



(51) International Patent Classification:

A61K 35/618 (2015.01) *A61P 29/00* (2006.01)
A61P 7/02 (2006.01)

(21) International Application Number:

PCT/AU2017/050041

(22) International Filing Date:

20 January 2017 (20.01.2017)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

2016900175 21 January 2016 (21.01.2016) AU

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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— with international search report (Art. 21(3))

(54) Title: BLACKLIP ABALONE (*HALIOTIS RUBRA*) EXTRACT

(57) Abstract: The present invention provides extracts and extract fractions of blacklip abalone (*Haliotis rubra*) processing waste. Processes for the preparation of the extract and fractions thereof and use of the extract or fraction as an ingredient, medicament, dietary supplement or nutraceutical are also described.



BLACKLIP ABALONE (HALIOTIS RUBRA) EXTRACT

Field of the Invention

[0001] The present invention relates to novel extracts and extract fractions of blacklip abalone (*Haliotis rubra*) processing waste. Processes for the preparation of the extract and fractions thereof and use of the extract or fraction as an ingredient, medicament, dietary supplement or nutraceutical are also described.

Background of the Invention

[0002] Seafood processing waste results in the generation of thousands of tonnes of skin, shell and offal which are discarded each year in Australia alone. In particular, the preparation of abalone for sale as a food product generates a disproportionate amount of processing waste as approximately only one third of the animal, i.e. the foot muscle, is marketable as abalone meat. The remaining two thirds comprise shell, meat trimmings and offal (viscera, internal organs), and are usually discarded.

[0003] The processing of abalone also requires washing steps which generate waste in the form of waste water streams that are also usually discarded. Furthermore, the preparation of some abalone meat for sale generates additional waste as it involves preservation in the form of cooking and packaging (canning or bagging) where liquids (or cooking juices) are liberated and also usually discarded. Processing waste may also comprise undersized whole abalone rejected during grading.

[0004] Transportation of the discarded abalone waste is costly and disposal of the waste is problematic and unpleasant. Furthermore, there is the additional problem of potential environmental impact relating to the disposal of large amounts of seafood waste.

[0005] There is a need to identify viable alternative uses for abalone processing waste in an effort to add value to the abalone resource, decrease waste disposal and minimise environmental impact. Furthermore there is a need to identify viable alternative uses for the waste processing stream to add value and improve economic return to the abalone meat processing industry.

Summary of the Present Invention

[0006] The present invention is predicated, at least in part, on the discovery that commercially viable biologically active components can be recovered from the non-shell processing waste from blacklip abalone (*Haliotis rubra*) meat production. Surprisingly, it was discovered that non-shell processing waste such as offal, meat trimmings, waste water streams and cooking juices generated from the processing of blacklip abalone are a source of anti-inflammatory and anti-thrombotic agents. In particular, extracts obtained from blacklip abalone processing waste produced significant and consistent anti-thrombotic and anti-inflammatory activity.

[0007] Based on these discoveries, the present inventors have developed extracts, extract fractions, compositions and methods for use in treating a range of conditions including inflammatory and thrombotic disorders as described hereafter.

[0008] Accordingly, in a first aspect, the present invention advantageously provides an extract of blacklip abalone (*Haliotis rubra*), or fraction thereof, comprising one or more biologically active components, which extract is derived from blacklip abalone processing waste. In one embodiment, the processing waste is raw (or fresh) processing waste.

[0009] In some embodiments the one or more biologically active components are selected from anti-thrombotic agents and anti-inflammatory agents.

[0010] In some embodiments the extract is fractionated to produce a fraction enriched in protein components. In some embodiments the extract is fractionated to produce a fraction enriched in anionic polysaccharide components.

[0011] In another aspect, the invention provides a use of an extract, or fraction thereof, according to the invention in the preparation or manufacture of a medicament, dietary supplement, nutraceutical, functional food or functional food ingredient.

[0012] In yet another aspect, the present invention provides a medicament, dietary supplement, nutraceutical, functional food or functional food ingredient comprising an extract of the invention, or fraction thereof.

[0013] In yet another aspect, the present invention provides a pharmaceutical composition comprising an extract according to the invention, or fraction thereof, and one or more pharmaceutically acceptable carriers or excipients.

[0014] In a further aspect, the present invention provides an anti-inflammatory agent comprising an extract or fraction enriched in protein components according to the invention.

[0015] In a further aspect, the present invention provides a method of treating an IL-6 mediated condition in a subject, the method comprising administering to the subject an effective amount of an extract or fraction enriched in protein components according to the invention.

[0016] In a yet further aspect, the present invention provides a method of treating an inflammatory condition in a subject, the method comprising administering to the subject an effective amount of an extract or fraction enriched in protein components according to the invention.

[0017] In another aspect, the present invention provides a method of treating an autoimmune condition in a subject, the method comprising administering to the subject an effective amount of an extract or fraction enriched in protein components according to the invention.

[0018] In a yet further aspect, the present invention provides a use of an extract or fraction enriched in protein components according to the invention in the manufacture of a medicament for treating an IL-6-mediated condition, an inflammatory condition or an autoimmune condition, for example rheumatoid arthritis.

[0019] The present invention further provides an anti-thrombotic or anti-coagulant agent comprising an effective amount of an extract or fraction enriched in anionic polysaccharide components according to the invention.

[0020] In a further aspect, the present invention provides a method of inhibiting thrombosis, or of inhibiting thrombin activity in a subject, the method comprising administering to the subject an effective amount of an extract or fraction enriched in anionic polysaccharide components according to the invention.

[0021] In another aspect, the present invention provides the use of an extract or fraction enriched in anionic polysaccharide components according to the invention in the manufacture of a medicament for inhibiting thrombosis or thrombin activity.

[0022] In yet another aspect, the present invention provides a process for preparing an extract or fraction thereof according to the invention, the method comprising digesting blacklip abalone processing waste using an enzymatic or chemical digestion process and isolating the

extract from the digested waste. The inventors have discovered that it may be advantageous to enrich, or increase the concentration of certain component or components, such as anionic polysaccharide components or protein components. Accordingly a process for preparing a fraction according to the invention may also comprise fractionating the extract, preferably to increase the concentration of anionic polysaccharide components or protein components.

Brief Description of the Drawings

[0023] Figure 1 shows graphically the *in vitro* anti-inflammatory activity in response to various abalone processing waste extracts. Anti-inflammatory activity indicated by a decrease in nitric oxide production by LPS stimulated mouse macrophages (RAW 264.7 cells). Data indicated by "*" display significant statistical differences ($p < 0.05$) to the assay positive control (quercetin) using a one-way ANOVA with Dunnett's multiple comparisons test.

[0024] Figure 2 shows graphically the *in vitro* thrombin inhibition activity in response to various abalone processing waste extracts. Thrombin inhibition activity indicated by a decrease in thrombin activity using the chromogenic substrate ChromozymTH. Data indicated by "*" display significant statistical differences ($p < 0.05$) to the assay positive control (0.0625 mg/mL heparin) using a one-way ANOVA with Dunnett's multiple comparisons test.

[0025] Figures 3A and 3B show graphically anion exchange separation of various abalone processing waste extracts. Abalone extracts 1-1 (Figure 3A) and 2-1 (Figure 3B) were separated into 30 fractions using anion exchange chromatography and a linear NaCl gradient. Total protein (black) and sulphated polysaccharides (grey) were estimated in the fractions.

[0026] Figure 4 shows graphically *in vitro* anti-inflammatory activity in response to anion exchange chromatography fractions obtained from various abalone processing waste extracts that were pooled. Anti-inflammatory activity indicated by a decrease in nitric oxide production by LPS stimulated mouse macrophages (RAW 264.7 cells). Data indicated by "*" display significant statistical differences ($p < 0.05$) to the assay positive control 10 μ M quercetin using a one-way ANOVA with Dunnett's multiple comparisons test. Data with ^ display significant statistical differences ($p < 0.05$) to the assay positive control 1 μ M quercetin using a one-way ANOVA with Dunnett's multiple comparisons test.

[0027] **Figure 5** shows graphically *in vitro* heparin cofactor II-mediated thrombin inhibition in response to pooled anion exchange chromatography fractions obtained from various abalone processing waste extracts. Thrombin inhibition indicated by a decrease in thrombin activity using the chromogenic substrate ChromozymTH. Data indicated by "*" display significant statistical differences ($p < 0.05$) to the assay positive control (0.0625 mg/mL heparin) using a one-way ANOVA with Dunnett's multiple comparisons test. Data indicated by "^" display significant statistical differences ($p < 0.05$) to the assay positive control (0.001 mg/mL heparin) using a one-way ANOVA with Dunnett's multiple comparisons test.

[0028] **Figures 6A and 6B** show graphically the clinical score in mice following collagen II induced arthritis (CIA). Mice were orally administered control feed; prophylactic/therapeutic abalone dose 1; prophylactic/therapeutic abalone dose 2 and therapeutic prednisolone. Prophylactic treatments are summarised in **Figure 6A**. Therapeutic treatments are summarised **Figure 6B**.

[0029] **Figure 7** shows graphically the individual clinical scores at Day 20 for each treatment group, including mean clinical scores (with data range).

[0030] **Figure 8** shows graphically the LSMEANS of clinical scores from onset of symptoms (Day 6) until Day 20 for CIA treatment groups. Statistical significance, as determined using the GLM Procedure of SAS to estimate separately the least squares means (LSMEANS) for CIA treatment (group) effect, is shown in comparison to control feed: **** ($p < 0.0001$); *** ($p < 0.001$) and * ($p < 0.05$).

[0031] **Figure 9** shows graphically the LSMEANS of Circulating IL-6 (cytokine) levels in blood serum collect on day 21. Statistical significance, as determined using the GLM Procedure of SAS to estimate separately the least squares means (LSMEANS) for treatment (group) effect, is shown in comparison to control feed + CIA: **** ($p < 0.0001$); ** ($p < 0.01$).

[0032] **Figure 10** shows graphically the serum collagen-II antibody levels in blood serum collected on day 21. Antibody levels expressed as a ratio to no CIA control: control diet; abalone dose 1; abalone dose 2 and prednisolone.

[0033] **Figure 11** shows graphically the LSMEANS total synovitis scores at Day 21 for CIA and non-CIA treatment groups. Statistical significance, as determined using the GLM Procedure of SAS to estimate separately the least squares means (LSMEANS) for treatment (group) effect, is shown in comparison to control feed + CIA: **** ($p < 0.0001$); ** ($p < 0.01$).

Detailed Description of the Preferred Embodiments

Definitions

[0034] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which the invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, preferred methods and materials are described. For the purposes of the present invention, the following terms are defined below.

[0035] The articles "a" and "an" are used herein to refer to one or to more than one (i.e., to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

[0036] As used herein, "and/or" refers to and encompasses any and all possible combinations of one or more of the associated listed items, as well as the lack of combinations when interpreted in the alternative (or).

[0037] As used herein, the term "about" refers to a quantity, level, concentration, value, size, or amount that varies by as much as 10% or even as much as 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, to a reference quantity, level, concentration, value, size, or amount.

[0038] The term "agent" is used herein to denote a chemical compound, a mixture of chemical compounds, a biological macromolecule (such as a nucleic acid, an antibody, a polysaccharide or oligosaccharide, a protein or portion thereof, e.g., a peptide), or an extract made from biological materials such as bacteria, plants, fungi, or animal (particularly mammalian) cells or tissues. The activity of such agents may render it suitable as a "therapeutic agent" which is a biologically, physiologically, or pharmacologically active substance (or substances) that acts locally or systemically in a subject.

[0039] As used herein, "autoimmune disease" refers to a disease or condition arising from an inappropriate immune response against the body's own components, such as tissues and other components. In some embodiments, IL-6 levels are elevated in autoimmune disease. Non-limiting exemplary autoimmune diseases that may be treated with the extracts described herein include rheumatoid arthritis, inflammatory bowel diseases (especially ulcerative colitis and Crohn's disease), systemic lupus erythematosus, systemic sclerosis, polymyositis,

vasculitis syndrome including giant cell arteritis, takayasu arteritis, cryoglobulinemia, myeloperoxidase-antineutrophilcytoplasmic, eczema, psoriasis, myasthenia gravis, antibody-associated crescentic glomerulonephritis, rheumatoid vasculitis, relapsing polychondritis, acquired hemophilia A, and autoimmune hemolytic anemia.

[0040] Throughout this specification, unless the context requires otherwise, the words "comprise," "comprises" and "comprising" will be understood to imply the inclusion of a stated step or element or group of steps or elements but not the exclusion of any other step or element or group of steps or elements.

[0041] The term "blacklip abalone processing waste" and the like when used herein refer to waste produced during the processing of blacklip abalone to produce blacklip abalone meat (muscle, foot) for consumption. Processing waste includes parts of the soft tissue of the abalone discarded during processing of the animal to produce abalone meat, including meat trimmings and internal organs as well as waste liquids generated during washing processes, cooking juices and undersized whole abalone rejected during grading. The processing waste may be in the form of, for example, a solid, powder, paste or liquid; it may be raw, treated or cooked; and may be fresh, or preserved for example by freezing, bagging, canning or drying.

[0042] The terms "biologically active components" or "bioactive molecules" and similar when used herein refers to components of the extract that have a physiological effect when administered to an animal or investigated *in vitro*.

