In vitro comparison of three common essential oils mosquito repellents as inhibitors of the Ross River virus

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Abstract

Background

The essential oils of Cymbopogon citratus (CC), Pelargonium graveolens (PG) and Vetiveria zizanioides (VZ) are commonly used topically to prevent mosquito bites and thus the risk of infection by their vectored pathogens such as arboviruses. However, since mosquito bites are not fully prevented, the effect of these products on the level of viral infection remains unknown.

Objectives

To evaluate in vitro the essential oils from Reunion Island against one archetypal arbovirus, the Ross River virus (RRV), and investigate the viral cycle step that was impaired by these oils.

Methods

The essential oils were extracted by hydrodistillation and analyzed by a combination of GC-FID and GC×GC-TOF MS techniques. In vitro studies were performed on HEK293T cells to determine their cytotoxicity, their cytoprotective and virucidal capacities on RRV-T48 strain, and the level of their inhibitory effect on the viral replication and residual infectivity prior, during or following viral adsorption using the reporter virus RRV-renLuc.

Results

Each essential oil was characterized by an accurate quantification of their terpenoid content. PG yielded the least-toxic extract ($CC_{50} > 1000 \mu g.mL^{-1}$). For the RRV-T48 strain, the monoterpane-rich CC and PG essential oils reduced the cytopathic effect but did not display virucidal activity. The time-of-addition assay using the gene reporter RRV-renLuc showed that
the CC and PG essential oils significantly reduced viral replication and infectivity when applied prior, during and early after viral adsorption. Overall, no significant effect was observed for the low monoterpene-containing VZ essential oil.

**Conclusion**

The inhibitory profiles of the three essential oils suggest the high value of the monoterpene-rich essential oils from CC and PG against RRV infection. Combined with their repellent activity, the antiviral activity of the essential oils of CC and PG may provide a new option to control arboviral infection.

**Introduction**

Management of arthropod-borne viruses (arboviruses) related to neglected tropical diseases has become a global health public concern [1]. The discovery of novel prophylactic or therapeutic treatments against arboviruses remain a continuous goal aimed to counter emerging virus or new viral strains [2]. Ross River virus (RRV) is a small enveloped positive-sense single-stranded RNA virus that belongs to the alphavirus genus, family Togarividae [3]. The RRV incubation period in humans was estimated to be from 7 to 9 days [4] and like the well-known Chikungunya virus that belongs to the same viral family, it typically causes fever, rash and polyarthralgia [5, 6]. RRV is endemic in Australia where it is the most common mosquito-borne pathogen with an average of 5000 cases annually [7, 8]. After the major outbreak in the Pacific area in 1979 and 1980, serological studies revealed the silent circulation of RRV in the Fiji islands [9] and more recently in French Polynesia [10, 11].

Two biological characteristics distinguish RRV from other alphaviruses: more than 40 species of mosquitoes can act as its vectors, thus, providing a large number of potential amplification cycles, and numerous warm blood host (mainly marsupials) support this virus’s replication [12]. This provides numerous opportunities for RRV to infect humans and initiate outbreak foci [13]. Indeed, during 2017, a large outbreak was observed in the South-West Region of Australia with more than 2 thousand cases reported in less than 2 months. Because the infection lead to very painful and debilitating joint, up to months after the initial onset, the disease has a direct impact on health services and calls for direct responses from the Australian authorities [14]. Consequently, RRV for which no efficient treatment is available, remains a major focus of basic research, and necessitates on-going surveys by the Australian health services [13, 15–18]. Mannose binding lectin (MBL) has been proposed as an efficient therapeutic target to alleviate RRV-induced arthritis but to date only pentosan sulfate, initially approved for the treatment of cystitis in U.S., is available [19, 20]. In an *in vitro* re-evaluation of 40 plants species used in Australian folk medicine, inhibition of RRV-induced cytopathic effect (25–50%) was observed with the ethanolic extract of *Myoporaceae* and *Pittosporaceae* species [21]. Essential oils are natural complex mixtures and their antiviral properties are due to complementary and overlapping mechanisms, as assumed for herpes simplex virus (HSV), influenza virus and yellow fever virus. To date, the anti-infective properties of essential oils, though of growing interest, have not been explored for RRV [22–24].

In arboviruses-related control measures, a number of essential oils are exploited as topical repellents to reduce the incidence of mosquito bites [25]. However, as yet these have not been investigated for antiviral activity at the site of infection, the skin, where they could be absorbed percutaneously. Such additional benefits of skin-applied essential oils may offer a great
opportunity to control the early stages of infection, even when their repelling action fails. *Cym-
blogon citratus*, *Vetiveria zizanioides* (family: Poaceae) and *Pelargonium graveolens* (family:
Geraniaceae) are distributed worldwide and their essential oils (denoted hereafter as CC, VZ
and PG, respectively), are readily available and have notable mosquito repellent properties
[26]. The aim of the present study was to investigate the inhibitory effects *in vitro* of these
three common essential oils at non-cytotoxic concentrations against RRV. We assessed their
effects on both virus entry using the wild-type of RRV-T48 strain (RRV-T48) and viral replica-
tion using a recombinant RRV expressing *Renilla reniformis* luciferase (RRV-renLuc).

