

## A METHOD FOR REPELLING INSECT PESTS

### FIELD OF THE INVENTION

The present invention relates to methods and compositions for repelling arthropod pests, more particularly for repelling the insect pests' mosquitoes and flies. The compositions of the invention include massoia lactone and/or a structurally related compound.

### BACKGROUND OF THE INVENTION

Mosquitoes are devastating disease vectors. They are able to transmit diseases from an infected host to another host during blood feeding that provides the female mosquitoes with the nutrient for egg production. *Aedes*, which is one of the genus of mosquito, are now found in all continents except for Antarctica, spreading diseases like chikungunya, dengue fever, yellow fever and Zika [Vector Borne Disease [www.dothdotint/en/news-room/fact-sheets/detail/vector-borne-diseases]]. Another genus of mosquito known as the *Anopheles* is the vector for transmitting Malaria which accounts for more than 400,000 deaths in the world annually [Bernier UR, Kline DL, *et al.*, *PLoS Negl Trop Dis.* 2019, 13(3): e0007188]. With vector-borne diseases accounting for more than 17% of all infectious diseases, the world health organization had responded by providing strategies and guidelines for vectors control, personal protection against vectors, management of outbreaks, training in diagnosis and development of new tools.

The use of insect repellent is an effective strategy to reduce mosquito bites and disease transmission. Commercially, there are many insect and mosquito repellents in the market that are being sold as applicable spray, aerosol spray, patch and cream. The active ingredients are usually compounds such as N,N-diethyl-*meta*-toluamide (DEET), citronella oil, lemon eucalyptus oil and picaridin. While DEET is still considered the 'golden standard' as the active compound in many commercially available mosquito repellents due to its effectiveness, it has adverse health effects like skin irritation, breathing difficulty, burning eyes and headaches [Legeay S, Clere N, Apace-Marchais V, Faure S, Lapied B: *Eur J Pharmacol.* 2018, 825: 92-98; Keith S, *et al.*, Toxicological profile for DEET (N, N-diethyl-*meta*-toluamide). Atlanta: Agency for Toxic Substances & Disease Registry. 2017, 1-263].

Therefore, there is a demand for novel insect repelling agents, particularly those with high repellency and lower health risk.

**SUMMARY OF THE INVENTION**

The present invention provides methods of using alternative compounds and compositions for repelling arthropod pests, more preferably insect pests.

5

The bark oil of massoia tree (*Cryptocaria massoia*) contains several lactones, notably C10 ((R)-5,6-Dihydro-6-pentyl-2H-pyran-2-one), C12 and C14 delta-lactone with a double bond at the C2 position in the lactone ring (Figure 1). Massoia lactones are very safe to humans as it has been used extensively as a natural flavouring agent for food and beverage. It has a sweet, creamy and milky odour that is similar to that of coconut. Massoia lactone repelled mosquitoes and flies and was at least as effective as DEET in repelling mosquitoes. Moreover, the repellent effect lasted longer than that for DEET. Other, massoia-like compounds tested were also effective in repelling mosquitoes.

15 According to a first aspect, the present disclosure provides a method of repelling an arthropod pest, the method comprising contacting a subject or article with a composition comprising massoia lactone compound ((R)-5,6-Dihydro-6-pentyl-2H-pyran-2-one), a massoia lactone-like compound or mixture thereof.

20 The arthropod may be a mosquito or fly, or other pest. Pests may be selected from the group comprising ants, cockroaches, fleas, flies such as sand flies, mites, mosquitos, spiders, ticks, termites and wasps.

According to a second aspect, the present disclosure provides a composition for use in repelling arthropod pests, comprising at least one massoia lactone compound, a massoia lactone-like compound or mixture thereof.

25

According to a third aspect, the present disclosure provides use of massoia lactone, a massoia lactone-like compound or mixture thereof as an arthropod pest repellent.

30

According to a fourth aspect, the present disclosure provides use of massoia lactone, a massoia lactone-like compound or mixture thereof, in the manufacture of an arthropod pest repellent.

Other aspects, features and embodiments of the invention will be more fully apparent from the ensuing disclosure.

#### BRIEF DESCRIPTION OF THE FIGURES

5 **Figure 1** shows the molecular structures of compounds tested in the present disclosure.

**Figure 2** is a schematic diagram of a modified cage for hand assay. **1.** Wiring mesh for viewing of mosquito and air exchange. **2.** 10% CO<sub>2</sub> and 90% air mixture are introduced at 3NI/min (normal liters per minute) into the cage via a fly pad placed at the top of the cage. **3.** Wire mesh from screen port is replaced with cling wrap for a better view of the opposite screen port. **4.** Posterior end of the cage contains an opening covered by sleeves that had been removed for illustration purposes. **5.** Wire mesh of screen port is replaced with a cut out of a cloth that is soaked with the tested compound. **6.** A 90 mm Petri dish cover with a cut out of 70 mm is placed over the screen port to create a gap  
10 between the cloth and the hand. **7.** Filter paper covering the side of the cage facing the tester to minimize air exchange and to block the vision.

