

Use of 3,6,7-trimethyluracil for ameliorating or treating MMP-9 associated conditions and inflammation

Related applications

This application derives priority from New Zealand patent application number 755138 incorporated herein by reference.

Field of Invention

The invention relates to compositions comprising 3,6,7-trimethyluracil, methods and uses thereof in preventing, ameliorating or treating inflammation and/or preventing, ameliorating or treating conditions associated with inflammation. More particularly, though not solely, the invention relates to compositions comprising 3,6,7-trimethyluracil and methods of use thereof in preventing, ameliorating or treating MMP-9 associated conditions, such as inflammation of the gastrointestinal tract and/or inflammatory conditions associated with the gastrointestinal tract.

Background of the Invention

Inflammation relating to the immune system can be beneficial but this is not always the case. It is often considered to be a negative reaction or a reaction to be avoided; especially in the context of the gastrointestinal system.

Inflammation is implicated in a wide range of gastrointestinal disorders. In a healthy gut, the intestinal mucosa is in a state of controlled response regulated by an intricate balance of pro-inflammatory and anti-inflammatory cytokines and cells. Disruptions to this balance can culminate in a sustained activation of the immune/non-immune responses, resulting in active inflammation and tissue destruction. Failure to prevent or resolve inflammation adequately is implicated in the pathogenesis of several diseases of the gastrointestinal tract including gastric ulcers, inflammatory bowel disease (IBD), Crohn's disease and ulcerative colitis.

Depending on the severity, extent and medical goals of treatment, conventional medications for inflammatory conditions such as sulfasalazine, mesalazine, corticosteroids, and methotrexate are primarily used to modulate immune and inflammatory responses. Limitations in both the safety and efficacy encountered with current medical approaches for inflammatory conditions continue to drive the search for better and safer alternative therapeutic agents. Consumers are also looking more generally for natural ways to support their health and wellbeing.

Although the specific causes of inflammation are yet to be identified in many diseases, cytokine activation in the intestinal mucosal system is a key target for modulating inflammation in gut inflammatory diseases.

Gastric ulcers are another common inflammation-associated gastrointestinal disorder. Gastric ulcers are benign mucosal lesions that penetrate deeply into the gut wall beyond the muscularis mucosae and form craters surrounded by acute and chronic inflammatory cell infiltrates. Many studies report that major risk factors for gastric ulcers include *Helicobacter pylori* infection, smoking, aspirin/Non-steroidal anti-inflammatory drugs (NSAIDs) use, alcohol abuse and stress.

Matrix metalloproteinases (MMPs) are a group of enzymes that exhibit pro-inflammatory effects. They have been shown to have a role in inflammation and to be involved in the inflammatory response to diseases (Stallmach, 2000) with MMP-1, MMP-2 and MMP-9 being shown to be important in inflammation (Manicone, 2008).

MMP-9 is a gelatinase-type enzyme which specifically regulates acute and chronic gastric ulcers (Swarnakar et al., 2005). Elevated MMP-9 activity (up to 10 times) has been reported in multiple studies during ethanol and indomethacin-induced gastric ulcers (Lempinen, Inkinen, Wolff, & Ahonen, 2000; Pradeepkumar Singh, Kundu, Ganguly, Mishra, & Swarnakar, 2007). MMP-9 is one of the key proinflammatory enzymes which can proteolytically process a number of cytokines and chemokines into more active forms, such as pro-IL-1 β and IL-8 (Van den Steen et al., 2000). MMP-9 is therefore a target of interest in the treatment of gastric ulcer to prevent excessive tissue degradation of the extracellular matrix.

Conventional treatment for gastric ulcers includes pharmaceutical management with medicines such as omeprazole and ranitidine. Such medicines can have severe side effects such as myelosuppression and abnormal heart rhythm and are known to have high relapse rates.

There is therefore an interest in identifying other anti-inflammatory agents for use in treating gastrointestinal inflammation and/or conditions associated with gastrointestinal inflammation, including MMP9-associated inflammatory conditions and other MMP-9 associated conditions.

Honey is well-known for its anti-microbial activities. It is also suggested in the art that honey possesses anti-inflammatory activity, although the reason for this has not been well characterised. One patent publication, WO2015/030609, which is hereby incorporated by reference, explores the anti-inflammatory activity of a specific fraction

of honey. This publication teaches that a low molecular weight fraction from honey has generalised anti-inflammatory effects and no immune-stimulatory effects. It does not discuss specific anti-inflammatory action.

It will be appreciated from the above that it would be useful to provide alternative methods of treating inflammatory conditions, including inflammatory conditions associated with the gastrointestinal tract.

It is an object of the invention to provide methods of treating inflammatory conditions, including inflammatory conditions associated with the gastrointestinal tract and/or to address the foregoing problems or at least to provide the public with a useful choice.

Further aspects and advantages of the product, compositions methods and uses will become apparent from the ensuing description that is given by way of example only.

Summary of the Invention

Described herein are compositions comprising 3,6,7-trimethylumazine, and methods of using the same for preventing, ameliorating or treating MMP-9 associated conditions, inflammation of the gastrointestinal tract, and/or inflammatory conditions associated with gastrointestinal tract. The inventors have identified that a pteridine from honey, 3,6,7-trimethylumazine, has anti-inflammatory activity and MMP-9 inhibitory activity. Being able to isolate the compound and characterise the anti-inflammatory and MMP-9 inhibitory activity provides the ability to produce medicaments for various uses including the treatment, prevention and amelioration of conditions associated with MMP-9, including inflammatory conditions of the gastrointestinal tract.

In a first particular aspect, the present invention provides a method of preventing, ameliorating or treating an MMP-9 associated condition in a subject, comprising administering to a subject in need thereof a composition comprising 3,6,7-trimethylumazine.

In one embodiment of the first aspect, the MMP-9 associated condition is selected from gastrointestinal inflammatory diseases, gastric ulcers (for example peptic ulcers), gastritis, MMP-associated inflammatory conditions, inflammatory bowel disease (IBD), Crohn's disease, ulcerative colitis, Irritable Bowel Syndrome (IBS), digestive diseases, Gastroesophageal Reflux Disease (GERD), heartburn, acid reflux, *Helicobacter pylori* infection, mouth ulcers, stomatitis, pharyngitis, gingivitis, esophageal ulcers, neuropsychiatric illnesses (such as schizophrenia, bipolar mood disorder, multiple

sclerosis), neurodegenerative disorders (such as traumatic brain injury, multiple sclerosis, and Alzheimer's disease), cardiovascular diseases, cancer and arthritis.

In a second particular aspect, the invention provides a method of preventing, ameliorating or treating an MMP-9 associated inflammatory condition in a subject, comprising administering to a subject in need thereof a composition comprising 3,6,7-trimethylumazine.

In one embodiment of the second aspect, the MMP-9 associated inflammatory condition is associated with inflammation of the gastrointestinal tract. In another embodiment, the MMP-9 associated inflammatory condition is selected from, gastrointestinal inflammatory diseases, gastric ulcers (for example peptic ulcers), gastritis, MMP-associated inflammatory conditions, inflammatory bowel disease (IBD), Crohn's disease, ulcerative colitis, Irritable Bowel Syndrome (IBS), digestive diseases, Gastroesophageal Reflux Disease (GERD), heartburn, acid reflux, *Helicobacter pylori* infection, mouth ulcers, stomatitis, pharyngitis, gingivitis and/or esophageal ulcers.

In one embodiment, the MMP-9 associated inflammatory condition is selected from neuropsychiatric illnesses (such as schizophrenia, bipolar mood disorder, multiple sclerosis), neurodegenerative disorders (such as traumatic brain injury, multiple sclerosis, and Alzheimer's disease), cardiovascular diseases, cancer and arthritis.

In a third particular aspect, the invention provides a method of preventing, ameliorating or treating inflammation in a subject comprising administering to a subject in need thereof a composition comprising 3,6,7-trimethylumazine.

In one embodiment of the third particular aspect, the inflammation is associated with the gastrointestinal tract of a subject. In one embodiment, the inflammation is associated with conditions selected from: gastrointestinal inflammatory diseases, gastric ulcers (for example peptic ulcers), gastritis, MMP-associated inflammatory conditions, inflammatory bowel disease (IBD), Crohn's disease, ulcerative colitis, Irritable Bowel Syndrome (IBS), digestive diseases, Gastroesophageal Reflux Disease (GERD), heartburn, acid reflux, *Helicobacter pylori* infection, mouth ulcers, stomatitis, pharyngitis, gingivitis, esophageal ulcers, neuropsychiatric illnesses (such as schizophrenia, bipolar mood disorder, multiple sclerosis), neurodegenerative disorders (such as traumatic brain injury, multiple sclerosis, and Alzheimer's disease), cardiovascular diseases, cancer and/or arthritis.

In one embodiment of aspects one to three, the origin of the 3,6,7-trimethylumazine is from *Leptospermum*. In one embodiment, the 3,6,7-trimethylumazine is substantially

from plants selected from the group comprising: *Leptospermum scoparium*, *Leptospermum polygalifolium*, *Leptospermum subtenue*, and combinations thereof. In one embodiment, the 3,6,7-trimethylumazine is from *Leptospermum scoparium*.

In one embodiment of aspects one to three, the origin of the 3,6,7-trimethylumazine is honey.

In one embodiment, the honey is of a floral origin substantially from the genus *Leptospermum*. In one embodiment, the honey is of a floral origin substantially from: *Leptospermum scoparium*, *Leptospermum polygalifolium*, *Leptospermum subtenue*, and/or combinations thereof. In one embodiment, the honey is of a floral origin substantially from *Leptospermum scoparium* (also referred to as Manuka).

In one embodiment of aspects one to three, the 3,6,7-trimethylumazine is derived directly from a plant of the genus *Leptospermum*. In one embodiment, the 3,6,7-trimethylumazine is derived directly from the flowers, nectar, roots, fruit, seeds, bark, oil, leaves, wood, stems or other plant material of a plant of the genus *Leptospermum*. In one embodiment, the 3,6,7-trimethylumazine is substantially from plants selected from the group comprising: *Leptospermum scoparium*, *Leptospermum polygalifolium*, *Leptospermum subtenue*, and combinations thereof.

In one embodiment of the above aspects, the composition comprising 3,6,7-trimethylumazine comprises honey. In one embodiment, the composition comprising 3,6,7-trimethylumazine consists of honey.

In one embodiment of the above aspects, the composition comprising 3,6,7-trimethylumazine comprises of a honey extract. In one embodiment of the above aspects, the composition comprising 3,6,7-trimethylumazine comprises a honey extract, wherein the honey extract comprises a concentration of 3,6,7-trimethylumazine that is higher than the concentration of 3,6,7-trimethylumazine found naturally occurring in honey. In one embodiment, the composition consists of a honey extract, wherein the honey extract comprises a concentration of 3,6,7-trimethylumazine that is higher than the concentration of 3,6,7-trimethylumazine found naturally occurring in honey. In one embodiment, the honey extract comprises a concentration of 3,6,7-trimethylumazine that is higher than the concentration of 3,6,7-trimethylumazine found naturally occurring in the honey from which the extract was derived.

In one embodiment, the honey from which the extract is derived is of a floral origin substantially from the genus *Leptospermum*. In one embodiment, the honey from

which the extract is derived is of a floral origin substantially from a genus selected from: *Leptospermum scoparium*, *Leptospermum polygalifolium*, *Leptospermum subtenue*, and combinations thereof. In one embodiment, the composition further comprises honey.

In one embodiment of the above aspects, the honey is raw honey, heat-treated honey or pasteurised honey.

In one embodiment of the above aspects, the composition comprises 3,6,7-trimethylumazine isolated from honey. In one embodiment, the honey is of a floral origin substantially from the genus *Leptospermum*. In one embodiment, the honey is of a floral origin substantially from the genus: *Leptospermum scoparium*, *Leptospermum polygalifolium*, *Leptospermum subtenue*, and/or combinations thereof. In one embodiment, the 3,6,7-trimethylumazine is isolated by subjection of the honey to solid phase extraction, followed by normal-phase flash chromatography and preparative thin layer chromatography.

In one embodiment of the above aspects, the 3,6,7-trimethylumazine is synthetic. In one embodiment, the composition further comprises honey.

In one embodiment, the composition comprises from about 2.5 µg/mL to about 1000 µg/mL 3,6,7-trimethylumazine. In one embodiment, the composition comprises 3,6,7-trimethylumazine from about 2.5 µg/mL, about 5 µg/mL, about 10 µg/mL, about 20 µg/mL, about 40 µg/mL, about 50 µg/mL, about 60 µg/mL, about 70 µg/mL, about 80 µg/mL, about 90 µg/mL, about 100 µg/mL, 150 µg/mL, about 200 µg/mL, about 250 µg/mL, about 300 µg/mL, about 350 µg/mL, about 400 µg/mL, about 450 about 500 µg/mL, about 550 µg/mL, about 600 µg/mL, about 650 µg/mL, about 700 µg/mL, about 750 µg/mL, about 800 µg/mL, about 850 µg/mL, about 900 µg/mL, about 950 µg/mL, to about 1000 µg/mL, or wherein the composition comprises 3,6,7-trimethylumazine from about 2.5 to 5 µg/mL, about 5 to 10 µg/mL, about 10 to 20 µg/mL, about 20 to 40 µg/mL, about 40 to 50 µg/mL, about 50 to 60 µg/mL, about 60 to 70 µg/mL, about 70 to 80 µg/mL, about 80 to 90 µg/mL, about 90 to 100 µg/mL, about 100 to 150 µg/mL, 150 to 200 µg/mL, about 200 to 250 µg/mL, about 250 to 300 µg/mL, about 300 to 350 µg/mL, about 350 to 400 µg/mL, about 400 to 450 µg/mL, about 450 to 500 µg/mL, about 500 to 550 µg/mL, about 550 to 600 µg/mL, about 600 to 650 µg/mL, about 650 to 700 µg/mL, about 700 to 750 µg/mL, about 750 to 800 µg/mL, about 800 to 850 µg/mL, about 850 to 900 µg/mL, about 900 to 950 µg/mL, about 950 to 1000 µg/mL.

In one embodiment, the composition comprises 3,6,7-trimethylumazine from about 5 mg/kg to about 3000 mg/kg. In one embodiment, the composition comprises 3,6,7-

trimethyllumazine from about 5 mg/kg, about 10 mg/kg, about 15 mg/kg, about 20 mg/kg, about 25 mg/kg, about 30 mg/kg, about 35 mg/kg, about 40 mg/kg, about 45 mg/kg, about 50 mg/kg, about 55 mg/kg, about 60 mg/kg, about 70 mg/kg, about 80 mg/kg, about 90 mg/kg, about 100 mg/kg, about 150 mg/kg, about 200 mg/kg, about 250 mg/kg, about 300mg/kg, about 350 mg/kg, about 400 mg/kg, about 450 mg/kg, about 500 mg/kg, about 550 mg/kg, about 600 mg/kg, about 650 mg/kg, about 700 mg/kg, about 750 mg/kg, about 800 mg/kg, about 850 mg/kg, about 900 mg/kg, about 950 mg/kg, about 1000 mg/kg, about 1100 mg/kg, about 1200 mg/kg, about 1300 mg/kg, about 1400 mg/kg, about 1500 mg/kg, about 1600 mg/kg, about 1700 mg/kg, about 1800 mg/kg, about 1900 mg/kg, about 2000 mg/kg, about 2100 mg/kg, about 2200 mg/kg, about 2300 mg/kg, about 2400 mg/kg, about 2500 mg/kg, about 2600 mg/kg, about 2700 mg/kg, about 2800 mg/kg, about 2900 mg/kg to about 3000 mg/kg or wherein the composition comprises a concentration of 3,6,7-trimethyllumazine of from 5 to 10 mg/kg, or from 10 to 15 mg/kg, or from 15 to 20 mg/kg, or from 20 to 25 mg/kg, or from 25 to 30 mg/kg, or from 30 to 35 mg/kg, or from 35 to 40 mg/kg, or from 40 to 45 mg/kg, or from 45 to 50 mg/kg, or from 50 to 55 mg/kg, or from 55 to 60 mg/kg, or from 60 70 mg/kg or from 70 to 80 mg/kg, about 90 to 100 mg/kg, about 100 to 150 mg/kg, about 150 to 200 mg/kg, about 200 mg/kg, about 250 to 300 mg/kg, about 300 to 350 mg/kg, about 350 to 400 mg/kg, about 400 to 450 mg/kg, about 450 to 500 mg/kg, about 500 to 550 mg/kg, about 550 to 600 mg/kg, about 600 to 650 mg/kg, about 650 to 700 mg/kg, about 700 to 750 mg/kg, about 750 to 800 mg/kg, about 800 to 850 mg/kg, about 850 to 900 mg/kg, about 900 to 950 mg/kg, about 950 to 1000 mg/kg, about 1000 to 1100 mg/kg, about 1100 to 1200 mg/kg, about 1200 to 1300 mg/kg, about 1300 to 1400 mg/kg, about 1400 to 1500 mg/kg, about 1500 to 1600 mg/kg, about 1600 to 1700 mg/kg, about 1700 to 1800 mg/kg, about 1800 to 1900 mg/kg, about 1900 to 2000 mg/kg, about 2000 to 2100 mg/kg, about 2100 to 2200 mg/kg, about 2200 to 2300 mg/kg, about 2300 to 2400 mg/kg, about 2400 to 2500 mg/kg, about 2500 to 2600 mg/kg, about 2600 to 2700 mg/kg, about 2700 to 2800 mg/kg, about 2800 to 2900 mg/kg, about 2900 to 3000 mg/kg.

In one embodiment of the above aspects, the composition comprises a therapeutically effective amount of 3,6,7-trimethyllumazine.

In one embodiment, the composition comprising 3,6,7-trimethyllumazine is formulated as a medicament, therapeutic product or health supplement. In one embodiment, the composition comprising 3,6,7-trimethyllumazine is formulated into a range of delivery systems, including but not limited to, liquid formulations, capsules, chewable tablet, tablets, suppositories, fast moving consumer goods, intravenous preparations,

intramuscular preparations, subcutaneous preparations, solutions, food, beverages, dietary supplements, cosmetic formulation, gels, lotions, powders or sprays.

In one embodiment of the above aspects, the method comprises administering the composition comprising 3,6,7-trimethylumazine one, two, three, four or five times daily.

In one embodiment of the above aspects, the method comprises administering the composition comprising 3,6,7-trimethylumazine one, two, three, four, five, six or seven times weekly.

In one embodiment, the composition comprising 3,6,7-trimethylumazine is administered as a single dose or as a divided dose. In one embodiment, the composition comprising 3,6,7-trimethylumazine is administered as one, two three or four separate doses.