**Materials and methods**

**Plant material**

Fresh leaves of *Cymbopogon citratus* (DC) Strapf and *Pelargonium graveolens* L’Hér were har-
vested in July 2014 and June 2015 in Reunion Island. Roots of *Vetiveria zizanioides* (L.) Nash
were harvested in December 2015. All plant samples were kindly provided by the CAHEB
(Coopérative Agricole des Huiles Essentielles de Bourbon), Le Tampon, Reunion Island.

**Essential oil isolation and analysis**

Essential oils were extracted in triplicate from 2.5 kg of aerial part (PG and CC) or roots (ZV)
by hydrodistillation during 3 h using a Clevenger-type apparatus. Essential oils were decanted
from aqueous phase, dried over anhydrous sodium sulfate and filtered using Minisart filters
(0.2 μm). Samples were then stored at 4˚C in darkness. The chemical composition of the essen-
tial oils was quantified by gas chromatography-flame ionization detector (GC-FID) on *Clarus*
500 gas chromatograph and identified by gas chromatography-time-of-flight-mass spectrome-
try on a GC×GC-TOF MS LECO Pegasus 4D system [27].

**Cell culture**

Human embryonic kidney cell line HEK293T (ATCC) and the kidney epithelial cell line Vero
(ATCC) were grown in Dulbecco’s modified Eagle’s medium (DMEM, Dutscher, Issy-les-
 Moulineaux, France) or modified Eagle’s medium (MEM, Dutscher) supplemented with 10%
fetal bovine serum heat inactivated (FBS, Dutscher) and completed with 2 mmol.L⁻¹ L-gluta-
amine (Dutscher), 100 U.mL⁻¹–0.1 mg.mL⁻¹ penicillin-streptomycin (Dutschers), 1 mmol.L⁻¹
sodium pyruvate (Dutschers) and 250 μg.mL⁻¹ amphotericin (Dutschers). Cells were maintained
in a humidified atmosphere of 5% CO₂ at 37˚C in Petri dishes.

**Cytotoxicity assay**

Cell viability was measured using the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-
2,5-diphenyltetrazolium bromide) assay [28]. Vero or HEK293T cells were seeded in 96-well
plates (2×10⁵ cells/well) and allowed to adhere overnight at 37˚C. Cells were then treated 21 h
at 37˚C with each essential oil within a wide range of concentrations (0.14–1130 μg.mL⁻¹). The
essential oils were solubilized in 0.4% DMSO (Sigma). Following the treatment, 20 μL of 5 mg.
mL⁻¹ MTT solution (Sigma, Saint-Quentin Fallavier France) were added on the 96-well plates
and cells were stored at 37˚C in the darkness. After 3 h of treatment, supernatant was removed
and replaced by 100 μL of DMSO. Plates were then read at 570 nm on a microplate reader
Tecan Sunrise™. The cytotoxic concentrations are defined as the concentration of the essential
oil that causes death to 50% (CC₅₀) or 10% (CC₁₀) of viable cells with respect to controls with-
out the essential oil (Table 1). Experiments were performed in hexaplicate from five indepen-
dent experiments (see S1 Fig).
Virus strains

Ross River virus derived from an infectious clone of strain T48 (RRV-T48), GenBank GQ433359 was generously provided by Professor Richard J. Khun, Purdue University [29]. The recombinant RRV-T48 expressing *Renilla reniformis* luciferase, as an integral part of the non-structural polyprotein precursor, was obtained according to the procedure previously described for Chikungunya virus [30]. Briefly, the resulting plasmids pRRV-renLuc was linearized and the corresponding RNA was transcribed *in vitro* using the kit mMESSAGE mMACHINES Kit (Ambion) before transfection into Vero cells to provide live RRV-renLuc viruses [31]. Supernatants were collected after 48 h (first passage) and used to infect Vero cells in order to grow final virus stocks for experiments (second passage).

Plaque reduction assay

Virus titers were determined by plaque assays in Vero cells growing in 48-well plates as previously described [32]. Briefly, cells at confluence were incubated (0.1 mL/well) in triplicate with a serial of ten-fold dilutions of RRV-renLuc containing samples for 2 h at 37°C. Then, 0.1 mL of 0.8% carboxymethylcellulose (CMC, Sigma) was added. After 48 h incubation at 37°C, CMC was removed and the monolayers were fixed with 3.7% v/v of paraformaldehyde (10 min at room temperature). Cells were then stained with 0.5% w/w crystal violet solution (10 min) and the virus titer in each well was estimated by counting the number of plaques observed. The results are expressed as plaque-formation unit per milliliter (PFU.mL⁻¹).