**Figure 3** is a schematic diagram of a setup for 2-choice assay for *Drosophila melanogaster* repellent assay.

20

**Figure 4** is a bar graph showing candidate repellent compounds used for hand-based repellent assay (n=3). Compounds were formulated in ethanol to 2% (v/v). x-axis represents repellency.

25 **Figure 5** shows a bar graph of the results of the 2-choice trap assay. Starved *Drosophila melanogaster* females (n=20, 3 repeats) are allowed to choose among the two baited traps, with one containing solvent (control) and one containing various concentrations (ranging from 0.5% to 10% (v/v)) of massoia lactone ("Lactone"). Majority of female flies avoided the trap that contains massoia lactone at all concentrations tested and entered  
30 trap with solvent (grey bar).

**Figure 6** shows bar graphs of the repellency of dose ranges of (A) DEET and (B) massoia lactone toward *Ae. aegypti* mosquitoes.

**Figure 7** shows a bar graph of the repellency over time of 2% (v/v) massoia lactone and 2% (v/v) DEET evaluated by hand-based pull-push assay immediately (0 h), 2.5 h and 5 h after application.

- 5 **Figure 8** shows a bar graph comparing repellency between massoia lactone and essential oils; eucalyptus and citronella. All tests were done with 2% (v/v) concentration in ethanol.

- 10 **Figure 9** shows a bar graph comparing repellency between 2% (v/v) massoia lactone-ethanol and a citronella oil based commercial anti-mosquito spray.

**Figure 10** shows bar graphs comparing the repellency of massoia lactone (ML) (A) and DEET (0.01% to 0.5%) (B) for *Culex quinquefasciatus* established using the hand assay.

15 Definitions

The terms "a" and "an" are used herein to refer to one or to more than one (i.e. at least one) of the grammatical object of the article.

- 20 The term "article" is used herein to mean an object such as a man-made product comprised of fabric or solid substrate that said pest comes in contact with or comes in close proximity to. The article may include items of furniture, items of clothing and the solid substrate may be a household surface such as a window or door frame or wall.

- 25 The term "comprising" is herein defined as "including principally, but not necessarily solely". Further, the term "comprising" will be automatically read by the person skilled in the art as including "consisting of". The variations of the word "comprising", such as "comprise" and "comprises", have correspondingly varied meanings.

- 30 As used herein, the term "arthropod pest repellent" or "insect repellent" or "repellent compound" or "repellent composition" will refer to a compound or composition which deters arthropods, more particularly insects, from subjects or articles of manufacture. Most known repellents are not active poisons at all, but rather prevent damage to plants/animals or articles of manufacture by making insect food sources or living conditions unattractive or offensive. Typically, repellents are a compound or composition  
35 that can be either topically applied to the subject; or, the compound or composition is

incorporated into an article to produce an insect repellent article that deters insects from the nearby 3-dimensional space in which the subject or article exists. In either case, the effect of the insect repellent is to drive the insects away from or to avoid: 1.) the subject, thereby minimizing the frequency of insect "bites" to the subject; or 2.) the article.

5 Repellents may be in various forms as described herein and as understood in the art.

The term "extract" refers to any compound, composition or material which is extracted from a plant or part(s) thereof. For example, massoia lactone compound may be extracted from the bark oil of massoia tree (*Cryptocaria massoia*). Alternatively, massoia  
10 lactone compound may be produced by yeast fermentation and solvent-extracted from yeast cells.

"Massoia lactone" or "Massoia lactone compound" is herein defined as C10 massoia lactone with IUPAC name (R)-5,6-Dihydro-6-pentyl-2H-pyran-2-one.

15 "Massoia lactone-like compound" is herein defined as a compound that has structural similarities with massoia lactone. For example, the compound may contain a delta-lactone ring. Examples are shown in Figure 1. As used herein, C12 massoia lactone (5,6-dihydro-6-heptyl-2H-pyran-2-one) and C14 massoia lactone (5,6-dihydro-6-nonyl-2H-  
20 pyran-2-one) are considered as massoia lactone-like compounds.

The term "pest" is herein defined as an organism, usually an arthropod, which has characteristics that are regarded by humans as detrimental or unwanted, for example by biting and in some cases causing disease. The term "pest" includes but is not limited to  
25 insects. In particular, the pest may be selected from the group comprising ants, cockroaches, fleas, flies, mites, mosquitoes, spiders, ticks, termites, wasps and the like. Mosquitoes and/or flies may represent a significant hazard in some environments.

The term "subject" includes both human and animal subjects and parts thereof. Typically,  
30 subjects are considered to be insect-acceptable food sources or insect-acceptable habitats. Human subjects may be vulnerable to, for example, mosquito-borne disease such as malaria, Dengue virus and Zika virus. Animal subjects include but are not limited to mammals, including household pets, livestock animals and birds that are vulnerable to insect-borne diseases and/or infestations.

