtdsdanvvc
35      901 rqlgcgwats apgnarfqqg sgpiavddvdr csgyesylws cphngwlshn cghsedagvi
      961 csaaahswstp spdtlptitl pastvgsses lalrlvnggd rcqgrvevly qsgwtvcdd
      1021 swdtdanvv crqpgcgwam sapgnarfqq gsgpivlddv rcsghesypw scphngwlsh
      1081 ncghsedagv icsasqsrpt pspdtwptsh astagsessl alrlvnggdr cqgrvevlyr
      1141 gswgtvcddy wtdndanvvc rqlgcgwams apgnarfqqg sgpiavddvdr csghesylws
40      1201 cphngwlshn cghhedagvi csasqsqtp spdtwptsha stagsessla lrlvnggdrc
      1261 qgrvevlyrg swgtvcddyw dtdndanvvc rqlgcgwatsa pgnarfqqgs gpivlddvrc
      1321 sghesylwsc phngwlshnc ghhedagvic sasqsqtp ptdwptshas tagssesslal
      1381 rlvnggdrcq grvevlyrgs wgtvcddywd tndanvvc rqlgcgwatsap gnarfqqgs

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1441 pivlddvrcs ghesylwscp hngwlshncg hhedagvics asqsgptpsp dtwptsrast
1501 agsestlaln lvnggdrcrg rvevlyqgsw gtvcddywdt ndanvvcrql gcgwamsapg
1561 naqfgqsgp ivlddvrcsg hesylwscph ngwlshncgh hedagvicsa aqsqstprpd
1621 twlttnlpal tvgsesslal rlvnggdrcr grvevlyrgs wgtvcddswd tndanvvcrq
5 1681 lgcgwamsap gnarfgqsg pivlddvrcs gnesylwscp hkgwlthncg hhedagvics
1741 atqinstttt dwwhpttttta rpssncggfl fyasgtfssp sypayypnna kcvweievns
1801 gyrinlgfsn lk Leahhncs fdyveifdgs lnsslllgki cndtrqifts synrmtihfr
1861 sdisfntqtf lawynsfpsd atlrlnvlns syglcagrve iyhggtwgtv cddswtqiea
1921 evvcrlgqcg ravsalignay fgsgsgpitl ddvecsgtes tlwqcrnrgw fshncnhred
10 1981 agvicsgnhl stpafpflnit rpntdyscgg flsqpsgdfs spfypgnypn nakcvwdiev
2041 qnnrvrtvif rdvqleggc n ydyievfdgp yrsspliarv cdgargsfts ssnfmsirfi
2101 sdhsitrrgf raeyyspsn dstnlclpn hmqsavrsy lqslgfsasd lvistwnyy
2161 ecrpqitpnl viftipysgc gtfkqadndt idysnfltaa vsggiikrrt dlrihvsrm
2221 lqntwvdtmy iandtihvan ntiqveevqy gnfdvnisfy tsssflypvt srpyyvdlnq
15 2281 dlyvqaeilh sdavltlfvd tcvaspysnd ftsltydlir sgcvrddtyg pysspslria
2341 rfrfrafhfl nrfpsvylrc kmvvcraydp ssrorygcvl rskrdvgsyq ekvdvvlgpi
2401 qlqtpprree epr

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An example of a complement protein binding moiety protein is the complement receptor

20 type 2 isoform 1 precursor [Homo sapiens]. The protein has the following sequence (SEQ ID No. 2) with a molecular weight of 119.18 kDa:

```

1 mgaagllgvf lalvapgvlg iscgspppil ngrisyystp iavgtvirys csgtfrlige
61 ksllcitkdk vdgtdwkpap kceyfnkyss cpepivpggy kirgstpyrh gdsvtfackt
121 nfmngnksv wcqannmwgp trlptcvsvf plecpalpmi hnghtsenv gsiapglsvt
25 181 yscesgyllv gekiinclss gkwsavpttc eearckslgr fpngkvkepp ilrvgtanfv
241 fcdegyrlqg ppssrcviag qgvawtkmpv ceeifcpspp pilngrhign slansygsi