Cytoprotective effect of the essential oils against RRV-T48

The capacity of the tested essential oils on RRV-T48 inducing cytopathic effect was assessed on HEK293T cells using MTT assay as described above. HEK293T were seeded in 96-well plates (2×10⁵ cells/well) and allowed to adhere overnight at 37°C. Cells were infected by RRV-T48 alone at MOI 2 or by RRV-T48 at MOI 2 and addition of the essential oil at the highest non-toxic concentration (1×CC₁₀) during 2 h at 37°C. After virus adsorption, supernatants were removed and cell culture medium or 1×CC₁₀ of the essential oil was added again. Cell viability was measured at different time points from incubation at 37°C (6;12;18;24;30;36;42;48 or 24;32;48 h post-infection). Controls consist in RRV-infected-HEK293T cells untreated with the essential oils. Chloroquine at non-toxic concentration was used as positive control.

Table 1. Main characteristics of the essential oils.

| Essential oil               | Major components %                          | CC₅₀ (µg.mL⁻¹)ᵃ | CC₁₀ (µg.mL⁻¹)ᵇ |
|-----------------------------|---------------------------------------------|------------------|------------------|
| *Cymbopogon Citratus* (CC)  | Geranial (45.11 ± 2.46%)                    | 49.5 ± 20.5      | 17.6 ± 8.2       |
|                             | Neral (32.16 ± 0.69%)                       |                  |                  |
|                             | Myrcene (7.85 ± 1.46%)                      |                  |                  |
| *Pelargonium Graveolens* (PG) | Citronellol (23.43 ± 0.14%)               | > 1000           | 533 ± 199        |
|                             | Geraniol (16.85 ± 0.05%)                    |                  |                  |
|                             | Citronellyl formate (12.29 ± 0.05%)        |                  |                  |
|                             | Linalool (10.79 ± 0.05%)                    |                  |                  |
|                             | Isomenthone (7.06 ± 0.02%)                 |                  |                  |
| *Vetiveria Zizanoides* (VZ) | Khusimol (23.78 ± 0.13%)                    | 169.9 ± 72.2     | 29.4 ± 13.5      |
|                             | (E)-Isovalencenol (6.79 ± 0.12%)           |                  |                  |
|                             | α-Vetivone (3.84 ± 0.04%)                   |                  |                  |

ᵃ Cytotoxic concentration CC₅₀ that cause death to 50%
ᵇ Cytotoxic concentration CC₁₀ that causes death to 10%. Results are expressed as mean ± SD from triplicate determination.

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Virucidal activity of the essential oils against RRV-T48 and entry assay

To determine the virucidal activity, each essential oil at concentrations 0.1×CC<sub>10</sub> or 1×CC<sub>10</sub> was mixed with the RRV-T48 strain (1×10<sup>5</sup> PFU) during 1 h at 37˚C. The residual infectivity was titrated by plaque assay on Vero cells with a serial of ten-fold dilutions. For the entry assay, Vero cells monolayers were pre-treated 3 h at 37˚C with the essential oils. The residual infectivity of RRV-T48 strain was then titrated by plaque assay with a serial of ten-fold dilutions on the pre-treated Vero cells. All the experiments were performed in triplicate from five independent experiments and controls consist in RRV-T48-infected Vero cells without treatment with the essential oils.

RRV-renLuc reporter assay

After infection with RRV-renLuc, the luciferase reporter is released in the cytoplasm during the polyprotein processing. HEK293T cells were seeded in 96well plates (2×10<sup>5</sup> cells/well) and allowed to adhere overnight at 37˚C. Cells were infected with RRV-renLuc at MOI 2 for 48 h. At different time points (6;12;18;24;30;36;42;48 h post-infection), supernatants were removed and cells were lysed using 20 μL of lysis buffer (0.4% CHAPS, 10% glycerol, 1 mmol.L<sup>-1</sup> EGTA, Tris-HCl, Sigma). 100 μL of the substrate coelenterazine (Euromedex, Souffelweyersheim, France) were then added and plates were immediately read by a luminescent plate-reader FLUOstar® Omega (BMG Labtech, Offenburg, Germany). Experiments were performed in hexaplicate from five independent experiments.

Time-of-addition assay

To assess the effect of the essential oils on the replication of RRV, HEK293T cells were seeded in 96-well plates (2×10<sup>5</sup> cells/well) and allowed to adhere overnight at 37˚C. Cells were infected with RRV-renLuc at MOI 2 in presence of the essential oil. The essential oils were added at the highest non-toxic concentration (1×CC<sub>10</sub>): (i) 3 h before virus incubation (pre-treatment) and after virus incubation; (ii) during virus incubation (co-treatment) and after virus incubation; (iii) 2 h, 4 h or 6 h after virus incubation (post-treatment). In all cases, the luciferase activity was measured in hexaplicate from five independent experiments by the RRV-renLuc assay. Virus titers were determined from five independent experiments from harvested supernatants. The end point of these two analyses was 24 h post-infection. Controls consist in RRV-infected-HEK293T cells untreated with the essential oils. Chloroquine at non-toxic concentration was used as positive control.

