Abstract:
The present invention relates to a tunicate hexane, acetone, and/or methanol extract obtained by extracting a Styela clava or Ciona intestinalis tunicate sample with hexane, acetone, and/or methanol; and collecting the tunicate hexane, acetone, and/or methanol extracts. Methods of inhibiting human mast cell degranulation and activation and inhibiting human mast cell expression of TNF and MCP-1, comprising administering the tunicate hexane, acetone, and/or methanol extract are also provided.
TUNICATE EXTRACTS AND USES THEREOF IN ANTI-ALLERGY APPLICATIONS

FIELD OF THE INVENTION

The present invention relates to tunicate extracts and uses thereof in anti-allergy applications. More specifically, the present invention relates to tunicate extracts and their use in allergic inflammation therapy.

BACKGROUND OF THE INVENTION

Mast cells play a central role in homeostasis and disease (Galli et al, 2005; Fukuishi et al, 2004). Located at mucosal surfaces and surrounding blood vessels and nerves, they are uniquely situated to regulate the function of the vasculature, to initiate the recruitment and activation of leukocytes into tissues and to trigger physiological responses that are mediated by the nervous system.

Mast cells are primarily responsible for the acute allergic response to allergen exposure, including broncho-constriction and edema (Howarth et al, 1987). Mast cells and immunoglobulin E (IgE) are also important in the evolution of allergic late-phase responses, which are largely responsible for the pathogenesis of chronic allergic diseases such as asthma, atopic dermatitis and rhinitis (Kulka and Befus, 2003). Allergens bind IgE and activate high affinity IgE receptors (FceRI) expressed by mast cells; this causes mast cells to degranulate and release mediators such as histamine, leukotrienes and several pro-inflammatory cytokines and chemokines.

There are few commercial products that can effectively inhibit mast cell degranulation and activation, particularly during an acute allergic response. Most medications currently used to treat allergic inflammation target mast cell mediators, not mast cells themselves. Glucocorticoids such as fluticasone, inhibit mast cell production of de novo synthesized pro-inflammatory mediators but are less effective at blocking mast cell release of stored mediators such as histamine. The rapid process of stored mediator release is called degranulation because it requires the molecular docking and fusion of phospholipids-encased vesicles (called granules) with the plasma membrane. Sodium cromoglycate and nedocromil sodium have been used clinically for several years to inhibit mucosal mast cell activation in allergic conjunctivitis, for example, but these medications are largely ineffective for the treatment of other types of allergic inflammation (asthma, for example) and are ineffective at blocking the production of de novo synthesized pro-inflammatory mediators.
Many prescription and over-the-counter medications are available for asthma, a very specific form of allergic inflammation. Asthma is treated with two types of medicines: long-term control and quick-relief medicines. Long-term control medicines help reduce airway inflammation and prevent asthma symptoms. Quick-relief, or "rescue," medicines relieve asthma symptoms that may flare up. Most people who have asthma need to take long-term control medicines daily to help prevent symptoms. The most effective long-term medicines reduce airway inflammation. These treatments prevent symptoms from starting but do not provide quick relief from symptoms.

Inhaled corticosteroids are the preferred medicines for long-term control of asthma because they are the most effective long-term control medicine to relieve airway inflammation and edema that can lead to airway hypersensitivity. Reducing inflammation helps prevent the localized inflammatory cell response and subsequent amplification of the allergic response. Most patients on daily inhaled corticosteroids report a reduction in symptom severity and occurrence. Inhaled corticosteroids are generally safe when taken as prescribed, although side-effects can occur in a small subset of patients. One common side effect from inhaled corticosteroids is a mouth infection called thrush. For severe asthma, corticosteroid pills or liquid for short periods are prescribed to get the asthma symptoms under control. If taken for long periods, these medicines raise the risk for cataracts and osteoporosis. Other long-term control medicines include Inhaled long-acting beta2-agonists. These treatments open the airways and may be added to low-dose inhaled corticosteroids to improve asthma control. An inhaled long-acting p2-agonist shouldn't be used alone. Leukotriene modifiers are oral medications, often in pill form, given to block the leukotriene receptors that mediated the edema and inflammation associated with asthma. Cromolyn and nedocromil are inhaled treatments that block chloride channels and thereby block the activation of allergic effector cells - possibly mast cells as mentioned above. These are usually only effective in patients with mild asthma. Theophylline is also an oral medication that acts on smooth muscle to open the airways and improve breathing.

Therefore, the search for novel, mast cell-specific inhibitors that can block both degranulation and production of de novo synthesized mediators has been the holy grail of allergy research for the past few decades.
SUMMARY OF THE INVENTION

The present invention relates to tunicate extracts and uses thereof in anti-allergy applications. More specifically, the present invention relates to tunicate extracts and their use in allergic inflammation therapy.

There is increasing interest in developing effective and safe natural health products that can be used as alternative treatment options or co-solutions in treating or preventing allergic reaction. The present invention provides methods and products as a means to attain this goal.

The present invention provides a method of preparing hexane and acetone tunicate extracts comprising:

a) extracting a *Styela clava* or *Ciona intestinalis* tunicate sample with hexane, acetone, and/or methanol; and

b) collecting the hexane, acetone, and/or methanol tunicate extracts obtained in step a).

The tunicate sample may be a *Styela clava* tunicate sample, or a *Ciona intestinalis* tunicate sample. In the method as just described, the tunicate sample may be dried or lyophilized prior to step a); optionally, the dried or lyophilized sample may be milled prior to step a). Additionally, the method as described herein may comprise sequentially extracting the tunicate sample with hexane, acetone, and methanol in step a).

In the method as described herein, the hexane, acetone, and/or methanol extract may be further processed by chromatography, drying by rotatory evaporator, centrifugal vacuum evaporator, or any combination thereof. Additionally, the acetone extract may be further fractionated by normal or reverse phase chromatography, silica gel chromatography, and/or thin layer chromatography to yield sub-fractions; the methanol extract may also be further fractionated by normal or reverse phase chromatography, silica gel chromatography, and/or thin layer chromatography to yield sub-fractions. The sub-fractions may be further processed by chromatography, drying by rotatory evaporator, centrifugal vacuum evaporator, or any combination thereof.

The present invention further provides an acetone tunicate extract, fraction, or sub-fraction thereof obtained from *Styela clava* or *Ciona intestinalis*. The acetone tunicate extract, fraction, or sub-fraction thereof may be obtained by a method as described herein.

The acetone tunicate extract, fraction, or sub-fraction thereof of the present invention may be characterized by any one of the proton NMR spectrum of Figure 1B (3), (4), or (5), or Figure
13. Additionally, the acetone tunicate extract, fraction, or sub-fraction thereof of the present invention may comprise hexadecanoic acid (palmitic acid), octadecanoic acid (stearic acid), eicosa-5,8,11,14,17-pentaenoic acid (EPA), and 4,7,10,13,16,19-docosahexaenoic acid (DHA) as the main fatty acid components.

5 The present invention also provides a methanol tunicate extract, fraction, or sub-fraction thereof obtained from Styela clava. The methanol tunicate extract, fraction, or sub-fraction thereof may be obtained by the method as described herein. Additionally, the methanol tunicate extract, fraction, or sub-fraction thereof of the present invention may be characterized by the proton NMR spectrum of Figure 1C (5).

10 The present invention also provides a hexane tunicate extract, fraction, or sub-fraction thereof obtained from Styela clava. The tunicate acetone extract, fraction, or sub-fraction thereof may be obtained using the methods as described herein. The tunicate hexane extract, fraction, or sub-fraction thereof of the present invention may be characterized by the proton NMR spectrum as shown in Figure 1A (3).