In one embodiment of the above aspects, the method comprises administration of the composition comprising 3,6,7-trimethylumazine at a dose from about 1mg to about 3000mg. In one particular embodiment, the method comprises administration of the composition comprising from about 1mg, 10mg, 20mg, 30mg, 40 mg, 50 mg, 60 mg, 70 mg, 80 mg, 90 mg, 100mg, 150 mg, 200mg, 250 mg, 300mg, 350mg, 400mg, 450 mg, 500mg, 550 mg, 600mg, 650mg, 700mg, 750 mg, 800mg, 850 mg, 900mg, 950 mg, 1000mg, 1100mg, 1200mg, 1300mg, 1400mg, 1500mg, 1600mg, 1700mg, 1800mg, 1900mg, 2000mg, 2100mg, 2200mg, 2300mg, 2400mg, 2500mg, 2600mg, 2700mg, 2800mg, 2900mg, 3000mg 3,6,7-trimethylumazine.

In one embodiment of the above aspects, the method comprises administering the composition at a dose of from about 5g to about 100g 3,6,7-trimethylumazine.

In one embodiment of the above aspects, the composition comprising 3,6,7-trimethylumazine has a standardised concentration of 3,6,7-trimethylumazine obtained by:

- selecting a first composition with a known concentration of 3,6,7-trimethylumazine;
- selecting at least one further composition with a known concentration of 3,6,7-trimethylumazine;
- combining the first composition with the second composition to obtain a final composition with a standardised 3,6,7-trimethylumazine concentration of from about 5 mg/kg to about 3000 mg/kg.

In one embodiment of the above aspects, the composition comprising 3,6,7-

trimethylumazine has a standardised concentration of 3,6,7-trimethylumazine obtained by:

- selecting a first composition with a known concentration of 3,6,7-trimethylumazine;
- combining the selected first composition with one or more of:
 - synthetic 3,6,7-trimethylumazine;
 - isolated 3,6,7-trimethylumazine;
 - a honey extract comprising 3,6,7-trimethylumazine; and/or
 - 3,6,7-trimethylumazine derived directly from a plant of the genus *Leptospermum*;

to form a composition with a standardised 3,6,7-trimethylumazine concentration of from about 5 mg/kg to about 3000 mg/kg.

In one embodiment, the composition comprises honey, a honey extract, isolated 3,6,7-trimethylumazine and/or synthetic 3,6,7-trimethylumazine.

In one embodiment, the 3,6,7-trimethylumazine derived directly from a plant is derived directly from the flowers, nectar, roots, fruit, seeds, bark, oil, leaves, wood, stems or other plant material of a plant of the genus *Leptospermum*.

In one embodiment, the standardised 3,6,7-trimethylumazine concentration is from about 5 mg/kg to about 3000 mg/kg. In one embodiment, the standardised 3,6,7-trimethylumazine concentration is from: about 5 mg/kg, about 10 mg/kg, about 15 mg/kg, about 20 mg/kg, about 25 mg/kg, about 30 mg/kg, about 35 mg/kg, about 40 mg/kg, about 45 mg/kg, about 50 mg/kg, about 55 mg/kg, about 60 mg/kg, about 70 mg/kg, about 80 mg/kg, about 90 mg/kg, about 100 mg/kg, about 150 mg/kg, about 200 mg/kg, about 250 mg/kg, about 300mg/kg, about 350 mg/kg, about 400 mg/kg, about 450 mg/kg, about 500 mg/kg, about 550 mg/kg, about 600 mg/kg, about 650 mg/kg, about 700 mg/kg, about 750 mg/kg, about 800 mg/kg, about 850 mg/kg, about 900 mg/kg, about 950 mg/kg, about 1000 mg/kg, about 1100 mg/kg, about 1200 mg/kg, about 1300 mg/kg, about 1400 mg/kg, about 1500 mg/kg, about 1600 mg/kg, about 1700 mg/kg, about 1800 mg/kg, about 1900 mg/kg, about 2000 mg/kg, about 2100 mg/kg, about 2200 mg/kg, about 2300 mg/kg, about 2400 mg/kg, about 2500 mg/kg, about 2600 mg/kg, about 2700 mg/kg, about 2800 mg/kg, about 2900 mg/kg to about 3000 mg/kg of 3,6,7-trimethylumazine.

In one embodiment, the concentration of the 3,6,7-trimethylumazine is determined by chromatography, analytical measurements, spectrophotometry and/or any other method known to a person skilled in the art. In one embodiment, the concentration of 3,6,7-

trimethyllumazine is determined by reverse-phase HPLC system.

In a fourth particular aspect the invention provides a method of making a composition with anti-inflammatory and/or MMP-9 inhibitory activity comprising:

- a. testing a first composition comprising honey for 3,6,7-trimethyllumazine concentration;
- b. testing at least one further composition comprising honey for 3,6,7-trimethyllumazine concentration;
- c. selecting a composition comprising honey with a 3,6,7-trimethyllumazine concentration greater than from about 5 mg/kg 3,6,7-trimethyllumazine;
- d. selecting at least one further composition comprising honey with a 3,6,7-trimethyllumazine concentration greater than about 5 mg/kg;
- e. combining the selected composition comprising honey to form a honey composition with a 3,6,7-trimethyllumazine concentration of at least from about 5 to about 80 mg/kg.

In one embodiment of the fourth aspect, the composition comprising honey is selected if it has a concentration of 3,6,7-trimethyllumazine greater than about 5 mg/kg, about 10 mg/kg, about 15 mg/kg, about 20 mg/kg, about 25 mg/kg, about 30 mg/kg, about 35 mg/kg, about 40 mg/kg, about 45 mg/kg, about 50 mg/kg, about 55 mg/kg, about 60 mg/kg, about 70 mg/kg or about 80 mg/kg.

In one embodiment of the fourth aspect, the composition is honey.

In one embodiment, the 3,6,7-trimethyllumazine concentration is determined by chromatography, analytical measurements, spectrophotometry and/or any other method known to a person skilled in the art. In one embodiment, the concentration of 3,6,7-trimethyllumazine is determined by reverse-phase HPLC system.

In one embodiment of the fourth aspect, the composition with anti-inflammatory activity is suitable for use in the method of any one of aspects one to three.

In a fifth particular aspect the invention provides a method of identifying a composition as having anti-inflammatory and/or MMP-9 inhibitory activity comprising:

- a. testing a composition for 3,6,7-trimethyllumazine concentration; and
 - i. identifying the composition as having anti-inflammatory and/or MMP-9 inhibitory activity if it contains a 3,6,7-trimethyllumazine concentration greater than from about 5 mg/kg; or

- ii. identifying the composition as not having anti-inflammatory and/or MMP-9 inhibitory activity if it contains a 3,6,7-trimethyllumazine concentration lower than from about 5 mg/kg.

In one embodiment, the composition comprises honey or a honey extract.

In one embodiment of the fifth particular aspect, the composition comprising honey is determined as having anti-inflammatory activity if it contains greater than about 5 mg/kg, about 10 mg/kg, about 15 mg/kg, about 20 mg/kg, about 25 mg/kg, about 30 mg/kg, about 35 mg/kg, about 40 mg/kg, about 45 mg/kg, about 50 mg/kg, about 55 mg/kg, about 60 mg/kg, about 70 mg/kg or about 80 mg/kg.

In one embodiment of the fifth aspect, the composition is honey or a honey extract.

In one embodiment of the fifth particular aspect, the composition with anti-inflammatory activity is suitable for use in any one of aspects one to three.

In a sixth particular aspect, the invention provides a method of identifying a composition with anti-inflammatory and/or MMP-9 inhibitory activity suitable for use in a method of any of aspects one to three, comprising:

- a. testing a composition for 3,6,7-trimethyllumazine concentration; and
 - i. identifying the composition as suitable for use in any of aspects one to three if it contains a 3,6,7-trimethyllumazine concentration greater than from about 5 to about 80 mg/kg 3,6,7-trimethyllumazine; or
 - ii. identifying the composition as not suitable for use in any of aspects one to three if it contains a 3,6,7-trimethyllumazine concentration lower than from about 5 mg/kg 3,6,7-trimethyllumazine.

In one embodiment of the sixth particular aspect, the composition is identified as suitable for use in a method of any one of aspects one to four if it contains a 3,6,7-trimethyllumazine concentration greater than about 5 mg/kg, about 10 mg/kg, about 15 mg/kg, about 20 mg/kg, about 25 mg/kg, about 30 mg/kg, about 35 mg/kg, about 40 mg/kg, about 45 mg/kg, about 50 mg/kg, about 55 mg/kg, about 60 mg/kg, about 70 mg/kg or about 80 mg/kg.

In one embodiment, the composition is honey or a honey extract.

In one embodiment, the 3,6,7-trimethyllumazine concentration is determined by

chromatography, analytical measurements, spectrophotometry and/or any other method known to a person skilled in the art. In one embodiment, the concentration of 3,6,7-trimethylumazine is determined by reverse-phase HPLC system.

In a seventh particular aspect, the invention provides a composition comprising 3,6,7-trimethylumazine suitable for use in the method of any of the above aspects.

In one embodiment of the seventh aspect, the origin of the 3,6,7-trimethylumazine is from *Leptospermum*. In one embodiment, the 3,6,7-trimethylumazine is substantially from plants selected from the group comprising: *Leptospermum scoparium*, *Leptospermum polygalifolium*, *Leptospermum subtenue*, and combinations thereof. In one embodiment, the 3,6,7-trimethylumazine is from *Leptospermum scoparium*.

In one embodiment of the seventh aspect, the origin of the 3,6,7-trimethylumazine is honey. In one embodiment, the honey is of a floral origin substantially from the genus *Leptospermum*. In one embodiment, the honey is of a floral origin substantially from: *Leptospermum scoparium*, *Leptospermum polygalifolium*, *Leptospermum subtenue*, and/or combinations thereof. In one embodiment, the honey is of a floral origin substantially from *Leptospermum scoparium* (Manuka).

In one embodiment of the seventh aspect, the 3,6,7-trimethylumazine is derived directly from a plant of the genus *Leptospermum*. In one embodiment, the 3,6,7-trimethylumazine is derived directly from the nectar, roots, fruit, seeds, bark, oil, leaves, wood, stems or other plant material of a plant of the genus *Leptospermum*. In one embodiment, the 3,6,7-trimethylumazine is substantially from plants selected from the group comprising: *Leptospermum scoparium*, *Leptospermum polygalifolium*, *Leptospermum subtenue*, and combinations thereof.

In one embodiment of the seventh aspect, the composition comprising 3,6,7-trimethylumazine comprises honey. In one embodiment, the composition comprising 3,6,7-trimethylumazine consists of honey.

In one embodiment of the above aspects, the composition comprising 3,6,7-trimethylumazine comprises of a honey extract. In one embodiment, the composition comprising 3,6,7-trimethylumazine comprises a honey extract, wherein the honey extract comprises a concentration of 3,6,7-trimethylumazine that is higher than the concentration of 3,6,7-trimethylumazine found naturally occurring in honey. In one embodiment, the composition consists of a honey extract, wherein the honey extract comprises a concentration of 3,6,7-trimethylumazine that is higher than the concentration of 3,6,7-trimethylumazine found naturally occurring in honey. In one

embodiment, the honey extract comprises a concentration of 3,6,7-trimethylumazine that is higher than the concentration of 3,6,7-trimethylumazine found naturally occurring in the honey from which the extract was derived.

In one embodiment, the honey from which the extract is derived is of a floral origin substantially from the genus *Leptospermum*. In one embodiment, the honey from which the extract is derived is of a floral origin substantially from a genus selected from: *Leptospermum scoparium*, *Leptospermum polygalifolium*, *Leptospermum subtenue*, and combinations thereof. In one embodiment, the composition further comprises honey.

In one embodiment, the honey is raw honey, heat-treated honey or pasteurised honey.

In one embodiment, the composition comprises 3,6,7-trimethylumazine isolated from honey. In one embodiment, the honey is of a floral origin substantially from the genus *Leptospermum*. In one embodiment, the honey is of a floral origin substantially from the genus: *Leptospermum scoparium*, *Leptospermum polygalifolium*, *Leptospermum subtenue*, and/or combinations thereof. In one embodiment, the 3,6,7-trimethylumazine is isolated by subsection of the honey to solid phase extraction, followed by normal-phase flash chromatography and preparative thin layer chromatography.

In one embodiment, the composition comprises synthetic 3,6,7-trimethylumazine. In one embodiment, the composition further comprises honey.

In one embodiment, the composition comprises from about 2.5 µg/mL to about 1000 µg/mL 3,6,7-trimethylumazine. In one embodiment, the composition comprises from about 2.5 µg/mL, about 5 µg/mL, about 10 µg/mL, about 20 µg/mL, about 40 µg/mL, about 50 µg/mL, about 60 µg/mL, about 70 µg/mL, about 80 µg/mL, about 90 µg/mL, about 100 µg/mL, 150 µg/mL, about 200 µg/mL, about 250 µg/mL, about 300 µg/mL, about 350 µg/mL, about 400 µg/mL, about 450 µg/mL, about 500 µg/mL, about 550 µg/mL, about 600 µg/mL, about 650 µg/mL, about 700 µg/mL, about 750 µg/mL, about 800 µg/mL, about 850 µg/mL, about 900 µg/mL, about 950 µg/mL, to about 1000 µg/mL 3,6,7-trimethylumazine, or wherein the composition comprises from about 2.5 to 5 µg/mL, about 5 to 10 µg/mL, about 10 to 20 µg/mL, about 20 to 40 µg/mL, about 40 to 50 µg/mL, about 50 to 60 µg/mL, about 60 to 70 µg/mL, about 70 to 80 µg/mL, about 80 to 90 µg/mL, about 90 to 100 µg/mL, about 100 to 150 µg/mL, 150 to 200 µg/mL, about 200 to 250 µg/mL, about 250 to 300 µg/mL, about 300 to 350 µg/mL, about 350 to 400 µg/mL, about 400 to 450 µg/mL, about 450 to 500 µg/mL, about 500 to 550 µg/mL, about 550 to 600 µg/mL, about 600 to 650 µg/mL, about 650 to 700 µg/mL,

about 700 to 750 µg/mL, about 750 to 800 µg/mL, about 800 to 850 µg/mL, about 850 to 900 µg/mL, about 900 to 950 µg/mL, about 950 to 1000 µg/mL 3,6,7-trimethylumazine.

In one embodiment, the composition comprises 3,6,7-trimethylumazine from about 5 mg/kg to about 3000 mg/kg 3,6,7-trimethylumazine. In one embodiment, the composition comprises 3,6,7-trimethylumazine from about 5 mg/kg, about 10 mg/kg, about 15 mg/kg, about 20 mg/kg, about 25 mg/kg, about 30 mg/kg, about 35 mg/kg, about 40 mg/kg, about 45 mg/kg, about 50 mg/kg, about 55 mg/kg, about 60 mg/kg, about 70 mg/kg, about 80 mg/kg, about 90 mg/kg, about 100 mg/kg, about 150 mg/kg, about 200 mg/kg, about 250 mg/kg, about 300mg/kg, about 350 mg/kg, about 400 mg/kg, about 450 mg/kg, about 500 mg/kg, about 550 mg/kg, about 600 mg/kg, about 650 mg/kg, about 700 mg/kg, about 750 mg/kg, about 800 mg/kg, about 850 mg/kg, about 900 mg/kg, about 950 mg/kg, about 1000 mg/kg, about 1100 mg/kg, about 1200 mg/kg, about 1300 mg/kg, about 1400 mg/kg, about 1500 mg/kg, about 1600 mg/kg, about 1700 mg/kg, about 1800 mg/kg, about 1900 mg/kg, about 2000 mg/kg, about 2100 mg/kg, about 2200 mg/kg, about 2300 mg/kg, about 2400 mg/kg, about 2500 mg/kg, about 2600 mg/kg, about 2700 mg/kg, about 2800 mg/kg, about 2900 mg/kg to about 3000 mg/kg or wherein the composition comprises a concentration of 3,6,7-trimethylumazine of 5 to 10 mg/kg, or from 10 to 15 mg/kg, or from 15 to 20 mg/kg, or from 20 to 25 mg/kg, or from 25 to 30 mg/kg, or from 30 to 35 mg/kg, or from 35 to 40 mg/kg, or from 40 to 45 mg/kg, or from 45 to 50 mg/kg, or from 50 to 55 mg/kg, or from 55 to 60 mg/kg, or from 60 70 mg/kg or from 70 to 80 mg/kg, about 90 to 100 mg/kg, about 100 to 150 mg/kg, about 150 to 200 mg/kg, about 200 mg/kg, about 250 to 300 mg/kg, about 300 to 350 mg/kg, about 350 to 400 mg/kg, about 400 to 450 mg/kg, about 450 to 500 mg/kg, about 500 to 550 mg/kg, about 550 to 600 mg/kg, about 600 to 650 mg/kg, about 650 to 700 mg/kg, about 700 to 750 mg/kg, about 750 to 800 mg/kg, about 800 to 850 mg/kg, about 850 to 900 mg/kg, about 900 to 950 mg/kg, about 950 to 1000 mg/kg, about 1000 to 1100 mg/kg, about 1100 to 1200 mg/kg, about 1200 to 1300 mg/kg, about 1300 to 1400 mg/kg, about 1400 to 1500 mg/kg, about 1500 to 1600 mg/kg, about 1600 to 1700 mg/kg, about 1700 to 1800 mg/kg, about 1800 to 1900 mg/kg, about 1900 to 2000 mg/kg, about 2000 to 2100 mg/kg, about 2100 to 2200 mg/kg, about 2200 to 2300 mg/kg, about 2300 to 2400 mg/kg, about 2400 to 2500 mg/kg, about 2500 to 2600 mg/kg, about 2600 to 2700 mg/kg, about 2700 to 2800 mg/kg, about 2800 to 2900 mg/kg, about 2900 to 3000 mg/kg.

In one embodiment, the composition comprises a therapeutically effective amount of 3,6,7-trimethylumazine.

In one embodiment, the composition comprising 3,6,7-trimethylumazine is formulated as a medicament, therapeutic product or health supplement. In one embodiment, the composition comprising 3,6,7-trimethylumazine is formulated into a range of delivery systems, including but not limited to, liquid formulations, fast moving consumer goods, capsules, chewable tablet, tablets, suppositories, intravenous preparations, intramuscular preparations, subcutaneous preparations, solutions, food, beverages, dietary supplements, cosmetic formulations, gels, lotions, powders or sprays.

In an eighth particular aspect, the present invention provides a use of a composition comprising 3,6,7-trimethylumazine in the manufacture of a medicament for preventing, ameliorating or treating an MMP-9 associated condition in a subject.