Statistical analysis

Where applicable, a one way analysis of variance (ANOVA) followed by Tukeys or Dunn’s post-test when relevant, using the GraphPad Prism software, version 7.01 (GraphPad Software Inc.). A p value lower than 0.05 was considered significant. Values are reported as the means standard errors (SEM) of n = 5 determinations unless otherwise stated. For the cytotoxicity assay, values were derived from dose-response curves and were calculated using GraphPad Prism.

Results

Chemical composition and cytotoxicity of essential oils

The combination of GC-FID and GC-TOF MS allowed identifying 37, 67 and 53 components of the essential oils from CC, PG and VZ, respectively, and their major components are listed in Table 1 (detailed chemical compositions are available in S1–S3 Tables). For the CC essential oil, the major components were the two isomeric monoterpene aldehydes geranial (45.11%)
and neroli (36.11%) and a high amount of the monoterpene hydrocarbon myrcene (7.85%) typical for essential oils of African origin [33]. The chemical composition of the PG oil was also found to be in accordance with the African type [34] with five major monoterpenes, including citronellol (23.43%), geraniol (16.85%) and linalool (10.79%) alcohols beside the citronellyl formate ester (12.29%) and isomenthone ketone (7.06%). The major components identified for the VZ extract include the sesquiterpenes khusimol (23.78%), (E)-isovalencenol (6.79%) and α-vetivone (3.84%), which are related to the Reunion chemotype [35]. The cytotoxic concentrations CC$_{50}$ and CC$_{10}$ of the three essential oils are reported in Table 1. The lowest CC values were found for the CC essential oil followed by that of VZ, with the PG essential oil proving to be far less toxic (CC$_{50}$ > 1000 µg.mL$^{-1}$; CC$_{10}$ = 533 ± 199 µg.mL$^{-1}$).

**Cytoprotective effect of the essential oils against RRV-T48**

The antiviral activity of the essential oils was first screened by assessing the reduction of the viral cytopathic effect through determination of the viability of HEK293T cells by an MTT assay after infection by RRV-T48 at MOI 2. As shown in Fig 1, the viability of the infected cells without treatment was dramatically reduced beyond 30 h post-infection. Upon treatment by the essential oils at the non-toxic concentration 1×CC$_{10}$, cell viability was determined at 24 h, 32 h or 48 h post-infection and compared with untreated cells. The results in Fig 1B showed that cell viability increases significantly at 32 h post-infection upon treatment with the essential oils of CC ($p < 0.01$) or PG ($p < 0.005$). However, there was no more significant difference at 48 h post-infection.

**Virucidal activity of essential oils against RRV-T48 and entry assay**

In order to determine whether the essential oils interfere with virus entry, the residual infectivity was determined by plaque assay on Vero cells incubated with a mixture of virus (1×10$^5$ PFU) and the essential oils (0.1×CC$_{10}$ or 1×CC$_{10}$). The infected cells without exposure to the essential oils were used as controls. As shown in Fig 2A, no significant effect was observed whichever essential oil was used. For the entry assay performed on cells pre-treated with the essential oils before infection, viral progeny production was not affected (Fig 2B).

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**Fig 1. Viability of HEK293T cells infected with RRV-T48.** A: without treatment by the essential oils; B: upon treatment with the essential oil at the concentration 1×CC$_{10}$ at 24, 32 or 48 h post-infection. Controls (CTL) consist in untreated cells with the essential oils and results are expressed as mean ± SEM (n = 5). Statistical analysis was performed with Prism 7 (*$p < 0.05$; **$p < 0.01$; ***$p < 0.005$).

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Inhibition of RRV-renLuc replication by the essential oils

In order to determine the inhibitory effects of the essential oils at the early stage of the viral replication, we used a luciferase-based monitoring method using RRV-renLuc and controlled the residual infectivity by plaque assay. The time course of infection of RRV-renLuc (MOI 2) on HEK293T cells was first determined to guide subsequent experiments. We observed that the luciferase activity increases until 36 h post-infection (Fig 3) while the viral progeny production reaches a maximum at 30 h post-infection (Fig 3). Thus, the effect of essential oils on RRV-renLuc replication levels was determined 24 h after infection.

![Graph showing the inhibition of RRV-renLuc replication by essential oils](https://doi.org/10.1371/journal.pone.0196757.g003)
In a preliminary assessment, we compared the inhibitory capacity of the essential oils with that of the broad-range viral replication inhibitor chloroquine [36]. As shown in Fig 4A, at the non-toxic concentration 1×CC10 for the essential oils and for chloroquine, cell viability was not affected. maximal reduction of luciferase activity and residual infectivity was observed for the PG essential oil (Fig 4B and 4C).