[0043] As used herein, "cancer" means a disease or condition involving unregulated and abnormal cell growth. Non-limiting cancers that may be treated with the extracts described herein include multiple myeloma, leukemia, pancreatic cancer, breast cancer, colorectal cancer, cachexia, melanoma, cervical cancer, ovarian cancer, lymphoma, gastrointestinal, lung cancer, prostate cancer, renal cell carcinoma, metastatic kidney cancer, solid tumors, non-small cell lung carcinoma, non-Hodgkin's lymphoma, bladder cancer, oral cancer, myeloproliferative neoplasm, B-cell lymphoproliferative disease, and plasma cell leukemia. Non-limiting exemplary cancer-related conditions include non-small cell lung cancer-related fatigue and cancer related anorexia.

[0044] As used herein, the terms "enrich", "enriched", "enriching" and the like refer to the process of increasing the concentration of a component (e.g., a protein component) of a composition. For example, a protein component of a composition can be enriched by

chromatographic techniques including fractionation such that the protein component of the composition is increased about 1.5-fold, about 2-fold, about 3-fold, about 5-fold, about 10-fold, about 15-fold, about 30-fold, about 50-fold, or about 100-fold.

[0045] As used herein, an "IL-6 mediated disease or condition" refers to a disease or condition in which at least some of the symptoms and/or progression of the disease or condition is caused by IL-6-mediated signaling. Non-limiting exemplary IL-6 mediated diseases or conditions include inflammatory diseases, malignant diseases (including cancer and cancer-related conditions), infections, and autoimmune diseases. Further non-limiting exemplary IL-6 mediated diseases include, but are not limited to, Castleman's disease, ankylosing spondylitis, coronary heart disease, cardiovascular disease in rheumatoid arthritis, pulmonary arterial hypertension, chronic obstructive pulmonary disease (COPD), atopic dermatitis, psoriasis, eczema, sciatica, type II diabetes, obesity, giant cell arteritis, inflammatory bowel diseases (especially ulcerative colitis and Crohn's disease), acute graft-versus-host disease (GVHD), non-ST elevation myocardial infarction, anti-neutrophil cytoplasmic antibody (ANCA) associated vasculitis, neuromyelitis optica, chronic glomerulonephritis, Takayasu arteritis, graft versus host disease and transplant rejection (including in renal transplantation).

[0046] As used herein, "infection" refers to a disease or condition caused by a pathogen, such as a bacteria, virus, fungus, etc. Non-limiting exemplary infections that may be treated with the extracts described herein include human immunodeficiency virus (HIV), human T-lymphotropic virus (HTLV), cerebral malaria, urinary tract infections, and meningococcal infections.

[0047] As used herein, "inflammatory disease" refers to a disease or condition involving an inflammatory response. The inflammatory response may be acute and/or chronic. In some embodiments, chronic inflammation involves an increase in the level of IL-6. Non-limiting inflammatory diseases that may be treated with the extracts described herein include rheumatoid arthritis, juvenile idiopathic arthritis, systemic-onset juvenile idiopathic arthritis, osteoarthritis, sepsis, asthma, interstitial lung disease, inflammatory bowel disease, systemic sclerosis, intraocular inflammation, Graves disease, endometriosis, systemic sclerosis, adult-onset still disease, amyloid A amyloidosis, polymyalgia rheumatic, remitting seronegative symmetrical synovitis with pitting edema, Behcet's disease, uveitis, graft-versus-host diseases, and TNFR-associated periodic syndrome.

[0048] The term "protein components" when used herein includes polypeptides and oligopeptides. In one aspect protein components comprise collagen.

[0049] As used herein, the term "anionic polysaccharide" refers to negatively charged polysaccharides such as sulphated polysaccharides, and includes glycosaminoglycan polysaccharides (GAG) and glycosaminoglycan-like polysaccharides (GAG-like).

[0050] The term "nutraceutical" when used herein refers to an edible product derived from food sources. Nutraceuticals are considered to offer health benefits, and can, for example, form the basis of functional food, food ingredients, dietary supplements, drinks, and animal feeds.

[0051] As used herein, the term "anti-thrombotic agent" is an agent that reduces the formation of blood clots and can be used for the prevention and treatment of, for example, arterial and venous thrombosis. Examples of anti-thrombotic agents include anti-coagulant agents and antiplatelet agents. The anti-thrombotic effect may, for example, be mediated through heparin co-factor II or antithrombin (AT).

[0052] As used herein the term "anticoagulant agent" is an agent which acts to reduce blood clotting. Anticoagulant agents find application in the prevention and initial treatment of venous thromboembolism, including deep vein thrombosis (DVT) and pulmonary embolism (PE). Anti-coagulant agents can also be used in prophylaxis of ischaemic complications, in treatment of unstable angina, and treatment of non-Q-wave myocardial infarction. Anti-coagulant agents can be used in prophylaxis or treatment of DVT following, for example, joint replacement surgery or abdominal surgery. They can also be used to prevent or treat DVT which may occur due to restricted mobility as a result of injury or illness; or which may be a complication of a malignant disease such as a cancer. Anti-coagulant agents may also be used as a prophylactic in situations where there is a patient history of DVT or pulmonary embolism. Anti-coagulant agents can also be used to inhibit blood clotting in a patient undergoing surgical intervention, for example intravascular catheterisation procedures or cardiac surgery. Other therapeutic uses of anti-coagulants include treatment of atrial fibrillation, congestive heart failure, myocardial infarction and genetic or acquired hypercoagulability.

[0053] As used herein, the term "antiplatelet agent" or "antiaggregant agent" is an agent which acts to reduce platelet aggregation in the blood, and thus inhibits thrombus formation.

Antiplatelet agents are effective in the arterial circulation, where anti-coagulants may not be significantly effective. Platelet aggregation can lead to, for example, heart attack, angina or stroke. Antiplatelet agents can be used in prophylaxis or treatment of thrombotic cerebrovascular disease, such as stroke, mini-stroke, ischaemic stroke or haemorrhagic stroke; or cardiovascular disease such as hypertensive heart disease, rheumatic heart disease, cardiomyopathy, atrial fibrillation, congenital heart disease, endocarditis, aortic aneurysms, peripheral artery disease, venous thrombosis or coronary artery disease, e.g. angina or myocardial infarction.

[0054] As used herein, the term "thrombosis" refers to intravascular coagulation of the blood in the circulatory system, being the heart, arteries, veins and capillaries. The term "thrombin" refers to an enzyme which acts as a catalyst for several transformations in the blood coagulation cascade. It also promotes platelet activation and aggregation.

[0055] The terms "individual", "patient" and "subject" are used interchangeably herein to refer to individuals of human or other animal origin and includes any individual it is desired to examine or treat using the methods of the invention. However, it will be understood that these terms do not imply that symptoms are present. Suitable animals that fall within the scope of the invention include, but are not restricted to, humans, primates, livestock animals (e.g., sheep, cows, horses, donkeys, pigs), laboratory test animals (e.g., rabbits, mice, rats, guinea pigs, hamsters), companion animals (e.g., cats, dogs) and captive wild animals (e.g., foxes, deer, dingoes, birds, reptiles).

[0056] As used herein, "malignant disease" includes cancer and cancer-related conditions.

[0057] As used herein, the terms "treatment," "treating," and the like, refer to administering an agent to obtain a desired pharmacologic and/or physiologic effect. The effect may be prophylactic in terms of completely or partially preventing a disease or symptom thereof and/or may be therapeutic in terms of effecting a partial or complete cure for a disease and/or symptoms of the disease. The effect may be therapeutic in terms of a partial or complete cure for a disease or condition (e.g., a disease or condition mediated by IL-6) and/or adverse effect attributable to the disease or condition. These terms also cover any treatment of a condition or disease in a mammal, particularly in a human, and include: (a) preventing the disease or condition or a symptom of a disease or condition from occurring in a subject which may be predisposed to the disease or condition but has not yet been diagnosed as having it (e.g., including diseases or conditions that may be associated with or caused by a primary disease

or condition; (b) inhibiting the disease or condition, *i.e.*, arresting its development; (c) relieving the disease or condition, *i.e.*, causing regression of the disease or condition; (d) relieving a symptom of the disease or condition and/or (e) reducing the frequency of a symptom of the disease or condition.

[0058] By "pharmaceutically acceptable carrier, excipient or diluent" is meant a solid or liquid filler, diluent or encapsulating substance that can be safely used in systemic administration.

Extracts of Blacklip Abalone

[0059] The inventors have discovered that extracts obtained from blacklip abalone processing waste, or fractions thereof, comprise biologically active components that have anti-thrombotic or anti-inflammatory activity.

[0060] Extracts are prepared from waste streams obtained from the processing of blacklip abalone meat for consumption. The extracts are prepared from the processing waste using a process involving enzyme or chemical digestion, heat inactivation and clarification steps.

[0061] Accordingly, in a first aspect there is provided an extract of blacklip abalone, or fraction thereof, comprising one or more biologically active components, which extract is derived from blacklip abalone processing waste. In some embodiments the extract is derived from offal. In some embodiments the processing waste is raw. In some embodiments the processing waste is preserved. In some embodiments, the processing waste is in the form of a liquid. In some embodiments, the processing waste is in the form of a solid, for example a paste or a powder. In some embodiments, the processing waste may be canned, chilled or frozen. In some embodiments, the blacklip abalone is wild harvest abalone.

[0062] In some embodiments the one or more biologically active components are selected from anionic polysaccharide components and protein components. In some embodiments the protein components comprise collagen. In some embodiments, the anionic polysaccharide components comprise one or more sulphated polysaccharide components. In some embodiments, the anionic polysaccharide components comprise one or more glycosaminoglycan-like polysaccharides.

[0063] In another aspect, the present invention provides a process for preparing an extract according to the invention, the method comprising digesting blacklip abalone processing

waste using an enzymatic or chemical digestion process and isolating the extract from the digested waste. In some embodiments, the process comprises enzymatic digestion. Exemplary enzymes for enzymatic digestion include bromelain and/or papain. In some embodiments the processing waste samples are digested for about 16-18 hours. In some embodiments the digest volume contains about 10% w/v processing waste. In some embodiments the digest volume contains about 2% w/v of one or more enzymes, for example about 1% w/v bromelain and about 1% w/v papain. In some embodiments the digests are heat inactivated for about 10 minutes at about 95°C before cooling. In some embodiments the digests are fractionated to separate different fractions of the digest. In some embodiments the fractionation produces a fraction enriched in protein components. In some embodiments the fractionation produces a fraction enriched in anionic polysaccharide components.

[0064] The fractionation may be achieved using any suitable technique. Suitable techniques are well known to the skilled person, and illustrative examples include centrifugation, extraction, continuous liquid-liquid extraction, pervaporation, filtration including membrane filtration, extractive filtration and ultrafiltration membrane separation, reverse osmosis, electrodialysis, distillation, crystallization, ion exchange chromatography, size exclusion chromatography (gel filtration, gel permeation) and absorption chromatography.

[0065] In some embodiments, the process comprises the steps of: digesting blacklip abalone processing waste in the presence of a digesting enzyme to produce an aqueous fraction and a precipitate; and separating the aqueous fraction from the precipitate to thereby produce the extract. In some embodiments, a process of the invention further comprises filtering the aqueous fraction to produce a filtrate. In some embodiments, the filtrate is desalted. In some embodiments the filtrate is desalted using a 3 kDa filter to produce the extract as a 3 kDa retentate.

[0066] Blacklip abalone extracts can be separated into fractions using, for example, ion exchange chromatography and size exclusion chromatography. In some embodiments, the process further comprises separating the extract into one or more fractions enriched in protein components or anionic polysaccharide components, preferably using ion exchange chromatography and/or size exclusion chromatography. The composition of an extract or extract fraction of the invention with respect to amount of protein components and anionic polysaccharide components, is readily determined by methods known in the art, such as those described in the Examples herein.

[0067] Accordingly, in a further aspect, the present invention provides a blacklip abalone extract, or extract fraction, enriched in protein components. In some embodiments, the protein components comprise protein collagen.

[0068] In a further aspect, the present invention provides a blacklip abalone extract, or extract fraction, enriched in anionic polysaccharide components. In some embodiments, the anionic polysaccharide components comprise one or more sulphated polysaccharides. In some embodiments, the anionic polysaccharide components comprise one or more glycosaminoglycan-like polysaccharide components.

[0069] In another aspect, the present invention provides a blacklip abalone extract, or extract fraction, obtained or obtainable by a process of the invention.

Methods of the Invention

[0070] The extracts and extract fractions of the present invention comprise one or more biologically active components. In some embodiments the one or more biologically active components are selected from anti-thrombotic agents and anti-inflammatory agents. In some embodiments the anti-thrombotic agents comprise one or more anionic polysaccharide components. In some embodiments the anti-thrombotic agents comprise one or more glycosaminoglycan-like polysaccharide components. In some embodiments the one or more biologically active components are selected from anti-inflammatory agents. In some embodiments the anti-inflammatory agents comprise one or more protein components.

[0071] In accordance with the present invention, it has been found that extracts and fractions obtained from blacklip abalone processing waste produce significant and consistent anti-thrombotic and anti-inflammatory activity *in vitro*. Furthermore, these extracts and fractions have also been found to significantly reduce the symptoms of severe arthritis *in vivo* in a mouse model of rheumatoid arthritis.

[0072] Of particular note, mice fed powdered abalone extracts prior to and during the onset of rheumatoid arthritis, experienced less severe symptoms than mice fed a basal diet.