301 vtytcdpdpe egvnfilige stlrctvdsq ktgtwsgpap rcelstsavq cphpqilrgr
361 mvsgqkdryt yndtvifacm fgftlkgksq ircnaqgtwe psapvcekec qappnilngq
421 kedrhmvrfd pgtsikyscn pgylvgees iqctsegvwt ppvpqckvaa ceatgrqlt
30 481 kpqhqvfrpd vnsscgegyk lsgsvyqecq gtipwfmeir lckeitcopp pviyngahtg
541 ssledfpygt tvtytcnpgp ergvefslig estirctsnd qergtwsgpa plcklslav
601 qcshvhiang ykisgkeapy fyndtvtfkc ysgftlkgss qirckadntw dpeipvcek
661 cqspplghh rhtggnvff vsgmtvdytc dpgyllvgnk sihcpsgnw spsaprceet
721 cqhvqrslqe lpagsrvelv ntscqdgyql tghayqmcqd aengwfkki plckvihchp
35 781 ppvivngkht gmmaenfily nevseycdqg fyllgekkk crsdskgghs wsgpspqclr
841 kppvtrcpnp evkhgyklk thsayshndi vyvdcnpgfi mngsrvirch tdntwvpgvp
901 tcikkafigc pppktpngn htggniarfs pgmsilyscd qgyllvgeal llcthegtws
961 qpaphckevn csspadmdgi qkgleprkmy qygavvtlec edgymlegsp qsqcqsdhqw
1021 npplavcrsr slapvlcgia aglilltfl vitlyviskh rarnyytdts qkeafhlear
40 1081 evysvdpynp as

```

An example of a lectin binding moiety protein is the c Killer cell lectin-like receptor subfamily B member 1. The protein has the following sequence (SEQ ID No. 3) with a molecular weight of 25.42 kDa:

```

45 1 mdqqaiyael nlptdsgpes sspsslprdv cggspwhqfa klscagiil lylvvtglsv
61 svtsliqkss iekcsvdiq srnkttterpg llncpiywqq lrekcillfsh tvnpwnnsia
121 dcstkessll lirdkdelih tqnlirdkai lfwiglfnsl seknwkwing sflnsndlei
181 rgdakensci sisqtsvyse ycsteirwic qkeltprvnrk vypds

```

50 An example of a ficolin binding moiety protein has the following sequence (SEQ ID No. 4) with a molecular weight of 35.08 kDa:

```

1 melsgatmar glavlllvfl hiknlpaqaa dtcpevkvvg legskltil rgcpglpgap
61 gpkgeagvig ergerglpga pgkagpvvgpk gdrgekmg rg ekgdagqsqs catgprnckd
121 lldrgyflsg whtiylpdc r pltlvcdmdt dgggwtvfqr rmdgsvdfyr dwaaykqgfg
181 sqlgefwnlg dnihaltaqg sselrvdlvd fegnhqfaky ksfskvadeae kyklvlgafv
5 241 ggsagnsltg hnnffstkd qdndvssnc aekfqqawwy adchasnng lylmgphesy
301 anginwsaak gykysykvse mkvrpa

```

An example of a collectin binding moiety protein is Collectin-12. The protein has the following sequence (SEQ ID No. 5) with a molecular weight of 81.53 kDa:

```

10 1 mkddfaeeee vqsfgykrfg iqegtqctkc knnwalkfsi illyilcall titvailgyk
61 vvekmdnvtg gmetstrqtyd dkltavesdl kklgdqtgkk aistnselst frsdildlrq
121 qlreitekts knkdtlekld asgdalvdrq sqlketlenn sflittvnkt lqayngyvt n
181 lqqdtsvlqg nlqnqmyshn vvimmnnln ltqvqqrnli tnlqrsvddt sqalqriknd
241 fqnlqqvflq akkdt dwlke kvqslqtlaa nnsalakann dtledmnsq l nsftggmeni
15 301 ttisqaneqn lkdlqdlhkd aenrtaikfn qleerfqlfe tdivniisni sytahhlrtl