To assess if the inhibitory effects of the essential oils is related to the viral absorption or entry we altered treatment timing (pre-treatment, co-treatment or post-treatment, Fig 5).

The overall results are presented in Fig 6. Using the CC essential oil, only pretreatment and co-treatment significantly decrease both luciferase activity and residual infectivity titer. The maximum inhibitory effect was observed for co-treatment, with both a luciferase activity level and a virus residual infectivity titer down close to 50% of the non-treated (p < 0.01 and p < 0.05 respectively). For the PG essential oil, all treatments induced a significant decrease of the virus activity with the lowest level of luciferase activity observed for pre-treatment (34%) but later was the treatment, more increased the luciferase activity to reach a high level (67%) for post-treatment at 6 h post-infection (ANOVA one-way test for trend slope = 13.2, p < 0.001). However, the reduction in virus residual infectivity titer is always significant (p < 0.05 to 0.01) compared to non-treated control, it varied in a non-regular manner not significantly different.
whatever was the timing of treatment (p > 0.058, Kruskal-Wallis test) even if maximum reduction was observed by co-treatment and post-treatment at 4 h post-infection (< 20%). In contrast, the VZ essential oil exhibited no significant effect either on luciferase activity or on viral progeny production whatever was the treatment timing. Thus, the inhibitory profiles were found to be significantly different for the three tested essential oils.

**Discussion**

Essential oils have demonstrated a wide range of biological activities (e.g. antibacterial, anti-fungal, antioxidants, etc.) and remain promising sources of new therapeutics [37, 38]. A growing attention has been given to the antiviral capacity of essential oils against arboviruses as illustrated for dengue serotype 2 (DEN-2) virus [39], Yellow Fever virus (YFV) [40], and Japanese Encephalitis virus (JEV) [41]. To the best of our knowledge, none of the available essential oils used as topical mosquito repellents have not yet been investigated for their antiviral capacity against mosquito-borne viruses. In here, we investigated such inhibitory effects of three common essential oils of CC, PG and VZ at non-toxic concentrations against RRV infection.

For this study, HEK293T cells were selected for their high sensitivity to the viral cytopathic effect, in contrast to the resistant skin cell lines [42]. In addition, we used a representative RRV strain to provide a fast and representative evaluation of essential oils against the alphaviruses family.

The cytopathic effect of RRV-T48 on HEK293T cells observed after 30 h post-infection (Fig 1A) and the time-limited cytoprotective effect of the tested essential oils at 32 h post-infection clearly indicated the best opportunity to control RRV infection at the early stages. Specific assays (Fig 2) showed that the essential oils do not have virucidal activity and could not interfere with virus entry at the two non-toxic concentrations tested (0.1×CC_{10} and 1×CC_{10}). Thus, we carried out further investigations to determine the inhibitory effect of the essential oil on viral replication. This was supported by the time course of infection of RRV-renLuc at MOI 2 that reached a maximum at 36 hours (Fig 3A), leading us to select the endpoint of monitoring at 24 h post-infection for both the luciferase activity and the residual infectivity upon different treatments by the essential oils (Fig 5).

Interestingly, the time-of-addition assay showed that the additional supply of the essential oils of CC and PG prior or during or the viral adsorption (pre-treatment or co-treatment) provides the most significant inhibition of the viral replication (Fig 6A). The viral replication was also reduced in post-treatment (Fig 6A), but only the PG essential oil exhibited a marked effect on the residual infectivity (Fig 6B). This latter result suggests that the PG essential oil may also interfere with the post-transcriptional stage of the virus life cycle.

Thus, the results showed the high potential of the PG essential oil against RRV, as it has low cytotoxicity (CC_{50} > 1000 μg.mL^{-1}) and displays noteworthy inhibitory effects when present prior, during or after infection. The CC essential oil, which presented the highest cytotoxicity (CC_{50} = 49.5 ± 20.5 μg.mL^{-1}), exhibited a moderate antiviral activity when introduced prior or during the viral absorption. In contrast, there was no evidence for an inhibitory effect from the VZ essential oil. The contribution of the major components of essential oils to their antiviral properties has been claimed in the case of HSV-1 [43–45]. PG and CC essential oils are
monoterpane-rich essential oils in contrast to the non-active VZ essential oil that is mainly constituted by sesquiterpenes. Thus, we propose that antiviral capacity is related to the monoterpane composition, and the actual molecular components deserve to be characterized and explored further.