35

**DETAILED DESCRIPTION OF THE INVENTION**

Bibliographic references mentioned in the present specification are for convenience listed in the form of a list of references and added at the end of the examples. The whole content of such bibliographic references is herein incorporated by reference. Any  
5 discussion about prior art is not an admission that the prior art is part of the common general knowledge in the field of the invention.

The present invention relates to the identification of novel insect repelling agents based on screening of massoia lactone and compounds that are structurally similar using *Ae.*  
10 *aegypti* and *Drosophila melanogaster* as the model insects.

According to a first aspect, the present disclosure provides a method of repelling an arthropod pest, the method comprising contacting a subject or article with a composition comprising a massoia lactone compound, a massoia lactone-like compound or mixture  
15 thereof.

Contacting of the composition may be by way of any method known in the art including, for example, spraying, pouring, dipping, in the form of concentrated liquids, solutions, suspensions, sprays, powders or the like, formulated to deliver a composition in an  
20 effective concentration to repel an arthropod pest.

In some embodiments, the pest is selected from the group comprising ants, cockroaches, fleas, flies, mites, mosquitoes, sand flies, spiders, ticks, termites and wasps. In some embodiments, the pest is an insect. In some preferred embodiments, the pest is a  
25 mosquito or fly. The mosquito may be from, for example, the *Aedes* genus, the *Culex* genus, or from the *Anopheles* genus. *Aedes* species are known to transmit diseases such as Dengue and Zika, *Culex* species are known to transmit Japanese encephalitis, whereas *Anopheles* species are known to transmit malaria. In some embodiments the mosquito is selected from the species *Ae. aegypti*, the species *C. quinquefasciatus*, the  
30 species *Anopheles sinensis* and *Anopheles gambiae*. The fly may be a biting fly and/or may carry a disease. Flies can spread diseases because they feed freely on human food and filthy matter alike. The diseases that flies can transmit include enteric infections (such as dysentery, diarrhoea, typhoid, cholera and certain helminth infections), eye infections (such as trachoma and epidemic conjunctivitis), poliomyelitis and certain skin  
35 infections (such as yaws, cutaneous diphtheria, some mycoses and leprosy). Biting flies

may also feed on animal blood and transmit disease such as Trypanosomiasis (sleeping sickness) transmitted by the tsetse fly and Leishmaniasis transmitted by infected sand flies.

- 5 It would be understood that animals susceptible to bites and possibly disease from arthropod pests would benefit from the use of a repellent composition. In some embodiments the subject is a human, or animal such as a household pet, livestock animal or bird.
- 10 Rather than direct contact of the composition with a subject, the composition may be applied to a surface of, or impregnated in, an article. In some embodiments, the article is comprised of fabric or other solid substrate that said pest comes in contact with or comes in close proximity to. The article may include items of furniture, items of clothing, such as bracelets, hairbands, waistbands, shoe laces, leather belts, caps and similar
- 15 items, candles, air diffusers, oil diffusers, incense, tablets, and the solid substrate may be a household surface such as a window or door frame or wall or even home paints.

- The formulation of the pest control composition can include combining the active produced in accordance with the invention with a suitable carrier or vehicular formulation
- 20 appropriate to the end-use administration of the pest control composition. For example, the pest control composition may be formulated with appropriate ingredients to provide a desired form of the composition, including, without limitation, lotions, oils, creams, cosmetics, gels, spray formulations, etc. In some embodiments, the composition is formulated for topical application. In some embodiments, the composition is a foam, gel,
- 25 cream, lotion, spray, patch, suspension, emulsion, microemulsion, emulsifiable concentrate or other appropriate form. In some embodiments, the composition is formulated as an aerosol or surface spray.

- In some embodiments, the composition comprises massoia lactone, a massoia lactone-like compound or mixture thereof is at least 0.001% (v/v), 0.01% (v/v), at least 0.1% (v/v), at least 0.5% (v/v), at least 1% (v/v), at least 5% (v/v), at least 10% (v/v), at least 15% (v/v). In some embodiments, the composition comprises massoia lactone, a massoia lactone-like compound or mixture thereof in the range of 0.001% to 15% (v/v), preferably in the range of 0.01% to 15% (v/v), 0.01% to 5% (v/v), more preferably in the range 0.05%
- 35 to 2% (v/v).

In some embodiments, the massoia lactone-like compound is selected from the group comprising: 5,6-dihydro-6-heptyl-2H-pyran-2-one, 5,6-dihydro-6-nonyl-2H-pyran-2-one, 6-Amy- $\alpha$ -pyrone, 4,6-Dimethyl- $\alpha$ -pyrone, 2,6-Dimethyl- $\gamma$ -pyrone, 2H-Pyran-2-one, 4-Hydroxy-6-methyl-2-pyrone, 4-Methoxy-6-methyl-2H-pyran-2-one,  $\delta$ -Valerolactone,  $\gamma$ -Hexalactone,  $\gamma$ -Octalactone,  $\gamma$ -Nonanoic lactone,  $\delta$ -Nonalactone,  $\delta$ -Decalactone, Eugenol, 2-Undecanone, ethyl pyruvate, and/or cyclopentanone. In some embodiments, the massoia lactone compound is C10 massoia lactone and/or the massoia-like compound is selected from one or more of C12 massoia lactone and C14 massoia lactone.