361 tsnlnevrtt ctdtltkhtd dltslnntla nirlsvslr mqqdlmrsl dtevanlsvi
421 meemklvdsk hqqliknfti lqgppgprgp rgdrgsqgpp gptgnkgqkg ekgepgppgp
481 agergpiga gppgerggkg skgsqgpkgs rgspgkpgpq gssgdpgppg ppgeglpgp
541 qgppgfqglq gtvgepgvpg prglpglpgv pgmpgkpgp gppgpgsavv plalqneptp
20 601 apedngcph wkntdkcyy fsvekeifed aklfcedkss hlvfintree qqwikqmvq
661 reshwigltd serenewkw l dgtspdyknw kagqpdnwg hghpgedcag liyagqwndf
721 qc edvnnfic ekdretvlss al

```

An example of a pentraxin binding moiety protein is the neuronal pentraxin receptor (homo sapiens). The protein has the following sequence (SEQ ID No. 6) with a molecular weight of 52.86 kDa:

```

1 mkflavllaa gmlaflgavi ciiasvplaa sparalpaga dnasvasgaa aspgpqrsls
61 alhgaggsag ppalpgapaa sahlpppgpl fsrflctpla aacpsgaqqg daagaapger
121 eellllqsta eqlrqtalq eariradqdt ireltgklgr cesglprglq gagprrdtma
30 181 dgpwdspali leledavral rdridrleqe lparvnlsaa papvsavptg lnskmdqleg
241 qllaqvlale kervalshss rrqrqeveke ldvlqgrvae lehgsaysp pda fkisipi
301 rnnymyarvr kalpelyaft acmwlrsrss gtgggt pfsy svpgqaneiv lleaghepme
361 llindkvaql plskdngwh hiciawttrd glwsayqdge lqsggenlaa whpikphgil
421 ilgqe qdtlg grfdatqafv gdiaqfnlwd haltpaqvlg ianctapllg nvlpwe dklv
35 481 eafggatkaa fdvckgraka

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Examples of suitable extraction solutions that may be used with the above binding moieties include:

Extraction solution 1: 50% to 90% of the lectin binding moiety and 10% to 50% of the opsonin binding moiety;

Extraction solution 2: 10% to 30% of the lectin binding moiety, 10% to 30% of the complement protein binding moiety, 10% to 30% of the opsonin binding moiety, 5% to 15% of the ficolin binding moiety, 5% to 15% of the collectin binding moiety, and 10% to 30% of the pentraxin binding moiety;

Extraction solution 3: 5% to 45% of the lectin binding moiety, 35% to 65% of the complement protein binding moiety, and 5% to 45% of the opsonin binding moiety;

Extraction solution 4: 10% to 90% of the opsonin binding moiety, and 10% to 90% of the pentraxin binding moiety;

- 5 Extraction solution 5: 30% to 50% of the ficolin binding moiety, 10% to 50% of the collectin binding moiety, and 10% to 50% of the pentraxin binding moiety;

Extraction solution 6: 55% to 85% of the complement protein binding moiety, 5% to 15% of the ficolin binding moiety, and 10% to 30% of the collectin binding moiety; and

- 10 Extraction solution 7: 5% to 15% of the lectin binding moiety, 10% to 30% of the opsonin binding moiety, and 55% to 85% of the collectin binding moiety.