15 Additionally, the present invention provides a method of inhibiting human mast cell degranulation and activation comprising administering one or more than one of the tunicate hexane, acetone, and/or methanol extract, fraction, or sub-fraction thereof as described herein, or any mix of compounds or single compounds obtained therefrom, or any combination thereof, to a subject in need thereof.

20 Furthermore, the present invention provides a method of inhibiting human mast cell expression of TNF and MCP-1 comprising administering one or more than one of the tunicate acetone extract, fraction, or sub-fraction thereof as described herein, or any mix of compounds or single compounds obtained therefrom, or any combination thereof, to a subject in need thereof.

It is presently shown that tunicate extracts PTC-1 100 and PTC-1200 inhibit human mast cell degranulation and activation. PTC-1200 is more potent than PC-1 100 and these extracts have different modes of action. PTC-1 100 may act directly on compound 48/80, thereby interfering with its ability to activate mast cell signalling pathways. The effect of PTC-1 100 on degranulation are transient and are sensitive to the "wash out" effect, while the effect of PTC-1200 on degranulation are more long-term and likely involve changes in human mast cell phenotype. PTC-1200 further inhibits mast cell expression of TNF and MCP-1, important pro-inflammatory mediators.

Additional aspects and advantages of the present invention will be apparent in view of the following description. The detailed description and examples, while indicating preferred
embodiments of the invention, are given by way of illustration only, as various changes and modifications within the scope of the invention will become apparent to those skilled in the art in light of the teachings of this invention.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features of the invention will now be described by way of example, with reference to the appended drawings, wherein:

FIGURE 1 shows proton NMR spectra for various extracts of the tunicate samples. FIGURE 1A shows hexane extracts (1- SH PTC-31 00; 2- BR PTC-21 00; 3- MB PTC-1 100; 4- INH-OS-79H; 5- INH-OS-78H); FIGURE 1B shows acetone extracts (1- SH PTC-3200; 2- BR PTC-2200; 3- MB PTC-1 200; 4- INH-OS-79A; 5- INH-OS-78A); FIGURE 1C shows methanol extracts (1- SH PTC-3300; 2- BR PTC-2300; 3- MB PTC-1 300; 4- INH-OS-79M; 5- INH-OS-78M); and FIGURE 1D shows hot water extracts (1- SH PTC-3400; 2- BR PTC-2400; 3- MB PTC-1 400; 4- INH-OS-79W; 5- INH-OS-78W).

FIGURE 2 is a bar graph showing PTC-1 100 dosage-dependent inhibition of human mast cell degranulation. Human mast cells were pre-treated with PC-1 100 for 30 min prior to activation with compound 48/80. Asterisk represents significance of p<0.01 when compared to DMSO control.

FIGURE 3 is a bar graph illustrating PTC-1 200 dosage-dependent inhibition of human mast cell degranulation. Human mast cells were pre-treated with PTC-1 200 for 30 min prior to activation with compound 48/80. Asterisk represents significance of p<0.01 when compared to DMSO control.

FIGURE 4 is a bar graph showing the synergistic effects of PTC-1 100 and PTC-1 200 on human mast cell degranulation. Human mast cells were pre-treated with extracts for 30 min prior to activation with compound 48/80. Asterisk represents significance of p<0.01 when compared to DMSO control.

FIGURE 5 is a bar graph showing the effect of tunicate extracts from INH-OS-78 and INH-OS-79 on LAD2 degranulation. The percentage of β-hexosaminidase release was calculated as a percent of total content. CTL is an untreated cells control. DMSO, 0.4% is a vehicle control where cells were treated with DMSO, 0.4%. Experiment was carried out in quadruplicate. Data are represented as mean ± SEM. *** p<0.001 compared to a vehicle control.
FIGURE 6 is a bar graph illustrating the wash-out effect of extract PTC-1 100 on mast cell activation. Human mast cells were pre-treated with PTC-1 200 for 30 min prior to activation with compound 48/80.

FIGURE 7 is a bar graph showing the effect of PTC-1 200 on human mast cell degranulation. Human mast cells were pre-treated with PTC-1 200 for indicated times prior to activation with compound 48/80. Asterisk represents significance of p<0.01 when compared to 72 hr DMSO control.

FIGURE 8 is a bar graph showing the synergistic effects of PTC-1 100 and PTC-1 200 on human mast cell degranulation. Human mast cells were pre-treated with extracts for indicated times, washed and activated with compound 48/80. Asterisk represents significance of p<0.01 when compared to 72 hr DMSO control.

FIGURE 9 is a bar graph illustrating cell viability of human mast cells as measured by MTT assay. Human mast cells were treated with extracts for indicated times either individually or in combination (n=3).

FIGURE 10 is a bar graph illustrating the effect extracts on compound 48/80-induced TNF expression by human mast cells. Human mast cells were pre-treated with PTC-1 100 or PTC-1 200 individually or in combination. Mast cells were activated with compound 48/80 for 3 hr and expression of TNF was determined by quantitative PCR. Asterisk represents significance of p<0.01 when compared to compound 48/80 alone.

FIGURE 11 is a bar graph showing the effect of extracts on compound 48/80-induced MCP-1 expression by human mast cells. Human mast cells were pre-treated with PTC-1 100 or PTC-1 200 individually or in combination. Mast cells were activated with compound 48/80 for 3 hr and expression of TNF was determined by quantitative PCR. (n=3) Asterisk represents significance of p<0.01 when compared to compound 48/80 alone.

FIGURE 12 is a bar graph showing the effect of extracts on compound 48/80-induced TNF production by human mast cells. Human mast cells were pre-treated with PTC-1 100 or PTC-1 200 individually or in combination. Mast cells were activated with compound 48/80 for 20 hr and TNF production was measured by ELISA. Asterisk represents significance of p<0.01 when compared to compound 48/80 alone.

FIGURE 13 is a comparison of proton NMR spectra from fraction 78-A-2 (3) and sub-fractions 78A-2-1 (2) and 78A-2-2 (1) of tunicate acetone extract (in CDCl₃, 600 MHz).
FIGURE 14 shows the effect the first sub-stage fractions of tunicate extracts on LAD2 degranulation. The percentage of β-hexosaminidase release was calculated as a percent of total content. CTL is an untreated cells control. DMSO, 0.1% is a vehicle control where cells were treated with DMSO, 0.1%. Data are represented as mean ± SEM. N=3. * p<0.05 compared to a vehicle control.

FIGURE 15 shows the effect of second sub-stage fractions tunicate extracts on LAD2 degranulation. FIGURE 15A shows samples 1 to 5, while FIGURE 15B shows samples 6 to 10. The percentage of β-hexosaminidase release was calculated as a percent of total content. CTL is an untreated cells control. DMSO, 0.1% is a vehicle control where cells were treated with DMSO, 0.1%. Data are represented as mean ± SEM. N=3. ** p<0.01 or *** p<0.001 compared to a vehicle control.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to tunicate extracts and uses thereof in anti-allergy applications. More specifically, the present invention relates to tunicate extracts and their use in allergic inflammation therapy.

The present invention provides a method of preparing hexane and acetone tunicate extracts comprising:

a) extracting a *Styela clava* or *Ciona intestinalis* tunicate sample with hexane, acetone, and/or methanol; and

b) collecting the tunicate hexane, acetone, and/or methanol extracts obtained in step a).