According to another aspect, the present disclosure provides a pest-repellent composition for use in repelling arthropod pests, comprising at least one massoia lactone compound, a massoia lactone-like compound or mixture thereof.

Liquid formulations of the composition according to any aspect of the present disclosure may be aqueous-based or non-aqueous (i.e. organic solvents), or combinations thereof, and may be employed as foams, gels, creams, lotions, sunblock, oils, gels, sprays (including aerosol and surface), suspensions, emulsions, microemulsions or emulsifiable concentrates or the like. The composition according to any aspect of the present disclosure may be formulated for dispersal. In particular, the composition according to any aspect of the present invention may be employed alone or in mixtures with one another and/or with such solid and/or liquid dispersible carrier vehicles as described herein or as otherwise known in the art.

In liquid formulations of the invention, it is possible to blend a massoia lactone compound, a massoia lactone-like compound or mixture thereof with commonly used adjuvants or auxiliary agents such as emulsifying or dispersing agent, spreading agent, wetting agent, suspending agent, preservative, propellant and film-forming agent. Examples of the emulsifying or dispersing agents usable in the present invention include soaps, polyoxyethylene fatty acid - alcohol ethers such as polyoxyethylene oleyl ether, polyoxyethylene alkylaryl ethers such as polyoxyethylene nonylphenyl ether, polyoxyethylene fatty acid esters, fatty acid glyceride, sorbitan fatty acid esters, sulfuric esters of higher alcohols, and alkylaryl sulfonates such as sodium dodecylbenzenesulfonate; examples of the spreading and wetting agents include

- glycerine and polyethylene glycol; examples of the suspending agents include casein, gelatin, alginic acid, carboxymethyl cellulose, gum arabic, hydroxypropyl cellulose and bentonite; examples of preservatives include methyl p-hydroxybenzoate, ethyl p-hydroxybenzoate, propyl p-hydroxybenzoate, and butyl p-hydroxybenzoate; examples of
- 5 propellants include dimethyl ether, chlorofluorocarbons and carbon dioxide; and examples of film forming agents include nitrocellulose, acetyl cellulose, acetylbutyl cellulose, methyl cellulose derivatives, vinyl resins such as vinyl acetate resin, and polyvinyl alcohol.
- 10 Carriers usable in the preparation of cream formulations include hydrocarbons such as liquid paraffin, vaseline and paraffin; silicones such as dimethylsiloxane, colloidal silica and bentonite; monohydric alcohols such as ethanol, stearyl alcohol, lauryl alcohol and cetyl alcohol; polyhydric alcohols such as polyethylene glycol, ethylene glycol and glycerin; carboxylic acids such as lauric acid and stearic acid; and esters such as
- 15 beeswax and lanoline. In cream formulations, there may also be blended the adjuvants or auxiliary agents same as used in any of the liquid formulations described herein.

In some embodiments, the massoia lactone-like compound is selected from the group comprising: 5,6-dihydro-6-heptyl-2H-pyran-2-one, 5,6-dihydro-6-nonyl-2H-pyran-2-one,

20 6-Amy- $\alpha$ -pyrone, 4,6-Dimethyl- $\alpha$ -pyrone, 2,6-Dimethyl- $\gamma$ -pyrone, 2H-Pyran-2-one, 4-Hydroxy-6-methyl-2-pyrone, 4-Methoxy-6-methyl-2H-pyran-2-one,  $\delta$ -Valerolactone,  $\gamma$ -Hexalactone,  $\gamma$ -Octalactone,  $\gamma$ -Nonanoic lactone,  $\delta$ -Nonalactone,  $\delta$ -Decalactone, Eugenol, 2-Undecanone, ethyl pyruvate, and/or cyclopentanone.

- 25 In some embodiments, the pest is selected from the group comprising ants, cockroaches, fleas, flies, mites, mosquitos, spiders, ticks, termites and wasps. In some embodiments, the pest is an insect. In some preferred embodiments, the pest is a mosquito or fly. The mosquito may be from, for example, the *Aedes* genus, the *Culex* genus, or from the *Anopheles* genus. *Aedes* species are known to transmit diseases such as Dengue and
- 30 Zika, *Culex* species are known to transmit Japanese encephalitis, whereas *Anopheles* species are known to transmit malaria. In some embodiments the mosquito is selected from the species *Ae. aegypti*, the species *C. quinquefasciatus*, the species *Anopheles sinensis* and the species *Anopheles gambiae*.

The chemical purity of massoia lactone extract may be in various ranges. In particular, the massoia lactone extract may have 0.1 to at least 95% chemical purity.

5 The concentration of agents in the composition according to any aspect of the present invention may vary widely depending upon the nature of the particular formulation, particularly whether it is a concentrate or to be used directly as known to a skilled person. The concentration of massoia lactone extract in the composition may be at least about 0.001% to 50% (v/v), 0.001% to 25% (v/v), 0.001% to 15% (v/v), 0.01% to 15% (v/v), 0.01% to 10% (v/v), 0.01% to 5% (v/v) or 0.1% to 2% (v/v) depending on the formulation  
10 and application. In some embodiments, the amount of massoia lactone, a massoia lactone-like compound or mixture thereof is in the range of 0.05% to 2% (v/v).