[0073] The present inventors have also found that, on a clinical, histological and immunological basis, blacklip abalone processing waste extract according to the present invention produced positive and significant results in a mouse model of rheumatoid arthritis. On the basis of clinical outcomes, from the onset of arthritis symptoms until the end of the

clinical trial, prophylactic administration of abalone extract significantly decreased toe, paw and carpus swelling and deformity (Figures 7 and 8). On a histological basis, prophylactic and therapeutic administration of abalone extract significantly reduced total synovitis observed in joints and paws (Figure 11). Finally, on an immunological basis, prophylactic administration of abalone extract and therapeutic administration of abalone extract, significantly decreased the circulating levels of IL-6 (Figure 9), a cytokine that plays a key role in chronic inflammation and rheumatoid arthritis (Gabay C, Interleukin-6 and chronic inflammation, *Arthritis Res Ther.*, 2006;8 Suppl 2:S3; Hennigan S *et al*, Interleukin-6 inhibitors in the treatment of rheumatoid arthritis. *Ther Clin Risk Manag*, 2008 Aug;4(4):767-75), indicating significant potential of blacklip abalone extract in the treatment of inflammatory disorders such as rheumatoid arthritis.

[0074] The extracts and fractions of the present invention are therefore considered to be useful in the treatment or prevention of IL-6 mediated diseases or conditions as described for example above. In some embodiments the fraction is a fraction enriched in protein components. In some embodiments, the IL-6 mediated disease or condition is an autoimmune disease or condition such as but not limited to rheumatoid arthritis, inflammatory bowel diseases (especially ulcerative colitis and Crohn's disease), eczema, psoriasis and myasthenia gravis, or transplant rejection (including in renal transplantation).

[0075] In some embodiments, the extracts and fractions of the invention are useful in treating or preventing rheumatoid arthritis.

[0076] Accordingly, the present invention provides a method of treating an IL-6 mediated condition in a subject, the method comprising administering to the subject an effective amount of the extract according to the invention, preferably a fraction enriched in protein components.

[0077] The present invention further provides a method of treating an inflammatory condition in a subject, the method comprising administering to the subject an effective amount of an extract according to the invention, preferably a fraction enriched in protein components. In one embodiment, the inflammatory condition is an autoimmune condition.

[0078] There is also provided a method of treating an autoimmune condition in a subject, the method comprising administering to the subject an effective amount of an extract according to the invention, preferably a fraction enriched in protein components.

[0079] In a yet further aspect, the present invention provides a use of an extract of the invention, preferably a fraction enriched in protein components, in the manufacture of a medicament for treating an IL-6-mediated condition, an inflammatory condition, or an autoimmune condition.

[0080] In some embodiments, the condition is rheumatoid arthritis.

[0081] Further investigations into *in vivo* anti-inflammatory activity using a mouse model of rheumatoid arthritis was used to assess the prophylactic and therapeutic administration of low and high doses of abalone extract. Oral administration significantly decreased symptoms of arthritis including reduced swelling and deformities whilst maintaining animal weight.

[0082] Accordingly, the present invention also provides for use of an extract or fraction of the invention as a medicament. In some embodiments, the medicament is formulated for oral administration. There is also provided the use of an extract of the invention in the manufacture of a dietary supplement, nutraceutical, functional food or functional food ingredient.

[0083] The present inventors also discovered that extracts and fractions from blacklip abalone processing waste produced significant and consistent anti-thrombotic activity *in vitro*. The extracts or fractions of the present invention are considered to have anti-thrombotic or anti-coagulant properties, and are therefore considered to be useful in treatment or prevention of thrombosis, or of inhibiting thrombin activity.

[0084] Accordingly, the present invention further provides an anti-thrombotic or anti-coagulant agent comprising an effective amount of an extract of the invention, preferably a fraction enriched in anionic polysaccharide components. In some embodiments, the anti-thrombotic effect is mediated through heparin cofactor II or antithrombin.

[0085] In another aspect, the present invention provides a method of inhibiting thrombosis, or of inhibiting thrombin activity in a subject, the method comprising administering to the subject an effective amount of an extract according to the invention, preferably a fraction enriched in anionic polysaccharide components.

[0086] In another aspect, the present invention provides the use of an extract according to the invention, preferably a fraction enriched in anionic polysaccharide components, in the manufacture of a medicament for inhibiting thrombosis or thrombin activity.

[0087] The subjects, individuals or patients to be treated are mammalian subjects including but not limited to humans, primates, livestock animals such as sheep, cattle, pigs, horses, donkeys and goats; laboratory test animals such as mice, rats, rabbits and guinea pigs; companion animals such as cats and dogs or captive wild animals such as those kept in zoos. In a particular embodiment, the subject is a human.

[0088] An "effective amount" means an amount necessary at least partly to attain the desired response, or to delay the onset or inhibit progression or halt altogether, the onset or progression of a particular condition being treated. The amount varies depending upon the health and physical condition of the individual to be treated, the taxonomic group of individual to be treated, the degree of protection desired, the formulation of the composition, the assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials. An effective amount in relation to a human patient, for example, may lie in the range of about 0.1 ng per kg of body weight to 1 g per kg of body weight per dosage. The dosage is preferably in the range of 1 µg to 1 g per kg of body weight per dosage, such as is in the range of 1 mg to 1 g per kg of body weight per dosage. In one embodiment, the dosage is in the range of 1 mg to 500 mg per kg of body weight per dosage. In another embodiment, the dosage is in the range of 1 mg to 250 mg per kg of body weight per dosage. In yet another embodiment, the dosage is in the range of 1 mg to 100 mg per kg of body weight per dosage, such as up to 50 mg per kg of body weight per dosage. In yet another embodiment, the dosage is in the range of 1 µg to 1 mg per kg of body weight per dosage. Dosage regimes may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily, weekly, monthly or other suitable time intervals, or the dose may be proportionally reduced according to the situation.

[0089] Reference herein to "treatment" and "prophylaxis" is to be considered in its broadest context. The term "treatment" does not necessarily imply that a subject is treated until total recovery. "Treatment" may also reduce the severity of an existing condition. The term "prophylaxis" does not necessarily mean that the subject will not eventually contract a disease condition. The term "prophylaxis" may be considered to include delaying the onset of a particular condition. Accordingly, treatment and prophylaxis include amelioration of the symptoms of a particular condition or preventing or otherwise reducing the risk of developing a particular condition.

[0090] In some embodiments, an extract of the invention may be administered together with another therapy. Administration may be in a single composition or in separate compositions simultaneously or sequentially such that each compound or therapy is active within the same time period in the body.

[0091] An extract of the invention may be administered with one or more further nutraceutical products such as, for example, Omega-3 oils, glucosamine or chondroitin sulphate. An extract, particularly an extract fraction enriched in protein components, may also be administered with anti-inflammatory agents including steroids, such as cortisone, hydrocortisone, prednisolone, methylprednisolone, prednisone, budesonide, mometasone, triamcinolone and aclometasone, and non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin, diflunisal, salsalate, ibuprofen, naproxen, fenoprofen, ketoprofen, flubiprofen, oxaprozin, loxoprofen, indomethacin, sulindac, etodolac, ketorolac, diclofenac, nabumetone, piroxicam, meloxicam, tenoxicam, droxicam, lornoxicam, metenamic acid, meclofenamic acid, flufenamic acid, tolfenamic acid, celecoxib, rofecoxib, valdecoxib, parecoxib, etoricoxib and ferocoxib.

[0092] The extracts and fractions of the invention are considered to be beneficial to health and/or well being, accordingly there is also provided a method for enhancing or maintaining the well being of a subject, the method comprising administering to the subject an effective amount of an extract or fraction of the invention.

Compositions of the Invention

[0093] The extract or fraction of the invention may be taken alone as a neat extract. However, in some circumstances it is preferable to formulate the extract for consumption. For example, the extract may be formulated as a dietary supplement, nutraceutical, functional food or functional food ingredient.

[0094] In a further aspect, the present invention also provides a medicament, dietary supplement, nutraceutical, functional food or functional food ingredient comprising an extract or fraction according to the invention.

[0095] The extract or fraction of the invention may be formulated as a food or drink product. Animal food is also contemplated as part of the invention.

[0096] In some embodiments, the dietary supplement, nutraceutical, functional food or functional food ingredient further comprises one or more acceptable excipients, fillers or carriers. In some embodiments, the extract or fraction of the invention may be used in combination with one or more further active ingredients selected from, for example, nutraceuticals, functional foods or functional food ingredients. The content ratio of active ingredients may be appropriately determined depending on the purpose of the use. The dietary supplement, nutraceutical, functional food or functional food ingredient may be appropriately used according to conventional methods. Generally, when producing a solid or liquid food, an extract of the present invention may be added at up to 50% w/w, and more preferably up to 30%, 20% or 10% w/w. Generally, when producing a drink, an extract of the present invention may be added at up to 20% w/w, and more preferably up to 15%, 10% or 5% w/v.

[0097] Suitable excipients, fillers or carriers are known in the art and include starches, sugars, edible oils, water, glycerine, gums, methyl cellulose and the like.

[0098] Dietary supplement, nutraceutical, functional food or functional food ingredient compositions of the invention may also comprise additional components such as flavouring agents, colourings, sweetening agents, thickeners, pH modifiers, stabilizers, or preservatives.

[0099] Dietary supplement, nutraceutical, functional food or functional food ingredient compositions of the present invention may further comprise additional active ingredients such as nutrients, vitamins, or electrolytes.

[00100] Nutraceutical, functional food or food ingredient compositions may be formulated, for example, as powders, granules, liquids, pastes, oils or gels.

[00101] Dietary supplements may be formulated, for example, as pills, tablets, capsules, softgels, gelcaps, pastes or powders. Dietary supplements may also be formulated as liquids, such as solutions or suspensions.

[00102] The required daily dose of an extract or fraction according to the present invention can be determined according to the specific needs of the individual. A typical daily intake is considered to be from 100 mg to 500 mg/kg body weight/day. In one aspect, the daily intake of a fraction is less than 100 mg/kg body weight/day

[00103] In some embodiments, the extract or fraction of the invention may be incorporated into conventional foodstuffs. Accordingly, the present invention further provides a foodstuff comprising an extract of the invention.

[00104] In another aspect of the invention there is provided a pharmaceutical composition comprising an extract or fraction according to the invention, and one or more pharmaceutically acceptable carriers or excipients. In some embodiments the composition is for use in treating or preventing an inflammatory disease or condition. In some embodiments the composition is for use in treating an autoimmune disease or condition such as rheumatoid arthritis. In some embodiments the fraction is enriched in protein components. In some embodiments the fraction is enriched in anionic polysaccharide components.

[00105] While it is possible that, for use in therapy, an extract or fraction of the invention may be administered alone, in some embodiments the active extract or fraction is provided in the form of a pharmaceutical composition.

[00106] Thus, in a further aspect of the invention, there is provided a pharmaceutical composition comprising an extract or fraction of the invention and at least one pharmaceutically acceptable carrier, excipient or diluent.

[00107] The pharmaceutical compositions of the present invention or the compositions used in the methods of the present invention may be formulated and administered using methods known in the art. Techniques for formulation and administration may be found in *Remington: The Science and Practice of Pharmacy*, Loyd V. Allen, Jr (Ed), The Pharmaceutical Press, London, 22nd Edition, September 2012.

[00108] The carrier(s) must be "acceptable" in the sense of being compatible with the other ingredients of the composition and not deleterious to the recipient thereof.

[00109] Pharmaceutical compositions or formulations include those suitable for oral, rectal, nasal, topical (including buccal and sub-lingual), vaginal or parenteral (including intramuscular, subcutaneous and intravenous) administration or in a form suitable for administration by inhalation or insufflation. The compounds of the invention, together with a conventional adjuvant, carrier, excipient, or diluent, may thus be placed into the form of pharmaceutical compositions and unit dosages thereof, and in such form may be employed as solids, such as tablets or filled capsules, or liquids such as solutions, suspensions, emulsions, elixirs, or capsules filled with the same, all for oral use, in the form of suppositories for rectal

administration; or in the form of sterile injectable solutions for parenteral (including subcutaneous) use. Such pharmaceutical compositions and unit dosage forms thereof may comprise conventional ingredients in conventional proportions, with or without additional active compounds or principles, and such unit dosage forms may contain any suitable effective amount of the active ingredient commensurate with the intended daily dosage range to be employed. Formulations containing 10 milligrams of active ingredient or, more broadly, 0.1 to 200 milligrams, per tablet, are accordingly suitable representative unit dosage forms. The compounds of the present invention can be administered in a wide variety of oral and parenteral dosage forms. It will be routine to those skilled in the art that the following dosage forms may comprise, as the active component, an extract of the invention.

[00110] For preparing pharmaceutical compositions from the extract or fraction of the present invention, pharmaceutically acceptable carriers can be either solid or liquid. Solid form preparations include powders, tablets, pills, capsules, cachets, suppositories, and dispersible granules. A solid carrier can be one or more substances which may also act as diluents, flavouring agents, solubilizers, lubricants, suspending agents, binders, preservatives, tablet disintegrating agents, or an encapsulating material.

[00111] In a preferred aspect, the extract or fraction is formulated for oral administration. Suitably the extract or fraction is formulated as a powder, tablet, pill, capsule, or dispersible granules.