The tunicate sample may be obtained or harvested from any suitable tunicate species. Tunicates, also referred to as "urochordates", are marine organisms belonging to a group of underwater filter feeders with incurrent and excurrent siphons. Of particular interest in the present invention are tunicates of species *Styela clava* and *Ciona intestinalis*, or any combination thereof. In one non-limiting example, the tunicate sample may be *Styela clava*; in another non-limiting example, the tunicate sample may be *Ciona intestinalis*. Tunicate samples may be harvested from coastal areas globally, including but not limited to the coastal areas of North America, Europe, Asia, and South America. For example and without wishing to be limiting in any manner, the tunicate sample may be obtained from coastal areas in eastern Canada or the north-eastern USA. The tunicate sample may be collected at any suitable time of year. For example, and without wishing to be limiting in any manner, the tunicate sample may be collected between about September and May. Alternatively, the tunicate samples may
be obtained from cultured tunicates. The tunicates may be cultured in an appropriate environment; methods for cultivating tunicates would be known to those of skill in the art. Optionally, the cultivated tunicates may be bioengineered (via hybridization or genetic engineering) to produce extracts exhibiting increased bioactivity characteristics.

5  The tunicate sample may be extracted directly with hexane, acetone, or methanol to obtain a hexane, acetone, or methanol extract, respectively; alternatively, the tunicate sample may be sequentially extracted with various solvents, hexane, acetone, and methanol. For example, and without wishing to be limiting in any manner, the tunicate sample may be sequentially extracted with hexane, acetone, methanol, and optionally hot water. By the term "sequentially extracted", and according to this example, it is meant that the tunicate sample is first extracted with hexane, and the resulting solid residue is then extracted with acetone; the solid residue resulting from acetone extraction is then extracted with methanol; optionally, the solid residue resulting from methanol extraction may be further extracted using hot water. The tunicate sample may be extracted with additional or different solvents, not limited to those included herein. As would be understood by a person of skill in the art, the sample may be extracted in a different order than that listed herein.

For hexane, acetone and methanol extractions, the tunicate sample or the solid residue may be mixed with a suitable amount of the respective solvent. For example, and without wishing to be limiting in any manner, the amount of solvent may be in a ratio of about 1:20 original sample weight:solvent, or a ratio in the range of about 1:5 to 1:100; in a non-limiting example, the ratio may be about 1:5, 1:10, 1:15, 1:20, 1:25, 1:30, 1:35, 1:40, 1:45, 1:50, 1:55, 1:60, 1:65, 1:70, 1:75, 1:80, 1:85, 1:90, 1:95, 1:100, or any ratio therebetween. As would be understood by one of skill in the art, the ratio is determined from the weight of the original tunicate sample. In a specific, non-limiting example, 1 g of sample or solid residue may be mixed with 20 ml solvent. The extraction may proceed for any suitable time; for example, and without wishing to be limiting, the extraction time may be about 30 minutes, or for a time in the range of about 5 min to 2 hrs; in a non-limiting example, the extraction time may be about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 70, 80, 90, 100, 110, 120 minutes, or any time therebetween. The extraction may proceed at any suitable temperature; for example and without wishing to be limiting, the temperature may be about room temperature, or between about room temperature and the boiling point of the solvents used. As would be known to a person of skill in the art, the extraction temperature will vary based on other conditions and solvents used; it would be within the capabilities of the skilled person to adjust the temperature appropriately. The extraction may incorporate any acceptable physical or mechanical method known in the art; for
example, and without wishing to be limiting, the extraction may incorporate stirring and/or sonication. Additionally, the extraction may also include immersing and/or refluxing.

For the optional water extraction step, the solid residue resulting from a previous extraction step may be mixed with hot water. The water may be at a temperature between about 30°C and 100°C; for example, and without wishing to be limiting, the water may be at about 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100°C, or any temperature there between or any range of temperatures defined by these values. In a specific, non-limiting example, the water may be at about 80 to 90°C, in another non-limiting example the water may be at 90°C. The water is mixed with the solid residue in an a ratio of about 1:20 original sample weight:water, or in a ratio in the range of about 1:5 to 1:100; in a non-limiting example, the ratio may be about 1:5, 1:10, 1:15, 1:20, 1:25, 1:30, 1:35, 1:40, 1:45, 1:50, 1:55, 1:60, 1:65, 1:70, 1:75, 1:80, 1:85, 1:90, 1:95, 1:100, or any ratio therebetween. In a specific, non-limiting example, 1 g of solid residue may be mixed with 20 mL water. The water extraction may proceed for any suitable time; for example and without wishing to be limiting, the water extraction may proceed for a time in the range of about 30 min up to several hrs. The water extraction may also incorporate microwave, sonication, or other approaches facilitating extraction that are known in the art. Following extraction, the sample may be cooled then filtered using any suitable filtration method known in the art, such as paper filtration or vacuum filtration. Alternatively, centrifugation may also be used for separation of water extract from solid residue.

Following each extraction step, the sample mixture may be filtered using any suitable filtration method known in the art, such as paper filtration or vacuum filtration. Alternatively, separation of liquid extract and solid residue may be done by centrifugation. Following filtration, the flow-through or supernatant fraction is collected and is labelled as the respective extract after solvent is removed, while the solid filtrate or precipitate (also referred to herein as the solid residue) is subjected to further extraction.

Prior to extraction, the fresh tunicate may be dried, lyophilized and/or homogenized by any suitable method known in the art. For example, and without wishing to be limiting in any manner, the tunicate sample may be homogenized using a commercially available homogenizer, grinder, blender, etc. The tunicate sample may be dried or lyophilized, also referred to herein as "freeze-dried", using any suitable method of freeze-drying or other drying methods known in the art, such as but not limited to oven drying, drum dryer, rotary evaporator, centrifugal evaporator, or conveyor dryer.
The dry tunicate tissue may optionally be milled by any suitable method known in the art. For example, and without wishing to be limiting in any manner, the tunicate sample may be milled using a commercially available grinder, or manually ground using a mortar and pestle. Subsequent to milling, the sample may be immediately subjected to extraction, or may be stored. The sample may be stored under any suitable conditions, for example and without wishing to be limiting in any manner, the sample may be stored at -80°C to room temperature. In a specific, non-limiting example, the sample may be stored at -80°C, -20°C, 4°C, 10°C, 25°C, or room temperature.

The acetone extract resulting from the extraction method described herein may optionally be processed or refined further using any suitable method in the art; for example, and without wishing to be limiting in any manner, the acetone extract may be subjected to chromatography, drying by using rotary evaporator or centrifugal vacuum evaporator, or any combination thereof. The acetone extract may optionally also be further extracted or fractionated. Such further fractionation may be accomplished using any suitable method known in the art, for example, but not limited to the method described herein, or the method as follows. In a non-limiting example, the acetone extract may be submitted to silica gel chromatography or thin layer chromatography using an appropriate solvent, for example but not limited to MeOH/CH₂Cl₂, to yield sub-fractions (first stage). Other chromatography methods, such as but not limited to normal or reverse phase chromatography, may also be used to yield first stage sub-fractions. Methods for silica gel chromatography would be known to those of skill in the art. The first stage sub-fractions may optionally be further processed by any suitable method known in the art, for example, but not limited to chromatography, drying by rotary evaporator, centrifugal vacuum evaporator, or any combination thereof; optionally, the first stage sub-fractions may also be further fractionated, using any suitable method known in the art, for example, but not limited to silica gel chromatography or thin layer chromatography using an appropriate solvent, for example but not limited to MeOH/CH₂Cl₂, to yield sub-fractions (second stage). Other chromatography methods, such as but not limited to normal or reverse phase chromatography, may also be used to yield second stage sub-fractions.