According to another aspect, the present disclosure provides use of massoia lactone, a massoia lactone-like compound or mixture thereof, for repelling an arthropod pest.

15 In some embodiments, the arthropod pest is selected from the group comprising ants, cockroaches, fleas, flies, mites, mosquitoes, spiders, ticks, termites and wasps. In some embodiments, the arthropod pest is an insect. In some embodiments, the pest is a mosquito or fly. The mosquito may be from, for example, the *Aedes* genus, the *Culex* genus, or from the *Anopheles* genus. *Aedes* species are known to transmit diseases such as Dengue and Zika, *Culex* species are known to transmit Japanese encephalitis, whereas *Anopheles* species are known to transmit malaria. In some embodiments the mosquito is selected from the species *Ae. aegypti*, the species *C. quinquefasciatus*, the species *Anopheles sinensis* and the species *Anopheles gambiae*.

25 In some embodiments, the amount of massoia lactone, a massoia lactone-like compound or mixture thereof is in the range of 0.001% to 15% (v/v). In some embodiments, the amount of massoia lactone is in the range of 0.01% to 15% (v/v), preferably 0.05% to 2% (v/v).

30 According to another aspect, the present disclosure provides use of massoia lactone, a massoia lactone-like compound or mixture thereof, in the manufacture of an arthropod pest repellent.

In some embodiments, the arthropod pest is selected from the group comprising ants, cockroaches, fleas, flies, mites, mosquitoes, spiders, ticks, termites and wasps. In some embodiments, the arthropod pest is an insect. In some embodiments, the pest is a mosquito or fly. The mosquito may be from, for example, the *Aedes* genus, the *Culex* genus, or from the *Anopheles* genus. *Aedes* species are known to transmit diseases such as Dengue and Zika, *Culex* species are known to transmit Japanese encephalitis, whereas *Anopheles* species are known to transmit malaria. In some embodiments the mosquito is selected from the species *Ae. aegypti*, the species *C. quinquefasciatus*, the species *Anopheles sinensis* and the species *Anopheles gambiae*.

10

In preferred embodiments, massoia lactone is used.

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention.

15

## EXAMPLES

### EXAMPLE 1

#### Material and Methods

##### 1.1 Mosquito species and rearing

The *Aedes aegypti* (*Ae. aegypti*) (Singapore strain) mosquitoes used were maintained at Temasek Life Sciences Laboratory (TLL) Arthropod facility. The strain was obtained from EHI/National Environment Agency in April 2017. Adult mosquitoes were reared in environmental chambers at  $28 \pm 1$  °C and 75 to 80% relative humidity on a 12:12 hour photoperiod. Adult mosquitoes were provided with 10% sugar solution and the larvae were fed with a mixture of ground fish food flakes (TETRAMIN®, Tetra, Spectrum Brands Pet LLC, VA, US) and dry yeast granule (2:1). Blood feedings were carried out using membrane feeding system (HEMOTЕК®, Hemotek Ltd, UK) with pathogen-free mini pig blood or rabbit blood and mouse skins as the membrane.

30

The *Culex quinquefasciatus* (Singapore strain) used were maintained at Temasek Life Sciences Laboratory (TLL) Arthropod facility. The strain was obtained from EHI/National Environment Agency in Feb 2020. Adult mosquitoes were reared in environmental chambers at  $28 \pm 1$  °C and 75 to 80% relative humidity on a 12:12 hour photoperiod. Adult mosquitoes were provided with 10% sugar solution and the larvae were fed with a

35

mixture of ground fish food flakes (TETRAMIN®, Tetra, Spectrum Brands Pet LLC, VA, US) and dry yeast granule (2:1). Blood feedings were carried out using membrane feeding system (HEMOTЕК®, Hemotek Ltd, UK) with pathogen-free rabbit blood and mouse skins as the membrane.

5

### 1.2 Mosquitoes (for Hand Assay)

A set of at least 3 repeats, 20 females *Ae. aegypti* or *C. quinquefasciatus* mosquitoes aged 5-7 days are placed in a cage (30 cm x 30 cm x 30 cm) and allowed to acclimatize for 10 minutes prior to the start of the experiment. The mosquitoes were starved for 12  
10 hours with access only to water. Female mosquitoes which exhibit host seeking behaviour were transferred from the rearing cage to the testing cage.

### 1.3 Cage

The cage (30 cm x 30 cm x 30 cm) was purchased from Bug Dorm  
15 (shopdotbugdormdotcom). It contains an opening with a sleeve for transferring of mosquitoes and two screen ports (wired screen with donut lid) on the left and right side of the cage that has a circumference of 9 cm (Figure 2). For the experiment, the wired screen of one screen port is replaced with cling wrap for a better view and the wired screen of the other screen port is replaced with a disc of fabric (circumference: 10 cm)  
20 that had been soaked with the test compound.