Extraction solution 8: 70% of the lectin binding moiety and 30% of the opsonin binding moiety;

- 15 Extraction solution 9: 20% of the lectin binding moiety, 20% of the complement protein binding moiety, 20% of the opsonin binding moiety, 10% of the ficolin binding moiety, 10% of the collectin binding moiety, and 20% of the pentraxin binding moiety;

Extraction solution 10: 25% of the lectin binding moiety, 50% of the complement protein binding moiety, and 25% of the opsonin binding moiety;

Extraction solution 11: 50% of the opsonin binding moiety, and 50% of the pentraxin binding moiety;

- 20 Extraction solution 12: 40% of the ficolin binding moiety, 30% of the collectin binding moiety, and 30% of the pentraxin binding moiety;

Extraction solution 13: 70% of the complement protein binding moiety, 10% of the ficolin binding moiety, and 20% of the collectin binding moiety; and

- 25 Extraction solution 14: 10% of the lectin binding moiety, 20% of the opsonin binding moiety, and 70% of the collectin binding moiety.

The percentage provided for each binding moiety is the percentage weight of each binding moiety relative to the total weight of the binding moieties present. The binding moiety/moieties may be dissolved in any suitable solvent or used as a mixture. Examples
30 of solvent includes water and buffer solutions.

After the contacting or mixing step, the mixture is homogenised with a homogeniser, treated with an acidic solution, and heated to 100°C to cause partial hydrolysis of the target compounds. The acid is preferably a food acid, for example ascorbic acid, citric acid, malic acid, acetic acid, tartaric acid, fumaric acid, and lactic acid. The mixture is cooled to room
5 temperature and neutralised to a pH of 7.

After hydrolysis, at least one binding moiety and bounded molecules can be separated from the mixture by any of the commonly known methods. Some of these methods include physicochemical fractionation, class-specific affinity and antigen-specific affinity.
10 Physicochemical fractionation includes differential precipitation, size-exclusion or solid-phase binding of immunoglobulins based on size, charge or other shared chemical characteristics of antibodies. Class-specific affinity includes solid-phase binding of particular antibody classes (e.g. IgG) by immobilised biological ligands that have specific affinity to immunoglobulins. Antigen-specific affinity includes using specific antigens to
15 purify antibodies through their specific antigen-binding domains.

The bounded compounds are released from the at least one binding moiety by adding excess larger peptides. For example, the larger peptides should have a minimum molecular weight of 50 kDa, and include natural glycoaminoglycans and other proteins.
20

The released compounds are subsequently isolated from the added peptides, enzymes and at least one binding moiety via the use of a dialysis bag.

Alternatively, the EBN containing mixture may be sequentially treated with extraction
25 solutions comprising a different binding moiety, and separated to extract out the desired compounds sequentially. This ensures optimal use of the EBN, seaweed and/or hasma, and avoids wastage.

The isolated/concentrated compounds can be further hydrolysed with vegetable and/or
30 food proteases at 45 °C for 1 hour at a pH of 6.5 to 9.0. The concentration of enzymes used should be at least 10 µg/mL for effective hydrolysis, and preferably up to 100 µg/mL. Examples of suitable enzymes include corn and maize terminal proteases. The enzymes are

subsequently denatured by heating the mixture at 70 °C for 5 minutes. The enzymes precipitate out at a temperature above 55 °C, hence the mixture can be filtered at a temperature above 55 °C to afford the desired compounds as a solution in the filtrate.

- 5 The solution of desired compounds is dried to give the compounds as a powder. Preferably, the compounds are dried by freeze drying or spray drying. The freeze drying is carried out by cooling the solution to a temperature between -180 °C to -70 °C with liquid nitrogen or dry ice, and submitting the frozen mixture to vacuum to sublime the ice. The freeze drying can be repeated if required to give a dried powdered product.

10

A bird's nest concentrate or extract obtained from the process described above comprise the bioactive molecules extracted from the process described. In some embodiments, the bioactive molecules may have been broken down and may be difficult to characterise. In some embodiments, the bioactive molecules may be largely intact.

15

As such, the bird's nest extract comprises a plurality of molecules selected from at least two of the following groups: opsonin, a complement protein, a lectin, a ficolin, a collectin, and a pentraxin. The bird's nest extract may further include extracts from hasma and/or seaweed.