The methanol extract resulting from the extraction method described herein may optionally be processed or refined further using any suitable method in the art; for example, and without wishing to be limiting in any manner, the methanol extract may be subjected to chromatography, drying by using rotary evaporator or centrifugal vacuum evaporator, or any combination thereof. The methanol extract may optionally also be further extracted or fractionated. Such further fractionation may be accomplished using any suitable method known in the art, for example, but not limited to the method described herein, or the method as
follows. In a non-limiting example, the methanol extract may be submitted to reverse phase chromatography (such as, but not limited to C18 column) or thin layer chromatography using an appropriate solvent, for example but not limited to MeOH/CH₂Cl₂, to yield sub-fractions (first stage). Other chromatography methods, such as but not limited to silica gel (or other resin) chromatography, may also be used to yield first stage sub-fractions. Methods for reverse phase or thin layer chromatography would be known to those of skill in the art.

The present invention also provides a hexane tunicate extract, fraction, or sub-fraction thereof obtained from *Styela clava*. The tunicate hexane extract, fraction, or sub-fraction thereof may be obtained using the methods as described herein. The tunicate hexane extract, fraction, or sub-fraction thereof of the present invention may be characterized by the proton NMR spectrum as shown in Figure 1A (3).

The present invention also provides an acetone tunicate extract, fraction, or sub-fraction thereof obtained from *Styela clava* or *Ciona intestinalis*. The tunicate acetone extract, fraction, or sub-fraction thereof may be obtained using the methods as described herein.

The tunicate acetone extract, fraction, or sub-fraction thereof of the present invention may be characterized by any one of the proton NMR spectrum as shown in Figure 1B (3), (4), or (5), or Figure 13. The acetone extract or sub-fraction thereof of the present invention may comprise hexadecanoic acid (palmitic acid), octadecanoic acid (stearic acid), eicosa-5,8,11,14,17-pentaenoic acid (EPA), and 4,7,10,13,16,19-docosahexaenoic acid (DHA) as the main fatty acid components.

The present invention additionally provides a methanol tunicate extract, fraction, or sub-fraction thereof obtained from *Styela clava*. The tunicate methanol extract, fraction, or sub-fraction thereof may be obtained using the methods as described herein. The tunicate methanol extract, fraction, or sub-fraction thereof of the present invention may be characterized by the proton NMR spectrum as shown in Figure 1C (5).

The present invention further provides a method of inhibiting human mast cell degranulation and activation comprising administering one or more than one of the hexane, acetone, or methanol tunicate extract, fraction, or sub-fraction thereof as described herein, any mix of compounds or single compounds obtained therefrom, or any combination thereof, to a subject in need thereof.

The present invention also provides a method of inhibiting mast cell expression of TNF and MCP-1 comprising administering one or more than one of the acetone tunicate extract,
fraction, or sub-fraction thereof as described herein, any mix of compounds or single compounds obtained therefrom, or any combination thereof, to a subject in need thereof.

The extract may be administered in an effective amount to obtain the desired effect. As would be known to those of skill in the art, a specific dosage will vary based on several factors such as age and body weight. For example, and without wishing to be limiting in any manner, an effective dosage may be in the micromolar range.

It is presently shown that tunicate extracts PTC-1 100 and PTC-1 200, INH-OS-78A, INH-OS-79A, and INH-OS-78M inhibit human mast cell degranulation and activation. PTC-1 200 is more potent than PTC-1 100 and these extracts have different modes of action. PTC-1 100 may act directly on compound 48/80, thereby interfering with its ability to activate mast cell signalling pathways. PTC-1 100 effects on degranulation are transient and are sensitive to the “wash out” effect, while the effect of PTC-1 200 on degranulation are more long-term and likely involve changes in human mast cell phenotype. PTC-1 200 further inhibits mast cell expression of TNF and MCP-1, important pro-inflammatory mediators. INH-OS-78A and -79A and INH-OS-78M inhibited mast cell degranulation by more than 80%, with the most potent effect noted by INH-OS-78-M - which reduced degranulation to background (unstimulated and resting) levels.

The present invention will be further illustrated in the following examples. However, it is to be understood that these examples are for illustrative purposes only and should not be used to limit the scope of the present invention in any manner.

Example 1: Preparation of tunicate extracts

Tunicate samples can be collected from coastal areas in eastern Canada (for example Nova-Scotia, New-Brunswick, Prince Edward Island, Newfoundland) or the northeastern USA (for example Maine, Massachusetts, Connecticut, Rhode Island, New York, New Jersey).

Three tunicate samples were obtained from PEI Aquaculture and Fisheries Research Initiatives, Inc from different sites in PEI, in 2008:

1. 081 006MB: *Styela clava* (Clubbed tunicate), collected on October 6, 2008 at Malpeque Bay;
2. 081112BR: *Ciona intestinalis* (Vase tunicate) with some *Styela clava*, collected on November 12, 2008 at Brudenell River; and
• 081203SH: mix of *Botryloides violaceus* (Violet tunicate) and *Botryllus schlosseri* (Golden star tunicate), collected on December 3, 2008 at Savage Harbour.

Two pure species of tunicate samples were obtained from PEI Aquaculture and Fisheries Research Initiatives, Inc in 2010:

5  
- INH-OS-78: *Styela clava*, collected in January/February; and
- INH-OS-79: *Ciona intestinalis*, collected in April/May.

These samples were extracted with hexane, acetone, methanol and water, sequentially as described below. All samples were subject to global profiling of bioactive compounds using NMR (see Example 2).

The samples were processed, freeze-dried, milled, and kept at -80°C. The dry powder of each tunicate sample was extracted sequentially using four different solvents: hexane, acetone, MeOH, and hot water. The tunicate sample was mixed 1:20 in hexane (1g in 20ml solvent), stirred for 30min at room temperature and then sonicated for 30min. The mixture was then filtered through filtration paper and the flow-through was collected. The extraction was repeated once and the solvent in the combined liquid extract was removed by rotary evaporator then centrifugal evaporator (Genevap) to yield the hexane extract (noted by an "H" suffix). The solid residue was then mixed 1:20 (original sample weight) in acetone and stirred and then sonicated for 30min; the mixture was filtered and the flow-through was collected. Similarly, the extraction was repeated once and the solvent in the combined liquid extract evaporated (acetone extract, noted by "A" suffix). The solid residue was then resuspended in MeOH (1:20), followed by 30min stirring and sonication; the mixture was then filtered and the flow-through was collected. The extraction was repeated once and the solvent in the combined liquid extract evaporated (MeOH extract, noted by "M" suffix). Finally, the solid residue was mixed with hot water (1:20) and stirred at 80-90°C for 5hrs. After cooling to room temperature and filtration, the flow-through was mixed with 3 volumes of 95% EtOH and put in ice bath for 2 hrs. The precipitate was then filtered, washed with EtOH, and collected by centrifugation. The solvent was removed from each extract by centrifugal evaporator (Genevap) and freeze-dryer to yield water extracts (noted by "W" suffix).

Results of the extractions are shown in Table 1.