### 1.4 Candidate insect repelling compounds

C10 massoia lactone was initially produced by yeast fermentation and extracted by H<sub>2</sub>SO<sub>4</sub> hydrolysis of yeast cells followed by ethyl acetate extraction according to the  
25 methods described previously [Ji L, Ngoh ST, WO2017030503 A1], incorporated herein by reference in their entirety. The C10 massoia lactone was purified by fractional distillation. The solvent was removed thoroughly using a rotary evaporator (Heidolph, Germany) set at 120 °C and massoia lactone was collected by heating the residual at 200°C under a vacuum of about 20 mBar or below. A second distillation may be  
30 performed until C10 massoia lactone reached at least 95% as judged by GCMS analysis. C10 massoia lactone was also purchased from Sigma-Aldrich (W374400, natural, ≥95%).

Other compounds, including DEET (97%, SKU: D100951), were purchased from Sigma-Aldrich (USA). The compounds were dissolved in 100% ethanol to make up 1 ml of  
35 solution according to the concentration stated in corresponding experiment. 1 ml of the

solution was added drop-wise onto a cut out of a piece of fabric with a circumference of 10 cm. The fabric was left to dry in a fume hood for 5 minutes prior to usage or longer in the case of duration experiments. The dried fabric was placed in between the donut lid and onto the cage.

5

### 1.5 Quantification

To quantify the protection percentage of the active compound, the number of mosquitoes landing and probing on the fabric were recorded every minute for a period of 5 minutes and the sum of landing and probing during these 5 minutes was divided by 5 to get an average. The average number for the treatment was then divided by the average number for the control treatment (solvent only) to get a landing percentage. The repellency is calculated by subtracting 100 percentage to this landing percentage.

10

## EXAMPLE 2

### 15 *Mosquito Hand Assay (push-pull)*

The hand repellent assay is a rapid screening method developed to screen compounds for their mosquito repellent property. Using a human hand as the attractant, the compounds are tested for their repellent property by creating a barrier with a piece of fabric soaked with 1 ml of diluted compound of interest. Mosquitoes that land on the fabric and probe are quantified every minute for 5 minutes to account for the percentage of repellency that the compound provides. The screening method allows for the study of different concentrations of the compounds and their effectiveness in repelling the mosquitoes. The effectiveness of the compound through time can also be determined with the assay.

20

To induce host seeking response, 10% CO<sub>2</sub> and 90% air mixture are introduced at 3 NI/min (normal liters per minute) into the cage via a fly pad placed at the top of the cage (Figure 2). Mosquitoes were allowed to acclimatize for 10 minutes prior to the start of the experiment. A timer of 5 minutes started once the hand was placed onto a 90 mm Petri dish cover that was placed over the screen port to create a gap between the fabric and the hand. A photograph is taken every minute for 5 minutes for quantification purpose. The control experiment is usually conducted with the fabric soaked with either the diluent or just the fabric only if the diluent cannot be determined in the case of commercial mosquito repellents. Each experiment consists of 3 repeats of control and 3 repeats of treatment. Pre-washed cages and fresh mosquito stock were used in each experiment.

30

35

Mosquitoes are more sensitive to massoia lactone than DEET

A titration test of repellency to *Ae. aegypti* at 0.01%, 0.02%, 0.05%, 0.1%, 0.2%, 0.5%, 1% and 2% (v/v) showed that both massoia lactone and DEET provided 100% protection at concentrations greater than 1%. However, at lower concentrations, massoia lactone exhibited better repellency than DEET (Figure 6).

Massoia lactone was also tested for repellency against *Culex quinquefasciatus* mosquitoes using the push-pull hand repellent assay. Our results show that massoia lactone exhibited strong repellency against *Culex quinquefasciatus* (Figure 10). A dosage-dependent response of *Culex quinquefasciatus* to massoia lactone and DEET was observed and showed *Culex quinquefasciatus* mosquitoes are more sensitive to the repellents than *Ae. aegypti* (cf. Fig. 10A and Fig. 6A). 0.5% (v/v) of massoia lactone provided 100% protection against *Culex quinquefasciatus* which is more effective when comparing to results from *Ae. Aegypti*.

During the course of hand-based “push-pull” experiments, two kinds of mosquito behaviour associated with massoia lactone application were observed. In control experiments, the majority of mosquitoes approached, landed on the fabric next to the hand position within minutes, and exhibited probing behaviour. After massoia lactone application, the majority of mosquitoes did not approach and stayed away from the fabric, suggesting that massoia lactone acts as a volatile repellent compound. A small number of mosquitoes did approach and land on fabric but immediately flew away from the fabric, suggesting a contact-based repellent activity, and massoia lactone may also act as a feeding deterrent. These observations suggest that, similar to DEET, massoia lactone may activate the odorant receptor as well as the gustatory receptor/inotropic receptor pathways.