In one embodiment of the eighth aspect, the MMP-9 associated condition is selected from gastrointestinal inflammatory diseases, gastric ulcers (for example peptic ulcers), gastritis, MMP-associated inflammatory conditions, inflammatory bowel disease (IBD), Crohn's disease, ulcerative colitis, Irritable Bowel Syndrome (IBS), digestive diseases, Gastroesophageal Reflux Disease (GERD), heartburn, acid reflux, *Helicobacter pylori* infection, mouth ulcers, stomatitis, pharyngitis, gingivitis, esophageal ulcers, neuropsychiatric illnesses (such as schizophrenia, bipolar mood disorder, multiple sclerosis), neurodegenerative disorders (such as traumatic brain injury, multiple sclerosis, and Alzheimer's disease), cardiovascular diseases, cancer and arthritis.

In a ninth particular aspect, the invention provides a use of a composition comprising 3,6,7-trimethylumazine in the manufacture of a medicament for preventing, ameliorating or treating an MMP-9 associated inflammatory condition in a subject.

In one embodiment of the ninth aspect, the MMP-9 associated inflammatory condition is associated with inflammation of the gastrointestinal tract. In another embodiment, the MMP-9 associated inflammatory condition is selected from, gastrointestinal inflammatory diseases, gastric ulcers (for example peptic ulcers), gastritis, MMP-associated inflammatory conditions, inflammatory bowel disease (IBD), Crohn's disease, ulcerative colitis, Irritable Bowel Syndrome (IBS), digestive diseases, Gastroesophageal Reflux Disease (GERD), heartburn, acid reflux, *Helicobacter pylori* infection, mouth ulcers, stomatitis, pharyngitis, gingivitis and/or esophageal ulcers.

In one embodiment, the MMP-9 associated inflammatory condition is selected from neuropsychiatric illnesses (such as schizophrenia, bipolar mood disorder, multiple sclerosis), neurodegenerative disorders (such as traumatic brain injury, multiple sclerosis, and Alzheimer's disease), cardiovascular diseases, cancer and arthritis.

In a tenth particular aspect, the invention provides a use of a composition comprising 3,6,7-trimethylumazine in the manufacture of a medicament for preventing, ameliorating or treating inflammation of the gastrointestinal tract in a subject.

In one embodiment of the tenth aspect, the invention provides a use of preventing, ameliorating or treating conditions associated with inflammation of the gastrointestinal tract. In one embodiment, the condition of the gastrointestinal tract is selected from: gastrointestinal inflammatory diseases, gastric ulcers (for example peptic ulcers), gastritis, MMP-associated inflammatory conditions, inflammatory bowel disease (IBD), Crohn's disease, ulcerative colitis, Irritable Bowel Syndrome (IBS), digestive diseases, Gastroesophageal Reflux Disease (GERD), heartburn, acid reflux, *Helicobacter pylori* infection, mouth ulcers, stomatitis, pharyngitis, gingivitis and/or esophageal ulcers.

In an eleventh particular aspect, the invention provides a use of a composition comprising 3,6,7-trimethylumazine in the manufacture of a medicament for preventing, ameliorating or treating inflammation in a subject.

In one embodiment of the eleventh particular aspect, the inflammation is associated with the gastrointestinal tract of a subject. In one embodiment, the inflammation is associated with conditions selected from: gastrointestinal inflammatory diseases, gastric ulcers (for example peptic ulcers), gastritis, MMP-associated inflammatory conditions, inflammatory bowel disease (IBD), Crohn's disease, ulcerative colitis, Irritable Bowel Syndrome (IBS), digestive diseases, Gastroesophageal Reflux Disease (GERD), heartburn, acid reflux, *Helicobacter pylori* infection, mouth ulcers, stomatitis, pharyngitis, gingivitis and/or esophageal ulcers.

In one embodiment, the inflammation is associated with a condition selected from: , neuropsychiatric illnesses (such as schizophrenia, bipolar mood disorder, multiple sclerosis), neurodegenerative disorders (such as traumatic brain injury, multiple sclerosis, and Alzheimer's disease), cardiovascular diseases, cancer and arthritis.

In a twelfth particular aspect, there is provided a method, use or composition of any one of the above aspects, wherein the composition further comprises a COX-2 inhibitor.

In a thirteenth particular aspect, there is provided a method or use of any one of the above aspects, further comprising co-administration of a COX-2 inhibitor.

Advantages of the above methods and uses may be varied. In some embodiments, the source of 3,6,7-trimethylumazine is naturally occurring and able to be manufactured on a sustainable basis. 3,6,7-trimethylumazine is not anticipated to have side effects

and it may be formulated in a wide variety of ways for various methods of administration.

This invention may also be said broadly to consist in the parts, elements and features referred to or indicated in the specification of the application, individually or collectively, and any or all combinations of any two or more of said parts, elements and features, and where specific integers are mentioned herein which have known equivalents in the art to which this invention relates, such known equivalents are deemed to be incorporated herein as if individually set forth.

Further aspects of the invention, which should be considered in all its novel aspects, will become apparent to those skilled in the art upon reading of the following description which provides at least one example of a practical application of the invention.

Brief Description of the Drawings

Embodiments of the invention will now be described, by way of example only, with reference to the accompanying drawings in which:

Figure 1 is a graph illustrating the fluorescence intensity generated by MMP-9 activity over the course of 10 min.

Figure 2 is a graph illustrating the percentage inhibition of MMP-9 activity from 3,6,7-trimethylumazine ranging from 2.5–40 µg/ml. Data are shown as mean ± SEM. n=4. ****p<0.0001.

Figure 3 is a graph illustrating the correlation between 3,6,7-trimethylumazine concentration and MMP-9 inhibition. The IC₅₀ was calculated as 11.5 µg/ml. Data are shown as mean ± SEM. n=4.

Figure 4 is a graph illustrating MMP-9 activity measured by absorbance at 412nm over 120 min.

Figure 5 is a graph illustrating the percentage inhibition by 3,6,7-trimethylumazine on the activity of MMP-9 (n=5, 2 reciprocal replicates each)

Figure 6 is a graph illustrating that there is no significant interaction between 3,6,7-trimethylumazine and the chromogenic substrate (A) or the reaction product (B) over the course of 20 min.

Figure 7 shows a typical gelatin gel zymography showing gels incubated in normal developing buffer (column 3-5), 3,6,7-trimethylumazine supplemented buffer

(column 6-8), and NNGH (column 9-11). The clear band on top represents gelatinase activity from the fibronectin domain of inactive MMP-9 (~47 kDa). The bottom band represents the gelatinase activity from active MMP-9, where the pro-domain is cleaved off (~37 kDa).

Figure 8 is a graph illustrating the percentage inhibition from 3,6,7-trimethylumazine using gelatin zymography (n=5). Percentage inhibition was calculated by comparing the optical density with the negative control (no inhibitor).

Figure 9 illustrates 3,6,7-trimethylumazine was docked into the S'1 substrate binding pocket of MMP-9. A hydrogen bond was found between the N-H group of 3,6,7-trimethylumazine and the Tyr420 residue of MMP-9.

Figure 10 illustrates the amount of 3,6,7-trimethylumazine (ng/mL) during the gastric digestion of four Manuka honey samples (A,B,C,D) as a function of digestion time. Data represent mean $\pm$ SD, n=3.

Figure 11 illustrates the amount of 3,6,7-trimethylumazine (ng/mL) during the intestinal digestion of four Manuka honey samples (A,B,C,D) as a function of digestion time. Data represent mean $\pm$ SD, n=3.

Figure 12 illustrates the amount of 3,6,7-trimethylumazine (ng/mL) during the gastric digestion of four 50% Manuka honey samples (A,B,C,D) as a function of digestion time. Data represent mean $\pm$ SD, n=3.

Figure 13 illustrates the amount of 3,6,7-trimethylumazine (ng/mL) during the intestinal digestion of four 50% Manuka honey samples (A,B,C,D) as a function of digestion time. Data represent mean $\pm$ SD, n=3.

Figure 14 illustrates the amount of 3,6,7-trimethylumazine (ng/mL) during the gastric digestion of pure 3,6,7-trimethylumazine as a function of digestion time. Data represent mean $\pm$ SD, n=3.

Figure 15 illustrates the amount of 3,6,7-trimethylumazine (ng/mL) during the intestinal digestion of pure 3,6,7-trimethylumazine as a function of digestion time. Data represent mean $\pm$ SD, n=3.

Figure 16 illustrates the effect of 3,6,7-trimethylumazine (2.5-40 μ g/mL) on cell viability. Data is presented as mean $\pm$ SD.

Figure 17 illustrates the effect of 3,6,7-trimethylumazine on lipopolysaccharide (055:B5, 1 μ g/mL) induced matrix metalloproteinase 9 (MMP-9) secretion in

differentiated THP-1 cells (n=2 replicates) (based on the raw values). Small letter represents significant differences between the treatments. a – 40µg/mL of 3,6,7-trimethylumazine inhibits mmp-9 secretion (P=0.02); b - 30µg/mL of 3,6,7-trimethylumazine inhibits mmp-9 secretion (P=0.02).

Figure 18 illustrates the effect of 3,6,7-trimethylumazine on lipopolysaccharide (055:B5, 1µg/mL) induced matrix metalloproteinase 9 (MMP-9) secretion in differentiated THP-1 cells (n=2 replicates) (based on the absolute values). Small represents significant differences between the treatments. a - 40µg/mL of 3,6,7-trimethylumazine inhibits mmp-9 secretion (P=0.00), b- 30µg/mL of 3,6,7-trimethylumazine inhibits mmp-9 secretion (P=0.04); c- Azithromycin inhibits mmp-9 secretion (P=0.00).

Detailed Description of Preferred Embodiments

Described herein are compositions comprising 3,6,7-trimethylumazine methods, and uses of the same for the preventing, ameliorating or treating inflammation and/or inflammatory conditions. In particular, inflammation or inflammatory conditions associated with the gastrointestinal tract, including MMP-9 associated inflammatory conditions.

Definitions

For the purposes of this specification, the term “comprising” as used in this specification means “consisting in whole or at least in part of”. When interpreting statements in this specification which include that term, the features, prefaced by that term in each statement, all need to be present, but other features can also be present. Related terms such as “comprise” and “comprised” are to be interpreted in the same manner.

The term “about” or “approximately” and grammatical variations thereof mean a quantity, level, degree, value, number, frequency, percentage, dimension, size, amount, weight or length that varies by as much as 30, 25, 20, 15, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1%.

The term “medicament” or grammatical variations thereof refers to medical products. The medical products include, but are not limited to, liquid formulations, capsules, tablets, chewable tablets, gels, lotions, powders, fast moving consumer goods, suppositories, cosmetic formulations, spray preparations, food preparations, beverages, intravenous preparations, intramuscular preparations, subcutaneous preparations, and solutions.

The term “therapeutic products” or grammatical variations thereof refer to products which help to support, heal or restore health. The products include, but are not limited to, fast moving consumer goods, liquid formulations, capsules, tablets, chewable tablets, gels, lotions, powders suppositories, spray preparations, food preparations, beverages, cosmetic formulations, intravenous preparations, intramuscular preparations, subcutaneous preparations and solutions.

The term “inflammatory condition” means a condition or disorder associated with unwanted and/or abnormal inflammation.

The term “inflammation” means a body’s reaction that produces redness, warmth, swelling and/or pain as the result of infection, irritation, injury, disease, condition or other cause. Inflammation can also be characterised at a cellular level. Cellular inflammation may be characterised by production of various inflammatory mediators such as cytokines, chemokines or reactive nitrogen and oxygen species.

The term “anti-inflammatory” or grammatical variations thereof refer to the prevention, mitigated, quenching, calming, suppression or reduction of inflammation associated cytokines, chemokines, reactive nitrogen and oxygen species, when compared to the duration, grade or situation, where no anti-inflammatory compound or compounds were added. It also refers to the inflammation being prevented, mitigated, quenched, calmed or suppressed to the extent that there is reduced redness, warmth, swelling and/or pain, the reduced amount being relative to the duration, grade or situation, where no anti-inflammatory compound or compounds were added.

The term “therapeutically effective” with reference to an amount or dosage of a composition or medicament refers to an amount of a composition that is sufficient to effectively prevent, ameliorate or eliminate inflammation in a subject. The term should not be seen as limiting. It may refer to an amount of a dosage of a composition or medicament that optimises the anti-inflammatory effects on a subject depending on desired application.

The term “health supplement” in the context of the invention means a product intended to be supplemented into the diet of a subject.

The term “treatment” is to be considered in its broadest context. The term does not necessarily imply that a subject is treated until total recovery. Accordingly, “treatment” includes reducing, alleviating or ameliorating the symptoms or severity of a particular condition or preventing or otherwise reducing the risk of developing a particular

condition. It may also include maintaining or promoting a complete or partial state of remission of a condition.

The term “raw honey” means honey which has either undergone minimal heat (for example <50 °C) treatment or not undergone any heat processing.

The term “standardised concentration” in the context of the invention means a concentration that has been determined to meet a pre-determined concentration range.

As used herein the term “and/or” means “and” or “or”, or both.

As used herein “(s)” following a noun means the plural and/or singular forms of the noun.

It is intended that reference to a range of numbers disclosed herein (for example, 1 to 10) also incorporates reference to all rational numbers within that range (for example, 1, 1.1, 2, 3, 3.9, 4, 5, 6, 6.5, 7, 8, 9 and 10) and also any range of rational numbers within that range (for example, 2 to 8, 1.5 to 5.5 and 3.1 to 4.7).

A “subject” may be human or a non-human animal. Non-limiting examples of non-human animals are companion animals (e.g. cats and dogs), horses, livestock such as cattle, sheep and deer.

As noted above, the inventors have identified that 3,6,7-trimethylumazine, for example 3,6,7-trimethylumazine found in honey, has anti-inflammatory activity. In particular, the inventors surprisingly discovered that 3,6,7-trimethylumazine has anti-inflammatory effects. In particular, the inventors discovered that 3,6,7-trimethylumazine has MMP-9 inhibitory activity. Being able to characterise the activity and stability of 3,6,7-trimethylumazine provides the ability to produce compositions for preventing, ameliorating or treating inflammation, including preventing, ameliorating or treating various MMP-9 associated conditions and inflammatory conditions, in particular, inflammatory conditions of the gastrointestinal tract.

Pteridines are a group of compounds based on a pyrimido[4,5-*b*]pyrazine ring structure. The bicyclic compounds are naturally produced by many living organisms and are often referred to as pterins. Pteridines and pteridine derivatives are also synthetically produced. Many pteridine derivatives play essential metabolic roles as enzymatic cofactors, including the synthesis of nucleic acids, amino acids, neurotransmitters, nitrogen monoxides as well as purine and aromatic amino acids.

Herein we report 3,6,7-trimethylumazine, a pteridine derivative from *Leptospermum* honey, compositions comprising the same, and methods of use. The isolation,

structural elucidation and synthesis of 3,6,7-trimethylumazine has previously been described in New Zealand Patent Application No. 722140 (NZ 722140) filed by the same applicant, herein incorporated by reference.

Inflammation is a multifactorial phenomenon implicated in a wide range of diseases. In a healthy gut, the intestinal mucosa is in a state of controlled response regulated by an intricate balance of pro-inflammatory cytokines (for example tumour necrosis factor, TNF- α , Interferon, IFN- γ , IL-1, IL-6) and anti-inflammatory cytokines (for example IL-4, IL-10). Defects in this can facilitate the complex interplay involved between genetic, microbial and environmental factors culminating in a sustained activation of the immune/non-immune responses, resulting in active inflammation and tissue destruction. Failure to resolve inflammation is implicated in the pathogenesis of gastrointestinal inflammatory related conditions such as gastric ulcers, inflammatory bowel disease (IBD), Crohn's disease and ulcerative colitis.

Matrix metalloproteinases (MMPs) are a group of zinc-dependent endopeptidases and are important elements involved in numerous biological and pathological processes, including inflammation and oxidative stress.

Inflammatory and oxidative stress related conditions which are associated with MMP-9 include a range of different conditions such as gastrointestinal inflammatory diseases, gastric ulcers (for example peptic ulcers), gastritis, MMP-associated inflammatory conditions, inflammatory bowel disease (IBD), Crohn's disease, ulcerative colitis, Irritable Bowel Syndrome (IBS), digestive diseases, Gastroesophageal Reflux Disease (GERD), heartburn, acid reflux, *Helicobacter pylori* infection, mouth ulcers, stomatitis, pharyngitis, gingivitis, esophageal ulcers, neuropsychiatric illnesses (such as schizophrenia, bipolar mood disorder, multiple sclerosis), neurodegenerative disorders (such as traumatic brain injury, multiple sclerosis, and Alzheimer's disease), cardiovascular diseases, cancer and arthritis.

One of the main roles of MMPs in inflammation is regulating physical barriers. Inflammatory cell migration is facilitated by MMPs due to their ability to digest intercellular junctions. Several major components of endothelial adherent junctions have been identified as substrates of MMPs. The disassembly of these cellular components increases vascular permeability thus allowing the influx of inflammatory cells and plasma proteins.

MMP-9 (also known as Gelatinase B) is a proinflammatory enzyme which can proteolytically process a number of cytokines and chemokines into more active forms, such as pro-IL-1 β and IL-8 (Schonbeck et al., 1998; Van den Steen, Proost, Wuyts,

Van Damme, & Opdenakker, 2000). It is also reported that MMP-9 can regulate epithelial barrier permeability by degrading occludins in tight junctions to facilitate the influx of inflammatory cells and proteins (Caron et al., 2005; Reijerkerk et al., 2006) and is highly involved in extracellular matrix (ECM) degradation which leads to mucosal damage and cellular remodelling (Swarnakar et al., 2007). MMP-9 is associated with a number of conditions including neuropsychiatric illnesses (such as schizophrenia, bipolar mood disorder, multiple sclerosis), neurodegenerative disorders (such as traumatic brain injury, multiple sclerosis, and Alzheimer's disease), cardiovascular diseases, cancer and arthritis (Rybakowski 2009, Fingleton (2007), Reinhard, 2015).

MMP-9 is also highly associated with the occurrence and severity of gastric ulcers. Numerous studies have reported elevated expression and activity of MMP-9 during the process of gastric ulceration (Pradeepkumar Singh, Kundu, Ganguly, Mishra, & Swarnakar, 2007; Swarnakar et al., 2005, 2007). It is also reported that ethanol-induced gastric ulcers are associated with the elevation of pro-MMP-9 activity in a dose-, time- and severity-dependent manner and that MMP-9 a risk factor for the reoccurrence of gastric ulcers (Li et al., 2013).