Table 1. Tunicate species and extracts
<table>
<thead>
<tr>
<th>Sample</th>
<th>Sample Mass</th>
<th>Extract</th>
<th>Extract Name</th>
<th>Extract Mass</th>
<th>% by mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>081006MB</td>
<td>40.204</td>
<td>Hexane</td>
<td>PTC-1 100 or MB-H</td>
<td>0.475</td>
<td>1.181</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Acetone</td>
<td>PTC-1 200 or MB-A</td>
<td>0.203</td>
<td>0.505</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methanol</td>
<td>PTC-1 300 or MB-M</td>
<td>8.509</td>
<td>21.165</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Water</td>
<td>PTC-1400 or MB-W</td>
<td>3.373</td>
<td>8.39</td>
</tr>
<tr>
<td>081112BR</td>
<td>39.995</td>
<td>Hexane</td>
<td>PTC-21 00 or BR-H</td>
<td>0.726</td>
<td>1.815</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Acetone</td>
<td>PTC-2200 or BR-A</td>
<td>0.454</td>
<td>1.135</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methanol</td>
<td>PTC-2300 or BR-M</td>
<td>11.582</td>
<td>28.959</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Water</td>
<td>PTC-2400 or BR-W</td>
<td>1.411</td>
<td>3.528</td>
</tr>
<tr>
<td>081203SH</td>
<td>40.015</td>
<td>Hexane</td>
<td>PTC-31 00 or SH-H</td>
<td>0.417</td>
<td>1.042</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Acetone</td>
<td>PTC-3200 or SH-A</td>
<td>0.257</td>
<td>0.642</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methanol</td>
<td>PTC-3300 or SH-M</td>
<td>11.835</td>
<td>29.576</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Water</td>
<td>PTC-3400 or SH-W</td>
<td>1.72</td>
<td>4.298</td>
</tr>
<tr>
<td>INH-OS-78</td>
<td>40.007</td>
<td>Hexane</td>
<td>INH-OS-78-H</td>
<td>0.424</td>
<td>1.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Acetone</td>
<td>INH-OS-78-A</td>
<td>0.224</td>
<td>0.56</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methanol</td>
<td>INH-OS-78-M</td>
<td>8.46</td>
<td>21.146</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Water</td>
<td>INH-OS-78-W</td>
<td>2.688</td>
<td>6.719</td>
</tr>
<tr>
<td>INH-OS-79</td>
<td>40.003</td>
<td>Hexane</td>
<td>INH-OS-79-H</td>
<td>0.151</td>
<td>0.377</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Acetone</td>
<td>INH-OS-79-A</td>
<td>0.127</td>
<td>0.317</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methanol</td>
<td>INH-OS-79-M</td>
<td>19.601</td>
<td>48.999</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Water</td>
<td>INH-OS-79-W</td>
<td>2.078</td>
<td>5.195</td>
</tr>
<tr>
<td>INH-OS-78</td>
<td>195.18</td>
<td>Hexane</td>
<td>INH-OS-78-H</td>
<td>1.15</td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Acetone</td>
<td>INH-OS-78-A</td>
<td>0.549</td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methanol</td>
<td>INH-OS-78-M</td>
<td>48.83</td>
<td>25.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Water</td>
<td>INH-OS-78-W</td>
<td>17.61</td>
<td>9.02</td>
</tr>
<tr>
<td>INH-OS-79</td>
<td>94.54</td>
<td>Hexane</td>
<td>INH-OS-79-H</td>
<td>0.363</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Acetone</td>
<td>INH-OS-79-A</td>
<td>0.64</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methanol</td>
<td>INH-OS-79-M</td>
<td>56.69</td>
<td>59.97</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Water</td>
<td>INH-OS-79-W</td>
<td>7.484</td>
<td>7.92</td>
</tr>
</tbody>
</table>

**Example 2: Proton NMR characterization of tunicate extracts**

The extracts obtained in Example 1 were submitted to Proton NMR profiling.

Briefly, 2 mg of the hexane, acetone, and MeOH extracts were separately dissolved in 100 µL DMSO-d6, while 2 mg of water extracts were dissolved in 100 µL D2O. Sample solutions were transferred to 1.7 mm NMR tubes and proton NMR spectra were acquired on Bruker Avance III 600 MHz NMR spectrometer (Bruker Corporation, East Milton, ON) operating at 600.28 MHz 1H observation frequency and a temperature of 25±0.2°C. The signals were acquired, processed and analyzed using TopSpin® NMR data acquisition and processing Software (Bruker Biospin Ltd, East Milton, ON) integrated with the spectrometer.
Results in the form of NMR spectra are shown in Figure 1. General proton NMR profiling indicated that there is certain level of similarity in the main components of extracts prepared from different tunicate species. Hexane and acetone extracts comprised fatty acids (including polyunsaturated FAs), while the water extracts showed the presence of polysaccharides or protein glycans as the main components.

Example 3: Effect of tunicate extracts on human mast cell activation

The tunicate extracts Example 1 were screened for their ability to inhibit human mast cell activation. Specifically, tunicate extracts were tested for their ability to inhibit human mast cells activated by compound 48/80, a "classical" non-receptor-mediated mast cell stimulus.

LAD2 cells (human mast cell line) were washed in StemPro media (containing 100 ng/mL human recombinant stem cell factor), re-suspended in buffer, and pre-treated with tunicate extracts at 100 µg/mL for 30 minutes at 37°C. Cells were then stimulated with compound 48/80 (Sigma-Aldrich; 1 µg/mL for 2008 samples; 0.3125 pg/mL for 2010 samples; unless otherwise stated) at 37°C for 0.5 hr. Degranulation was determined by measuring the release of β-hexosaminidase, a mast cell granule marker. The β-hexosaminidase released into the supernatants and in cell lysates was quantified by measuring the hydrolysis of p-nitrophenyl N-acetyl-p-D-glucosamide (Sigma Aldrich) in 0.1 M sodium citrate buffer (pH 4.5) for 90 min at 37°C. Hydrolysis was measured as a change in absorbance at Aβ650 on a Varioskan spectrophotometric plate reader. The percentage of β-hexosaminidase release was calculated as a percent of total content.

Figure 2 shows that PTC-1100 dose-dependently inhibited human mast cell degranulation, with statistically significant inhibition occurring at 25 pg/mL tunicate fraction (inhibition of approximately 20%). This is a relatively low dose for a crude extract and implies that the compound(s) of interest may be very potent. It is also worth noting that the human mast cells were pre-treated with the crude extract for only 30 min prior to activation. Many second messenger-specific inhibitors (such as inhibitors of signalling molecules) require at least 30 min to inhibit mast cell degranulation. As a result, it is entirely possible that the compound(s) of interest in this extract binds to and inhibits important signalling molecules involved in the degranulation process.

Compared to PTC-1100, PTC-1200 was a more potent inhibitor of human mast cell degranulation (Fig. 3). PTC-1200 dose-dependently inhibited human mast cell degranulation, with statistically significant inhibition occurring at 25 µg/mL of crude extract (inhibition of approximately 30%).
Since both PTC-1 100 and PTC-1 200 exhibited inhibitory activity, the combined effects PTC-
1 100 and PTC-1 200 on human mast cell activation was explored next. Human mast cells
were pre-treated with PTC-1 200 for 30 min then activated with compound 48/80 (0.3125
µg/mL) and β-hex release was measured as described previously. Together, the two extracts
synergistically inhibited human mast cell activation (Fig. 4). At 50 µg/mL, PTC-1 100 and PTC-
1 200 together inhibited degranulation by 55% which was more than their additive effect. The
effect of PTC-1 100 and PTC-1 200 at 25 µg/mL was only significant when the extracts were
added together.

The results for fractions of INH-OS-78 and -79 (Figure 5) indicate that INH-OS-78A and -79A
and INH-OS-78M inhibited mast cell degranulation by more than 80%, meaning reduced the
release of granule-associated mediators that cause an allergic reaction (eg. histamine). INH-
OS-78-M was the most potent inhibitor of mast cell activation and reduced degranulation to
background (unstimulated and resting) levels.