[00112] In tablets, the active component is mixed with the carrier having the necessary binding capacity in suitable proportions and compacted in the shape and size desired.

[00113] The powders and tablets preferably contain from 5-70% percent of the active compound. Suitable carriers are magnesium carbonate, magnesium stearate, talc, sugar, lactose, pectin, dextrin, starch, gelatin, tragacanth, methylcellulose, sodium carboxymethylcellulose, a low melting wax, cocoa butter, and the like. The term "preparation" is intended to include the formulation of the active compound with encapsulating material as carrier providing a capsule in which the active component, with or without carriers, is surrounded by a carrier, which is thus in association with it.

[00114] Liquid form preparations for oral administration include solutions, suspensions, and emulsions, for example, water or water-propylene glycol solutions. Aqueous solutions suitable for oral use can be prepared by dissolving the active component in water and adding

suitable colorants, flavours, stabilizing and thickening agents, as desired. Aqueous suspensions suitable for oral use can be made by dispersing the finely divided active component in water with viscous material, such as natural or synthetic gums, resins, methylcellulose, sodium carboxymethylcellulose, or other well known suspending agents.

[00115] Also included are solid form preparations which are intended to be converted, shortly before use, to liquid form preparations for oral administration. Such liquid forms include solutions, suspensions, and emulsions. These preparations may contain, in addition to the active component, colorants, flavors, stabilizers, buffers, artificial and natural sweeteners, dispersants, thickeners, solubilizing agents, and the like.

[00116] Alternatively the active ingredients may be provided in the form of a dry powder, for example a powder mix of the compound in a suitable powder base such as lactose, starch, starch derivatives such as hydroxypropylmethyl cellulose and polyvinylpyrrolidone (PVP).

[00117] When desired, formulations adapted to give sustained release of the active ingredient may be employed.

[00118] The pharmaceutical preparations are preferably in unit dosage forms. In such form, the preparation is subdivided into unit doses containing appropriate quantities of the active component. The unit dosage form can be a packaged preparation, the package containing discrete quantities of preparation, such as blister pack tablets, capsules, and powders in vials or ampoules. Also, the unit dosage form can be a capsule, tablet, cachet, or lozenge itself, or it can be the appropriate number of any of these in packaged form.

[00119] Persons skilled in the art will appreciate that numerous variations and modifications will become apparent. All such variations and modifications which become apparent to persons skilled in the art should be considered to fall within the spirit and scope that the invention broadly appearing before described.

[00120] In order that the invention may be readily understood and put into practical effect, particular preferred embodiments will now be described by way of the following non-limiting examples.

EXAMPLES

[00121] The following blacklip abalone processing waste samples were used:

Sample 1: liquid; processed and bagged abalone offal; wild harvest abalone

Sample 2: Solid (paste); processed and canned abalone offal; wild harvest abalone

Sample 3: Solid (powder); processed abalone offal; wild harvest abalone

Sample 4: Solid; raw (fresh) abalone offal; wild harvest abalone

[00122] All of the samples, except the powdered sample, were either shipped fresh at 4°C, or frozen and transported on dry ice before being stored at -20°C until required. The powdered samples were stored at room temperature (~21°C).

Extract Preparation

[00123] Extracts were prepared from the processing waste using a method comprising digestion, centrifugation, filtration and desalting. All processing waste samples were digested overnight (16 - 18 hours) at 50°C using two food grade enzymes, bromelain and papain (commercially available from, for example, Enzyme Solutions Pty Ltd, Vic 3136). All solid waste samples were cut into small pieces (1 - 2 cm²). Digests volumes ranged from 50 - 1000 mL (in water) and contained 10 - 20 % w/v processing waste (5 - 200 g) and 0.5 - 1% w/v addition of each enzyme (0.25 - 10.0 g) (Osborne SA *et al*, Glycobiology. 2008 Mar; 18(3):225-34). Following the overnight incubation, digests were heat inactivated at 95°C for 10 - 20 minutes and then cooled on ice before being centrifuged at 5,940 - 50,000 g for 10 - 30 minutes to remove any fat (top) layer and undigested sediment (pellet). The aqueous layer (supernatant) was retained and sequentially filtered using 2 µm and 1 µm (Whatman glass microfiber) pre-filters followed by a final filtration with a 0.45 µm (Millipore mixed cellulose ester) filter. Filtered samples were then desalted using a 3 kDa (Amicon Ultra-15, Ultracel low binding regenerated cellulose membrane) centrifugal filter device producing retentate (>3 kDa and containing low/no salt) and filtrate (<3 kDa and containing salt) extract samples. The retentate samples were selected for further analysis due to the possible interference of salt in quantitative and bioactivity assays.

[00124] Throughout this specification the term 'extract' will refer to the 3 kDa retentate samples, unless otherwise specified.

Total Protein Estimation

[00125] Total protein content was estimated in the extracts using the Pierce BCA (bicinchoninic acid) Protein Assay Kit (Thermo Fisher Scientific, VIC, 3179, Australia) according to manufacturer's instructions. Briefly, 25 µl of standards (bovine serum albumin) and extracts were diluted in water and added in triplicate to a 96 well plate (Nunc®, polystyrene). 200 µl of BCA working reagent was prepared (by mixing Reagent A with Reagent B using 50:1 ratio) and added to each well. The plate was mixed and incubated at 37°C for 30 minutes before the plate absorbance was read at 562 nm. A linear standard curve was constructed and used to interpolate the total protein concentration in the diluted extracts.

Total Collagen Estimation

[00126] Total collagen content was estimated in the extracts using the QuickZyme Biosciences Total Collagen Assay Kit (Bio-Scientific, Kirrawee, NSW, Australia) according to manufacturer's instructions. Briefly, 125 µl standards (rat tail collagen) and extracts were added to 125 µl 12 M HCl and hydrolysed for 20 hours at 95°C in screw capped tubes. Following the hydrolysis, 35 µl hydrolysed samples and standards were transferred to the provided 96 well plate, mixed with 75 µl assay buffer and incubated at room temperature (~21°C) for 20 minutes. 75 µl detection reagent was then added to each well and the resulting solution mixed and incubated at 60°C for 30 minutes. The plate absorbance was read at 570 nm. A linear standard curve was constructed and used to interpolate the total collagen concentration in the extracts. All assays were performed in either duplicate or triplicate.

Sulphated Polysaccharide Estimation

[00127] Sulphated polysaccharide content was estimated using two different assays.

[00128] A basic 1,9-dimethylmethylene blue assay (DMMB) was used initially to estimate the sulphated polysaccharide contents and determine the dilutions needed to more accurately measure the sulphated polysaccharide content using the Blyscan Sulphated Glycosaminoglycan Assay (Labtek Pty Ltd, QLD 4500, Australia).

[00129] The DMMB dye is a metachromatic dye that detects sulphated glycosaminoglycans (Osborne SA *et al*, Glycobiology. 2008 Mar; 18(3):225-34) and many other types of sulphated and anionic polysaccharides (Cinelli LP *et al*,. Comp Biochem Physiol B Biochem

Mol Biol. 2009 Sep;154(1):108-12; Keler T *et al*, Anal Biochem. 1986 Jul; 156(1):189-93. 19-20).

[00130] The Blyscan Sulphated Glycosaminoglycan Assay is also based on the DMMB dye. Sulphated polysaccharide concentration was estimated in the extracts according to manufacturer's instructions. Briefly, 25 µl standard (chondroitin sulphate from bovine cartilage) and samples were added in triplicate to a V bottom polypropylene plate (PerkinElmer, Melbourne 3150, Australia). 250 µl Blyscan dye reagent was added to each well. The plate was then placed on an orbital shaker for 30 minutes at room temperature before being centrifuged at 2090 g for 10 minutes. The supernatant (liquid) was removed and replaced with 250 µl Blyscan dye dissociation reagent. The plate was mixed on an orbital shaker for 30 minutes before 200 µl of each standard and sample was transferred to a polystyrene 96 well plate (Nunc®) for absorbance readings at 656 nm. A linear standard curve was constructed and used to interpolate the sulphated polysaccharide concentration in the extracts.

***In Vitro* Anti-Inflammatory Activity**

[00131] Bioactivity, in terms of anti-inflammatory activity, was indicated using an *in vitro* cell based assay. Bacterial lipopolysaccharide (LPS) was used to induce an inflammatory cellular state in the murine macrophage cell line, RAW 264.7 (Sosroseno W *et al*, Oral Microbiol Immunol. 2002 Apr;17(2):72-8). Inflammation was indicated by the cellular production of nitric oxide (NO) as measured using the Griess assay. A decrease in the production of NO by a positive control, or by the abalone processing waste extracts, indicated anti-inflammatory activity.

[00132] Anti-inflammatory activity was measured in response to the quercetin positive control (Sigma Aldrich Corporation) and extracts as previously described (Cho JY *et al*, Eur J Pharmacol. 2000 Jun 23;398(3):399-407; Kim AR *et al*, Arch Pharm Res. 2005 Mar;28(3):297-304), with some minor modifications. The RAW 264.7 cells (American Type Culture Collection, www.ATCC.com) were routinely cultured in RPMI-1640 media (Thermo Fisher Scientific, Vic 3179, Australia) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin (Life Technologies, Thermo Fisher Scientific, Vic 3179, Australia) and 10% v/v fetal bovine serum (*in vitro*) and grown at 37°C and 5% CO₂ in humidified air. The anti-inflammatory assay was performed over two days. On day one the cells were seeded into 96 well plates at a density of 6x10⁴ cells/well. 300 ng/mL LPS in culture media was added to the

cells with either the positive control (six point dose response of quercetin using a 0 – 100 μ M concentration range) or extracts. The cells were cultured for 48 hours and on day two, NO secreted by the RAW 264.7 cells was measured in the cell media by the Griess reaction (50 μ L of cell media was added to 50 μ L Griess reagent (Sigma Aldrich Corporation). A standard curve was also prepared with sodium nitrite (0 – 50 μ M) and used to interpolate the NO production in the cell media. All assays were performed in triplicate (n=3). Cell viability was also measured in response to the LPS, quercetin and extracts using the MTS CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega Corporation, VIC 3122, Australia) according to the manufacturer's instructions.

***In Vitro* Thrombin Inhibition**

[00133] Bioactivity, in terms of thrombin inhibition was measured using an *in vitro* kinetic assay with the chromogenic substrate, Chromozym TH (CH-TH) (Dupouy D *et al*, Thromb Haemost. 1988 Oct 31;60(2):236-9). Thrombin is a serine protease that is involved in blood clotting by converting soluble fibrinogen into insoluble strands of fibrin. CH-TH is cleaved by thrombin producing a yellow coloured compound (called p-nitroaniline). Thrombin can be inhibited by heparin cofactor II (HCII) and antithrombin (AT) through a variety of sulphated polysaccharides. A decrease in the production of p-nitroaniline indicates anti-thrombotic activity, that may also lead to anti-clotting activities

[00134] Heparin cofactor II (HCII)-mediated thrombin inhibition was measured in a 96 well plate (polystyrene kinetic assay as previously described (Dupouy D *et al*, Thromb Haemost. 1988 Oct 31;60(2):236-9), with modifications (Osborne SA *et al*, Glycobiology. 2008 Mar;18(3):225-34). Briefly, HCII, assay buffer (0.02 M Tris-HCl pH 7.4/0.15 M NaCl/1 mg/mL PEG), and either extracts or commercial standards (porcine heparin from Sigma Aldrich Corporation) were mixed and incubated at room temperature for 2 min before 150 nM thrombin was added. This solution was mixed gently for 1 min prior to the addition of 100 μ M CH-TH. The assay was incubated at 37°C for 40 min with the absorbance measured at 405 nm (SpectraMax PLUS 384 microplate reader, Molecular Devices) at 2 minute intervals. The HCII-mediated thrombin inhibition by the extracts or heparin standard was expressed as the percentage decrease in thrombin activity after 40 minutes. Each sample was assayed once in triplicate (n=3) and expressed as the mean the standard error.

[00135] Antithrombin (AT)-mediated thrombin inhibition was measured in the extracts and fractions using a 384 well-format, robot-assisted assay. In this kinetic assay, thrombin

cleaves Chromozym TH, producing two molecules: a residual peptide and 4-nitraniline that can be measured by absorbance change at 405 nm. The assay standard heparin (final concentration range: 0.016 – 1.04 µg/mL) or molecules present within the samples bind to ATIII (0.2 µg/mL) forming a ternary complex with thrombin (2.03 ng/mL) preventing it from cleaving chromozym TH and forming 4-nitraniline. A heparin standard was diluted and with final concentrations 0.016 – 1.04 µg/mL. The ATIII-mediated thrombin inhibition by the extracts or heparin standard was expressed as the percentage decrease in thrombin activity after 40 minutes. Each sample was assayed once in triplicate (n=3) and expressed as the mean the standard error.

***In Vitro* Plasma and Blood Clotting Assays**

Prothrombin time (PT) assay

[00136] Prothrombin time (PT) was measured by adding 100 µL of citrated plasma to a glass clotting tube and incubating at 37°C on the heating block of a Hyland-Clotek clotting machine (Hyland, USA) for 5 minutes. The machine measures time in seconds until clot formation. 50 µL of different concentrations of abalone extracts were added to the tube, using saline as a blank. The total volume was adjusted to 150 µL with plasma before adding 100 µL of Thromborel S® to initiate clotting (Siemens, 545477, USA).