The expression and secretion of MMP-9 is very low in normal healthy tissues. During the formation of a gastric ulcer, the induction of oxidative stress intensifies the secretion of MMP-9 and leads to mucosal damage (Ganguly & Swarnakar, 2012; Li et al., 2013). MMP-9 is thus a known therapeutic target in preventing and healing gastric ulceration.

MMP-9-associated conditions are therefore conditions in which there is an increase in expression of MMP-9, and include inflammatory conditions in which there is an increase in expression or overexpression of MMP-9. Such conditions include, but are not limited to, gastric ulcers (for example peptic ulcers), gastritis, other MMP-associated inflammatory conditions, inflammatory bowel disease (IBD), Crohn's disease, ulcerative colitis, Irritable Bowel Syndrome (IBS), digestive diseases, Gastroesophageal Reflux Disease (GERD), heartburn, acid reflux, *Helicobacter pylori* infection, mouth ulcers, stomatitis, pharyngitis, gingivitis and/or esophageal ulcers. In one particular embodiment, the MMP-9 associated inflammatory condition is gastric ulcers or gastritis. MMP-9 associated conditions also include other conditions such as including neuropsychiatric illnesses (such as schizophrenia, bipolar mood disorder, multiple sclerosis), neurodegenerative disorders (such as traumatic brain injury,

multiple sclerosis, and Alzheimer's disease), cardiovascular diseases, cancer and arthritis.

As will be appreciated from the above, MMP-9 is a desirable target for preventing, ameliorating or treating inflammation and/or preventing, ameliorating or treating conditions associated with inflammation. In particular, for preventing, ameliorating or treating conditions associated with inflammation of the gastrointestinal tract. MMP-9 is also a desirable target for treating other conditions which are associated with MMP-9, such as neuropsychiatric illnesses (such as schizophrenia, bipolar mood disorder, multiple sclerosis), neurodegenerative disorders (such as traumatic brain injury, multiple sclerosis, and Alzheimer's disease), cardiovascular diseases, cancer and arthritis.

The inventors have found that 3,6,7-trimethylumazine and compositions comprising the same have MMP-9 inhibitory activity and are therefore useful in methods of preventing, ameliorating or treating MMP-9 associated conditions, such as those related to inflammation and/or oxidative stress. The inventors found that surprisingly, 3,6,7-trimethylumazine both inhibits the activity and the expression of MMP-9. The MMP-9 inhibitory effects are significant, suggesting good efficacy and potentially a broad range of applications and uses, in particular in the prevention and/or treatment of inflammation and/or inflammatory conditions, such as gastrointestinal inflammatory conditions. In particular, gastritis and gastric ulcers.

In one aspect, the invention provides a method of preventing, ameliorating or treating an MMP-9 associated condition in a subject, comprising administering to a subject in need thereof a composition comprising 3,6,7-trimethylumazine.

In one embodiment, the MMP-9 associated condition is selected from gastrointestinal inflammatory diseases, gastric ulcers (for example peptic ulcers), gastritis, MMP-associated inflammatory conditions, inflammatory bowel disease (IBD), Crohn's disease, ulcerative colitis, Irritable Bowel Syndrome (IBS), digestive diseases, Gastroesophageal Reflux Disease (GERD), heartburn, acid reflux, *Helicobacter pylori* infection, mouth ulcers, stomatitis, pharyngitis, gingivitis, esophageal ulcers, neuropsychiatric illnesses (such as schizophrenia, bipolar mood disorder, multiple sclerosis), neurodegenerative disorders (such as traumatic brain injury, multiple sclerosis, and Alzheimer's disease), cardiovascular diseases, cancer and arthritis.

In one aspect, the invention provides a method of preventing, ameliorating or treating an MMP-9 associated inflammatory condition in a subject, comprising administering to a subject in need thereof a composition comprising 3,6,7-trimethylumazine.

In one embodiment, the MMP-9 associated inflammatory condition is associated with inflammation of the gastrointestinal tract. In one embodiment, the MMP-9 associated inflammatory condition is selected from, gastrointestinal inflammatory diseases, gastric ulcers (for example peptic ulcers), gastritis, MMP-associated inflammatory conditions, inflammatory bowel disease (IBD), Crohn's disease, ulcerative colitis, Irritable Bowel Syndrome (IBS), digestive diseases, Gastroesophageal Reflux Disease (GERD), heartburn, acid reflux, *Helicobacter pylori* infection, mouth ulcers, stomatitis, pharyngitis, gingivitis and esophageal ulcers.

In one embodiment, the MMP-9 associated inflammatory condition is selected from neuropsychiatric illnesses (such as schizophrenia, bipolar mood disorder, multiple sclerosis), neurodegenerative disorders (such as traumatic brain injury, multiple sclerosis, and Alzheimer's disease), cardiovascular diseases, cancer and arthritis.

In another aspect, the invention provides a method of preventing, ameliorating or treating inflammation of the gastrointestinal tract in a subject comprising administering to a subject in need thereof a composition comprising 3,6,7-trimethylumazine.

In another aspect, the invention provides a method of preventing, ameliorating or treating conditions associated with inflammation of the gastrointestinal tract.

In one aspect, the invention provides a method of preventing, ameliorating or treating inflammation in a subject comprising administering to a subject in need thereof a composition comprising 3,6,7-trimethylumazine. In one embodiment, the inflammation is inflammation of the gastrointestinal tract.

In one embodiment, the invention provides a method of preventing, ameliorating or treating inflammation associated with conditions such as, gastrointestinal inflammatory diseases, gastric ulcers (for example peptic ulcers), gastritis, MMP-associated inflammatory conditions, inflammatory bowel disease (IBD), Crohn's disease, ulcerative colitis, Irritable Bowel Syndrome (IBS), digestive diseases, Gastroesophageal Reflux Disease (GERD), heartburn, acid reflux, *Helicobacter pylori* infection, mouth ulcers, stomatitis, pharyngitis, gingivitis, esophageal ulcers, neuropsychiatric illnesses (such as schizophrenia, bipolar mood disorder, multiple sclerosis), neurodegenerative disorders (such as traumatic brain injury, multiple sclerosis, and Alzheimer's disease), cardiovascular diseases, cancer and arthritis.

In one embodiment, the invention provides a method of preventing, ameliorating or treating conditions such as, gastrointestinal inflammatory diseases, gastric ulcers (for example peptic ulcers), gastritis, MMP-associated inflammatory conditions,

inflammatory bowel disease (IBD), Crohn's disease, ulcerative colitis, Irritable Bowel Syndrome (IBS), digestive diseases, Gastroesophageal Reflux Disease (GERD), heartburn, acid reflux, *Helicobacter pylori* infection, mouth ulcers, stomatitis, pharyngitis, gingivitis, esophageal ulcers, neuropsychiatric illnesses (such as schizophrenia, bipolar mood disorder, multiple sclerosis), neurodegenerative disorders (such as traumatic brain injury, multiple sclerosis, and Alzheimer's disease), cardiovascular diseases, cancer and arthritis.

As will be appreciated from the above, 3,6,7-trimethylumazine, and compositions comprising the same, may be useful in a wide range of other uses, including for supporting or maintaining a subject's normal digestion, supporting or maintaining a subject's healthy digestion and supporting or maintaining a subject's general gut health and wellbeing.

In one embodiment of the invention, the origin of the 3,6,7-trimethylumazine in the methods, uses and compositions disclosed herein is from *Leptospermum*. In one embodiment, the 3,6,7-trimethylumazine is substantially from plants selected from the group comprising: *Leptospermum scoparium*, *Leptospermum polygalifolium*, *Leptospermum subtenue*, and combinations thereof. In one embodiment, the 3,6,7-trimethylumazine is from *Leptospermum scoparium* (Manuka).

In one embodiment of the invention, the origin of the 3,6,7-trimethylumazine is honey. In one embodiment, the honey is of a floral origin substantially from the genus *Leptospermum*. In one embodiment, the honey is of a floral origin substantially from: *Leptospermum scoparium*, *Leptospermum polygalifolium*, *Leptospermum subtenue*, and combinations thereof.

In one embodiment, the honey is of a floral origin substantially from *Leptospermum scoparium* (also referred to as Manuka).

In one particular embodiment, the 3,6,7-trimethylumazine is derived directly from a plant of the genus *Leptospermum*. In one embodiment of the invention, the 3,6,7-trimethylumazine is derived directly from the nectar, roots, fruit, seeds, bark, oil, leaves, wood, stems or other plant material of a plant of the genus *Leptospermum*. In one embodiment of the invention, the 3,6,7-trimethylumazine is derived directly from the nectar of a plant of the genus *Leptospermum*. In one aspect, the 3,6,7-trimethylumazine is substantially from plants selected from the group comprising: *Leptospermum scoparium*, *Leptospermum polygalifolium*, *Leptospermum subtenue*, and combinations thereof.

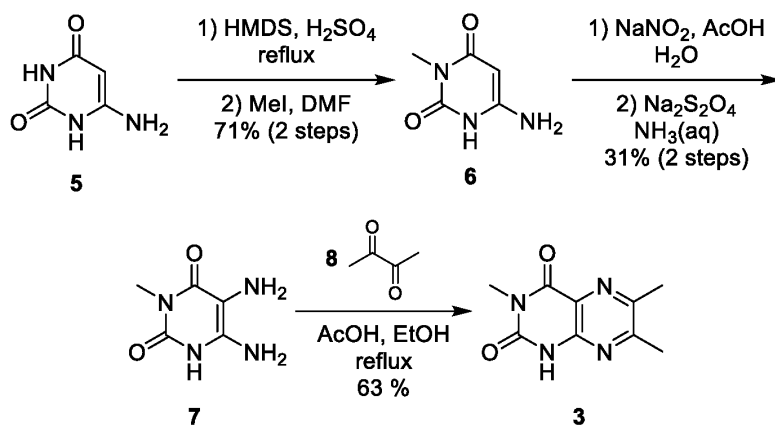
In one embodiment of the invention, the 3,6,7-trimethyluracine is synthetic.

For example, the 3,6,7-trimethyluracine may be synthesised as described in the applicant's earlier patent published as NZ 722140, incorporated herein by reference and as shown below.

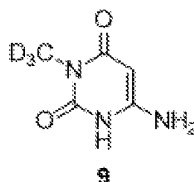
Referring to the below scheme, and following the work of Gala *et al*, (1997) *N*-methylation of 6-aminouracil (**5**) at position 3 was accomplished via silylation of the exocyclic amino and carbonyl groups upon treatment with hexamethyldisilazane (HMDS) in the presence of a catalytic amount of sulfuric acid (H_2SO_4). Ammonium sulfate could also be used as a catalyst. Methylation was then effected using iodomethane (MeI) in the presence of dimethylformamide (DMF) as an organic solvent in a 71% yield over two steps. Dimethylsulfate could also be used as a methylating agent. Subsequent desilylation during aqueous workup afforded 6-amino-3-methyluracil (**6**) in 78% yield.

Aminouracil (**6**) was then treated with sodium nitrite (NaNO_2) and acetic acid (AcOH) solution, followed by reduction with sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$) in the aqueous solvent ammonia (NH_3) at 70 °C (Chaudhari *et al*, 2009) to give 5,6-diamino-3-methyluracil (**7**) in 31% yield over two steps. Alternative acids which could be used in the nitrosation first step include hydrochloric acid. An alternative to the first step reduction with sodium nitrite and acetic acid is catalytic hydrogenation using a catalyst such as palladium on carbon or platinum dioxide in an aqueous or organic solvent.

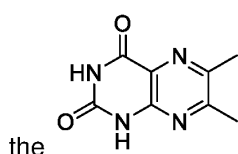
Condensation of diamino uracil (**7**) with 2,3-butanedione (**8**) in ethanol (EtOH) and acetic acid (AcOH) solution gave 3,6,7-trimethyluracine (**3**) as a colourless solid. An alternative acid for use in the condensation step is hydrochloric acid. Spectroscopic data (UV-vis, IR, ^1H NMR, ^{13}C NMR) of synthetic 3,6,7-trimethyluracine was in excellent agreement with that of the isolated natural product. Furthermore, the ^1H NMR



spectrum of both the natural and synthetic products was identical to the ^1H NMR spectra of separate natural and synthetic material. Thus the structure of 3,6,7-trimethyluracil (**3**) was definitively established as 3,6,7-trimethyluracil.

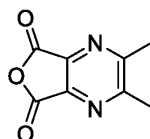


Alternative synthesis of compound (**9**) is via methylation at N-3 of



the

intermediate compound shown below or via transformation of the intermediate compound shown below into a transient isocyanate species, including but not limited to those generated by a Curtius, Hofmann, Lossen or Schmidt rearrangement.



Referring to the above, *N*-deuteromethylation of 6-aminouracil (**5**) at position 3 was accomplished via silylation of the exocyclic amino and carbonyl groups upon treatment with hexamethyldisilazane (HMDS) in the presence of a catalytic amount of sulfuric acid (H_2SO_4). Methylation was then effected using iodomethane- d_3 (CD_3I) in the presence of dimethylformamide (DMF) as an organic solvent in a 71% yield over two steps. Subsequent desilylation during aqueous workup afforded 6-amino-3-($^2\text{H}_3$)methyluracil (**9**) in 78% yield.

Amino uracil (**6**) was then treated with sodium nitrite (NaNO_2) and acetic acid (AcOH) solution, followed by reduction with sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$) in the aqueous solvent ammonia (NH_3) at 70°C (Chaudhari *et al.*, 2009) to give 5,6-diamino-3-($^2\text{H}_3$)methyluracil (**10**) in 31% yield over two steps. Alternative acids which could be used in the nitrosation first step include hydrochloric acid. An alternative to the first step reduction with sodium nitrite and acetic acid is catalytic hydrogenation using a catalyst such as palladium on carbon or platinum dioxide in an aqueous or organic solvent.

Condensation of diamino uracil (**10**) with 2,3-butanedione (**8**) in ethanol (EtOH) and acetic acid (AcOH) solution gave 3,6,7-(3-²H₃)trimethylumazine (**11**) as a colourless solid.

Materials and Methods: All reactions were carried out in flame- or oven-dried glassware under a dry nitrogen atmosphere. All reagents were purchased as reagent grade and used without further purification. Dimethyl formamide was degassed and dried using an LC Technical SP-1 solvent purification system. Ethanol was distilled over Mg(OEt)₂. Ethyl acetate, methanol, and petroleum ether were distilled prior to use. All other solvents were used as received unless stated otherwise. Solid Phase Extraction (SPE) was performed using Strata C₁₈ E 70 Å, 55 µm 20 g/60 mL columns. RP-HPLC was performed with an Agilent 1100 using a Jupiter C₁₈ 300 Å, 5 µm, 2.0 mm x 250 mm column at a flow rate of 0.2 mLmin⁻¹ with a DAD Detector operating at 262, 280 and 320 nm. A suitably adjusted gradient of 5% B to 100% B was used, where solvent A was 0.1% HCOOH in H₂O and B was 20 % A in MeCN. Flash chromatography was carried out using 0.063-0.1 mm silica gel with the desired solvent. Thin layer chromatography (TLC) was performed using 0.2 mm Kieselgel F254 (Merck) silica plates and compounds were visualised using UV irradiation at 254 or 365 nm and/or staining with a solution of potassium permanganate and potassium carbonate in aqueous sodium hydroxide. Preparative TLC was performed using 500 µm, 20 x 20 cm Uniplate™ (Analtech) silica gel TLC plates and compounds were visualised using UV irradiation at 254 or 365 nm. Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. Infrared spectra were obtained using a Perkin-Elmer Spectrum 100 FTIR spectrometer on a film ATR sampling accessory. Absorption maxima are expressed in wavenumbers (cm⁻¹). NMR spectra were recorded as indicated on either a Bruker Avance 400 spectrometer operating at 400 MHz for ¹H nuclei and 100 MHz for ¹³C nuclei, a Bruker DRX-400 spectrometer operating at 400 MHz for ¹H nuclei, 100 MHz for ¹³C nuclei, a Bruker Avance AVIII-HD 500 spectrometer operating at 500 MHz for ¹H nuclei, 125 MHz for ¹³C nuclei or a Bruker Avance 600 spectrometer operating at 600 MHz for ¹H nuclei, 150 MHz for ¹³C nuclei. ¹H and ¹³C chemical shifts are reported in parts per million (ppm) relative to CDCl₃ (¹H and ¹³C) or (CD₃)₂SO (¹H and ¹³C). ¹⁵N chemical shifts were referenced using the unified δ_f scale (Harris *et al.*, 2008) as implemented by the Bruker library function “xiref.” ¹H NMR data is reported as chemical shift, relative integral, multiplicity (s, singlet; assignment). Assignments were made with the aid of COSY, NOESY, HSQC and HMBC experiments where required. High resolution mass spectra were recorded on a Bruker micrOTOF-Q II mass spectrometer with ESI ionisation source. Ultraviolet-

visible spectra were run as H₂O solutions on a Shimadzu UV-2101PC scanning spectrophotometer.

In one embodiment, the invention provides a composition comprising 3,6,7-trimethylumazine for use in the methods described above. In one embodiment, the composition comprises a therapeutically effective amount of 3,6,7-trimethylumazine.

In one embodiment of the invention, the composition comprising 3,6,7-trimethylumazine comprises honey. In one particular embodiment, the composition comprising 3,6,7-trimethylumazine consists of honey.

In one embodiment, the honey is of a floral origin substantially from the genus *Leptospermum*. In one embodiment, the honey is substantially from plants selected from the group comprising: *Leptospermum scoparium*, *Leptospermum polygalifolium*, *Leptospermum subtenue*, and combinations thereof.

In one embodiment, the composition comprises from about 2.5 µg/mL to about 80 µg/mL 3,6,7-trimethylumazine. In one embodiment, the composition comprises about 2.5 µg/mL, about 5 µg/mL, about 10 µg/mL, about 20 µg/mL, about 40 µg/mL, about 50 µg/mL, about 60 µg/mL, about 70 µg/mL or about 80 µg/mL 3,6,7-trimethylumazine, or wherein the composition comprises a concentration of 3,6,7-trimethylumazine of from 2.5 µg/mL to 5 µg/mL, or from 5 µg/mL to 10 µg/mL, or from 10 µg/mL to 20 µg/mL, or from 20 µg/mL to 40 µg/mL, or from 40 µg/mL to 50 µg/mL, or from 50 µg/mL to about 60 µg/mL, or from 60 µg/mL to 70 µg/mL, or from 70 µg/mL to 80 µg/mL 3,6,7-trimethylumazine.