The assays that produced the data in Figures 2-4 were designed to determine the effects of
tunicate extracts on human mast cell in the presence of activator (compound 48/80). However,
a direct interaction between compound 48/80 and the extracts could compromise the
stimulatory activity of compound 48/80 itself, irrespective of effects on the cells.

For this reason, cells were pre-treated with extracts for different time points and then activated
with compound 48/80. This is referred to as the "wash-out" experiments because the mast
cells are activated after the extracts have been removed by washing the cells. PTC-1 100 did
not have a significant effect on mast cell degranulation when it was washed out (Fig. 6), i.e.,
PTC-1 100 likely has a direct effect on compound 48/80. It is also likely that PTC-1 100 inhibits
an intracellular signalling molecule, but binds with a high Kd and therefore must be in culture in
order to mediate its effect. Interestingly, PTC-1 200 was remarkably different in its effect on
human mast cell degranulation (Fig. 7). When human mast cells are pre-treated with PTC-
1 200 for 30 min and then PTC-1 200 was removed prior to activation with compound 48/80,
PTC-1 200 did not inhibit mast cell degranulation.

However, pre-treatment of human mast cells with PTC-1 200 for 2 hr, removal of PTC-1 200
and subsequent activation by compound 48/80 inhibited degranulation. This suggests that
PTC-1 200 may have two independent effects - one similar to the properties of PTC-1 100 and
another inhibitory effect that depends upon a fundamental change in cell phenotype (possibly
due to changes in gene expression), which would ultimately result in a decreased sensitivity to
activation signals. Interestingly, 8 hr is the optimum pre-treatment time for PTC-1 200's
inhibitory effects - once again suggesting that gene expression systems may be involved. PTC-1 200 retains its inhibitory potency even after 72 hr of pre-treatment.

The interaction between PTC-1 100 and PTC-1 200 was tested as a function of the wash-out effect over time. When human mast cells were pre-treated with both extracts for only 30 min, washed and stimulated with compound 48/80, there was no significant effect on degranulation (Fig. 8). However, when human mast cells were pre-treated with the combined extracts for 2 hr, washed and stimulated with compound 48/80, the extracts significantly inhibited degranulation. This inhibitory effect was observable after 72 hr of pre-treatment.

These results indicate that the extracts have two effects on human mast cell degranulation. The first effect is acute, occurs within 30 min and may be directly related to interference with compound 48/80. The second effect is long-term and may involve changes in mast cell protein synthesis and phenotypic changes that result in a decreased sensitivity to stimulus.

**Example 4: Long-term effect of tunicate extracts on human mast cell function**

The long-term effects of tunicate extracts were tested on mast cell functions. The degranulation assay has a built-in cytotoxicity control such that cytotoxic effects of compounds can be monitored. Based on degranulation results of Example 3, it was unlikely that the extracts were cytotoxic to human mast cells. However, to confirm these results, the cytotoxicity effects on human mast cells were determined using the MTT assay and microscopic observation.

**MTT protocol:** 5 x 10⁴ cells/well were plated into a 96 well plate in StemPro media containing 100 ng/mL stem cell factor to a final volume of 100 µL/well. Cells were rested for 2 hr, then drug was added to each well and cells were incubated at 37°C, 5% CO₂ for indicated time periods. 10 µL of MTT solution was added to each well and thoroughly mixed into the media. Plate was incubated at 37°C, 5% CO₂ for 2 hr to allow the MTT to be metabolized. Formazan crystals were solubized by adding solubization reagent (10% Triton X-100 and 0.1 N HCl in anhydrous isopropanol) and read at 570 nm with a background absorbance at 690 nm for standardization.

Human mast cells treated with both PTC-1 100 and PTC-1 200 for up to 72 hr showed no changes in cell viability (as measured by trypan blue) or morphology. Similarly, MTT assay showed no changes in cell viability even after 72 hr of treatment with extracts (Fig. 9).

The effect of extracts on human mast cell gene expression of a pro-inflammatory cytokine (tumor necrosis factor; TNF) and chemokine (monocyte chemoattractant protein-1 ; MCP-1)
was analyzed. Human mast cells were pre-treated with extracts for 30 min, activated with compound 48/80 for 3 hr and TNF and MCP-1 expression was analyzed by quantitative PCR using minor-binding groove primer/probe sets.

RNA Extraction: Total RNA was isolated from each preparation using the guanidine thiocyanate method. One mL of a mixture of guanidine thiocyanate and phenol in a monophase solution, was added to 1 x 10^6 cells and vortexed at 150 rpm effectively dissolving DNA, RNA, and protein. Two hundred µl of chloroform was added and the sample for vortexed (150 rpm) for 15 seconds and allowed to stand at room temperature for 2-15 minutes. Sample was centrifuged at 12,000 x g for 15 minutes at 4°C separating the sample into 3 phases: an aqueous phase containing the RNA, the interphase containing DNA, and an organic phase containing proteins. Each component can then be isolated after separating the phases. Aqueous phase was removed, mixed with 500 µl of isopropanil and allowed to stand for 10 minutes at room temperature. Sample was centrifuged at 12,000 x g for 10 minutes at 4°C. RNA pellet was washed with 75% ethanol and dried under vacuum for 10 min. RNA was resuspended in sterile, DNase- and RNase-free H₂O and quantified by A₂₆₀/A₂₈₀ readings.

cDNA synthesis and qPCR: Five micrograms of total cellular RNA was reverse transcribed using M-MLV reverse transcriptase and OligoDT primers (Invitrogen). Gene expression was analyzed using realtime PCR on an ABI7500 SDS system. Fifty ng of cDNA was used in each quantitative PCR assay. Primer sets for PCR amplifications were designed using the Primer Express software (Perkin-Elmer Applied Biosystems). All reactions were performed in triplicate for 40 cycles as per the manufacturer’s recommendation. Results are expressed as relative mRNA corrected with reference to GAPDH mRNA as an internal control (Vandesompele et al., 2002).

At 50 µg/ml, PTC-1200 inhibited human mast cell expression of TNF, while PTC-1 100 did not have a significant effect (Fig. 10). Together, the extracts inhibited compound 48/80-induced TNF expression by more than 50%. The PTC-1 100 and PTC-1200 combination activated some TNF expression from resting cells but this value (52.72 fold) was relatively minor compared to compound 48/80 stimulated cells (18,000-fold). Similar to the data for TNF, PTC-1200 significantly inhibited compound 48/80-induced MCP-1 expression by human mast cells (Fig. 11). Once again, PTC-1 100 had no effect on compound 48/80 induced MCP-1 expression. Individually and in combination with PTC-1200, PTC-1 100 had no significant effect on MCP-1 expression.

These data suggest that PTC-1200 is inhibiting the pathways leading to TNF and MCP-1 gene expression, both of which are controlled by the NF-κB signalling pathways.
Further to the data showing PTC-1 200 inhibition of TNF gene expression by quantitative PCR, the effect of tunicate extracts on TNF protein expression was confirmed using ELISA analysis.