Activated Partial Thromboplastin Time (aPTT) Assay

[00137] Activated partial thromboplastin time (aPTT) was determined by adding 100 µL of citrated plasma, 100 µL Triniclot (TriniCLOT aPTT, HS, Tcoag, Ireland Limited) and different concentrations of abalone extracts to a clotting tube. The final volume was adjusted to 250 µL by adding saline. The clotting tube was incubated at 37°C in the heating block of a Hyland-Clotek clotting machine for 5 minutes before 50 µL 50 mM Ca²⁺ was added to initiate clotting.

Ion Exchange Chromatography

[00138] To separate the biologically active molecules and prepare fractions enriched with collagen or anionic polysaccharides, anion exchange chromatography was used to separate the biologically active molecules on the basis of charge.

[00139] Anionic separations were performed on abalone processing waste extracts using columns packed with Q Sepharose Fast Flow or Q Sepharose Big Beads (GE Healthcare,

Little Chalfont, UK). Extracts were applied to the column in Buffer A (water) and eluted in Buffer B (2M NaCl) using a linear gradient from 0-100% Buffer B. The sample application and flow through following a wash in Buffer A (i.e. unbound molecules) were collected along with the gradient fractions.

Mouse Model of Rheumatoid Arthritis

[00140] Bioactivity, in terms of *in vivo* anti-inflammatory activity, was investigated using an established and validated animal model of rheumatoid arthritis, namely a collagen type II-induced arthritis (CIA) model in mice (van Duivenvoorde LM *et al*, Arthritis. Rheum. 2004 Oct;50(10):3354-64; Leung BP *et al*, J. Immunol. 2004 Jul 1;173(1):145-50; van Holten J *et al*, Arthritis Res. Ther. 2004;6(3):R239-49). Two doses of abalone extract, 100 mg and 500 mg/kg body weight/day (dose 1 and 2 respectively) were incorporated into pelleted feed and administered 21 days prior to and during collagen II induced arthritis (CIA) (prophylactic dose), or after the induction and onset of CIA symptoms (therapeutic dose). Additionally, both doses of the abalone extract were administered to mice without CIA to monitor any adverse effects of the abalone extract.

[00141] Specifically, 108 male DBA-1 mice (10 – 12 week old) were weighed and divided into 9 groups (n=12 mice/group). The trial was performed in two batches of 54 mice (n=6 mice/group). Induction of CIA was achieved by anesthetising the animals with isoflurane before intradermally injecting chicken collagen type II in Freund's complete adjuvant (Sigma Aldrich Company) at two sites approximately 1 cm from the tail base. Animals were then monitored every 3 days and weighed weekly before a booster immunization was given 21 days after the first injection (at two sites approximately 2 cm from the tail base). Following the boost immunization, the animals were monitored, weighed and arthritis symptoms scored every 3 days for a total of 3 weeks. An anti-inflammatory drug, prednisolone, was also included in the trial as a positive treatment control.

[00142] The groups received the following treatments:

1. Control feed; no CIA
2. Dose 1; no CIA
3. Dose 2; no CIA
4. Control feed + CIA
5. Dose 1 (prophylactic) + CIA

6. Dose 2 (prophylactic) + CIA
7. Dose 1 (therapeutic) + CIA
8. Dose 2 (therapeutic) + CIA
9. Prednisolone (therapeutic + CIA)

[00143] Trial Time Line

-29 days	Prophylactic Feed start
-21 days	Initial immunization
0 days	Boost immunization
6 days	Onset of symptoms and therapeutic feed start
21 days	Termination

Clinical scores were determined using a standard arthritis scoring system as outlined below:

Score 0: No arthritis

Score 1: 1-2 toes affected only

Score 2: 3 or more toes and/or swelling of the paw (metacarpus/metatarsus)

Score 3: swelling of the carpus/tarsus

Score 4: deformity with ankylosis of the carpus/tarsus.

[00144] On termination day, blood samples were collected by cardiac puncture under anesthesia prior to euthanasia. Blood samples were allowed to clot at room temperature for 1 hour, spun and serum collected. Anti-collagen II antibody and cytokines were determined via ELISA. Also on termination day, one knee joint and rear paw were harvested from each animal, fixed in 10% neutral buffered formalin, decalcified in 10% formic acid in 5% formalin and paraffin embedded. Sagittal sections of the knee were stained with haematoxylin and eosin, as well as toluidine blue and fast green. Histology sections were scored using a standard histopathological grading system.

Statistical Analyses

[00145] Unless otherwise specified, all statistical analyses were conducted using a one-way ANOVA followed by post hoc comparisons using Tukey's or Dunnett's multiple comparisons test. P-values less than 0.05 were considered significant. These calculations were performed using GraphPad Prism 6 Software for Windows (GraphPad, San Diego California USA, www.graphpad.com).

Estimates of Total Protein, Collagen and Sulphated Polysaccharide Contents

[00146] Estimates of total protein, collagen and sulphated polysaccharide contents in abalone processing waste extracts prepared are detailed in Table 1. In total, four different types of abalone processing waste were investigated.

***In Vitro* Anti-Inflammatory Activity**

[00147] Anti-inflammatory activity, measured in response to the abalone processing waste extracts, a commercial abalone extract and an assay positive control (quercetin), is presented in Table 2. Unless specified, cell viability remained above 80% (or below 20% cell death or toxicity) following application of the abalone extracts or other samples. Given that a 70% decrease in nitric oxide production was the maximum effect of the positive control, results comparable to this decrease were considered positive. All abalone extracts and the commercial abalone extract produced positive results decreasing the production of nitric oxide in the RAW 264.7 cells by 40 – 80%. In particular, sample 2-2 displayed the greatest anti-inflammatory activity by significantly decreasing nitric oxide production more than the assay positive (quercetin) control (Figure 1).

***In Vitro* Thrombin Inhibition**

[00148] Heparin cofactor II-mediated thrombin inhibition, measured in response to the abalone processing waste extracts and an assay positive control (heparin), is presented in Table 3. In these assays, the abalone extracts were assayed without dilution to observe maximum anti-thrombotic potential.

[00149] All abalone extracts except sample 3-1 displayed thrombin inhibition comparable to the assay positive control (heparin), (Figure 2).

[00150] Antithrombin and heparin cofactor II-mediated thrombin inhibition, measured in response to the abalone processing waste extracts and an assay positive control (heparin), is presented in Table 4. In these assays, the abalone extracts were assayed with several dilutions to observe the full anti-thrombotic potential.

Table 1: Estimated total protein, collagen and sulphated polysaccharide contents in abalone processing waste samples

Sample number	Sample description	Batch number	Yield of bioactive molecules in processing waste expressed in grams per kilogram of waste (g/kg)		
			Total protein	Total collagen	Sulphated polysaccharides
1-1	Processed abalone offal - liquid	Batch 1	17.1±0.5	0.7±0.03	4.0±0.3
1-2	Processed abalone offal - liquid	Batch 2	14.2±0.4	0.6±0.03	3.6±0.3
2-1	Processed abalone offal - canned	Batch 1	10.3±0.1	0.5±0.03	2.1±0.12
2-2	Processed abalone offal - canned	Batch 2	15.1±0.6	0.6±0.03	7.0±0.3
3-1	Processed abalone offal - powder	Batch 1	9.7±0.2	0.0±0.0	1.1±0.05
4-1	Fresh abalone offal	Batch 1	54±1.4	1.0±0.5	1.6±0.02
4-2	Fresh abalone offal	Batch 2	60.4±0.5	5.25±1.0	3.0±0.1

Table 2: *In vitro* anti-inflammatory activity from extracts prepared from abalone processing

Sample number	Sample concentration ($\mu\text{g/mL}$ collagen)	Anti-inflammatory activity (% decrease in nitric oxide production)
1-1	58.0	62.5 $\pm$ 1.4
	17.4	55.6 $\pm$ 1.8
	5.8	47.8 $\pm$ 1.0
	1.7	40.6 $\pm$ 2.0
1-2	47.0	55.2 $\pm$ 1.0
	14.1	55.7 $\pm$ 1.0
	4.7	45.4 $\pm$ 3.6
	1.4	47.8 $\pm$ 1.8
2-1	45.2	61.1 $\pm$ 1.0
	13.6	50.5 $\pm$ 2.5
	4.5	47.2 $\pm$ 1.5
	1.4	41.9 $\pm$ 1.9
2-2	52.7	79.0 $\pm$ 0.1
	15.8	57.9 $\pm$ 2.3
	5.3	42.9 $\pm$ 1.6
	1.6	40.8 $\pm$ 1.1
3-1*	16.7 mg/mL ¹	58.0 $\pm$ 1.7
	5 mg/mL ¹	45.0 $\pm$ 0.8
	1.7 mg/mL ¹	37.8 $\pm$ 2.8
4-1	115.0	77.9 $\pm$ 5.5 ²
Other commercial products		
Commercial abalone extract	100 mg/mL ¹	43.7 $\pm$ 0.7
	33 mg/mL ¹	23.8 $\pm$ 2.9
	10 mg/mL ¹	18.6 $\pm$ 2.4
	3.3 mg/mL ¹	18.6 $\pm$ 1.2
Assay positive control		
Quercetin	10 μM ³	70.1 $\pm$ 1.0
	3 μM ³	46.5 $\pm$ 2.0
	1 μM ³	41.4 $\pm$ 1.4
	0.3 μM ³	43.0 $\pm$ 2.0

Table notes: (1) Sample 3-1 (powder) and a commercial abalone extract (powder) were dosed into the assay on a dry weight basis (mg powder dissolved in water); (2) Significant cell toxicity was observed following application of sample 4-1; (3) Quercetin was dosed into the assay on a micromolar (μM) basis.

Table 3: *In vitro* HCII-mediated thrombin inhibition from extracts prepared from abalone processing waste

Sample number	Sample concentration (mg/mL sulphated polysaccharides)	HCII thrombin inhibition (% decrease in thrombin activity)
1-1	1.0	88.2±2.1
1-2	0.9	94.4±0.9
2-1	0.5	72.9±3.2
2-2	1.7	89.2±3.3
3-1	0.06	7.3±3.0
4-1	0.18	32.8±9.0
Assay positive control		
Heparin	0.0625	90.0±1.8

Table 4: *In vitro* antithrombin (AT) and heparin cofactor II (HCII)-mediated thrombin inhibition from extracts prepared from abalone processing waste

Concentration (µg/mL sulphated polysaccharides)	HCII thrombin inhibition (% decrease in thrombin activity)	AT thrombin inhibition (% decrease in thrombin activity)
Sample 4.2		
100	92.0±1.5	59.19±1.4
50	41.4±1.3	49.8±1.1
10	23.5±0.9	14.1±0.3
Assay positive control - heparin		
1.04	90.9±1.3	98.9±0.9

Ion Exchange Chromatography

[00151] On the assumption that the sulphated polysaccharide content in the abalone extracts contributed a negative charge to the abalone extracts, anion exchange chromatography was selected to fractionate the abalone extracts into different molecular pools. An estimation of total protein content and sulphated polysaccharides was determined in the pooled fractions using the Pierce BCA Total Protein and DMMB assays. Anionic separations of abalone extracts 1-1 and 2-1 are presented in Figure 3. The elution profiles indicated that most of the protein weakly bound to the column eluting with low NaCl concentrations, whereas the sulphated carbohydrates were more strongly bound to the column requiring higher concentrations of NaCl to elute from the column. To collect similarly charged groups of molecules, the flow through was collected and fractions pooled as detailed below. Prior to further analyse, all of the anion exchange samples were desalted using a 3 kDa centrifugal filter device (Amicon Ultra-15, Ultracel low binding regenerated cellulose membrane) to remove the NaCl added by the linear salt gradient. A wash step was also performed after the sample was loaded and before the NaCl gradient commenced, using only water. The wash samples were also collected, analysed and found not to contain protein or sulphated polysaccharides.

1. Flow through
2. Pool 1: fractions 1 – 4
3. Pool 2: fractions 5 – 18
4. Pool 3: fractions 19 – 25
5. Pool 4: fractions 26 – 30

***In Vitro* Anti-Inflammatory Activity**

[00152] Anti-inflammatory activity, measured in response to the abalone anion exchange chromatography pooled fractions and an assay positive control (quercetin), is presented in Table 5. Cell viability remained above 80% (or below 20% cell death or toxicity) following application of the pooled fractions and positive control.

[00153] The majority of the anti-inflammatory activity was retained in the anion exchange flow through samples (flow) that decreased nitric oxide production by 47 – 64% (Figure 4). Anti-inflammatory activity in response to 1-1, 2-1 and 2-2 flow samples was either comparable to higher than the highest positive assay control (10 μ M quercetin). The Flow

sample from 1-2 was significantly lower than the highest assay positive control, but still more active than the lowest positive assay control (quercetin 1 μ M). The pooled fractions decreased nitric oxide production by 23 – 38%. The anti-inflammatory response to all of the pooled fractions was either comparable to or significantly less than the lowest assay positive control, further confirming the retention of anti-inflammatory activity in the anion exchange flow samples.