In one embodiment, the composition comprises 3,6,7-trimethylumazine from about 5 to about 80 mg/kg. In one embodiment, the composition comprises about 5 mg/kg, about 10 mg/kg, about 15 mg/kg, about 20 mg/kg, about 25 mg/kg, about 30 mg/kg, about 35 mg/kg, about 40 mg/kg, about 45 mg/kg, about 50 mg/kg, about 55 mg/kg, about 60 mg/kg, about 70 mg/kg or about 80 mg/kg of 3,6,7-trimethylumazine or wherein the composition comprises a concentration of 3,6,7-trimethylumazine of 5 to 10 mg/kg, or from 10 to 15 mg/kg, or from 15 to 20 mg/kg, or from 20 to 25 mg/kg, or from 25 to 30 mg/kg, or from 30 to 35 mg/kg, or from 35 to 40 mg/kg, or from 40 to 45 mg/kg, or from 45 to 50 mg/kg, or from 50 to 55 mg/kg, or from 55 to 60 mg/kg, or from 60 to 70 mg/kg or from 70 to 80 mg/kg.

In one embodiment the, honey is raw honey. In one embodiment, the honey is heat-treated or pasteurised according to methods that would be well known to a person skilled in the art.

In one particular embodiment, the composition comprises a honey extract. In one embodiment, the composition consists of a honey extract.

In one embodiment, the honey extract comprises a concentration of 3,6,7-trimethylumazine that is higher than the concentration of 3,6,7-trimethylumazine found naturally occurring in honey.

In one embodiment, the honey extract comprises a concentration of 3,6,7-trimethylumazine that is higher than the concentration of 3,6,7-trimethylumazine found naturally occurring in the honey from which the extract was derived.

In one embodiment, the honey from which the extract is derived is of a floral origin substantially from the genus *Leptospermum*. In one embodiment, the honey from which the extract is derived is substantially from plants selected from the group comprising: *Leptospermum scoparium*, *Leptospermum polygalifolium*, *Leptospermum subtenue*, and combinations thereof.

In one embodiment, the extract comprises from about 2.5 µg/mL to about 1000 µg/mL 3,6,7-trimethylumazine. In one embodiment, the extract comprises about 2.5 µg/mL, about 5 µg/mL, about 10 µg/mL, about 20 µg/mL, about 40 µg/mL, about 50 µg/mL, about 60 µg/mL, about 70 µg/mL, about 80 µg/mL, about 90 µg/mL, about 100 µg/mL, 150 µg/mL, about 200 µg/mL, about 250 µg/mL, about 300 µg/mL, about 350 µg/mL, about 400 µg/mL, about 450 µg/mL, about 500 µg/mL, about 550 µg/mL, about 600 µg/mL, about 650 µg/mL, about 700 µg/mL, about 750 µg/mL, about 800 µg/mL, about 850 µg/mL, about 900 µg/mL, about 950 µg/mL, to about 1000 3,6,7-trimethylumazine, or wherein the composition comprises 3,6,7-trimethylumazine from about 2.5 to 5 µg/mL, about 5 to 10 µg/mL, about 10 to 20 µg/mL, about 20 to 40 µg/mL, about 40 to 50 µg/mL, about 50 to 60 µg/mL, about 60 to 70 µg/mL, about 70 to 80 µg/mL, about 80 to 90 µg/mL, about 90 to 100 µg/mL, about 100 to 150 µg/mL, 150 to 200 µg/mL, about 200 to 250 µg/mL, about 250 to 300 µg/mL, about 300 to 350 µg/mL, about 350 to 400 µg/mL, about 400 to 450 µg/mL, about 450 to 500 µg/mL, about 500 to 550 µg/mL, about 550 to 600 µg/mL, about 600 to 650 µg/mL, about 650 to 700 µg/mL, about 700 to 750 µg/mL, about 750 to 800 µg/mL, about 800 to 850 µg/mL, about 850 to 900 µg/mL, about 900 to 950 µg/mL, about 950 to 1000 µg/mL.

In one embodiment, the extract comprises 3,6,7-trimethylumazine from about 5 to about 3000 mg/kg. In one embodiment, the extract comprises about 5 mg/kg, about 10 mg/kg, about 15 mg/kg, about 20 mg/kg, about 25 mg/kg, about 30 mg/kg, about 35 mg/kg, about 40 mg/kg, about 45 mg/kg, about 50 mg/kg, about 55 mg/kg, about 60 mg/kg, about 70 mg/kg, about 80 mg/kg, about 90 mg/kg, about 100 mg/kg, about 150

mg/kg, about 200 mg/kg, about 250 mg/kg, about 300mg/kg, about 350 mg/kg, about 400 mg/kg, about 450 mg/kg, about 500 mg/kg, about 550 mg/kg, about 600 mg/kg, about 650 mg/kg, about 700 mg/kg, about 750 mg/kg, about 800 mg/kg, about 850 mg/kg, about 900 mg/kg, about 950 mg/kg, about 1000 mg/kg, about 1100 mg/kg, about 1200 mg/kg, about 1300 mg/kg, about 1400 mg/kg, about 1500 mg/kg, about 1600 mg/kg, about 1700 mg/kg, about 1800 mg/kg, about 1900 mg/kg, about 2000 mg/kg, about 2100 mg/kg, about 2200 mg/kg, about 2300 mg/kg, about 2400 mg/kg, about 2500 mg/kg, about 2600 mg/kg, about 2700 mg/kg, about 2800 mg/kg, about 2900 mg/kg to about 3000 mg/kg of 3,6,7-trimethylumazine or wherein the extract comprises a concentration of 3,6,7-trimethylumazine of 5 to 10 mg/kg, or from 10 to 15 mg/kg, or from 15 to 20 mg/kg, or from 20 to 25 mg/kg, or from 25 to 30 mg/kg, or from 30 to 35 mg/kg, or from 35 to 40 mg/kg, or form 40 to 45 mg/kg, or from 45 to 50 mg/kg, or from 50 to 55 mg/kg, or from 55 to 60 mg/kg, or from 60 70 mg/kg or from 70 to 80 mg/kg, about 90 to 100 mg/kg, about 100 to 150 mg/kg, about 150 to 200 mg/kg, about 200 mg/kg, about 250 to 300 mg/kg, about 300 to 350 mg/kg, about 350 to 400 mg/kg, about 400 to 450 mg/kg, about 450 to 500 mg/kg, about 500 to 550 mg/kg, about 550 to 600 mg/kg, about 600 to 650 mg/kg, about 650 to 700 mg/kg, about 700 to 750 mg/kg, about 750 to 800 mg/kg, about 800 to 850 mg/kg, about 850 to 900 mg/kg, about 900 to 950 mg/kg, about 950 to 1000 mg/kg, about 1000 to 1100 mg/kg, about 1100 to 1200 mg/kg, about 1200 to 1300 mg/kg, about 1300 to 1400 mg/kg, about 1400 to 1500 mg/kg, about 1500 to 1600 mg/kg, about 1600 to 1700 mg/kg, about 1700 to 1800 mg/kg, about 1800 to 1900 mg/kg, about 1900 to 2000 mg/kg, about 2000 to 2100 mg/kg, about 2100 to 2200 mg/kg, about 2200 to 2300 mg/kg, about 2300 to 2400 mg/kg, about 2400 to 2500 mg/kg, about 2500 to 2600 mg/kg, about 2600 to 2700 mg/kg, about 2700 to 2800 mg/kg, about 2800 to 2900 mg/kg, about 2900 to 3000 mg/kg.

In one embodiment, the composition comprises at least 0.1%, 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% 3,6,7-trimethylumazine.

In one embodiment, the composition comprises a honey extract and further comprises honey.

In one embodiment, the composition comprises isolated 3,6,7-trimethylumazine that is isolated from honey. In one embodiment, the honey is of a floral origin substantially from the genus *Leptospermum*. In one embodiment, the honey is substantially from plants selected from the group comprising: *Leptospermum scoparium*, *Leptospermum polygalifolium*, *Leptospermum subtenue*, and combinations thereof.

In one embodiment, the 3,6,7-trimethylumazine is isolated by any method well known to a person skilled in the art. In one embodiment, the 3,6,7-trimethylumazine is isolated by subjection of the honey to SPE (solid phase extraction), followed by normal-phase flash chromatography and preparative TLC (thin layer chromatography).

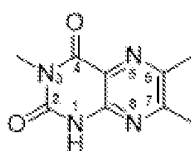
In one embodiment, the 3,6,7-trimethylumazine is isolated by a method as described in the applicant's earlier patent published as NZ 722140, incorporated herein by reference and as shown below. incorporated herein by reference, and as shown below.

Chemical isolation of 3,6,7-trimethylumazine

Raw manuka honey (51.3 g) was dissolved in H₂O + 0.1% HCOOH (150 mL) and sonicated for 20 min. The resulting suspension was filtered through Celite and the filtrate used in the next step.

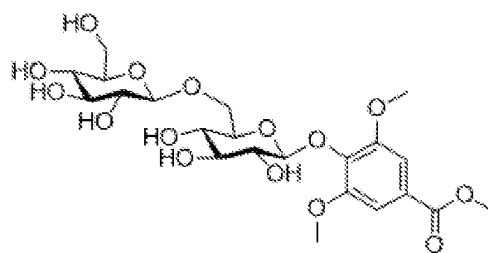
The filtrate was divided into two portions of 100 mL and each portion was subjected to SPE using MeOH-H₂O + 0.1% HCOOH (1:9, 80 mL) to remove undesired substances. The desired fraction was then eluted using MeOH-H₂O + 0.1% HCOOH (4:1, 80 mL). The two fractions were combined and concentrated to give the crude extract (0.23 g) which was further purified by flash chromatography (pet. ether-EtOAc 1:4) to give purified extract (3 mg) as a brown solid.

Several purified extracts were combined (6 mg total) and further purified by preparative TLC (pet. ether-EtOAc 1:3, 4 runs) to give **3** (4 mg) (as shown below) as a colourless solid.



3

While using HPLC to examine New Zealand and Australian honeys derived from species of *Leptospermum*, *Eucalyptus*, *Kunzea* and *Knightia* for the presence of leptosperin (**4**) (Kato *et al.* 2012 and 2014; Aitken, *et al.* 2013; structure as shown below) a proposed biomarker for *Leptospermum* honey, an unexpected UV absorbance was noted at 320 nm.



4

This peak was observed only in *Leptospermum* honeys (*L. scoparium*, *L. scoparium* var. *exinium*, *L. polygalifolium*, *L. subtenue*), including honey derived from *L. subtenue* in which no leptosperin was detected. The use of solid phase extraction (SPE) followed by reverse-phase HPLC enabled purification of the compound that exhibited the UV absorbance at 320 nm. However this method was time consuming, low yielding and not scalable, hence a more efficient isolation method was sought. Subjection of manuka honey to SPE, followed by normal-phase flash chromatography and preparative TLC enabled isolation of 3,6,7-trimethylleucospermin as a colourless solid in sufficient quantity to conduct spectroscopic analysis.

In one embodiment of the invention, the composition comprises synthetic 3,6,7-trimethylleucospermin or isolated 3,6,7-trimethylleucospermin. In one embodiment, the composition further comprises honey. In one embodiment, the composition consists of synthetic 3,6,7-trimethylleucospermin and honey. In one embodiment, the composition consists of isolated 3,6,7-trimethylleucospermin and honey.

In one embodiment, the honey is of a floral origin substantially from the genus *Leptospermum*. In one embodiment, the honey is substantially from plants selected from the group comprising: *Leptospermum scoparium*, *Leptospermum polygalifolium*, *Leptospermum subtenue*, and combinations thereof.

In one embodiment, the composition comprises synthetic 3,6,7-trimethylleucospermin or isolated 3,6,7-trimethylleucospermin from about 2.5 µg/mL to about 1000 µg/mL 3,6,7-trimethylleucospermin. In one embodiment, the composition comprises synthetic 3,6,7-trimethylleucospermin or isolated 3,6,7-trimethylleucospermin from about 2.5 µg/mL, about 5 µg/mL, about 10 µg/mL, about 20 µg/mL, about 40 µg/mL, about 50 µg/mL, about 60 µg/mL, about 70 µg/mL, about 80 µg/mL, about 90 µg/mL, about 100 µg/mL, 150 µg/mL, about 200 µg/mL, about 250 µg/mL, about 300 µg/mL, about 350 µg/mL, about 400 µg/mL, about 450 about 500 µg/mL, about 550 µg/mL, about 600 µg/mL, about 650 µg/mL, about 700 µg/mL, about 750 µg/mL, about 800 µg/mL, about 850 µg/mL, about 900 µg/mL, about 950 µg/mL, to about 1000 µg/mL or wherein the composition

comprises synthetic 3,6,7-trimethylumazine or isolated 3,6,7-trimethylumazine of from about 2.5 to 5 µg/mL, about 5 to 10 µg/mL, about 10 to 20 µg/mL, about 20 to 40 µg/mL, about 40 to 50 µg/mL, about 50 to 60 µg/mL, about 60 to 70 µg/mL, about 70 to 80 µg/mL, about 80 to 90 µg/mL, about 90 to 100 µg/mL, about 100 to 150 µg/mL, 150 to 200 µg/mL, about 200 to 250 µg/mL, about 250 to 300 µg/mL, about 300 to 350 µg/mL, about 350 to 400 µg/mL, about 400 to 450 µg/mL, about 450 to 500 µg/mL, about 500 to 550 µg/mL, about 550 to 600 µg/mL, about 600 to 650 µg/mL, about 650 to 700 µg/mL, about 700 to 750 µg/mL, about 750 to 800 µg/mL, about 800 to 850 µg/mL, about 850 to 900 µg/mL, about 900 to 950 µg/mL, about 950 to 1000 µg/mL.

In one embodiment, the composition comprises synthetic 3,6,7-trimethylumazine or isolated 3,6,7-trimethylumazine from about 5 mg/kg to about 3000 mg/kg. In one embodiment, the composition comprises synthetic 3,6,7-trimethylumazine or isolated 3,6,7-trimethylumazine from about 5 mg/kg, about 10 mg/kg, about 15 mg/kg, about 20 mg/kg, about 25 mg/kg, about 30 mg/kg, about 35 mg/kg, about 40 mg/kg, about 45 mg/kg, about 50 mg/kg, about 55 mg/kg, about 60 mg/kg, about 70 mg/kg, about 80 mg/kg, about 90 mg/kg, about 100 mg/kg, about 150 mg/kg, about 200 mg/kg, about 250 mg/kg, about 300mg/kg, about 350 mg/kg, about 400 mg/kg, about 450 mg/kg, about 500 mg/kg, about 550 mg/kg, about 600 mg/kg, about 650 mg/kg, about 700 mg/kg, about 750 mg/kg, about 800 mg/kg, about 850 mg/kg, about 900 mg/kg, about 950 mg/kg, about 1000 mg/kg, about 1100 mg/kg, about 1200 mg/kg, about 1300 mg/kg, about 1400 mg/kg, about 1500 mg/kg, about 1600 mg/kg, about 1700 mg/kg, about 1800 mg/kg, about 1900 mg/kg, about 2000 mg/kg, about 2100 mg/kg, about 2200 mg/kg, about 2300 mg/kg, about 2400 mg/kg, about 2500 mg/kg, about 2600 mg/kg, about 2700 mg/kg, about 2800 mg/kg, about 2900 mg/kg to about 3000 mg/kg or wherein the composition comprises synthetic 3,6,7-trimethylumazine or isolated 3,6,7-trimethylumazine of from 5 to 10 mg/kg, or from 10 to 15 mg/kg, or from 15 to 20 mg/kg, or from 20 to 25 mg/kg, or from 25 to 30 mg/kg, or from 30 to 35 mg/kg, or from 35 to 40 mg/kg, or form 40 to 45 mg/kg, or from 45 to 50 mg/kg, or from 50 to 55 mg/kg, or from 55 to 60 mg/kg, or from 60 70 mg/kg or from 70 to 80 mg/kg, about 90 to 100 mg/kg, about 100 to 150 mg/kg, about 150 to 200 mg/kg, about 200 mg/kg, about 250 to 300 mg/kg, about 300 to 350 mg/kg, about 350 to 400 mg/kg, about 400 to 450 mg/kg, about 450 to 500 mg/kg, about 500 to 550 mg/kg, about 550 to 600 mg/kg, about 600 to 650 mg/kg, about 650 to 700 mg/kg, about 700 to 750 mg/kg, about 750 to 800 mg/kg, about 800 to 850 mg/kg, about 850 to 900 mg/kg, about 900 to 950 mg/kg, about 950 to 1000 mg/kg, about 1000 to 1100 mg/kg, about 1100 to 1200 mg/kg, about 1200 to 1300 mg/kg, about 1300 to 1400 mg/kg, about 1400 to

1500 mg/kg, about 1500 to 1600 mg/kg, about 1600 to 1700 mg/kg, about 1700 to 1800 mg/kg, about 1800 to 1900 mg/kg, about 1900 to 2000 mg/kg, about 2000 to 2100 mg/kg, about 2100 to 2200 mg/kg, about 2200 to 2300 mg/kg, about 2300 to 2400 mg/kg, about 2400 to 2500 mg/kg, about 2500 to 2600 mg/kg, about 2600 to 2700 mg/kg, about 2700 to 2800 mg/kg, about 2800 to 2900 mg/kg, about 2900 to 3000 mg/kg.

In one embodiment, the composition comprises 0.1% to 100% 3,6,7-trimethylumazine.

In one embodiment, the composition comprises at least 0.1%, 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or substantially pure 3,6,7-trimethylumazine.

Compositions comprising honey-derived 3,6,7-trimethylumazine and/or synthetic 3,6,7-trimethylumazine are not anticipated to have side effects. 3,6,7-trimethylumazine is naturally occurring in some honey and such honey containing 3,6,7-trimethylumazine has been sold and consumed for many years.

The composition comprising 3,6,7-trimethylumazine may be formulated as a medicament, therapeutic product or health supplement.

In one embodiment, the composition comprising 3,6,7-trimethylumazine is formulated as a medicament, therapeutic product or health supplement. In one embodiment, the composition comprising 3,6,7-trimethylumazine is formulated into a range of delivery systems, including but not limited to, liquid formulations, capsules, fast moving consumer goods, chewable tablet, tablets, suppositories, intravenous preparations, intramuscular preparations, subcutaneous preparations, solutions, food, beverages, dietary supplements, cosmetic formulations, gels, lotions, powders or sprays.

In one particular embodiment, the method of the invention as described above comprises administration of the composition comprising 3,6,7-trimethylumazine from about 1mg to about 3000mg. In one particular embodiment, the method of the invention as described above comprises administration of the composition comprising 3,6,7-trimethylumazine from about 1 mg, 10 mg, 20 mg, 30 mg, 40 mg, 50 mg, 60 mg, 70 mg, 80 mg, 90 mg, 100mg, 150 mg, 200mg, 250 mg, 300mg, 350 mg, 400mg, 450 mg, 500mg, 550 mg, 600mg, 650mg, 700mg, 750 mg, 800mg, 850 mg, 900mg, 950 mg, 1000mg, 1100mg, 1200mg, 1300mg, 1400mg, 1500mg, 1600mg, 1700mg, 1800mg, 1900mg, 2000mg, 2100mg, 2200mg, 2300mg, 2400mg, 2500mg, 2600mg, 2700mg, 2800mg, 2900mg, 3000mg.