Massoia lactone provides longer protection than DEET

The duration of effective repellency is an important considering factor for making a mosquito repellent. To test this, 2% massoia lactone was compared to 2% of DEET in the hand-based “push-pull” repellent assay. After application onto the fabrics, the samples were left to dry under the fume hood (with constant airflow) for 5 minutes (defined as 0 h), 150 minutes (defined as 2.5 h) or 300 minutes (5 h) before repellency test. Although 2% DEET showed 100% repellency (or protection) at 0 h, its repellency

dropped to 92.3% by 2.5 h and further to 74.07% by 5 h (Figure 7). In contrast, 2% massoia lactone gave 100% repellency throughout the 5 h testing period. These results indicate that massoia lactone has longer protection efficacy under the “push-pull” testing conditions.

5

Massoia lactone-based formulation out-performed popular essential oil-based products

Some essential oils have been reported to have mosquito repellent activity. The repellency of massoia lactone -ethanol was compared with eucalyptus oil and citronella oil. Both are commonly found in some repellent products. In the hand-based “push-pull” assay, 2% (v/v) eucalyptus oil only exhibits 25.56% repellency (Figure 8). A 2% (v/v) citronella oil in ethanol showed strong repellency at 96.18% at 0 h, however, it dropped quickly to 26.21% after 2.5 h. In contrast, 2% (v/v) massoia lactone in ethanol maintained 100% efficacy throughout the 5 h test period. Notably, massoia lactone -ethanol compared favourably to a commercial mosquito repellent spray, which contains 9% of citronella oil and 3% camphor and exhibited 85.33% and 62.35% repellency at 2.5 h and 5h after application, respectively, much lower than our massoia lactone formulation (Figure 9).

**EXAMPLE 3**

*2-choice trap assay for Drosophila melanogaster*

The assay consisted of 2 traps (control, various concentration of massoia lactone) placed within a 150 mm × 150 mm × 150 mm acrylic plastic cage. Each trap (cylindrical vial; 23 mm D × 72 mm H) was filled with 1 ml of apple cider vinegar as bait and stoppered with a cotton plug (Figure 2). C10 massoia lactone was diluted to a concentration ranging from 0.5% to 50% (v/v) with ethanol absolute. 100 µl of the diluted massoia lactone was applied onto a filter paper disc (25 mm diameter with a 6 mm hole at the centre), and the ethanol was evaporated before the filter paper was placed on the cotton plug of the trap. Control trap was set up similarly with just the solvent (EtOH). A trimmed 200 µl pipette tip (25 mm; opening diameter: 6 mm; exit diameter: 3 mm) was inserted through both the hole of filter paper and the cotton plug to form a channel extending into the trap which the flies could enter but not leave. The 2 traps were then positioned separately at opposite corners of the cage (Figure 3). 40 - 50 females were placed in each cage and were allowed to freely choose between these 2 traps. The assay was performed in the dark at 25 °C and the number of flies in and outside the traps were counted after 24 h.

35

To see if massoia lactone could function as a repellent to other insects, we performed a repellency assay using *Drosophila melanogaster*. A 2-choice assay was set up (Figure 3). Our results showed that massoia lactone displayed clear repellency against female fruit flies in a dosage-dependent manner, between 0.5% and 10% (v/v) (Figure 5).

5

## SUMMARY

### ***Identification of massoia lactone as an effective repellent against Aedes aegypti, Culex quinquefasciatus and Drosophila melanogaster***

To identify potential *Aedes* mosquito repellents, we first established a hand-based “push-pull” assay using DEET (Figure 1), the gold standard mosquito repellent, and a modified cage (Figure 2). Various concentrations of DEET (dissolved in ethanol) were tested and the repellent efficiency was established (Figure 6). Our results showed that, under our assay conditions, DEET gave 100% repellency (or protection) against the *Ae. aegypti* mosquitoes when applied at 1% (v/v) or higher concentrations in a 5-min assay window. In subsequent screening, 2% (v/v) concentration was used for all other candidate repellents (Figure 4), which were selected based on structural similarity to the “head” of delta-lactone ring and DEET (Figure 1). Among the compounds tested, C10 massoia lactone (ML) and its saturated derivative,  $\delta$ -decalactone, showed excellent repellency. Interestingly, gamma-lactone, such as  $\gamma$ -nonanoic lactone and  $\gamma$ -octalactone showed comparable repellency at this concentration. Eugenol is more similar to DEET and also exhibited strong repellent activity against *Aedes aegypti* mosquitoes (Figure 4). Undecanone, which resembles to the tail region of lactones, showed significant repellency. Molecules with pyrone rings also showed significant repellent activity. Surprisingly, ethyl pyruvate, a reported strong inhibitor of the *Ae. aegypti* CO<sub>2</sub> receptor in mosquito olfactory neuron [Tauxe GM, *et al.*, *Cell* 2013, 155(6):1365-1379], showed weak activity. This could be due to different assay conditions and setup used for assay. Notably, cyclopentanone is a strong attractant although it has the same ring structure of gamma-lactones (Figure 1) [Tauxe GM, *et al.*, *Cell* 2013, 155(6): 1365-1379]. Therefore, an effective repellent molecule requires both a hexa or penta carbon/oxygen ring and an alkyl or alkene tail of at least 3 carbons. The double bond inside the ring structure appeared to play a small role, possibly through modulating boiling point to affect protection duration.