**Conclusion**

The repellent activity of the three essential oils from CC, PG and VZ was known from folk medicine. The present study provides the first investigation of their antiviral activity against RRV infection. The different inhibitory profiles of these three essential oils suggest a relationship with their chemical compositions. Exposure to these oils prior or at the same time as viral infection resulted in the highest inhibitory effects and suggest that the primary application of these essential oils as repellents may further provide an additional valuable preventive effect against the viral infection. Our findings demonstrate the value of re-evaluating essential oils mosquito repellents for their antiviral capacity, as this might provide a novel, eco-friendly and cost-effective strategy in the prevention of arboviruses infection.

**Supporting information**

S1 Fig. Determination of essential oils cytotoxicity on HEK293T. Viability of HEK293T cells was determined by MTT assay upon treatment by the essential oils of CC (A); PG (B); VZ (C). Values are expressed as mean ± SEM (n = 3). Dashed line indicated the CC_{10}.

S1 Table. Chemical composition of the leaf Cymbopogon citratus (CC) essential oil from Reunion Island, area percentage mean ± standard deviation (n = 9).

S2 Table. Chemical composition of the leaf Pelargonium graveolens (PG) essential oil from Reunion Island, area percentage mean ± standard deviation (n = 9).

S3 Table. Chemical composition of the roots Vetiveria zizanioides (ZV) essential oil from Reunion Island, area percentage mean ± standard deviation (n = 6).

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References

1. Dye C, Mertens T, Himschall G, Mpanju-Shumbusho W, Newman RD, Raviglione MC, et al. WHO and the future of disease control programmes. Lancet. 2013; 381(9864):413–8. Epub 2013/02/05. https://doi.org/10.1016/S0140-6736(12)61812-1 PMID: 23374479

2. Liang G, Gao X, Gould EA. Factors responsible for the emergence of arboviruses; strategies, challenges and limitations for their control. Emerg Microbes Infect. 2015; 4(3):e18. Epub 2015/06/04. https://doi.org/10.1038/emi.2015.18 PMID: 26039768

3. Atkins GJ. The Pathogenesis of Alphaviruses. International Scholarly Research Notices. 2012; 2013. https://doi.org/10.5402/2013/861912

4. Fraser JR, Cunningham AL. Incubation time of epidemic polyarthritis. Med J Aust. 1980; 1(11):550–1. Epub 1980/05/31. PMID: 6104774

5. Suhrbier A, Jaffar-Bandjee MC, Gasque P. Arthritogenic alphaviruses—an overview. Nat Rev Rheumatol. 2012; 8(7):420–9. Epub 2012/05/09. https://doi.org/10.1038/nrrheum.2012.64 PMID: 22565316

6. Liu X, Thamararajah K, Taylor A. Ross River virus disease clinical presentation, pathogenesis and current therapeutic strategies. Microbes Infect. 2017; 19(11):496–504. Epub 2017/07/30. https://doi.org/10.1016/j.micinf.2017.07.001 PMID: 28754345

7. Harley D, Sleigh A, Ritchie S. Ross River virus transmission, infection, and disease: a cross-disciplinary review. Clin Microbiol Rev. 2001; 14(4):909–32, table of contents. Epub 2001/10/05. https://doi.org/10.1128/CMR.14.4.909-932.2001 PMID: 11585790

8. Mackenzie JS, Lindsay MDA, Smith DW, Imrie A. The ecology and epidemiology of Ross River and Murray Valley encephalitis viruses in Western Australia: examples of One Health in Action. Trans R Soc Trop Med Hyg. 2017; 111(6):248–54. Epub 2017/10/19. https://doi.org/10.1093/trstmh/trx045 PMID: 29044370

9. Klapsing P, MacLean JD, Glaze S, McClean KL, Drebot MA, Lanciotti RS, et al. Ross River virus disease reemergence, Fiji, 2003–2004. Emerg Infect Dis. 2005; 11(4):613–5. Epub 2005/04/15. https://doi.org/10.3201/eid1104.041070 PMID: 15829203

10. Aubry M, Finke J, Teissier A, Roche C, Broult J, Paulous S, et al. Silent Circulation of Ross River Virus in French Polynesia. Int J Infect Dis. 2015; 37:19–24. Epub 2015/06/19. https://doi.org/10.1016/j.ijid.2015.06.005 PMID: 26086687

11. Aubry M, Teissier A, Huart M, Merceron S, Vanhornwegen J, Roche C, et al. Ross River Virus Seroprevalence, French Polynesia, 2014–2015. Emerg Infect Dis. 2017; 23(10):1751–3. Epub 2017/09/21. https://doi.org/10.3201/eid2310.170583 PMID: 28930020

12. Claffin SB, Webb CE. Ross River Virus: Many Vectors and Unusual Hosts Make for an Unpredictable Pathogen. PLoS Pathog. 2015; 11(9):e1005070. Epub 2015/09/04. https://doi.org/10.1371/journal.ppat.1005070 PMID: 26335937
13. Files EJ, Weinstein P, Anderson SJ, Koolhof I, Foufopoulos J, Williams CR. Ross River virus and the necessity of multi-scale, eco-epidemiological analyses. J Infect Dis. 2017. Epub 2017/12/08. https://doi.org/10.1093/infdis/jix156 PMID: 29216368

14. Government of Western Australia. Ross River and Barmah Forest virus disease risk warning 2017. Available from: http://ww2.health.wa.gov.au/Media-releases/2017/Ross-River-and-Barmah-Forest-virus-disease-risk-warnings.