Enzyme-linked immunosorbent assay (ELISA): The capture antibody (anti-TNF or anti-MCP-1) was diluted in 0.1 M bicarbonate buffer, pH 9.2 and then 50 μl was added to each well of a 96 well microtiter plate (Corning Costar 9018). The antibody coated plate was covered with Paraffin and incubated at 4°C for 12 hr. The plate was washed 5 times with wash buffer (100 mM phosphate buffer, 150 mM NaCl, 0.2% BSA and 0.05% Tween 20) and non-specific protein binding was blocked with 100 μl of blocking buffer (100 mM phosphate buffer, pH 7.2, 1% BSA and 0.5% Tween-20) for 30 min at room temperature. The plate was emptied and washed three times with wash buffer. The antigen solution (human recombinant TNF or MCP-1) was diluted in antigen buffer (100 mM phosphate buffer, 150mM NaCl) and then added to the plate in a volume of 50 μl per well. The plate was incubated at room temperature for one hour and washed three times with wash buffer. The enzyme-labelled antibody against antigen (TNF or MCP-1) was diluted appropriately in 0.1 M bicarbonate buffer, pH 9.2 and then 50 μl is added to each well and incubated at room temperature for 30 min. The plate was washed 7 times with wash buffer and 100 μl per well of substrate solution was added to each well. Plates were incubate at room temperature for 15 minutes and 50 μl of stop solution was added to each well. Plates were read at 450 nm with a reference absorbance of 570 nm.

Compound 48/80 induced significant production of TNF; this was inhibited almost completely by both PTC-1 100 and PTC-1 200 - a reduction of over 95% in most cases (Fig. 12).

Example 5: Further fractionation and characterization of tunicate extracts

Based on the results of Example 5, the acetone extracts (INH-OS-78A and INH-OS-79A), and MeOH extract INH-OS-78M were selected for further fractionation.

For INH-OS-78A, the extract (0.4 g) was loaded to silica gel column (12 g), eluted with three times column volume each of 2%, 5%, 10%, 15%, and 25% MeOH in CH₂Cl₂ to obtain INH-OS-78A-1 (11 mg), 78A-2 (34 mg), 78A-3 (155 mg), 78A-4 (128 mg), and 78A-5 (30 mg).

As INH-OS-78A-2 was shown to have higher bioactivity (see Example 7), further fractionation was done on preparative silica gel TLC plate. INH-OS-78A-2 (30 mg) was dissolved in acetone, loaded to 20 cm * 20 cm silica gel TLC plate and then developed with CH₂Cl₂-MeOH (10:1). Ten sub-fractions were obtained after cutting the silica gel bands and eluting with CH₂Cl₂-MeOH (1:1); notably INH-OS-78A-2-1 (6 mg) at Rₗ 0.06 were found to be the most active fraction (see Example 7).
For INH-OS-79A, the extract (0.4 g) was loaded to silica gel column (12 g), eluted with three times of column volume each of 2%, 5%, 10%, 15%, and 25% MeOH in CH₂Cl₂ to obtain INH-OS-79A-1 (4.5 mg), 79A-2 (69.2 mg), 79A-3 (5.5 mg), 79A-4 (27.1 mg), and 79A-5 (29.6 mg).

For INH-OS-78M, 2 g of the extract was fractionated on a C18 column, eluted with aqueous MeOH (5, 25, 50, and 100%), and MeOH-CH₂Cl₂ to obtain INH-OS-78M-1 (1.5 g), 78M-2 (355.3 mg), 78M-3 (87.5 mg), 78M-4 (156.2 mg), and 78M-5 (226.9 mg).

For proton NMR characterization, samples were dissolved in CDCl₃, and analyzed on a Bruker Avance III 600 MHz NMR spectrometer. Proton NMR spectral comparison of all the acetone extracts, presented in Example 2, showed that they have similar composition profiles (Figure 1B). Most of the peaks indicate the presence of fatty acids, including unsaturated fatty acids. Proton NMR spectra (Figure 13) of the bioactive fraction (78A-2) and the sub-fractions (78A-2-1 and 78A-2-2) of acetone extract (INH-OS-78A) further confirmed the presence of saturated and unsaturated fatty acids in these fractions.

GC-MS analysis of the bioactive fraction (78A-2) and the sub-fractions (78A-2-1 and 78A-2-2) of acetone extract (INH-OS-78A) was also performed. 1 mg sample was dissolved in 1 mL hexane; 100 μL 2N potassium hydroxide in MeOH was added and the mixture vortexed for 30 sec. After centrifugation, the supernatant was transferred to autosampler vial for analysis. The GC-MS analyses were performed on an Agilent 6890GC equipped with a 5973 MSD. Agilent 122-2361 DB-23 capillary column (60 m x 250 μm, 0.15pm film thickness) was used under the following conditions: oven temperature program from 130°C (1 min) to 170°C at 6.5°C/min, then 215°C at 2.75°C (12 min), then to 230°C at 40.0°C/min (3.1 1 min), total run time 39 min; "split mode" ratio 1:10; carrier gas helium, flow rate 1.2 mL/min; temperature of injector 250°C; ion source temperature 230°C, mass range of m/z 100-400 and transfer line temperature 250°C. The constituents were identified in comparison with their spectra of mass with those gathered in NIST library.

The GC-MS analysis results of the bioactive fraction (78A-2) and its sub-fractions (78A-2-1 and 78A-2-2) are shown in Table 2. The major fatty acid components in these bioactive fractions include hexadecanoic acid (palmitic acid), octadecanoic acid (stearic acid), and polyunsaturated fatty acids eicosa-5,8,11,14,17-pentaenoic acid (EPA) and 4,7,10,13,16,19-docosahexaenoic acid (DHA).

Table 2. GC-MS results of INH-OS-78-A-2, INH-OS-78-A-2-1, and INH-OS-78-A-2-2 tunicate acetone extract

<table>
<thead>
<tr>
<th>Peak R.T.</th>
<th>78A- area</th>
<th>78A- area</th>
<th>78A- area</th>
<th>Compound Name</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
For MS/MS analysis, sub-fractions 78A-2-1 and 78A-2-2 were analysed on an Agilent 1200 HPLC coupled with an Applied Biosystems 4000 QTRAP LC/MS/MS system equipped with a Turbo V ion source. The mobile phase was 10mM ammonium acetate in MeOH, flow rate 0.2ml/min and sample volume was 0.2uL. Samples were introduced by flow injection analysis with no LC column. Typical 4000 QTRAP parameters were as follows: current gas 30, GS1 40, GS2 60, CAD gas 6, DP 100 and CE 25. The ion source temperature was typically 400°C. Based on the fatty acid distribution obtained from the GC analyses, precursor ion scans were conducted using m/z for the deprotonated free acid, and results are shown in Table 3.

Table 3. Fragments observed from MS/MS

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Precursor scan m/z</th>
<th>Results m/z</th>
<th>MS2 scan m/z</th>
<th>Results m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexadecanoic acid</td>
<td>255.4</td>
<td>589.7</td>
<td>589.7</td>
<td>255.1, 333.3</td>
</tr>
<tr>
<td>Eicosanoic acid</td>
<td>311.5</td>
<td>645.9</td>
<td>645.9</td>
<td>311.2, 333.4</td>
</tr>
<tr>
<td>Octadecanoic acid</td>
<td>283.5</td>
<td>618.0</td>
<td>618.0</td>
<td>283.1, 333.4</td>
</tr>
<tr>
<td></td>
<td>281</td>
<td>615.8</td>
<td>616</td>
<td>281.2, 333, 350</td>
</tr>
<tr>
<td></td>
<td>297.5</td>
<td>631.7</td>
<td>631.7</td>
<td>297.4, 333.2</td>
</tr>
</tbody>
</table>
The 3 major fatty acids in this sub-fraction are all linked to a molecular fragment with m/z 334, however, the detailed structure of this fragment could not be characterized at this stage.

**Example 7: Effect of tunicate extract sub-fractions on human mast cell activation**

The ability of the sub-fractions of water extract from Example 6 to inhibit human mast cell activation was tested. The methods utilized are essentially as described in Example 5. Results are shown in Figures 14-15.