20

In an embodiment, the bird's nest extract comprises any one selected from:

- i) the lectin and the opsonin;
- ii) the lectin, the complement protein, the opsonin, the ficolin, the collectin, and the pentraxin;
- 25 iii) the lectin, the complement protein, and the opsonin;
- iv) the opsonin and the pentraxin;
- v) the ficolin, the collectin, and the pentraxin;
- vi) the complement protein, the ficolin, and the collectin; and
- vii) the lectin, the opsonin, and the collectin.

30

In particular, the bird's nest extract comprises any one selected from:

- i) 50% to 90% of the lectin, and 10% to 50% of the opsonin

- ii) 10% to 30% of the lectin, 10% to 30% of the complement protein, 10% to 30% of the opsonin, 5% to 15% of the ficolin, 5% to 15% of the collectin, and 10% to 30% of the pentraxin;
 - iii) 5% to 45% of the lectin, 35% to 65% of the complement protein, and 5% to 45% of the opsonin;
 - iv) 10% to 90% of the opsonin, and 10% to 90% of the pentraxin;
 - v) 30% to 50% of the ficolin, 10% to 50% of the collectin, and 10% to 50% of the pentraxin;
 - vi) 55% to 85% of the complement protein, 5% to 15% of the ficolin, and 10% to 30% of the collectin;
 - viii) 5% to 15% of the lectin, 10% to 30% of the opsonin, and 55% to 85% of the collectin.
- The percentage given is the percentage weight of the particular bioactive molecule relative to the total weight of bioactive molecules present.
- The following bird's nest extracts 1 to 7 were prepared based on the process described above using the extraction solutions 8 to 14 respectively, and are reflective of the properties possess by extracts with ranges encompassing these examples:
- Extract 1 was prepared using EBN and comprises: 70% of lectins, and 30% of opsonins;
- Extract 2 was prepared using EBN, hasma and seaweed, and comprises: 20% of lectins, 20% of complement proteins, 20% of opsonins, 10% of ficolins, 10% of collectins, and 20% of pentraxins;
- Extract 3 was prepared using EBN and seaweed, and comprises: 25% of lectins, 50% of complement proteins, and 25% of the opsonin;
- Extract 4 was prepared using EBN and seaweed and comprises: 50% of opsonins, and 50% of pentraxins;
- Extract 5 was prepared using EBN and comprises: 40% of ficolins, 30% of collectins, and 30% of pentraxins;
- Extract 6 was prepared using EBN and hasma, and comprises: 70% of complement proteins, 10% of ficolins, and 20% of collectins;
- Extract 7 was prepared using EBN and hasma, and comprises: 10% of lectins, 20% of opsonins, and 70% of collectins.

The dried powdered product may be mixed with other additives to give a food or pharmaceutical product. Alternatively, the product may be dissolved in water along with other additives.

- 5 The dried product may be mixed with maltodextrin in various formulations as follows:
1. 75% of EBN/EBN mixture concentrate/extract product and 25% maltodextrin;
 2. 50% of EBN/EBN mixture concentrate/extract product and 45% maltodextrin;
 3. 50% of EBN/EBN mixture concentrate/extract product and 50% maltodextrin;
 4. 30% of EBN/EBN mixture concentrate extract product and 70% maltodextrin.

10

It may be seen that the product comprises EBN concentrate/extract product and maltodextrin. Preferably, 30 to 75 wt. % of the EBN concentrate/extract and 25 to 70 wt.% of maltodextrin.

- 15 Bioavailability of these bioactive molecules are generally very poor due to their high water solubility. Common administration routes via topical application to the skin and joints, or oral administration is hampered by the poor permeability through the skin or hydrophobic membranes in the intestine. It is difficult for the bioactive molecules to reach the requisite sites in the body to have the desired effect. Other methods of administration are available
- 20 but is generally not suitable to be administered without a health professional. The hydrolysis of the bioactive molecules partially by acidic and/or enzymatic hydrolysis may prove to be beneficial to break down the molecules into more easily absorbable compounds making the extract more beneficial.