15. Faddy HM, Tran TV, Hoad VC, Seed CR, Vien net E, Chan HT, et al. Ross River virus in Australian blood donors: possible implications for blood transfusion safety. Transfusion. 2018. Epub 2018/01/20. https://doi.org/10.1111/trf.14472 PMID: 29350414

16. Gunn BM, Jones JE, Shabman RS, Whitmore AC, Sarkar S, Blevins LK, et al. Ross River virus envelope glycoproteins contribute to disease through activation of the host complement system. Virology. 2018; 515:250–60. Epub 2018/01/13. https://doi.org/10.1016/j.virol.2017.12.022 PMID: 29324290

17. Mazzon M, Castro C, Thaa B, Liu L, Musso M, Liu X, et al. Alphavirus-induced hyperactivation of PI3K/AKT directly pro-viral metabolic changes. PLoS Pathog. 2018; 14(1):e1006835. Epub 2018/01/30. https://doi.org/10.1371/journal.ppat.1006835 PMID: 29377936

18. Haist KC, Burrack KS, Davenport BJ, Morrison TE. Inflammatory monocytes mediate control of acute alphavirus infection in mice. PLoS Pathog. 2017; 13(12):e1006748. Epub 2017/12/16. https://doi.org/10.1371/journal.ppat.1006748 PMID: 29244871

19. Gunn BM, Morrison TE, Whitmore AC, Blevins LK, Hueston L, Fraser RJ, et al. Mannose binding lectin is required for alphavirus-induced arthritis/myositis. PLoS Pathog. 2012; 8(3):e1002586. Epub 2012/03/30. https://doi.org/10.1371/journal.ppat.1002586 PMID: 22457620

20. Herrero LJ, Foo SS, Sheng KC, Chen W, Forwood MR, Bucala R, et al. Pentosan Polysulfate: a Novel Glycosaminoglycan-Like Molecule for Effective Treatment of Alphavirus-Induced Cartilage Destruction and Inflammatory Disease. J Virol. 2015; 89(15):8063–76. Epub 2015/05/29. https://doi.org/10.1128/JVI.00224-15 PMID: 26018160

21. Semple SJ, Reynolds GD, O’Leary MC, Flower RL. Screening of Australian medicinal plants for antiviral activity. J Ethnopharmacol. 1998; 60(2):163–72. Epub 1998/05/15. PMID: 9582007

22. Bakkali F, Averbeck S, Averbeck D, Idaomar M. Biological effects of essential oils—a review. Food Chem Toxicol. 2008; 46(2):446–75. Epub 2007/11/05. https://doi.org/10.1016/j.fct.2007.09.105 PMID: 17996351

23. Jassim SA, NajI MA. Novel antiviral agents: a medicinal plant perspective. J Appl Microbiol. 2003; 95(3):412–27. Epub 2003/08/13. PMID: 12911688

24. Raut JS, Karuppayil SM. A status review on the medicinal properties of essential oils. Industrial Crops and Products. 2014; 62:250–64. https://doi.org/10.1016/j.indcrop.2014.05.055

25. Nerio LS, Olivero-Verbel J, Shashenko E. Repellent activity of essential oils: a review. Bioresour Technol. 2010; 101(1):372–8. Epub 2009/09/05. https://doi.org/10.1016/j.biortech.2009.07.048 PMID: 19729299

26. Pohlit AM, Lopes NP, Gama RA, Tadei WP, Neto VF. Patent literature on mosquito repellent inventions which contain plant essential oils—a review. Planta Med. 2011; 77(6):598–617. Epub 2011/02/18. https://doi.org/10.1055/s-0030-1270723 PMID: 21328177

27. Baranauskiene R, Rutkaite R, Peciulyte L, Kazernaviciute R, Venskutonis PR. Preparation and characterization of single and dual propylene oxide and octenyl succinic anhydride modified starch carriers for the microencapsulation of essential oils. Food Funct. 2016; 7(8):3555–65. Epub 2016/07/29. https://doi.org/10.1039/c6fo00775a PMID: 27465989

28. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 1983; 65(1–2):55–63. Epub 1983/12/16. PMID: 6606882

29. Kuhn RJ, Niesters HG, Hong Z, Strauss JH. Infectious RNA transcripts from Ross River virus cDNA clones and the construction and characterization of defined chimeras with Sindbis virus. Virology. 1991; 182(2):430–41. Epub 1991/06/01. PMID: 1673812