In one particular embodiment, the method of the invention as described above comprises administration of composition comprising 3,6,7-trimethylumazine, including

wherein the composition is honey or a honey extract. In one embodiment, the honey in the method of the invention is administered at a dose of from about 5g to about 100g. In one embodiment, the honey is administered at a dose of from about 5g, 10g, 15g, 20g, 25g, 30g, 40g, 50g, 60g, 70g, 80g, 90g, 100g. In one embodiment, the honey is administered at a dose of equivalent to about 1 teaspoon to about 5 tablespoons of honey. In one embodiment, the honey is administered as a single dose or in multiple doses.

In one embodiment, the composition comprising 3,6,7-trimethylumazine is administered as a single dose or as a divided dose. In one embodiment, the composition comprising 3,6,7-trimethylumazine is administered as one, two three or four separate doses.

In one particular embodiment, the method of the invention as described above comprises administration of the composition comprising 3,6,7-trimethylumazine one, two, three or four times daily. In another embodiment, the method of the invention as described above comprises administration of the composition comprising 3,6,7-trimethylumazine one, two, three, four, five, six or seven times weekly.

The concentration of 3,6,7-trimethylumazine can vary significantly from honey sample to honey sample. Therefore, in one particular embodiment of the invention described herein, the composition comprising honey has a standardised concentration of 3,6,7-trimethylumazine.

In one embodiment, the composition comprising 3,6,7-trimethylumazine has a standardised concentration of 3,6,7-trimethylumazine obtained by:

- selecting a first composition with a known concentration of 3,6,7-trimethylumazine;
- selecting at least one further composition with a known concentration of 3,6,7-trimethylumazine;
- combining the first composition with the second composition to obtain a final composition with a standardised 3,6,7-trimethylumazine concentration of from about 5 mg/kg to about 3000 mg/kg.

In one embodiment, the composition comprising 3,6,7-trimethylumazine has a standardised concentration of 3,6,7-trimethylumazine obtained by:

- selecting a first composition with a known concentration of 3,6,7-trimethylumazine;
- combining the selected first composition with one or more of:

- synthetic 3,6,7-trimethylumazine;
- isolated 3,6,7-trimethylumazine;
- a honey extract comprising 3,6,7-trimethylumazine; and/or
- 3,6,7-trimethylumazine derived directly from a plant of the genus

Leptospermum

to form a composition with a standardised 3,6,7-trimethylumazine concentration of from about 5 mg/kg to about 3000 mg/kg.

In one embodiment, the composition comprising 3,6,7-trimethylumazine has a standardised concentration of 3,6,7-trimethylumazine obtained by:

- selecting a first composition comprising honey with a known concentration of 3,6,7-trimethylumazine;
- combining the selected first composition comprising honey with one or more of:
 - synthetic 3,6,7-trimethylumazine;
 - isolated 3,6,7-trimethylumazine; and
 - a honey extract comprising 3,6,7-trimethylumazine; and/or
 - 3,6,7-trimethylumazine derived directly from a plant of the genus

Leptospermum

to form a composition with a standardised 3,6,7-trimethylumazine concentration of from about 5 to about 3000 mg/kg.

In one embodiment, the composition comprises honey, a honey extract, isolated 3,6,7-trimethylumazine and/or synthetic 3,6,7-trimethylumazine.

In one embodiment, the 3,6,7-trimethylumazine derived directly from a plant is derived directly from the flowers, nectar, roots, fruit, seeds, bark, oil, leaves, wood, stems or other plant material of a plant of the genus *Leptospermum*.

In one embodiment, the standardised 3,6,7-trimethylumazine concentration is from: about 2.5 µg/mL to about 1000 µg/mL 3,6,7-trimethylumazine. In one embodiment, the standardised 3,6,7-trimethylumazine concentration is from: about 2.5 µg/mL, about 5 µg/mL, about 10 µg/mL, about 20 µg/mL, about 40 µg/mL, about 50 µg/mL, about 60 µg/mL, about 70 µg/mL, about 80 µg/mL, about 90 µg/mL, about 100 µg/mL, 150 µg/mL, about 200 µg/mL, about 250 µg/mL, about 300 µg/mL, about 350 µg/mL, about 400 µg/mL, about 450 about 500 µg/mL, about 550 µg/mL, about 600 µg/mL, about 650 µg/mL, about 700 µg/mL, about 750 µg/mL, about 800 µg/mL, about 850 µg/mL, about 900 µg/mL, about 950 µg/mL, to about 1000 3,6,7-trimethylumazine.

In one embodiment, the standardised 3,6,7-trimethylumazine concentration is from: about 5 mg/kg to about 3000 mg/kg. In one embodiment, the standardised 3,6,7-trimethylumazine concentration is from: about 5 mg/kg, about 10 mg/kg, about 15 mg/kg, about 20 mg/kg, about 25 mg/kg, about 30 mg/kg, about 35 mg/kg, about 40 mg/kg, about 45 mg/kg, about 50 mg/kg, about 55 mg/kg, about 60 mg/kg, about 70 mg/kg, about 80 mg/kg, about 90 mg/kg, about 100 mg/kg, about 150 mg/kg, about 200 mg/kg, about 250 mg/kg, about 300mg/kg, about 350 mg/kg, about 400 mg/kg, about 450 mg/kg, about 500 mg/kg, about 550 mg/kg, about 600 mg/kg, about 650 mg/kg, about 700 mg/kg, about 750 mg/kg, about 800 mg/kg, about 850 mg/kg, about 900 mg/kg, about 950 mg/kg, about 1000 mg/kg, about 1100 mg/kg, about 1200 mg/kg, about 1300 mg/kg, about 1400 mg/kg, about 1500 mg/kg, about 1600 mg/kg, about 1700 mg/kg, about 1800 mg/kg, about 1900 mg/kg, about 2000 mg/kg, about 2100 mg/kg, about 2200 mg/kg, about 2300 mg/kg, about 2400 mg/kg, about 2500 mg/kg, about 2600 mg/kg, about 2700 mg/kg, about 2800 mg/kg, about 2900 mg/kg to about 3000 mg/kg of 3,6,7-trimethylumazine.

In one embodiment, the concentration of the 3,6,7-trimethylumazine is determined by chromatography, analytical measurements, spectrophotometry and/or any other method known to a person skilled in the art. In one embodiment, the concentration of 3,6,7-trimethylumazine is determined by reverse-phase HPLC system.

In one embodiment, the 3,6,7-trimethylumazine concentration in the honey is determined by a method as previously described in NZ 722140, herein incorporated by reference.

In another particular aspect, the invention provides a method of making a composition with anti-inflammatory and/or MMP-9 inhibitory activity comprising:

- a. testing a first composition comprising honey for 3,6,7-trimethylumazine concentration;
- b. testing at least one further composition comprising honey for 3,6,7-trimethylumazine concentration;
- c. selecting a composition comprising honey with a 3,6,7-trimethylumazine concentration greater than from about 5 mg/kg 3,6,7-trimethylumazine;
- d. selecting at least one further composition comprising honey with a 3,6,7-trimethylumazine concentration greater than from about 5 mg/kg 3,6,7-trimethylumazine;

- e. combining the selected composition comprising honey to form a honey composition with a 3,6,7-trimethylumazine concentration of at least from about 5 to about 80 mg/kg.

In one embodiment, the compositions comprising honey are selected if they have a concentration of 3,6,7-trimethylumazine greater than: about 5 mg/kg, about 10 mg/kg, about 15 mg/kg, about 20 mg/kg, about 25 mg/kg, about 30 mg/kg, about 35 mg/kg, about 40 mg/kg, about 45 mg/kg, about 50 mg/kg, about 55 mg/kg, about 60 mg/kg, about 70 mg/kg or about 80 mg/kg.

In one embodiment, the method further comprises a step of packaging the composition identified as having anti-inflammatory activity with a label identifying that it has a 3,6,7-trimethylumazine concentration of at least from about 5 to about 80 mg/kg. In one particular embodiment, at least from: about 5 mg/kg, about 10 mg/kg, about 15 mg/kg, about 20 mg/kg, about 25 mg/kg, about 30 mg/kg, about 35 mg/kg, about 40 mg/kg, about 45 mg/kg, about 50 mg/kg, about 55 mg/kg, about 60 mg/kg, about 70 mg/kg or about 80 mg/kg 3,6,7-trimethylumazine. In one embodiment, at least from 5 to 10 mg/kg, or from 10 to 15 mg/kg, or from 15 to 20 mg/kg, or from 20 to 25 mg/kg, or from 25 to 30 mg/kg, or from 30 to 35 mg/kg, or from 35 to 40 mg/kg, or from 40 to 45 mg/kg, or from 45 to 50 mg/kg, or from 50 to 55 mg/kg, or from 55 to 60 mg/kg, or from 60 to 70 mg/kg or from 70 to 80 mg/kg 3,6,7-trimethylumazine.

In one particular embodiment, the composition is honey or a honey extract.

In one embodiment, the composition with anti-inflammatory activity is suitable for use in any one of the methods as described above and below.

In one embodiment, the concentration of the 3,6,7-trimethylumazine is determined by chromatography, analytical measurements, spectrophotometry and/or any other method known to a person skilled in the art. In one embodiment, the concentration of 3,6,7-trimethylumazine is determined by reverse-phase HPLC system.

In one embodiment, the 3,6,7-trimethylumazine concentration is determined by a method as previously described in NZ 722140, herein incorporated by reference. In another particular aspect, the invention provides a method of identifying a composition as having anti-inflammatory and/or MMP-9 inhibitory activity comprising:

- a. testing a composition for 3,6,7-trimethylumazine concentration; and
 - i. identifying the composition as having anti-inflammatory activity if it contains a 3,6,7-trimethylumazine concentration greater than from about 5 to about 80 mg/kg 3,6,7-trimethylumazine; or

- ii. identifying the composition as not having anti-inflammatory activity if it contains a 3,6,7-trimethylumazine concentration lower than from about 5 mg/kg 3,6,7-trimethylumazine.

In one embodiment, the composition comprises honey, a honey extract, isolated 3,6,7-trimethylumazine and/or synthetic 3,6,7-trimethylumazine.

In one embodiment, the composition is determined as having anti-inflammatory activity if it contains greater than: about 5 mg/kg, about 10 mg/kg, about 15 mg/kg, about 20 mg/kg, about 25 mg/kg, about 30 mg/kg, about 35 mg/kg, about 40 mg/kg, about 45 mg/kg, about 50 mg/kg, about 55 mg/kg, about 60 mg/kg, about 70 mg/kg or about 80 mg/kg.

In one embodiment, the method further comprises a step of packaging the composition identified as having anti-inflammatory activity with a label identifying that it has a 3,6,7-trimethylumazine concentration of at least from about 5 to about 80 mg/kg and as having anti-inflammatory activity.

In one embodiment, the composition with anti-inflammatory activity is suitable for use in any one of the methods as described above and below.

In one particular embodiment, the composition is honey or a honey extract.

In another particular aspect, the invention provides a method of identifying a composition with anti-inflammatory and/or MMP-9 inhibitory activity suitable for use in a method of preventing, ameliorating or treating a condition associated with inflammation of the gastrointestinal tract comprising:

- a. testing a composition for 3,6,7-trimethylumazine concentration; and
 - i. identifying the composition as suitable for use in a method of preventing, ameliorating or treating a condition associated with inflammation of the gastrointestinal tract if it contains a 3,6,7-trimethylumazine concentration greater than from about 5 to about 80 mg/kg 3,6,7-trimethylumazine; or
 - ii. identifying the composition as not suitable for use in a method of preventing, ameliorating or treating a condition associated with inflammation of the gastrointestinal tract if it contains a 3,6,7-trimethylumazine concentration lower than from about 5 mg/kg 3,6,7-trimethylumazine.

In one embodiment, the composition comprises honey, a honey extract, isolated 3,6,7-trimethylumazine and/or synthetic 3,6,7-trimethylumazine.

In one embodiment, the conditions associated with the gastrointestinal tract is selected from gastrointestinal inflammatory diseases, gastric ulcers (for example peptic ulcers), gastritis, MMP-associated inflammatory conditions, inflammatory bowel disease (IBD), Crohn's disease, ulcerative colitis, Irritable Bowel Syndrome (IBS), digestive diseases, Gastroesophageal Reflux Disease (GERD), heartburn, acid reflux, *Helicobacter pylori* infection, mouth ulcers, stomatitis, pharyngitis, gingivitis, esophageal ulcers, neuropsychiatric illnesses (such as schizophrenia, bipolar mood disorder, multiple sclerosis), neurodegenerative disorders (such as traumatic brain injury, multiple sclerosis, and Alzheimer's disease), cardiovascular diseases, cancer and arthritis.

In one embodiment, the method further comprises a step of packaging the composition identified by the method above with a label identify it has having a 3,6,7-trimethylumazine concentration of at least from about 5 to about 80 mg/kg.

In another particular aspect, the invention provides a method of identifying a composition with anti-inflammatory and/or MMP-9 inhibitory activity suitable for use in a method of preventing, ameliorating or treating inflammation of the gastrointestinal tract comprising:

- a. testing a batch of honey for 3,6,7-trimethylumazine concentration; and
 - i. identifying the composition as suitable for use in a method of preventing, ameliorating or treating inflammation of the gastrointestinal tract if it contains a 3,6,7-trimethylumazine concentration greater than from about 5 to about 80 mg/kg 3,6,7-trimethylumazine; or
 - ii. identifying the composition as not suitable for use in a method of preventing, ameliorating or treating inflammation of the gastrointestinal tract if it contains a 3,6,7-trimethylumazine concentration lower than from about 5 mg/kg 3,6,7-trimethylumazine.

In one embodiment, the composition comprises honey, a honey extract, isolated 3,6,7-trimethylumazine and/or synthetic 3,6,7-trimethylumazine.

In one embodiment, the gastrointestinal inflammation is associated with a conditions selected from: gastrointestinal inflammatory diseases, gastric ulcers (for example peptic ulcers), gastritis, MMP-associated inflammatory conditions, inflammatory bowel

disease (IBD), Crohn's disease, ulcerative colitis, Irritable Bowel Syndrome (IBS), digestive diseases, Gastroesophageal Reflux Disease (GERD), heartburn, acid reflux, *Helicobacter pylori* infection, mouth ulcers, stomatitis, pharyngitis, gingivitis, esophageal ulcers, neuropsychiatric illnesses (such as schizophrenia, bipolar mood disorder, multiple sclerosis), neurodegenerative disorders (such as traumatic brain injury, multiple sclerosis, and Alzheimer's disease), cardiovascular diseases, cancer and arthritis.

In one embodiment, the method further comprises a step of packaging the composition identified by the method above with a label identifying that it has a 3,6,7-trimethylumazine concentration of at least from about 5 to about 80 mg/kg.

In one embodiment, the concentration of 3,6,7-trimethylumazine may be determined by chromatography, analytical measurements, spectrophotometry and/or any other method known to a person skilled in the art. In one embodiment, the concentration of 3,6,7-trimethylumazine is determined by reverse-phase HPLC system.

Quantification of 3,6,7-trimethylumazine in manuka honey using mass spectrometry

In one embodiment of the invention, the 3,6,7-trimethylumazine concentration is determined by a method as previously described in NZ 722140 filed by the same applicant, herein incorporated by reference and copied below:

Described is a quantitative technique to measure 3,6,7-trimethylumazine concentration using tandem mass spectrometry (LC-MS/MS). A heavier 3,6,7-trimethylumazine isotope was synthesized and employed as an internal standard to compensate the matrix effect from manuka honey. There was no interference from endogenous compound in manuka honey and the 3 Da mass difference can be clearly distinguished on the mass spectrum. The results described further below of LC-MS/MS strongly correlates with previous data from HPLC quantification and fluorescence spectrometry. Therefore 3,6,7-trimethylumazine can be accurately determined using all three methods. Results from LC-MS/MS quantification was comparatively lower than previous data from HPLC, this may be resulted from minor co-eluting compounds under the same HPLC peak. These findings demonstrate that quantitative mass spectrometry may be used as a standalone or complimentary approach for manuka honey authentication.

To validate the LC-MS/MS method, the mass spectrum of a typical manuka honey was obtained before and after the supplementation of the heavier 3,6,7-trimethylumazine

isotope. As shown, there was no significant interfering peaks from endogenous compounds in manuka honey from m/z 210-212. The 3 Da mass difference between the isotopes may be clearly identified on the mass spectrum. The final testing concentration of manuka honey was determined at 0.2% w/v to reduce sugar concentration while retaining relatively high mass spectrum resolution.

LC-MS/MS quantification

During the LC-stage, the endogenous 3,6,7-trimethylumazine and the heavier isotope co-eluted at the exact same time (12.85 min). These isomers displayed almost identical MS/MS spectrum, while only differentiated by a 3 Da mass shift from m/z 189 to m/z 192. The most abundant common ion was observed at 148.05 m/z . The heavy isotopes were not present on the part of the structure represented by this fragment ion. This common ion is employed for 3,6,7-trimethylumazine quantification to reduce background interference.

Comparing LC-MS/MS and HPLC quantification

Endogenous 3,6,7-trimethylumazine concentration was quantified as 3-44 mg/kg using mass spectrometry quantification. The results demonstrated strong linear correlation with previous data from HPLC analysis on the same set of manuka honey samples ($R^2=0.9517$). It should be noted that the mass spectrometry result was comparably lower than previous HPLC quantification (5-52 mg/kg). This suggests that other UV-absorbing compounds may have co-eluted with 3,6,7-trimethylumazine under the same HPLC peak.

The results from mass spectrometry quantification also correlates well with the signature fluorescence at $\lambda_{ex}330\text{nm}$ - $\lambda_{em}470\text{nm}$ ($R^2=0.8995$).

Structure Elucidation of 3,6,7-trimethylumazine

The chemical structure elucidation of 3,6,7-trimethylumazine was described in NZ 722140, incorporated herein by reference, and as shown below.