Table 5: *In vitro* anti-inflammatory activity from anion exchange chromatography fractions obtained from abalone extracts

Sample number	Sample concentration (μ g/mL collagen)	Anti-inflammatory activity (% decrease in nitric oxide production)
1-1	58.0	62.5 $\pm$ 1.4
1-1 Flow through	157.8	53.9 $\pm$ 3.7
1-1 Pool 1	8.9	33.6 $\pm$ 1.3
1-1 Pool 2	8.7	34.5 $\pm$ 0.8
1-1 Pool 3	7.9	37.6 $\pm$ 0.8
1-1 Pool 4	5.6	29.2 $\pm$ 3.1
1-2	47.0	55.2 $\pm$ 1.0
1-2 Flow through	56.0	47.6 $\pm$ 2.9
1-2 Pool 1	0	23.8 $\pm$ 4.5
1-2 Pool 2	0	26.6 $\pm$ 2.5
1-2 Pool 3	0	29.6 $\pm$ 1.9
1-2 Pool 4	0	29.3 $\pm$ 1.9
2-1	45.2	61.1 $\pm$ 1.0
2-1 Flow through	167.0	63.8 $\pm$ 3.8
2-1 Pool 1	18.8	26.4 $\pm$ 0.9
2-1 Pool 2	11.1	26.2 $\pm$ 3.0
2-1 Pool 3	3.7	26.9 $\pm$ 2.2
2-1 Pool 4	6.3	24.9 $\pm$ 2.8
2-2	52.7	79.0 $\pm$ 0.1
2-2 Flow through	101.1	51.6 $\pm$ 1.0
2-2 Pool 1	9.6	24.1 $\pm$ 0.1
2-2 Pool 2	8.6	29.0 $\pm$ 3.2
2-2 Pool 3	3.7	29.8 $\pm$ 3.3
2-2 Pool 4	5.0	27.9 $\pm$ 1.5
Assay positive control		
Quercetin	10 μ M	70.1 $\pm$ 1.0
	3 μ M	46.5 $\pm$ 2.0
	1 μ M	41.4 $\pm$ 1.4
	0.3 μ M	43.0 $\pm$ 2.0

***In Vitro* Thrombin inhibition**

[00154] Heparin cofactor II-mediated thrombin inhibition measured in response to the abalone anion exchange chromatography flow and pooled fractions, and an assay positive control (heparin), is presented in Table 6. In these assays, the abalone extracts were assayed without dilution to observe maximum anti-thrombotic potential.

Table 6: *In vitro* heparin cofactor II-mediated thrombin inhibition from anion exchange chromatography fractions obtained from abalone extracts

Sample number	Sample concentration (mg/mL sulphated polysaccharides)	HCII-thrombin inhibition (% decrease in thrombin activity)
1-1	1.0	88.2±2.1
1-1 Flow through	0.38	6.5±1.6
1-1 Pool 1	0	3.6±0.8
1-1 Pool 2	0.67	67.8±11.2
1-1 Pool 3	0.15	89.1±1.0
1-1 Pool 4	0.04	54.8±5.5
1-2	0.9	94.4±0.9
1-2 Flow through	0.6	1.4±1.1
1-2 Pool 1	0	3.9±1.6
1-2 Pool 2	0.55	81.5±4.1
1-2 Pool 3	0.23	93.2±0.7
1-2 Pool 4	0.02	90.1±1.3
2-1	0.5	72.9±3.2
2-1 Flow through	0	9.4±2.0
2-1 Pool 1	0.008	4.5±2.2
2-1 Pool 2	0.88	14.3±7.9
2-1 Pool 3	0.18	50.8±4.4
2-1 Pool 4	0.03	11.0±3.1
2-2	1.7	89.2±3.3
2-2 Flow through	0.12	6.2±2.1
2-2 Pool 1	0.006	2.8±1.4
2-2 Pool 2	0.34	75.4±6.4
2-2 Pool 3	0.33	91.8±0.8
2-2 Pool 4	0.05	88.1±1.3
Assay positive control		
Heparin	0.0625	90.0±1.8

[00155] The majority of thrombin inhibition was retained in pools 2 – 4 of the anion exchange samples. In particular pool 3 decreased thrombin activity by 50 – 93% (Figure 5). Thrombin inhibition in response to the 1-1, 1-2 and 2-2 pool 3 samples was comparable to the highest positive assay control (0.0625 mg/mL heparin). The pool 3 sample from 2-1 was significantly lower than the highest assay positive control, but still more active than the

lowest positive assay control (0.001 mg/mL heparin). The pool 1 and flow samples only decreased thrombin activity by 1 – 9%, significantly less than the lowest assay positive control and further confirming the retention of thrombin inhibition by pools 2 – 4 of the anion exchange samples.

In Vivo Anti-Inflammatory Activity

[00156] *In vivo* anti-inflammatory activity was assessed using a collagen induced mouse model of rheumatoid arthritis (CIA). The large scale abalone extract, sample 3-1, was included in a basal diet at two doses (100 and 500 mg/kg body weight/day) and fed to the mice prior to and after arthritis induction. A positive drug control, prednisolone, was also fed to mice after arthritis induction at a dose of 1 mg/kg body weight/day. The effects of the abalone and prednisolone treatments on arthritis severity were assessed via clinical outcomes, disease incidence, final body weight and levels of circulating collagen II antibodies. Clinical outcomes, using a standard arthritis scoring system, showed a wide range of symptom severity throughout the trial (Figure 6).

[00157] On the final day of the trial (day 20), no adverse symptoms were observed in mice that were not induced to develop arthritis (Figure 7), indicating that oral consumption of the abalone did not produce pro-inflammatory symptoms. Comparisons of the treatment groups with the CIA negative control were also made throughout the progression of the disease by comparing the least squares means of clinical scores from the day of onset (day 6) until day 20, using the GLM Procedure of SAS (<http://support.sas.com/en/support-home.html>; https://support.sas.com/documentation/cdl/en/statug/63033/HTML/default/viewer.htm#glm_toc.htm) (Figure 8). These comparisons revealed that prophylactic treatment with the abalone extract, dose 1 and 2, decreased mean clinical scores by ~50% ($p<0.0001$) and 20% ($p<0.05$). Therapeutic treatment with the abalone extract dose 1 decreased mean clinical scores by ~30% ($p<0.001$), however therapeutic treatment with abalone extract dose 2 increased the mean clinical score by 5% (not significant, $p=0.4374$). Therapeutic treatment with prednisolone significantly decreased the clinical score by ~70% ($p<0.0001$).

[00158] Animal weight was collected throughout the trial and remained steady in the CIA groups regardless of treatment type (data not shown). Circulating levels of cytokines (IL2, IL-6, IL-10, IFN- γ and TNF- α) and collagen type II antibodies were also measured in the serum samples collected on Day 21. IL-2, IL-10, IFN- γ and TNF- α remained low in all treatment groups (data not shown), however IL-6 was significantly higher following arthritis

induction (Figure 9). Following administration of the abalone extract and prednisolone, circulating levels of IL-6 significantly decreased in all treatment groups except following therapeutic administration of abalone extract dose 1. Circulating collagen type II antibody levels remained high in all CIA groups (Figure 10). Histological scoring also revealed a decrease in total synovitis following prophylactic abalone dose 2 (Figure 11).

Anti-Coagulant Activity

[00159] *In vitro* anti-coagulant activity was measured using the blood and plasma clotting assays prothrombin time (PT) and activated partial thromboplastin time (aPTT). Sample 1-2 and 2-2 delayed clotting times in both assays in a dose dependent manner (presented in Table 7) indicating that abalone extracts act as anti-coagulant molecules in blood and plasma.

[00160] *In vitro* anti-coagulant activity was also measured in response to fractions prepared from abalone extracts. Anion exchange fractions were prepared from sample 2-2 and pooled as described in Table 8. The pools that contained the highest amounts of sulphated carbohydrates and lower amounts of protein delayed clotting times the most in the aPTT and PT assays (presented in Table 9), indicating that anti-coagulant activity is consistent with sulphated polysaccharide content rather than protein content.

Table 7: Prothrombin (PT) and activated partial thrombin time (aPTT) in response to abalone processing extracts

Sample	Sample concentration ($\mu\text{g/mL}$ sulphated polysaccharides)	Prothrombin time (PT)	Sample concentration ($\mu\text{g/mL}$ sulphated polysaccharides)	Activated partial thrombin time (aPTT)
1-2	0	15.9 $\pm$ 0.4	0	35.2 $\pm$ 1.4
	0.09	25.7 $\pm$ 0.3	0.4	52.1 $\pm$ 0.9
	0.13	34.1 $\pm$ 0.2	0.8	64.3 $\pm$ 0.6
2-2	0	15.9 $\pm$ 0.4	0	35.2 $\pm$ 1.4
	0.12	30.5 $\pm$ 0.9	0.5	43.8 $\pm$ 1.6
	0.16	40.1 $\pm$ 0.4	1.0	61.1 $\pm$ 1.4

Table 8: Description of pooled samples from anion exchange separated abalone processing waste extract (sample 2-2)

Pool number	Description of pooled samples	Protein concentration (mg/mL)	Sulphated polysaccharide concentration (mg/mL)
1	Linear NaCl gradient fractions 1-13	1.3	0.4
2	Linear NaCl gradient fractions 14-19	1.9	2.0
3	Linear NaCl gradient fractions 20-25	0.4	2.5
4	Linear NaCl gradient fractions 26-31	0.3	2.0
5	Linear NaCl gradient fractions 31-37	0.05	0.4
6	Initial sample application (unbound)	8.9	0.7
7	Initial sample wash (unbound)	2.5	0.03

Table 9: Prothrombin (PT) and activated partial thrombin time (aPTT) in response to pooled samples from anion exchange separated abalone processing waste extract (sample 2-2)

Sample description	Sulphated polysaccharide concentration (mg/mL)	Protein concentration (mg/mL)	Seconds
Prothrombin time (PT)			
Control	0	0	17.4±0.3
Pool 1	0.08	0.26	14.2±1.2
Pool 2	0.40	0.38	17.3±0.6
Pool 3	0.19	0.02	20.75±0.3
	0.29	0.04	28.95±0.4
Pool 4	0.24	0.03	28.25±0.9
	0.32	0.05	38.65±0.6
Pool 5	0.07	0.01	13.6±0.8
Pool 6	0.13	1.78	13.2±0.2
Pool 7	0.006	0.50	13.4±1
Activated partial thrombin time (aPTT)			
Control	0	0	36.15±0.1
Pool 3	0.98	0.01	56.35±1.5
	1.96	0.02	98.4±1.3
Pool 4	0.8	0.01	48.6±1.2
	1.6	0.02	81.1±0.9

[00161] The disclosure of every patent, patent application, and publication cited herein is hereby incorporated by reference in its entirety.

[00162] The citation of any reference herein should not be construed as an admission that such reference is available as "Prior Art" to the instant application.

[00163] Throughout the specification the aim has been to describe the preferred embodiments of the invention without limiting the invention to any one embodiment or specific collection of features. Those of skill in the art will therefore appreciate that, in light of the instant disclosure, various modifications and changes can be made in the particular embodiments exemplified without departing from the scope of the present invention. All such modifications and changes are intended to be included within the scope of the appended claims.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

- 1) An extract of blacklip abalone (*Haliotis rubra*), or fraction thereof, comprising one or more biologically active components, which extract is derived from blacklip abalone processing waste.
- 2) An extract or fraction according to claim 1 wherein the extract or fraction is derived from raw processing waste.
- 3) An extract or fraction according to claim 1 or claim 2 wherein the one or more biologically active components are selected from anti-thrombotic agents and anti-inflammatory agents.
- 4) An extract or fraction of claim 1 or claim 2 wherein the one or more biologically active components are selected from proteins and anionic polysaccharides.
- 5) An extract fraction of any one of claims 1 to 4 which is enriched in protein components.
- 6) An extract fraction of any one of claims 1 to 4 which is enriched in anionic polysaccharide components.
- 7) Use of an extract or fraction according to any one of claims 1 to 6 as a medicament, dietary supplement, nutraceutical, functional food or functional food ingredient.
- 8) A medicament, dietary supplement, nutraceutical, functional food or functional food ingredient comprising an extract or fraction of any one of claims 1 to 6.
- 9) A pharmaceutical composition comprising an extract or fraction according to any one of claims 1 to 6, and one or more pharmaceutically acceptable carriers or excipients.
- 10) An anti-inflammatory agent comprising an extract or fraction of any one of claims 1 to 5.
- 11) A method of treating an IL-6 mediated condition in a subject, the method comprising administering to the subject an effective amount of an extract or fraction according to any one of claims 1 to 5.
- 12) A method of treating an inflammatory condition in a subject, the method comprising administering to the subject an effective amount of an extract or fraction according to any one of claims 1 to 5.
- 13) A method according to claim 12, wherein the inflammatory condition is an autoimmune condition.
- 14) A method of treating an autoimmune condition in a subject, the method comprising administering to the subject an effective amount of an extract or fraction according to any one of claims 1 to 5.