Eugenol, which was reported to act either as an insect attractant or repellent [Hao H, Wei J, Dai J, Du J: *J Med Entomol.* 2008, 45(3): 533-539; Chokechaijaroenporn O,

35

Bunyaphrathasara N, Kongchuensin S. *Phytomedicine* 1994, 1(2): 135-139; Fletcher BS, Bateman MA, Hart NK, Lamberton JA. *Journal of Economic Entomology* 1975, 68(6): 815-816; Klein MG, Tumlinson JH, Ladd Jr TL, Doolittle RE. *J Chem Ecol.* 1981, 7(1): 1-7], showed very good repellent activity against *Ae. aegypti*. Massoia lactone has a boiling point of 287 °C, almost identical to DEET (288 °C). Nevertheless, C10 massoia lactone gave better repellency at 0.5-1% (v/v) (Figure 6) and at prolonged time lapse (Figure 7) suggesting that massoia lactone is a significantly more potent repellent for *Ae. aegypti* mosquitoes than DEET. As massoia lactone has a boiling point significantly higher than that of eugenol (254 °C), we consider massoia lactone is more stable than eugenol and would be a better ingredient for insect repellents as it will last longer. Furthermore, an efficient bio-production method has been established for massoia lactone [Ji L, Ngoh ST. WO2017030503 A1], therefore, massoia lactone has an advantage of being an environmentally-friendly and a potentially safer active ingredient for mosquito repelling formulations.

Massoia lactone may function as a repellent in many more insect species, including other mosquito species such as *Culex* and *Anopheles*. Indeed, in the “push-pull” hand-based assay, massoia lactone also repels *Culex quinquefasciatus* (Figure 10), suggesting it likely functions as a general mosquito repellent. To see if massoia lactone could function as a repellent to other insects, we performed a repellency assay using *Drosophila melanogaster*. A 2-choice assay was set up (Figure 3). Our results showed that massoia lactone displayed clear repellency against female fruit flies in a dosage-dependent manner, between 0.5% and 10% (v/v) (Figure 5).

Data on the performance of our prototypes of massoia lactone -based mosquito repellent in comparison to popular essential oils and a commercial product (Figures 8 and 9) further support that massoia lactone is a strong candidate for novel anti-mosquito formulations and products. Several other molecules demonstrated here (see Figure 4) repelled mosquitoes.

## References

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

- 5 1. Bernier UR, Kline DL, Vazquez-Abad A, Perry M, Cohnstaedt LW, Gurman P, D'hers S, Elman NM: A combined experimental-computational approach for spatial protection efficacy assessment of controlled release devices against mosquitoes (Anopheles). *PLoS Negl Trop Dis*. 2019, 13(3): e0007188.
- 10 2. Chokechaijaroenporn O, Bunyapraphatsara N, Kongchuensin S: Mosquito repellent activities of ocimum volatile oils. *Phytomedicine* 1994, 1(2): 135-139.
3. Fletcher BS, Bateman MA, Hart NK, Lamberton JA: Identification of a fruit fly attractant in an Australian plant, *Zieria smithii*, as O-methyl eugenol. *Journal of Economic Entomology* 1975, 68(6): 815-816.
- 15 4. Hao H, Wei J, Dai J, Du J: Host-seeking and blood-feeding behavior of *Aedes albopictus* (Diptera: Culicidae) exposed to vapors of geraniol, citral, citronellal, eugenol, or anisaldehyde. *J Med Entomol*. 2008, 45(3): 533-539.
5. Ji L, Ngoh ST: Methods for fermentative production of massoia lactone. In.: WIPO (PCT); 2017: WO2017030503 A1.
- 20 6. Keith S, Harper C, Ashizawa A, Williams RL, Lladós F, Coley C, Carlson-Lynch H: Toxicological profile for DEET (N, N-diethyl-meta-toluamide). Atlanta: Agency for Toxic Substances & Disease Registry. 2017, 1-263.
7. Klein MG, Tumlinson JH, Ladd Jr TL, Doolittle RE: Japanese beetle (Coleoptera: Scarabaeidae): response to synthetic sex attractant plus phenethyl propionate: Eugenol. *J Chem Ecol*. 1981, 7(1): 1-7.
- 25 8. Legeay S, Clere N, Apaire-Marchais V, Faure S, Lapied B: Unusual modes of action of the repellent DEET in insects highlight some human side effects. *Eur J Pharmacol*. 2018, 825: 92-98.
9. Tauxe GM, MacWilliam D, Boyle SM, Guda T, Ray A: Targeting a dual detector of skin and CO<sub>2</sub> to modify mosquito host seeking. *Cell* 2013, 155(6):1365-1379.
- 30 10. Vector Borne Disease [www.doh.gov.sg/en/news-room/fact-sheets/detail/vector-borne-diseases]