- 25 Especially with a suitable formulation, the respective proprietary immunological concentrates can be delivered in effective and useful doses to the sites of pain and inflammation.

- Formulated in lipid forms, the present concentrate/extract may be used to produce a first
- 30 economical product in the market that is transdermal and is composed of safe and sustainable bioactive molecules, purified/extracted from natural but abundant supplies of EBN/EBN mixture recycled crumbs.

Extracts 1 to 7 (E1-E7) were tested on macrophages/B cells cell culture *in vitro* to determine their effects on the production of Type-1 IFN, cytokine production, and dengue virus replication. Macrophages/B cells in this application refer to differentiated primary
5 macrophages/B cells derived from bone-marrow progenitor's stem cells from mouse femurs. All these features are commonly used indicators of protective immune response of macrophages/B cells *in vitro*.

Methods and Results of Biological Assays

10 1. Method of cell culture and dengue virus replication assays

Bone marrow-derived macrophage culture

Bone marrow cells were obtained by injecting culture media into the femur and tibia. All cells were spun down by centrifuging 1000 rpm for 5 mins at 4 °C. To eliminate erythrocytes, cells were treated with 1 ml of red blood cells lysis buffer for 5 mins by
15 incubating on ice. The cells were washed in 10 ml culture media and collected by centrifuging at 1000 rpm for 5 mins at 4 °C. Bone marrow cells were counted using haemocytometer and 106 cells were differentiated on 10cm culture plate containing 10 ml of MCSF containing media for 6 days.

20 *Dengue virus infection of macrophages*

Dengue type 1 virus (Singapore Strain S275/90, D1) was propagated in C6/36 cells. Bone marrow-derived macrophages (3x10⁶) were seeded into each well of 6-well tissue-culture plate (NUNC). After overnight incubation, macrophages were infected with D1 at a multiplicity of infection (MOI) of 1 for 2 days. For detection of negative-strand D1 RNA,
25 total RNA was extracted from D1-infected cells using TRIzol (Invitrogen). 1µg of total RNAs was subject to reverse transcription with primer 5'-GTGCTGCCTGTGGCTCCATC-3', and was subsequently used as a template for synthesis of a PCR fragment with the primer pair 5'-AGAACCTGTTGATTCAACAGCACC-3' and 5'-CATGGAAGCTGTACGCATGG-3'. For detection of GAPDH by Reverse-Transcriptase PCR, cellular cDNAs were synthesized from above total
30 RNA with oligodT primer. GAPDH fragments were synthesized with the following primers, murine GAPDH, 5'-GACAACTTTGGCATTGTGGAA-3' and 5'-CCAGGAAATGAGCTTGACA-3', respectively.

Referring to Figure 2, it may be seen that the middle lane with the macrophages treated with Extract 1 (E1) in the above assay shows the absence or weak presence of a white band, indicating that Extract 1 restricts the replication of the dengue virus in macrophages. This
5 may be used as a means to control the dengue virus replication.

2. Method of cell culture, real-time genes expression quantitative-PCR (qPCR)

Bone marrow-derived macrophage culture

Bone marrow cells were obtained by injecting culture media into the femur and tibia. All
10 cells were spun down by centrifuging 1000 rpm for 5 mins at 4 °C. To eliminate erythrocytes, cells were treated with 1 ml of red blood cells lysis buffer for 5 mins by incubating on ice. The cells were washed in 10 ml culture media and collected by centrifuging at 1000 rpm for 5 mins at 4 °C. Bone marrow cells were counted using haemocytometer and 10⁶ cells were differentiated on 10cm culture plate containing 10 ml
15 of MCSF containing media for 6 days.