All fractions inhibited mast cell degranulation to certain a degree (Figure 14), with INH-OS-78A-2 yielding the best results - greater than 80% inhibition of mast cell degranulation - followed by INH-OS-79A-3, INH-OS-78A-5, and INH-OS-78A-4. Second sub-stage fractions of INH-OS-78A-2 (10 samples) were prepared and tested on mast cell degranulation (Figures 15A-B). The INH-OS-78A-2-XH-29-92-1 fraction yielded the best results among the second-stage fractions.

The embodiments and examples described herein are illustrative and are not meant to limit the scope of the invention as claimed. Variations of the foregoing embodiments, including alternatives, modifications and equivalents, are intended by the inventors to be encompassed by the claims. Furthermore, the discussed combination of features might not be necessary for the inventive solution.

**REFERENCES**

All patents, patent applications and publications referred to herein are hereby incorporated by reference.


CLAIMS:
1. A method of preparing hexane, acetone, and/or tunicate extracts comprising:
   a) extracting a *Styela clava* or *Ciona intestinalis* tunicate sample with hexane, acetone, and/or methanol; and
   b) collecting the hexane, acetone, and/or methanol tunicate extracts obtained in step a).

2. The method of claim 1, wherein the tunicate sample is a *Styela clava* tunicate sample.

3. The method of claim 1, wherein the tunicate sample is a *Ciona intestinalis* tunicate sample.

4. The method of any one of claims 1 to 3, wherein the tunicate sample is dried or lyophilized prior to step a).

5. The method of claim 2, wherein the dried or lyophilized sample is milled prior to step a).

6. The method of any one of claims 1 to 5, wherein step a) comprises sequentially extracting the tunicate sample with hexane, acetone, and methanol.

7. The method of any one of claims 1 to 6, wherein the hexane, acetone, and/or methanol extract is further processed by chromatography, drying by rotatory evaporator, centrifugal vacuum evaporator, or any combination thereof.

8. The method of any one of claims 1 to 7, wherein the acetone extract is further fractionated by reverse phase chromatography, silica gel chromatography, and/or thin layer chromatography to yield sub-fractions.

9. The method of any one of claims 1 to 7, wherein the methanol extract is further fractionated by reverse phase chromatography, silica gel chromatography, and/or thin layer chromatography to yield sub-fractions.

10. The method of claim 8 or 9, wherein the sub-fractions are further processed by chromatography, drying by rotatory evaporator, centrifugal vacuum evaporator, or any combination thereof.

11. An acetone tunicate extract, fraction, or sub-fraction thereof obtained from *Styela clava* or *Ciona intestinalis*.

12. An acetone tunicate extract, fraction, or sub-fraction thereof obtained by the method of any one of claims 1 to 8 or 10.
13. The acetone tunicate extract, fraction, or sub-fraction thereof of claim 11 or 12, characterized by any one of the proton NMR spectrum of Figure 1B (3), (4), or (5), or Figure 13.

14. The acetone tunicate extract, fraction, or sub-fraction thereof of any one of claims 11 to 13, comprising hexadecanoic acid (palmitic acid), octadecanoic acid (stearic acid), eicosa-5,8,11,14,17-pentaenoic acid (EPA), and 4,7,10,13,16,19-docosahexaenoic acid (DHA) as the main fatty acid components.

15. A methanol tunicate extract, fraction, or sub-fraction thereof obtained from *Styela clava*.

16. A methanol tunicate extract, fraction, or sub-fraction thereof obtained by the method of any one of claims 1 to 7 or 9 to 10.

17. The methanol tunicate extract, fraction, or sub-fraction thereof of claim 15 or 16, characterized by the proton NMR spectrum of Figure 1C (5).

18. A method of inhibiting human mast cell degranulation and activation comprising administering one or more than one of the tunicate extract, fraction, or sub-fraction thereof of any one of claims 11 to 17, any mix of compounds or single compounds obtained therefrom, or any combination thereof, to a subject in need thereof.

19. A method of inhibiting human mast cell expression of TNF and MCP-1 comprising administering one or more than one of the tunicate extract, fraction, or sub-fraction thereof of any one of claims 11 to 17, any mix of compounds or single compounds obtained therefrom, or any combination thereof, to a subject in need thereof.
FIG. 2

FIG. 3

5 / 12
FIG. 6

FIG. 7

7 / 12
FIG. 10

FIG. 11
FIG. 14

% β-hex release


unstimulated  stimulated
**INTERNATIONAL SEARCH REPORT**

International application No. PCT/CA2011/000205

A. **CLASSIFICATION OF SUBJECT MATTER**

IPC: A61K 35/56 (2006.01) . A61P 37/08 (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)

IPC: A61K 35/56 (2006.01) . A61P 37/08 (2006.01)

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

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

Canadian Patents Database, Total patent, Medline, Scopus (keywords: styela, clava, ciona, intestinalis, extract, tunicate, hexane, acetone, methanol, human, mast, cell, TNF, MCP-1).

C. **DOCUMENTS CONSIDERED TO BE RELEVANT**

<table>
<thead>
<tr>
<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>KR1020080018590A (INDUSTRY-ACADEMIC COOPERATION FOUNDATION OF KYUNGNAM UNIVERSITY) 28 February, 2008 (28-02-2008) Abstract</td>
<td>1, 2 and 4-17</td>
</tr>
<tr>
<td>X</td>
<td>JIN-TU, K. ET AL. Antioxidant and anticancer activities of extracts from Styela clava according to the processing methods and solvents. Journal of the Korean Society of Food Science and Nutrition. 8 May, 2006 (08-05-2006). Vol. 35, no. 3, pgs 278-283. ISSN: 1226-3311, Abstract.</td>
<td>1, 2, 4, 5 and 7-17</td>
</tr>
</tbody>
</table>

[ ] Further documents are listed in the continuation of Box C. [X] See patent family annex.

- * Special categories of cited documents:
  - “A” document defining the general state of the art which is not considered to be of particular relevance
  - “B” earlier application or patent but published on or after the international filing date
  - “C” document which may (but not: doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
  - “O” document referring to an oral disclosure, use, exhibition or other means
  - “P” document published prior to the international filing date but later than the priority date claimed
  - “Y” 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
  - “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
  - “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
  - “Z” document member of the same patent family

Date of the actual completion of the international search: 13 May 2011 (13-05-2011)

Date of mailing of the international search report: 6 June 2011 (06-06-2011)

Name and mailing address of the ISA/CA

Canadian Intellectual Property Office

Place du Portage 1, C114 - 1st Floor, Box PCT

50 Victoria Street

Gatineau, Quebec K1A 0C9

Facsimile No.: 001-819-953-2476

Authorized officer

Seema Bissoon-Haqqani (819) 934-7932
INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA20 11/000205

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of the first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claim Nos. 18 and 19

   because they relate to subject matter not required to be searched by this Authority, namely:

   Although claims 18 and 19 are directed to a method of treatment of the human/animal body, which this authority is not obliged to search under Rule 39. l(iv) of the PCT, the search has been carried out based on the alleged effects of the compositions referred to therein.

2. [ ] Claim Nos. :

   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claim Nos. :

   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claim Nos. :

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim Nos. :

   **Remark on Protest** [ ] The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

   [ ] The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

   [ ] No protest accompanied the payment of additional search fees.
<table>
<thead>
<tr>
<th>Patent Document</th>
<th>Publication Date</th>
<th>Patent Family Member(s)</th>
<th>Publication Date</th>
</tr>
</thead>
</table>