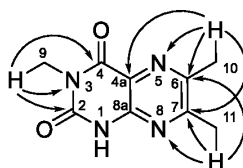
Table 1. ^1H , ^{13}C and ^{15}N NMR data for **3**^a

Position	$\delta_{\text{C}}/\delta_{\text{N}}$, type	δ_{H}	HMBC ^b
1	NH	8.42 br	
2	149.9, C		
3	154.1, N		
4	161.1, C		
4a	123.7, C		

5	292.0, N		
6	158.9, C		
7	150.6, C		
8	329.9, N		
8a	144.8, C		
9	28.5, CH ₃	3.50, s	2, 3, 4
10	22.8, CH ₃	2.63, s	4a, 5, 6, 7
11	21.9, CH ₃	2.67, s	6, 7, 8

^a ¹H (400 MHz); ¹³C (100 MHz); ¹⁵N (60.8 MHz), chemical shift indirectly determined from ¹H-¹⁵N HMBC NMR data. ^b HMBC correlations are from protons stated to the indicated carbon or nitrogen.

Referring to Table 1 above, the molecular formula of the unknown compound was established as C₉H₁₀N₄O₂ by positive ion HRESIMS. The compound was soluble in CD₃OD and CDCl₃; the latter was used for recording NMR spectra due to the presence of a broad resonance at δ 8.55 ppm (H-1) that was not present in spectra recorded in CD₃OD. This peak was assigned as an amide proton on the basis of its chemical shift and the absence of a distinctive hydroxyl absorption in the IR spectrum. Two singlets at δ 2.63 ppm (H-10) and δ 2.67 ppm (H-11) were assigned as heteroaryl methyl groups on the basis of their chemical shift, and the remaining singlet at δ 3.50 ppm (H-9) was assigned as an *N*-methyl group due to HMBC correlations of equal intensity to two quaternary carbonyl ¹³C signals (C-2, C-4, see below) and an HSQC correlation to a carbon signal at δ 28.5 ppm (C-9).



3

¹H-¹⁵N HMBC correlations from H-10 and H-11 to N-5 and N-8 at δ 292.0 ppm and δ 329.9 ppm respectively, suggested that these two methyl groups were attached to a pyrazine ring. A 2,3-dimethyl substitution pattern was assigned based on ¹H-¹³C HMBC correlations from H-10 to C-7 and from H-11 to C-6.

Given the high degree of unsaturation in the structure and the presence of a pyrazine ring, a fused heterocyclic structure was proposed for the unknown compound.

Furthermore, a similarity was noted between the chemical shifts of carbons C-2, C-4 and C-4a and shifts reported for analogous carbons in natural products containing lumazine structures (Pfleiderer, 1984; Kakoi, *et al.* 1995; Voerman, *et al.*, 2005; Meyer, *et al.* 2010; Chen, *et al.* 2014). This observation, coupled with HMBC correlations from H-9 to C-2 and C-4 and an additional four bond coupling from H-10 to C-4a, led to the tentative assignment of the structure of the isolated compound as 3,6,7-trimethylumazine (**3**).

3,6,7-Trimethylumazine (**3**) was first synthesized in 1958 (Curran & Angier, 1958). Since then it has been reported in several studies on related lumazines (Pfleiderer & Fink, 1963; Pfleiderer & Hutzenlaub, 1973; Ritzmann & Pfleiderer, 1973; Ram, *et al.* 1977; Southon & Pfleiderer, 1978; Uhlmann & Pfleiderer, 1981; Ram, *et al.* 1982; Bartke & Pfleiderer, 1989; Acuña-Cueva, *et al.* 2000). Characterisation data for lumazine **3** is limited to a melting point (Curran & Angier, 1958; Pfleiderer & Hutzenlaub, 1973), elemental analysis (Curran & Angier, 1958) and UV-vis peaks (Pfleiderer & Hutzenlaub, 1973; Ritzmann & Pfleiderer, 1973; Uhlmann & Pfleiderer, 1981); no NMR, MS or IR data have been reported to date.

The entire disclosures of all applications, patents and publications cited above and below, if any, are herein incorporated by reference.

For the avoidance of doubt, the term “composition” includes, but is not limited to, honey, honey extracts, or dried honey.

Reference to any prior art in this specification is not, and should not be taken as, an acknowledgement or any form of suggestion that that prior art forms part of the common general knowledge in the field of endeavour in any country in the world.

The invention may also be said broadly to consist in the parts, elements and features referred to or indicated in the specification of the application, individually or collectively, in any or all combinations of two or more of said parts, elements or features.

Wherein the foregoing description reference has been made to integers or components having known equivalents thereof, those integers are herein incorporated as if individually set forth.

It should be noted that various changes and modifications to the presently preferred embodiments described herein will be apparent to those skilled in the art. Such changes and modifications may be made without departing from the spirit and scope of the invention and without diminishing its attendant advantages. It is therefore intended that such changes and modifications be included within the scope of the invention.

WORKING EXAMPLES

The above-identified compositions, medicaments and methods of use are now described by reference to the Figures and specific Examples.

EXAMPLE 1 - Fluorometric assay

In this example, fluorometric inhibitor screening provides a rapid, sensitive and high throughput method to identify potential inhibitors of MMP-9.

Methods and materials

The MMP-9 inhibitor screening assay (fluorometric) kits were purchased from Abcam (Melbourne, Australia). The fluorometric kit contains the recombinant MMP-9 enzyme, MMP inhibitor NNGH (*N*-isobutyl-*N*-[4-methoxyphenylsulfonyl]glycyl hydroxamic acid), MMP fluorogenic substrate solubilised in DMSO, the fluorometric assay buffer and 96-well clear microplate.

Inhibitory activity on MMP-9 was assessed using the commercial MMP-9 inhibitor screening assay kit. MMP-9 activity was expressed as a change in fluorescence intensity measured using SpectraMax iD3 multi-mode microplate reader (Molecular Devices, San Jose, USA).

The assay employs a FRET-tagged (fluorescence resonance energy transfer) substrate, which can be hydrolysed by MMP-9 at a specific site (Abcam, 2018). The cleavage of the FRET substrate releases the quenched fluorescent Mca (7-methoxycoumarin-4-yl)-acetyl group (Abcam, 2018). The kit employs a quenched fluorogenic substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂, where the Mca fluorescence is quenched by Dpa until cleavage by MMPs. The amount of fluorescent product yielded by MMP-9 can be detected fluorometrically and it is proportional to the enzyme activity. Fluorescence were measured at λ_{ex} 320nm – λ_{em} 395nm to minimise fluorescence interference from 3,6,7-trimethylumazine at λ_{ex} 330nm – λ_{em} 470nm. Assays were performed on a 96-well clear microplate included in the kit with a final reaction volume of 100µL. Before adding the substrate, MMP-9 enzymes were incubated with testing samples and inhibitor control for 60 min at 37°C. The fluorescent substrate was added into each well prior to the assay to initiate the reaction. The assay was allowed to run for 20 min and the temperature in the reaction chamber was set to 37°C.

Testing samples were prepared comprising 3,6,7-trimethylumazine prepared as

outlined above.

A positive control was included with only MMP-9 and the fluorescent substrate, used as a reference to calculate the percentage inhibition. A broad spectrum MMP inhibitor NNGH was included as the negative control. A range of test controls were also included with 3,6,7-trimethylumazine at the testing concentration without MMP-9 and the fluorescent substrate, which is essential to measure the autofluorescence generated by 3,6,7-trimethylumazine.

Results

Synthesised 3,6,7-trimethylumazine was supplemented into the reaction mix at the concentrations found in Manuka honey from 2.5–40 µg/ml (3–44 µg/ml measured by LC-MS/MS). As shown in Figure 1, the change in fluorescence intensities were linear for all 3,6,7-trimethylumazine samples and controls. There was almost no change in fluorescence in the NNGH positive control. In contrast, a steady increase in fluorescence was observed for the negative control without inhibitor. 3,6,7-trimethylumazine samples displayed higher initial fluorescence due to its autofluorescence nature. A fluorescence control was included in this assay for each 3,6,7-trimethylumazine concentration.

In this study, 3,6,7-trimethylumazine at all tested concentrations exhibited inhibitory activities on MMP-9 ranging between 12% to 99%, as shown in Figure 2. In comparison with the negative control with no inhibitor, MMP-9 activities were significantly inhibited by 3,6,7-trimethylumazine at concentrations higher or equal to 5 µg/ml (All $p < 0.05$). 3,6,7-trimethylumazine at 2.5 µg/ml inhibited MMP-9 activity by 12%, but the inhibition was not significant ($p > 0.05$). 3,6,7-trimethylumazine almost completely inhibited MMP-9 at 40 µg/ml, there was no significant difference compared to the NNGH control ($p > 0.05$). The inhibition of MMP-9 appeared to be dose-dependent on 3,6,7-trimethylumazine concentration, as higher 3,6,7-trimethylumazine concentration always displayed stronger inhibition comparing to the lower concentrations (all $p < 0.05$).

The percentage inhibition of MMP-9 positively correlated with the concentration of 3,6,7-trimethylumazine as shown in Figure 3. The correlation fits best into a second-order polynomial model with an R^2 of 0.9965. Based on these data, the IC₅₀ of 3,6,7-trimethylumazine was calculated as 11.5 µg/ml.

EXAMPLE 2 - Colorimetric assay

In this example, an MMP-9 colorimetric inhibitor screening kit is used to further investigate the bioactivity of 3,6,7-trimethylumazine.

Methods and materials

The MMP-9 inhibitor screening assay (colorimetric) kits were purchased from Abcam (Melbourne, Australia). The kit contains the recombinant MMP-9 enzyme, MMP inhibitor NNGH, MMP chromogenic substrate, the colorimetric assay buffer and 96-well clear microplate.

The colorimetric kit uses a thiopeptide as a chromogenic substrate (Ac-PLG-[2-mercapto-4-methyl-pentanoyl]-LG-OC₂H₅), which can be hydrolysed by MMPs to produce a sulfhydryl group. This intermediate product further reacts with DTNB [5,5'-dithiobis(2-nitrobenzoic acid), Ellman's reagent] to form 2-nitro-5-thiobenzoic acid, which can be detected by absorbance at 412nm. The change in absorbance was measured using the SpectraMax iD3 multi-mode microplate reader (Molecular Devices, San Jose, USA). The assays are performed on a convenient 96-wells microplate with a final reaction volume of 100µL. Prior to the assay, all testing samples and inhibitor controls were incubated with MMP-9 for 60 min at 37°C. The chromogenic substrate was added into each well to initiate the reaction. The assay was allowed to run for 120 min at 37°C. The absorbance was measured at 1 min intervals during the first 20 min, then 10 min intervals till the end of assay.

Recombinant MMP-9 and the chromogenic substrate were used as the positive control to represent 100% enzyme activity. NNGH was used as a negative control. A range of 3,6,7-trimethylumazine concentrations were diluted with the colorimetric assay buffer to measure the absorbance of the reaction product.

Results

The underlying inhibitory bioactivity of 3,6,7-trimethylumazine was further investigated using the MMP-9 colorimetric inhibitor screening kit. The colorimetric kit uses a thiopeptide substrate that can be hydrolysed by MMPs to produce a sulfhydryl group intermediate, which further reacts with Ellman's reagent to form 2-nitro-5-thiobenzoic acid. The Ellman's reagent is used to detect the concentration of protein sulfhydryls, and the reaction product can be detected by absorbance at 412 nm (Riener, Kada, & Gruber, 2002).

The inhibitory bioactivity was first investigated by supplementing 3,6,7-trimethylumazine (40 µg/ml) into the reaction mix (Figure 4). In comparison with the negative control with no inhibitor, the rate of change in absorbance was slightly less in the 3,6,7-trimethylumazine supplemented sample. The NNGH was employed as the positive control which inhibited most of the MMP-9 activity. NNGH is not expected to completely

inhibit MMP-9 at 1.3 μ M (Abcam, 2019). The change in absorbance was linear for the 3,6,7-trimethylumazine sample and controls during the first 40 min. The product appeared to be unstable and begin to breakdown after 40 min. The first 20 min of the reaction was selected for further calculation.

3,6,7-trimethylumazine displayed inhibitory bioactivity against MMP-9 at concentrations between 2.5–80 μ g/ml. The percentage inhibition was calculated by comparing the absorbance change in 3,6,7-trimethylumazine samples against the negative control (no inhibitor, 100% MMP-9 activity). As shown in Figure 5, all 3,6,7-trimethylumazine samples inhibited MMP-9 by 3.5% to 10%. Compared to the negative control, 3,6,7-trimethylumazine at higher concentrations (20–80 μ g/ml) demonstrated significant inhibition on MMP-9 (all $p < 0.0001$). At lower 3,6,7-trimethylumazine concentrations (2.5–10 μ g/ml), the level of MMP-9 inhibition was insignificant (all $p > 0.05$). Increasing 3,6,7-trimethylumazine concentration from 40 μ g/ml to 80 μ g/ml did not further inhibit MMP-9 (both 10% inhibition, $p > 0.05$). This suggests that 3,6,7-trimethylumazine may have a relatively lower binding affinity (K_i) to the MMP-9 enzyme [E] or the enzyme-substrate complex [ES] compared to the chromogenic substrate.

In the absence of MMP-9, 3,6,7-trimethylumazine did not interfere with the absorbance signal generated by the chromogenic substrate and the reaction product. This was investigated by incubating 3,6,7-trimethylumazine (40 μ g/ml and 80 μ g/ml) with the substrate (Figure 6A) and the reaction product (Figure 6B) for 20 min. In both cases, 3,6,7-trimethylumazine did not significantly interfere with the absorbance signal.

Example 3: Gelatin gel zymography

To confirm the inhibition of MMP-9 by 3,6,7-trimethylumazine the inventors performed Gelatin gel zymography to detect the activity of MMP-9. Gelatin gel zymography is uniquely designed to detect the activity MMP-9 (gelatinase) due to its ability to digest gelatin.

Methods and materials

Novex™ 10% Zymogram Plus (Gelatin) Protein Gels (15 wells) were purchased from Thermo Fisher Scientific Inc. (Auckland, New Zealand). All chemicals required for the zymogram analysis were also purchased from Thermo Fisher, these include Novex™ Sharp Pre-stained Protein Standard, Novex Tris-Glycine SDS sample buffer, Novex Tris-Glycine SDS running buffer, Novex Zymogram renaturing buffer and Novex Zymogram

developing buffer. Double distilled water was purified from a Sartorius Arium® Pro (18.2 MΩ cm) water purification system. Gelatin gel zymography was performed as an independent technique to confirm the inhibition of MMP-9 from 3,6,7-trimethylumazine. This technique uses a non-reducing SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) gel embedded with gelatin. Proteins are migrated and separated during electrophoresis. The SDS is removed after electrophoresis and the gel is then incubated with essential cofactors required for enzymatic activity. The embedded gelatin can be digested by MMP-9, resulting in clear bands on a dark blue background after staining with Coomassie blue dyes. The gelatinase activity is represented by band densitometry, which can be assessed with image analysis software. Gelatin gel zymography is a highly sensitive technique at a relatively low cost (Leber & Balkwill, 1997). Additionally, this approach can simultaneously detect the gelatinase activity of both pro- and active MMPs, as they can be distinguished based on their migration distance through the gel (Rossano et al., 2014).

MMP-9 enzyme was diluted to a final testing concentration of 5µg/mL. The MMP-9 enzyme was gently mixed with loading buffer and water to achieve a total loading volume of 10µL per well. Gel electrophoresis was performed using the XCell Surelock™ Mini-Cell system (Thermo Fisher Scientific, Auckland, New Zealand). The upper chamber was filled with 200mL of 1X Tris-Glycine SDS running buffer, and the lower chamber with 600mL. The gel was running at a constant voltage of 125V and 30mA (starting current) for 105min. After electrophoresis, the gel was removed and incubated in 1X renaturing buffer for 30min with gentle agitation. Following the incubation, the gel was carefully cut into smaller pieces and further incubated separately in 1X developing buffer or 3,6,7-trimethylumazine supplemented developing buffer for 30 min under gentle agitation. The gel was further incubated overnight for 13 hours at 37°C with fresh developing buffer with or without 3,6,7-trimethylumazine. NNGH were also added into the developing buffer at 2.6µM as a positive control.

After incubation, the gelatin gel was rinsed with water for three times (5min each) under gentle agitation. The gel was stained by adding 20mL of SimplyBlue Safestain and incubated for 2 hours at room temperature under gentle agitation. It was destained by removing the SimplyBlue Safestain and rinsed with water for 2 hours at room temperature under gentle agitation. MMP-9 activities were analysed using densitometry on ImageJ Version 1.52a.

Results

The bioactivity of 3,6,7-trimethylumazine on MMP-9 was further examined using gelatin

zymography, by comparing gelatin gels incubated in normal developing buffer with 3,6,7-trimethylumazine-supplemented and NNGH-supplemented buffer. The MMP-9 enzyme used in this study were partially activated by 4-aminophenylmercuric acetate (4-APMA) to give more information on molecular interaction. The clear bands on the gel represent gelatinase activity from MMP-9 as shown in Figure 7. The bottom band represents gelatinase activity from active MMP-9 (~37 kDa). The top band represents gelatinase activity from partially activated pro-MMP-9 (~48 kDa). During electrophoresis, pro-MMP-9 was denatured by SDS, then renatured by removal of SDS with detergents such as Triton X-100 (Ren, Chen, & Khalil, 2017). This refolding process autoactivates a proportion of pro-MMP-9 without cleaving the pro-domain (Woessner, 1995). However, the autoactivated pro-MMP-9 may not represent the true activity in vivo.

3,6,7-trimethylumazine appeared to have reduced gelatinase activity from both active and inactive MMP-9 using gelatin zymography. In comparison with the negative control with no inhibitor (Figure 7, column 3–5), the area of both clear bands appeared to be reduced in 3,6,7-trimethylumazine-treated gels (Figure 7, column 6–8). The positive control NNGH completely inhibited the gelatinase activity from active MMP-9 (Figure 7, column 9–10). There appeared to be some gelatinase activity in NNGH-treated gels from the inactive MMP-9, which is likely a result of the residue gelatinase activity from the fibronectin domain.

3,6,7-trimethylumazine significantly reduced the gelatinase activity from both active and inactive MMP-9 (Figure 8, both $p < 0.001$). Percentage inhibition from 3,6,7-trimethylumazine and NNGH were analysed by densitometry and plotted in Figure 8. As shown, 3,6,7-trimethylumazine significantly inhibited the activity of active and inactive MMP-9 by 31% and 17%, respectively (both $p < 0.01$). It should be noted that 3,6,7-trimethylumazine displayed significantly stronger inhibition on the active MMP-9 compared to the inactive MMP-9 ($p < 0.05$). This suggests that 3,6,7-trimethylumazine is likely to interact more with the zinc active site of MMP-9. The same pattern can also be observed with NNGH treatment, where NNGH specifically interacts with the zinc ion (Bertini et al., 2005).

EXAMPLE 4 - Molecular docking of 3,6,7-trimethylumazine to MMP-9

In this example, a molecular docking study was carried out to predict the non-covalent interactions between 3,6,7-trimethylumazine and MMP-9.