- 15) Use of the extract or fraction of any one of claims 1 to 5 in the manufacture of a medicament for treating an IL-6-mediated condition.
- 16) Use of the extract or fraction of any one of claims 1 to 5 in the manufacture of a medicament for treating an inflammatory condition.
- 17) Use of the extract of any one of claims 1 to 5 in the manufacture of a medicament for treating an autoimmune condition.
- 18) A method or use according to any one of claims 11 to 17 wherein the condition is rheumatoid arthritis.
- 19) An anti-thrombotic or anti-coagulant agent comprising an extract or fraction of any one of claims 1 to 4 or claim 6.
- 20) An anti-thrombotic agent according to claim 19 wherein the anti-thrombotic effect is mediated through heparin cofactor II or antithrombin.
- 21) A method of inhibiting thrombosis in a subject, the method comprising administering to the subject an effective amount of the extract or fraction according to any one of claims 1 to 4 or claim 6.
- 22) A method of inhibiting thrombin activity in a subject, the method comprising administering to the subject an effective amount of an extract or fraction according to any one of claims 1 to 4 or claim 6.
- 23) Use of an extract or fraction according to any one of claims 1 to 4 and 6 in the manufacture of a medicament for inhibiting thrombosis or thrombin activity.
- 24) A process for preparing an extract or fraction according to any one of claims 1 to 6, the method comprising digesting blacklip abalone processing waste using an enzymatic or chemical digestion process and isolating the extract from the digested waste.
- 25) A process according to claim 23 comprising the steps of:
digesting blacklip abalone processing waste in the presence of a digesting enzyme to produce an aqueous fraction and a precipitate; and
separating the aqueous fraction from the precipitate to thereby produce the extract.
- 26) A process according to claim 24, further comprising filtering the aqueous fraction to produce a filtrate.
- 27) A process according to claim 25, further comprising desalting the filtrate.
- 28) A process according to any one of claims 23 to 26, further comprising separating the extract into one or more fractions enriched in protein components or anionic polysaccharide components.

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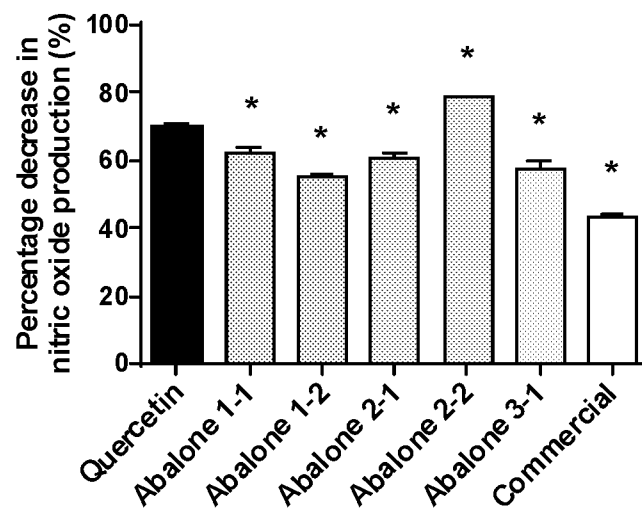


Figure 1

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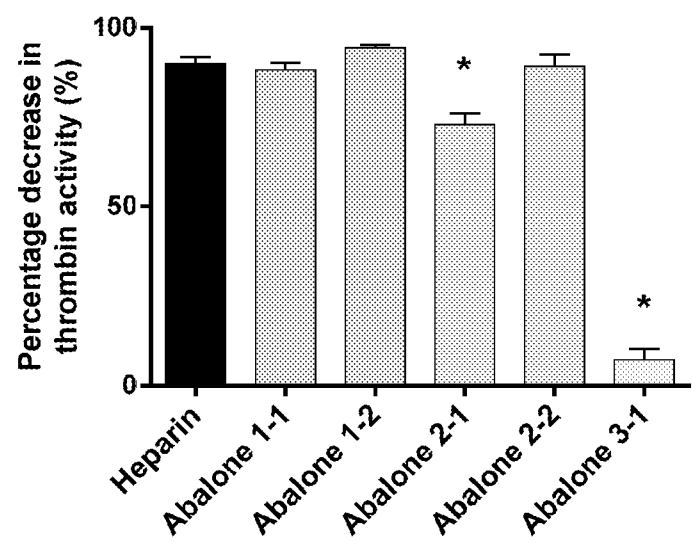


Figure 2

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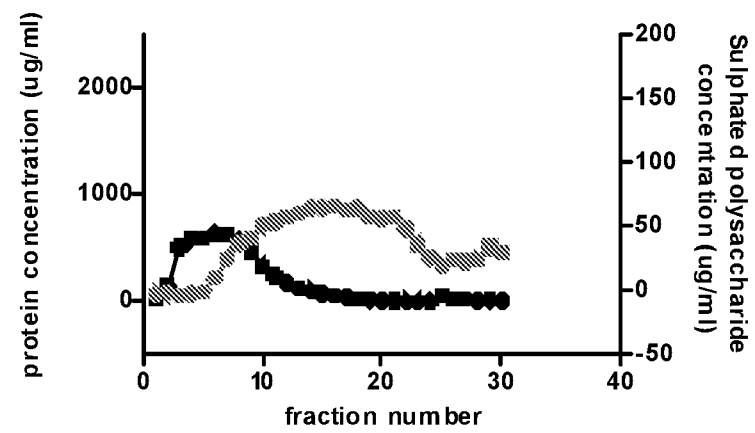


Figure 3A

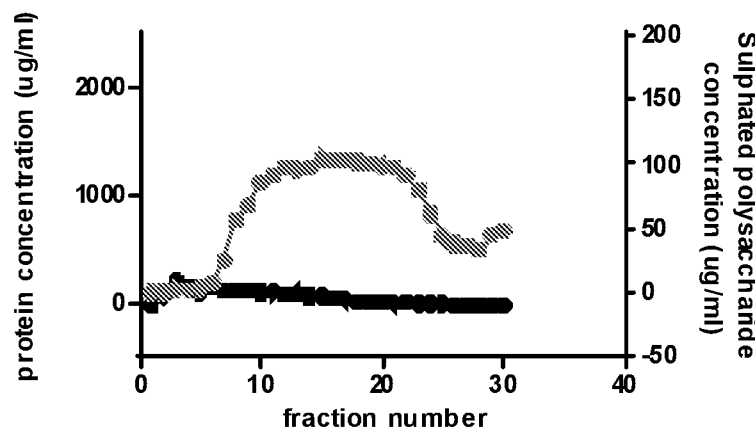


Figure 3B

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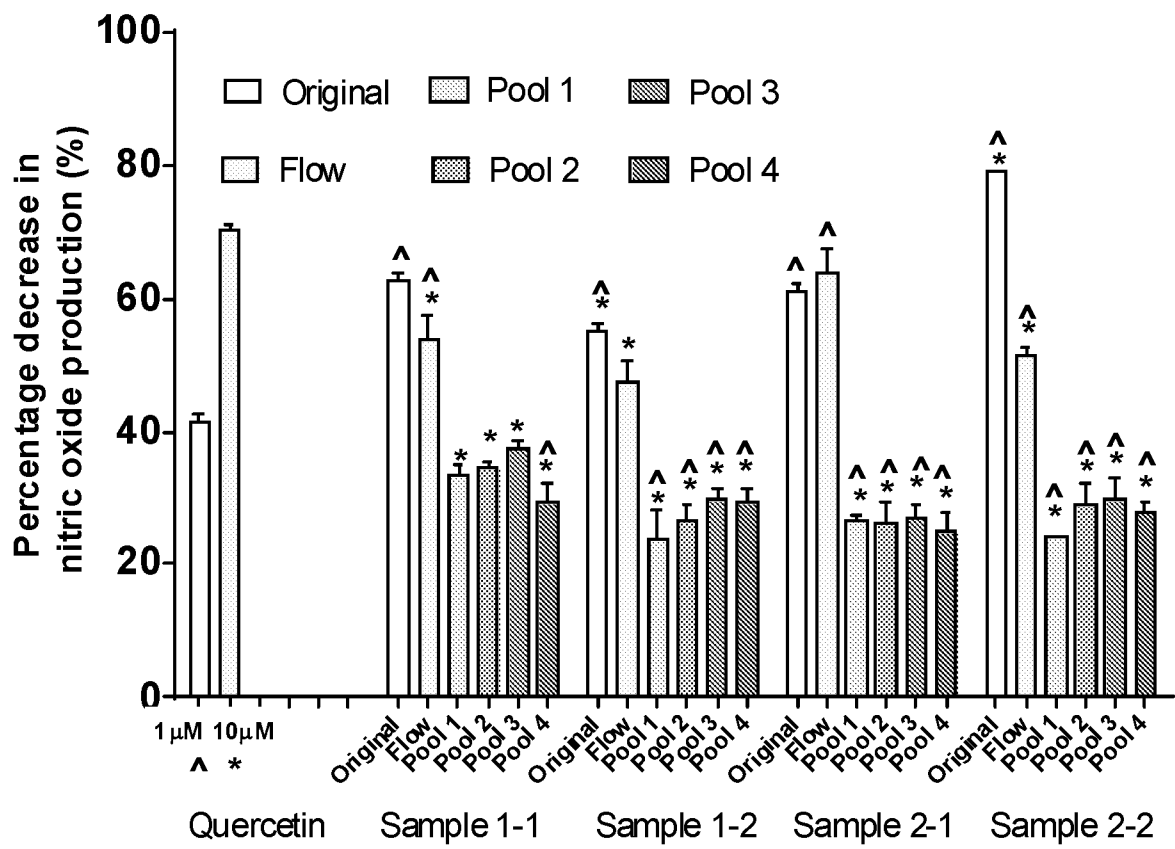


Figure 4

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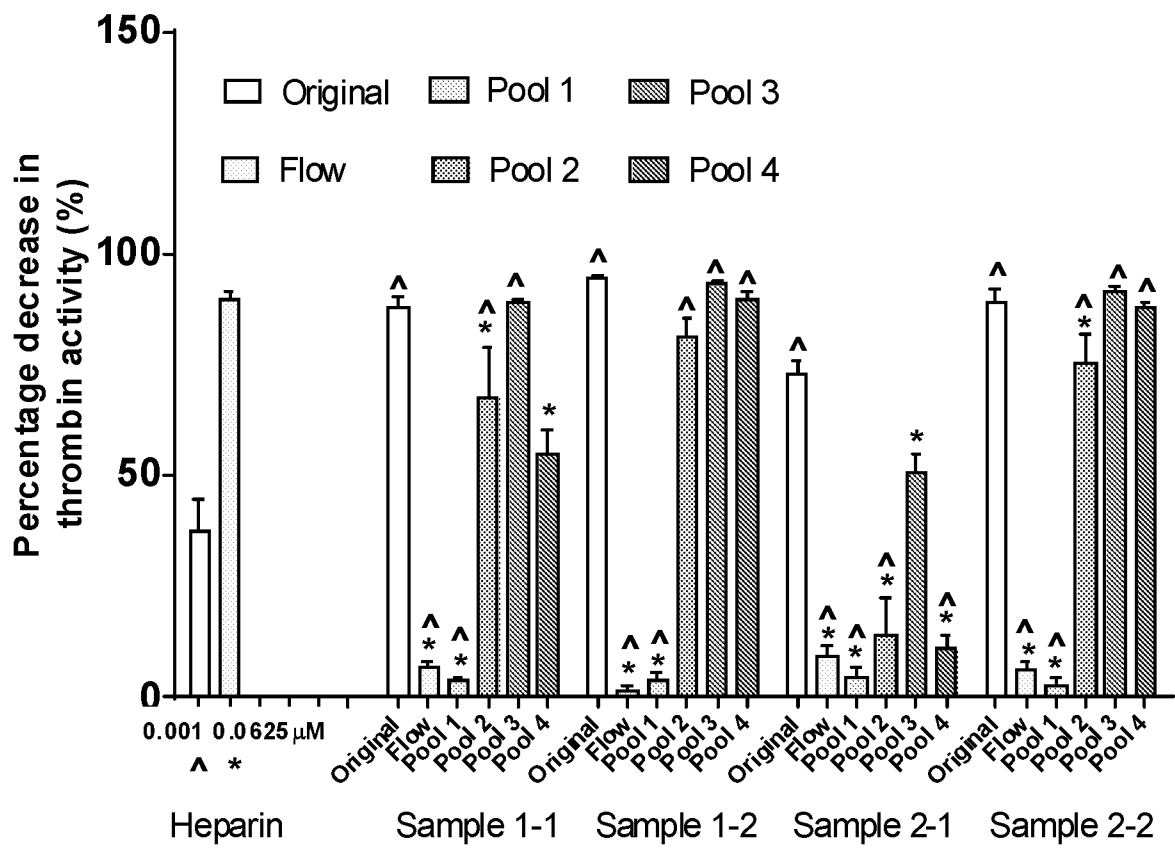


Figure 5

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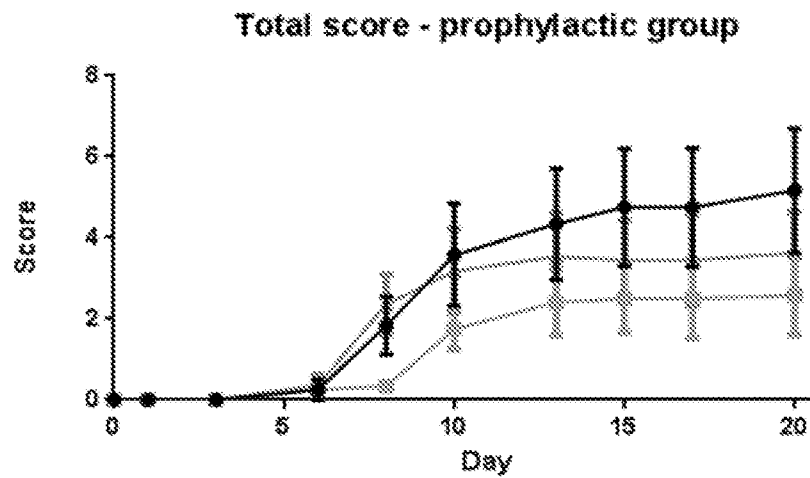