30. Henrik Gad H, Paulous S, Belarbi E, Dioncourt L, Drosten C, Kummerer BM, et al. The E2-E166K substitution restores Chikungunya virus growth in OAS3 expressing cells by acting on viral entry. Virology. 2012; 434(1):27–37. Epub 2012/08/15. https://doi.org/10.1016/j.virol.2012.07.019 PMID: 22888614

31. Krejbich-Trotot P, Belarbi E, Ramondbrairay M, El-Kalamouni C, Viranacke W, Roques P, et al. The growth of arthralgia Ross River virus is restricted in human monocytic cells. Virus Res. 2016; 225:64–8. Epub 2016/09/18. https://doi.org/10.1016/j.virusres.2016.09.007 PMID: 27637347

32. Frumence E, Roche M, Krejbich-Trotot P, El-Kalamouni C, Nativel B, Rondeau P, et al. The South Pacific epidemic strain of Zika virus replicates efficiently in human epithelial A549 cells leading to IFN-beta production and apoptosis induction. Virology. 2016; 493:217–26. Epub 2016/04/10. https://doi.org/10.1016/j.virol.2016.03.006 PMID: 27060565
33. Avoseh O, Oyedeji O, Rungqu P, Nkhe-Chungag B, Oyedeji A. Cymbopogon species; ethnopharmacology, phytochemistry and the pharmacological importance. Molecules. 2015; 20(5):7438–53. Epub 2015/04/29. https://doi.org/10.3390/molecules20057438 PMID: 25915460

34. Sharopov FS, Zhang H, Setzer WN. Composition of geranium (Pelargonium graveolens) essential oil from Tajikistan American Journal of Essential Oils and Natural Products. 2014; 2(2):13–6.

35. Chahal KK, Bhardwaj U, Kaushal S, Sandhu K. Chemical composition and biological properties of Chrysopogon zizanioides (L.) Roberty syn. Vetiveria zizanioides (L.). Indian Journal of Natural Products and Resources. 2015; 6:251–60.

36. Savarino A, Boelaert JR, Cassone A, Majori G, Cauda R. Effects of chloroquine on viral infections: an old drug against today's diseases? Lancet Infect Dis. 2003; 3(11):722–7. Epub 2003/11/01. PMID: 14592603

37. Nakatsu T, Lupo AT, Chinn JW, Kang RKL. Biological activity of essential oils and their constituents. In: Atta ur R, editor. Studies in Natural Products Chemistry. 21: Elsevier; 2000. p. 571–631.

38. Properzi A, Angelini P, Bertuzzi G, Venanzoni R. Some Biological Activities of Essential Oils. Medicinal & Aromatic Plants. 2013; 2:136. https://doi.org/10.4172/2167-0412.1000136

39. Garcia CC, Talarico L, Almeida N, Colombres S, Duschatzky C, Damonte EB. Virucidal activity of essential oils from aromatic plants of San Luis, Argentina. Phytother Res. 2003; 17(9):1073–5. Epub 2003/11/05. https://doi.org/10.1002/ptr.1305 PMID: 14595590

40. Meneses R, Ocazione RE, Martinez JR, Stashenko EE. Inhibitory effect of essential oils obtained from plants grown in Colombia on yellow fever virus replication in vitro. Ann Clin Microbiol Antimicrob. 2009; 8:8. Epub 2009/03/10. https://doi.org/10.1186/1476-0711-8-8 PMID: 19267922

41. Roy S, Chaurvedi P, Chowdhary A. Evaluation of antiviral activity of essential oil of Trachyspermum Ammi against Japanese encephalitis virus. Pharmacognosy Res. 2015; 7(3):263–7. Epub 2015/07/02. https://doi.org/10.4103/0974-8490.157977 PMID: 26130938

42. Assi M, Thon-Hon VG, Jaffar-Bandjee MC, Martinez A, Gasque P. Regulation of type I-interferon responses in the human epidermal melanocyte cell line SKMEL infected by the Ross River alphavirus. Cytokine. 2015; 76(2):572–6. Epub 2015/07/15. https://doi.org/10.1016/j.cyto.2015.07.003 PMID: 26159111

43. Astani A, Reichling J, Schnitzler P. Comparative study on the antiviral activity of selected monoterpenes derived from essential oils. Phytother Res. 2010; 24(5):673–9. Epub 2009/08/05. https://doi.org/10.1002/ptr.2955 PMID: 19653195

44. Astani A, Reichling J, Schnitzler P. Screening for antiviral activities of isolated compounds from essential oils. Evid Based Complement Alternat Med. 2011; 2011:253643. Epub 2009/12/17. https://doi.org/10.1093/ecam/nep187 PMID: 20008902

45. Astani A, Schnitzler P. Antiviral activity of monoterpenes beta-pinene and limonene against herpes simplex virus in vitro. Iran J Microbiol. 2014; 6(3):149–55. Epub 2015/04/15. PMID: 25870747