Principles

Molecular docking is a computational procedure that attempts to predict non-covalent interaction of ligands with biomacromolecular targets. AutoDock and AutoDock Vina are commonly used computational tools to assist researchers in the determination of biomolecular complexes. The software calculates the minimal interaction energy between targeted protein and ligand while efficiently exploring all torsional freedom. AutoDock is based on an empirical free energy force field and rapid Lamarckian genetic algorithm search method (Goodsell & Olson, 1990; Morris et al., 2009). AutoDock Vina uses a simpler scoring function and rapid gradient-optimisation conformational search, which significantly improves the speed and accuracy (Trott & Olson, 2010).

Methods and materials

Molecular docking study on MMP-9 and 3,6,7-trimethylumazine was carried out using AutoDock Vina v1.1.2. Docking preparation, post-docking analysis and visualisation were performed on Chimera v1.13.1 (Pettersen et al., 2004). The 3D structure of 3,6,7-trimethylumazine was drawn on Avogadro v1.2.0 (Hanwell et al., 2012). The full 3D crystallographic structure of MMP-9 (PDB ID: 1L6J) was retrieved through the RCSB Protein Data Bank (Elkins et al., 2002).

Docking preparation was performed for both compounds using Chimera. 3,6,7-trimethylumazine structure was minimised by employing the Smart Minimizer Algorithm. Detection of torsion angles and assignment of Gasteiger charges were also performed on Chimera. The MMP-9 structure was prepared by adding hydrogen atoms, merging non-polar hydrogen atoms, checking missing atom and assign Gasteiger charges. A grid box was defined on the catalytic domain of the MMP-9 enzyme with a volume of Å³=35, 45, 48 (x,y,z coordinates=30, 30, 35). This defines the area of the protein involved in the docking calculation.

Molecular docking was performed on AutoDock Vina with exhaustiveness set as 8 and the number of binding modes as 10. The best binding conformation with the highest score were listed on AutoDock Vina and visualised on Chimera. Potential inter-molecular hydrogen bond for each binding pose was also analysed on Chimera.

Results

Molecular docking predicted significant binding affinity between 3,6,7-trimethylumazine and MMP-9. 3,6,7-trimethylumazine was successfully docked into the active site of MMP-9 with the best docking score of -7.9 using AutoDock Vina (Table 2). The AutoDock

Vina score represents the predicted energy required for two compounds to bind, by considering a combination of hydrogen bonds, hydrophobic interactions and torsional penalty (Chang, Ayeni, Breuer, & Torbett, 2010). As a result, the most favourable binding conformation is represented as a negative score. In comparison, the docking score of the most active synthetic MMP-9 inhibitors ranged between -7.6 to -8.9 using AutoDock Vina (Rathee et al., 2018).

Table 2. The prediction score calculated by Autodock Vina.

Score	RMSD l.b.	RMSD u.b.	Number of Hbonds
-7.9	0	0	1
-6.7	2.283	5.418	1
-6.5	2.986	4.239	1
-6.5	3.634	5.699	1
-6.5	3.673	5.052	1
-6.4	16.1	18.083	0
-5.9	3.855	6.35	0
-5.8	4.987	7.423	0
-5.7	15.9	18.022	0
-5.7	2.361	4.741	0

3,6,7-trimethylumazine was docked into the S'1 substrate binding site by forming a hydrogen bonding with the Tyr⁴²⁰ residue. The S'1 substrate binding site is framed in the centre of the active site cleft closest in proximity to active site zinc. Compared to other binding pockets, the S'1 pocket varies among MMPs in both the amino acid makeup and depth of the pocket (Aureli et al., 2008). As a result, the S'1 pocket determines the substrate binding specificity and is a target for many MMP inhibitors. In particular for MMP-9, co-crystallisation with different inhibitors revealed that the Arg⁴²⁴ residue is highly flexible, which allows some MMP inhibitors to move into the S1' pocket (Tochowicz et al., 2007).

In a previous docking study, synthetic inhibitors with carboxylic acid and sulfonamide hydroxamate group were bound to the S'1 pocket; while the thio-ester group interacts with both S'1 and S1 pocket (Tandon & Sinha, 2011). A potential hydrogen bond was found between the N-H group of 3,6,7-trimethylumazine and Tyr⁴²⁰ near the wall of the S'1 cavity (Figure 9). The S'1 wall residues often act as hydrogen acceptors for inter main chain hydrogen bonds to substrates or inhibitors (Tyr⁴²⁰, Pro⁴²¹, Tyr⁴²³) (Tandon & Sinha, 2011). Zinc binding inhibitors with a carbonyl group or N-H groups offer opportunities for hydrogen bonding interactions with the S1' pocket. (Tandon & Sinha,

2011). Both structures are present in 3,6,7-trimethylumazine.

These results further supported the binding of 3,6,7-trimethylumazine at the exosite of MMP- located within the fibronectin type II domain. The inventors further identified high gold scores (53.4) of 3,6,7-trimethylumazine with MMP-9 (Docking was performed with GOLD v5.7.3 with a total of 10 GA runs per ligand and maximum search efficiency. Docked poses were scored with GoldScore). These findings suggested that 3,6,7-trimethylumazine may interact with the exosite of MMP-9 by disrupting the binding of gelatin. The results from molecular docking analysis further supported the binding of 3,6,7-trimethylumazine at the exosite of MMP-9 located within the fibronectin type II domain.

Example 5 - Simulation of gastrointestinal environment

The extent to which the anti-inflammatory bioactivity of comprising 3,6,7-trimethylumazine can be retained during the gastrointestinal digestion is unknown. It is possible that this bioactive molecule undergoes modification at low pH or by digestive enzymes, and partially or fully lose its biological activity.

A simulated gastric digestion followed by a simulated intestinal digestion of the 3,6,7-trimethylumazine -containing honey samples was conducted *in vitro*. At pre-determined time points during this process, the gastric or intestinal digesta was removed to analyse the remaining amount of 3,6,7-trimethylumazine.

Materials

Simulation of gastrointestinal environment:

The simulated gastrointestinal digestion was carried out using a static model. The simulated gastric fluid (SGF) and the simulated intestinal fluid (SIF) were prepared in accordance with a global consensus protocol (Minekus et al 2014). The SGF has a pH 3 to mimic the fed-state of the stomach. When mixed with Manuka honey (or a honey solution), the final mixture contains 2000 U/mL of pepsin. The SIF has pH 7 to mimic the fed-state of the small intestine, containing 2 mg/mL of pancreatin (8 x USP, or based on a protease activity of 200 U/mL) and 20 mM of porcine bile extract before use.

Gastric digestion:

In the simulated gastric digestion, 2 g of Manuka honey was incubated in 2 mL of SGF at 37°C under 95 rpm shaking for a period of 2 h (triplicates). At selected time points (0, 30, 60 and 120 min), a predetermined volume (0.1 mL) of the mixture was withdrawn for

3,6,7-trimethylllumazine analysis. The solution for analysis was kept on ice with an addition of 0.1 mL SIF (pH 7) to stop pepsin activity. As a control group, a pure 3,6,7-trimethylllumazine solution was treated in the same way for comparative purposes.

Intestinal digestion:

Following the 2 h gastric digestion, the resulting solution was mixed with SIF (pH 7) at a volume ratio of 1:1, to have a final mixture that contains 1 mg/mL of pancreatin and 10 mM of porcine bile extract. This mixture was incubated at 37 °C under 95 rpm shaking for 4 h (triplicates). At selected time points (0, 60, 120 and 240 min), a predetermined volume (0.1 mL) of the mixture was withdrawn for 3,6,7-trimethylllumazine concentration analysis. The pancreatin activity in the withdrawn solution was quenched by adding 5mmol/L Pefabloc ® (Egger et al 2019).

Analysis of 3,6,7-trimethylllumazine retention:

The gastric and intestinal digesta for 3,6,7-trimethylllumazine analysis was treated to remove insoluble fractions (e.g. pancreatin) before HPLC analysis. In brief, all samples were diluted with 0.1% formic acid and then centrifuged at 14,000 rpm for 10 min. Supernatant was taken for analysis. The amount of 3,6,7-trimethylllumazine at different time points was analysed using a reverse-phase HPLC system, which has been previously used to analyse 3,6,7-trimethylllumazine and leptosperin as reported in the literature (Bin Lin et al 2017). In brief, the samples were diluted 5 times in 0.1% v/v formic acid. A Hypersil GOLD column (150 x 2.1 mm, 3 µM particle size) was used as the stationary phase (25 °C), and the mobile phase will consist of 0.1% formic acid (phase A), and 80:20 acetonitrile: 0.1% formic acid (phase B). The injection volume was 3 µL, flow rate 0.200 mL, and a gradient elution as follow was used to separate 3,6,7-trimethylllumazine and others: initial 2 min (5% phase B), at 7 min (25% B), 14 min (50% B), 16 min (100% B), 19 min (5% B) and 20 min (5% B, held 10 min). The signal of 3,6,7-trimethylllumazine was detected at 320 nm.

Statistical analysis:

The significance of difference between two mean values was analysed using a two-tailed unpaired Student's t-test. When more than two mean values were compared, significant differences were analysed by a one-way analysis of variance followed by a Bonferroni's multiple comparison test (SPSS Statistics Version 24, IBM). Differences were considered to be statistically significant at $p < 0.05$.

Results

The results from the simulated gastrointestinal digestion of four honey samples indicate

that 3,6,7-trimethylxanthine from Manuka honey is highly stable in the harsh environment of the digestive tract. Up to the end of the study, i.e. 2h gastric digestion plus 4h intestinal digestion, nearly 100% of the initial 3,6,7-trimethylxanthine amount from the four honey samples could be fully recovered in the digesta. No evident degradation is observed. The detailed dynamics are shown in Figure 10 and 11, where in the first 2h 3,6,7-trimethylxanthine is incubated in simulated gastric fluids, while the subsequent 4h represents the intestinal digestion stage. The raw data are summarised in Table 3.

Details of experimental results

Table 3

Phase	Time (h)	A	B	C	D
SGF	0	167 ± 10	363 ± 21	608 ± 16	858 ± 3
	0.5	156 ± 17	371 ± 13	620 ± 26	840 ± 46
	1	167 ± 11	386 ± 13 *	632 ± 15	838 ± 34
	2	169 ± 6	367 ± 25	632 ± 27	819 ± 44
SIF	0	179 ± 17	384 ± 28	612 ± 2	885 ± 6
	1	161 ± 18	360 ± 26	600 ± 12	864 ± 21
	2	161 ± 14	375 ± 9	612 ± 10	874 ± 3
	4	161 ± 15	384 ± 17	596 ± 12	862 ± 9

* Based on two replicates instead of three due to technical issue.

Table 3: The remaining amount of 3,6,7-trimethylxanthine during the gastrointestinal digestion of four Manuka honey samples (A, B, C, D) Data represent mean ± SD, n=3.

In a subsequent study, we conducted the gastrointestinal digestion of diluted Manuka honey samples to understand the stability of 3,6,7-trimethylxanthine in different concentrations of the Manuka honey. The stability of 3,6,7-trimethylxanthine (pure compound) was also tested using the same *in-vitro* digestion protocol. Our results indicate that the stability of 3,6,7-trimethylxanthine is unchanged, either in 50% (w/w) Manuka honey solutions (Figure 12 and 13) or directly exposed to digestive media (Figure. 14 and 15), when compared with the stability profile of non-digested raw honeys. No significant degradation is observed. The raw data showing the detailed dynamics are summarised in Table 4.

Table 4

Phase	Time (h)	A	B	C	D	3,6,7-trimethylxanthine
SGF	0	171 ± 9	383 ± 2	587 ± 6	869 ± 21	682 ± 90
	0.5	186 ± 16	385 ± 1	583 ± 22	879 ± 22	703 ± 57
	1	187 ± 8	397 ± 4	592 ± 21	864 ± 31	728 ± 28
	2	183 ± 13	387 ± 9	586 ± 11	878 ± 6	688 ± 72

SIF	0	171 ± 3	384 ± 10	573 ± 9	838 ± 5	736 ± 6
	1	167 ± 10	380 ± 3	578 ± 19	866 ± 25	740 ± 12
	2	189 ± 6	377 ± 18	569 ± 26	863 ± 25	732 ± 10
	4	175 ± 7	381 ± 13	578 ± 12	865 ± 1	730 ± 3

Table 4: The remaining amount of 3,6,7-trimethylumazine during the gastrointestinal digestion of 50% (w/w) Manuka honey solutions (A, B, C, D) and 3,6,7-trimethylumazine compound. Data represent mean ± SD, n=3.

The *in-vitro* studies on the fate of 3,6,7-trimethylumazine in the digestive tract clearly indicate high stability of the bio-active compound, 3,6,7-trimethylumazine, as tested from the, diluted (50% dilution) Manuka honey samples A, B, C and D and in its purest form.

EXAMPLE 6 - Effect of 3,6,7-trimethylumazine on matrix metalloproteinase-9 (MMP-9) in human macrophage cell lines

The inventors investigated the efficacy of 3,6,7-trimethylumazine, present in Manuka honey, to inhibit lipopolysaccharides (LPS) induced MMP-9 secretion in human macrophage cell lines (THP-1) using Enzyme Linked Immunosorbent Assay (ELISA) technique.

Macrophages are a potential source of gastric MMPs, as they are known to respond to both bacterial factors and pro-inflammatory cytokines with an increased MMP-9 secretion. Therefore, MMP-9 secretion from THP-1 can be used as a marker of gastric inflammation.

Concentrations between 2.5-40 µg/mL of 3,6,7-trimethylumazine were tested for MMP-9 inhibitory activity. Azithromycin was selected as a positive control. 3,6,7-trimethylumazine at 40 µg/mL (38% reduction) and 30 µg/mL (23% reduction) significantly ($P < 0.05$) reduced MMP-9 secretion from the LPS (1 µg/mL) treated differentiated THP-1 cells compared to 20 and 5 µg/mL. 3,6,7-trimethylumazine at 40 µg/mL reduced MMP-9 and this reduction was slightly higher than Azithromycin over 30 µg/mL. However, based on the cell viability reports, 30 µg/mL (13% cell death) is slightly safer than 40 µg/mL (20% cell death).

Methods

Dose:

3,6,7-trimethylumazine was tested at doses between 2.5-40 µg/ml for its inhibition of

MMP-9 inflammation response using differentiated THP-1 cells.

Cell culture:

THP-1 cells (ATCC, ATCTIB202) were grown in RPMI-1640 (Gibco, 11875093) + 0.05 mM 2-mercaptoethanol + 10% fetal calf serum (FCS) + 1% pen-strep. For experiments, the cells were cultured in RPMI-1640 medium with 10% fetal bovine serum (FBS) only.

THP-1 monocyte cells were seeded at a density of 2.5×10^5 cells/ml in 96-well plates and differentiated into macrophages using 10 ng/ml of phorbol 12-myristate 13-acetate (PMA) (Bergin et al) (Sigma, P1585-1MG, Lot# SLBX889, 100% purity) for 72 hours. PMA media was then removed from the differentiated THP-1 cells, the cells were then washed once in RPMI-1640 media and then left to rest for ~ 5hours.

LPS stimulation and treatment with 3,6,7-trimethylumazine:

The differentiated THP-1 cells were stimulated with LPS from *E. coli* 055:B5 (Sigma, L6529; Lot#037K4068). LPS tested at a concentration of 1 µg/ml (Kong et al). The cells were incubated with LPS alone or in combination with 3,6,7-trimethylumazine (received from University of Auckland and diluted in RPMI-1640 at a stock concentration of 1 mg/ml, stock was stored in fridge for 2 days before use) at a concentration range between 2.5-40 µg/ml. 6 µM Azithromycin (Sigma, Cat#75199-25MG, Lot#069M4826V) was used as a positive control (Vandooren et al). The cells were then incubated with the different treatments for 24 hours (Kong et al). After 24 hours, the cell culture media was collected and measured for MMP-9 concentration using MMP-9 ELISA (R&D systems, RDSY91105 lot# P239459 and DY008 Lot #P239900). The cells were then incubated with WST-1 for cell viability.

Two additional 96-well plates were treated as above, media removed and the plate containing cells were frozen in -80°C for future RT-PCR experiments.

Elisa Specificity:

This human MMP-9 assay measures the 92 kDa Pro-MMP-9 and the 82 kDa active MMP-9. It does not measure the 65 kDa form. This assay also recognizes human MMP-9 when complexed to Lipocalin-2/NGAL, isolated from human source material.

The following factors prepared at 50 ng/mL were assayed and exhibited no cross-reactivity or interference: MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-10, MMP-12, MMP-13, MMP-14, TIMP-2, TIMP-3, TIMP-4, TIMP-4, recombinant mouse MMP-9.

Recombinant human TIMP-1 does not cross-react in this assay but does interfere at

concentrations > 1.56 ng/ml.

Cell viability and preliminary MMP-9 secretion test:

To measure cytotoxicity of 3,6,7-trimethylumazine at different doses, 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-1) (Roche, 11644807001, Lot#45255800) was used. WST-1 is a cell proliferation reagent for measurement of cellular proliferation, viability, and cytotoxicity using a colorimetric assay (Gosert (2011); Peskin (2000)).

After incubation, a portion of the culture media was taken and stored for MMP-9 secretion testing using ELISA. The remaining media in the plates was then removed and 100 µl of WST-1 in RPMI-1640 media (1:10 dilution) was added to each cell well and incubated at 37°C for a further 4 hours. The plates were then read using a plate reader at wavelength of 450 nm. Cytotoxicity was calculated as follows:

$$[\text{WST-1 score for each sample} / \text{WST-1 score for the control}] \times 100$$

A WST-1 score below 80% will be considered cytotoxic.

Statistical analysis:

In order to better capture the variability, each treatment was done at least in triplicate in each plate (2 plates). Media from the triplicate wells were pooled and analysed in duplicates for the MMP-9 ELISA. A student's test was performed in excel between media with LPS and the different treatments.

Results

3,6,7-trimethylumazine at concentration 40µg/mL has slightly more %cell toxicity than the other concentrations selected in the study (2.5-30µg/mL) (Figure 16). However, it is borderline for consideration (79.8) for it to be toxic. 3,6,7-trimethylumazine at 40µg/mL (38% reduction) and 30µg/mL (23% reduction) significantly ($P < 0.05$) reduced MMP-9 secretion from the LPS (1µg/mL) treated differentiated THP-1 cells compared to that at 20 and 5 µg/mL (Figure 17). 3,6,7-trimethylumazine at 40µg/mL reduced MMP-9 greater than Azithromycin over 30µg/mL. Adjusted values (in relation to LPS) are presented in Figure 18.

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