Figure 6A

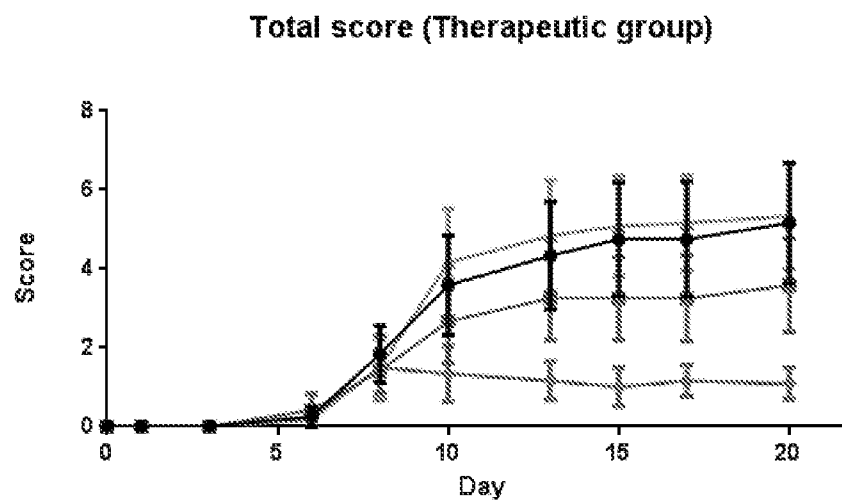


Figure 6B

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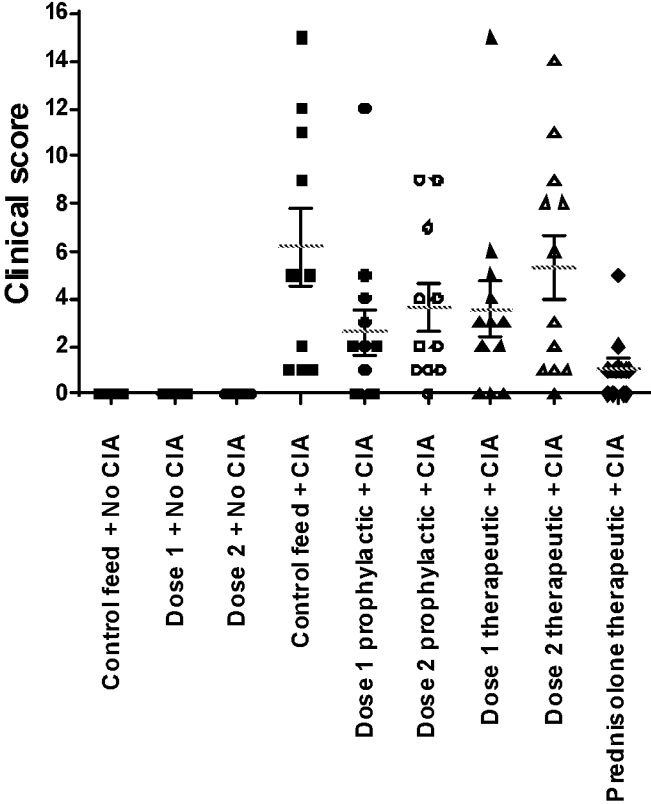


Figure 7

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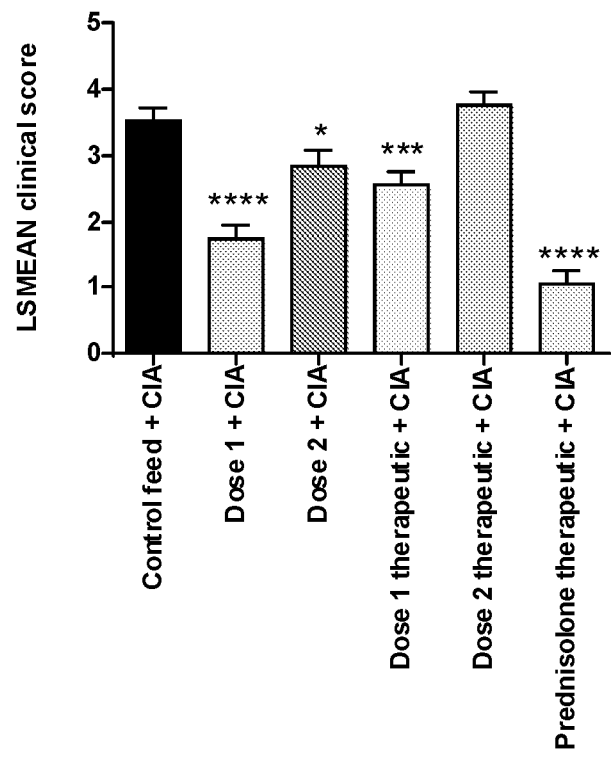


Figure 8

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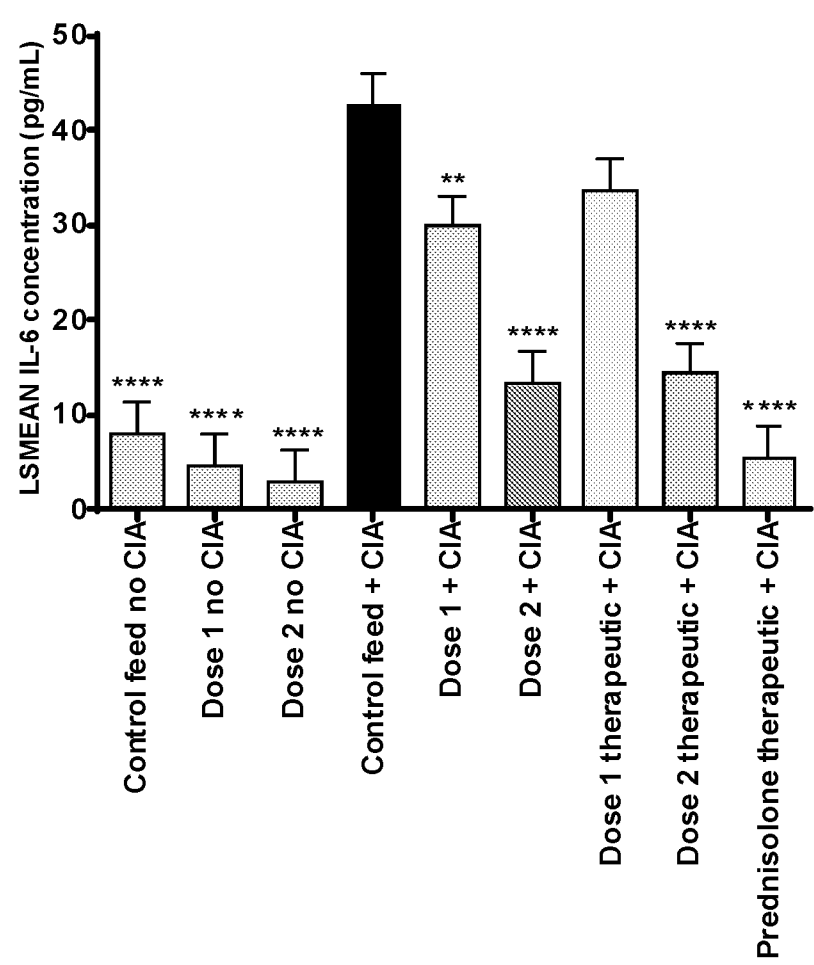


Figure 9

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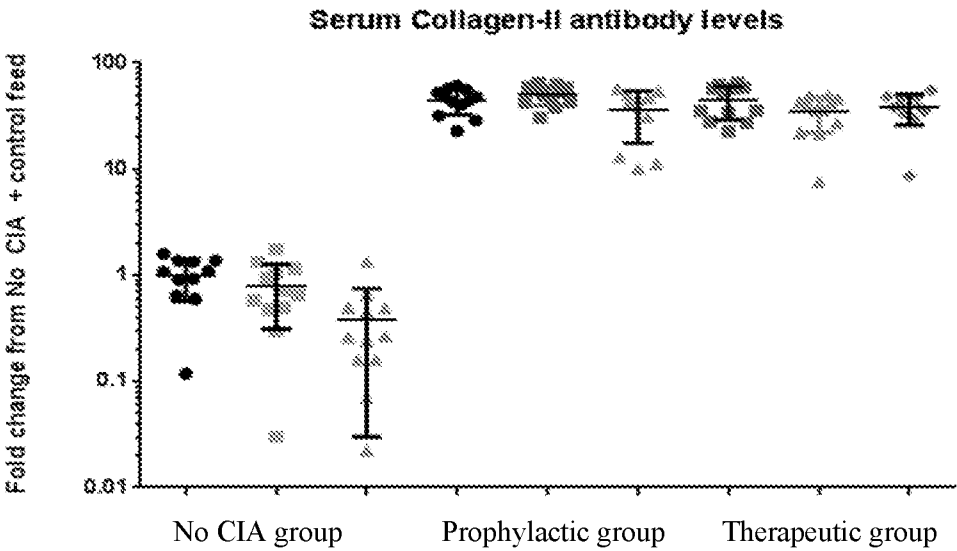


Figure 10

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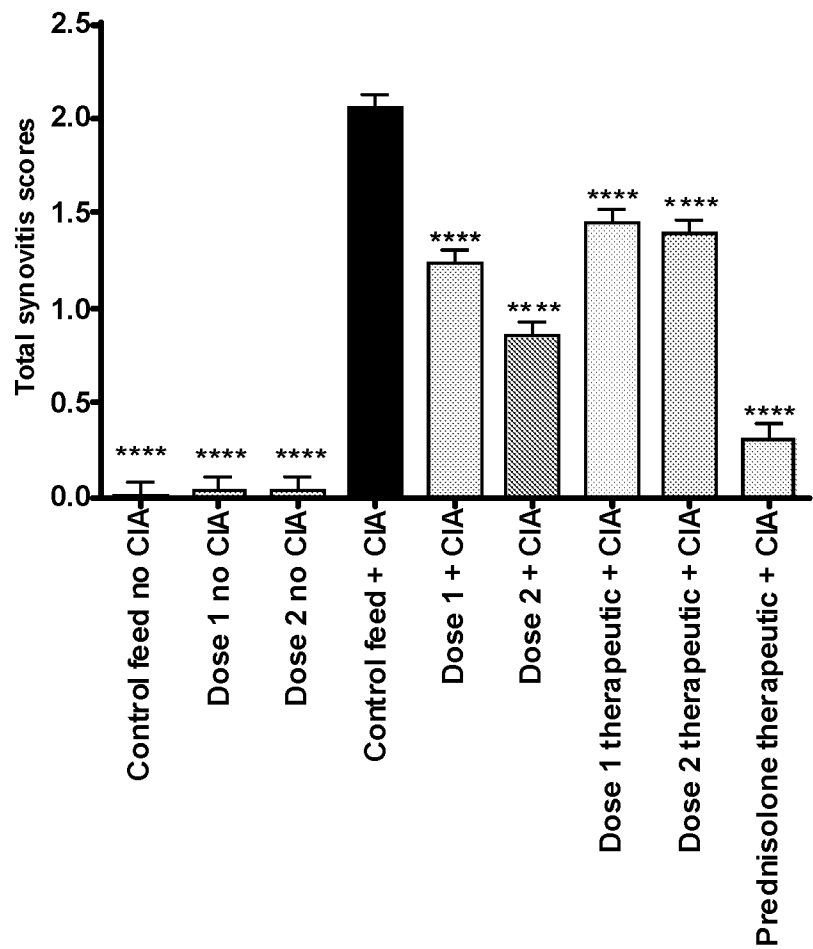


Figure 11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2017/050041

A. CLASSIFICATION OF SUBJECT MATTER

A61K 35/618 (2015.01) A61P 7/02 (2006.01) A61P 29/00 (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Databases Searched: WPIAP, EPODOC, Medline, Agricola, CAPlus, Biosis and Embase, Keywords Used: Abalone, extract, haliotis rubra, blacklip abalone, processing waste, offal, viscera, thrombosis, collagen, polysaccharide, inflammatory, A61K 35/618 and like terms

Patentscope, Esp@cenet and Auspat: Applicant and inventor name search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

☒ Further documents are listed in the continuation of Box C ☒ See patent family annex

* "A"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E"	earlier application or patent but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 2 May 2017	Date of mailing of the international search report 02 May 2017
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustalia.gov.au	Authorised officer Arati Sardana AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. 0262832905

INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation).		PCT/AU2017/050041
DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2002/102844 A1 (QUEENSLAND BIOPROCESSING TECHNOLOGY PTY LTD) 27 December 2002 Abstract, page 4 lines 10-21, page 2 lines 13-23, example 2, page 13 to line 3 on page 14, page 6 lines 20-34, page 25 and claims 1, 10, 22-23	1, 2, 4-5 and 7-9
X	WO 2002/102851 A1 (QUEENSLAND BIOPROCESSING TECHNOLOGY PTY LTD) 27 December 2002 Abstract, page 6 lines 1-20, example 1, example 8A, Table 16 on page 45, example 9, page 6 lines 13-30, page 54 lines 17-21 and claims 6-12	1, 2, 4, 7-9, 24-25 and 28
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