Quantitative real-time PCR

2x10⁶cells/ml of bone marrow macrophages were seeded onto 6 well plate in Opti-MEM (Gibco,US). Cells were stimulated with 50µg/ml Poly(I:C) (InvivoGen, US), 1µg/ml LPS
20 0111:B4 (purified from LPS 0111:B4) and 1µg/ml LPS 055:B5 (Sigma, US) for 2 hours before RNA extraction using TRIzol (Invitrogen, US). 1µg cDNA was synthesized from total RNA with Superscript III First Strand Synthesis System (Invitrogen, US) according to manufacturer's protocol. qPCR was performed on Applied Biosystems QuantStudio 6 Flex Real Time PCR system with the cytokine specific primers.

25

Referring to Figure 3, it can be seen that the positive controls elicit the production of IFNβ, TNFα, IL-10, IL-6, and IL-12 to different extents. Extract 2 (E2) elicit the production of IFNβ and inflammatory cytokines in macrophages, and provide a most complete immune balancing platform whereby key immune molecules could be harvested to influence the
30 orchestration of various pro-inflammatory and anti-inflammatory molecules to achieve a balanced state of immune system, manifested not just by macrophages but other important cells as well. The symphony of immune molecules that would produce enhanced

effects of prevention of microbial infections as well as better management of cancers. Extract 2 could possibly possess anti-dengue properties as well.

3. Phosphorylation of IRF3 in macrophages

5 Extract 3 (E3) when applied to macrophages induce phosphorylation of IRF3 in macrophages as shown in Figure 4 panel B. The lane marked E3 in the western bolt analysis shows the phosphorylation of IRF3 same as the positive controls poly(I:C) and LPS. Panel A of Figure 4 shows the negative control in the SF marked lane. Activated IRF3 has been
10 IRF7. Modulated properly via the adoption of the different ranges of triggering immune peptides, this might also be employed successfully in post-myocardial infarction cardioprotection.

The negative control (SF) is the edible bird's nest which had not undergo the extraction
15 process, and may be prepared by soaking the edible bird's nest in water and made up to the same mass concentration as the extract being tested. The SF control may be prepared similarly for the other assays.

4. Induction of NF- κ B and MAPK pathway in immune cells

20 Extract 4 (E4) when applied to the immune cells elicit the induction of the NF κ B and MAPK pathway in immune cells as shown in Figure 5. It can be seen in the Western bolt analysis that the immune cells treated with Extract 4 induces phosphorylation of p65, JNK, ERK, and p38 as shown in the lanes marked with Extract 4 while those with the negative control (SF lane) do not show the phosphorylated band.

25

NF- κ B plays a key role in regulating the immune response to infection. Incorrect regulation of NF- κ B has been linked to cancer, inflammatory and autoimmune diseases, septic shock, viral infection, and improper immune development. NF- κ B has also been implicated in processes of synaptic plasticity and memory.

30

MAPKs are involved in directing cellular responses to a diverse array of stimuli, such as mitogens, osmotic stress, heat shock and proinflammatory cytokines. They regulate cell

functions including proliferation, gene expression, differentiation, mitosis, cell survival, and apoptosis.

Extract 4 may be used in enhanced management of the various forms of stress, heat shock,
5 and septic shock.

5. Induction of B cells to produce cytokines

Extract 5 (E5) could induce B cells to produce cytokines in culture as shown in Figure 6. It can be seen in Figure 6 that the lane marked 10% Extract 5 (10% wt. of Extract 5 in aqueous
10 solution) shows increased production of IFN β , TNF α , IL-10, IL-6, and IL-12 compared to the negative control (media) and 10% SF (a negative control). Although pentraxin is associated with B cells, ficolins and collectins may not be. However, the presence of the three different type of bioactive molecules could induce a wide pool of different B cells to produce a huge diversity of cytokines to cope better with the very challenging various chronic diseases such
15 as diabetes, arthritis, multiple sclerosis, etc.

6. Dose-dependent effect on B cells for cytokine production

Extract 6 (E6) when applied to B cells shows dose-dependent effects on B cells for cytokine
20 production as shown in Figure 7, in particular for IFN β , IL-10, and IL-6. By replacing pentraxin with complement proteins, with a combination of immune peptides that may not be closely associated to B cells, dose dependent effects on B cells for cytokine production may be achieved. These dose dependent and prolonged overnight effects may be manifested using Extract 6 instead of Extract 5 above. The rationale is that since all
25 ficolins, collectins and complement proteins are components of the non-humoral arm of innate immunity, the earlier stage of the innate immunity, their overall effects could be more sustainable as the span of operational period of this composition could be longer, since the time covered by the earlier stage could be more accounted for.

30 7. Selective induction of IFN β , TNF α , and IL-6, but not IL-10 and IL-12, in B cells

Extract 7 (E7) when applied to B cells selectively induce IFN β , TNF α , and IL-6 in B cells, but not IL-10 and IL-12, as shown in Figure 8. The increased expression of IFN β , TNF α , and IL-6 are marked with a box to indicate the beneficial effects of Extract 7.

- 5 The primary role of TNF is in the regulation of immune cells. TNF, being an endogenous pyrogen, is able to induce fever, apoptotic cell death, cachexia, inflammation and to inhibit tumorigenesis and viral replication and respond to sepsis via IL1 & IL6 producing cells.

10 Interferon beta (IFN β) balances the expression of pro- and anti-inflammatory agents in the brain, and reduces the number of inflammatory cells that cross the blood brain barrier. Overall, therapy with interferon beta leads to a reduction of neuron inflammation. Moreover, it is also thought to increase the production of nerve growth factor and consequently improve neuronal survival.

15 Interleukin 6 (IL-6) is an interleukin that acts as both a pro-inflammatory cytokine and an anti-inflammatory myokine. Interleukin 6 is secreted by T cells and macrophages to stimulate immune response, e.g. during infection and after trauma, especially burns or other tissue damage leading to inflammation. IL-6 also plays a role in fighting infection. In addition, osteoblasts secrete IL-6 to stimulate osteoclast formation. Smooth muscle cells
20 in the tunica media of many blood vessels also produce IL-6 as a pro-inflammatory cytokine. IL-6's role as an anti-inflammatory cytokine is mediated through its inhibitory effects on TNF-alpha and IL-1, and activation of IL-1ra and IL-10.

25 TNF, IFN β and IL-6 together present a very novel but balanced immune modulating platform whereby there could be self-balancing of pro-inflammatory and anti-inflammatory factors. The novelty of combining the likely presence of opsonins, lectins and collectins (although they are associated peptides) in the immunological concentrate has yielded the ability of B cells to have induced expression of TNF, IFN β and IL-6 together. When there is a balanced immune modulating platform, symptoms such as loss of weight,
30 muscle atrophy, fatigue, weakness, significant loss of appetite, bone loss or even neurodegeneration would be alleviated. Cancer would also be less likely to occur as a result of a healthy and balanced immune system.

As can be seen from the assays and results discussed, the bird's nest Extracts 1 to 7 are able to induce various immune cells to increase production of cytokines, both pro and anti-inflammatory cytokines. This modulates the immune system as both types of cytokines are
5 boosted balancing the cytokines present in the body. This prevents the overproduction of a particular cytokine.

The bird's nest extract whether as defined by the process or by the product itself, are suitable to be consumed as a nutraceutical or health supplement. It may potentially be
10 usable as a medicament in certain compositions.

The process described herein allows for valuable bioactive molecules to be extracted from EBN, and provide for a cost effective and important source of immune boosters.

15 Whilst there has been described in the foregoing description preferred embodiments of the invention, it will be understood by those skilled in the field concerned that many variations or modifications in details of design or construction may be made without departing from the present invention.