Potential of garlic oil as a biopesticide against all *Aedes aegypti* life stages

Renata Garcia Dusi[a,b], Lais da Silva Morais[a], Natália Mendes Gomes Magalhães[a], Lorena Carneiro Albernaz[a], Chris J. Hamilton[b], Laila Salmen Espindola[a]*

[a]Laboratório de Farmacognosia, Universidade de Brasília, Campus Universitário Darcy Ribeiro, 70910-900, Brasília, DF, Brazil
[b]School of Pharmacy, University of East Anglia, Norwich Research Park, Norwich, NR4 7TJ, UK
*Corresponding author: darvenne@unb.br (L.S. Espindola)

Abstract

Vector control remains the most effective approach to prevent dengue, chikungunya and Zika arboviruses transmission. Conventional insecticides have historically failed to control the *Aedes aegypti* mosquito due to acquired resistance, environmental impact and toxicity. This study evaluated the potential of garlic oil as a biopesticide against the eggs, larvae, pupae and adult forms of *Ae. aegypti* eggs, in accordance with the World Health Organization recommendations. The larvicidal and pupicidal LC_{50} values were 1.0 ppm and 20.3 ppm after 72 h, respectively. The oil maintained its activity in simulated field trials, killing all larvae and pupae at the tested concentrations. At 100 ppm, garlic oil inhibited 59.6 ± 10.6% of egg hatching. Toxicity against the adult form was observed as was its potent spatial repellency. Garlic oils composed of different diallyl polysulfide ratios did not significantly impact insecticidal activity although the garlic oil polysulfide mixtures were more potent than the individual polysulfides. The ovicidal, larvicidal, pupicidal, adulticidal and repellent assays showed the broad activity of garlic oil against *Ae. aegypti*. These results, together with the activity in simulated field trials, support the applicability of garlic oil in integrated mosquito vector control programs.

Keywords

Aedes aegypti; garlic oil; biopesticide; diallyl polysulfides; vector control; spatial repellency

1. Introduction

Aedes aegypti L. is the primary mosquito species responsible for the transmission of dengue fever. Worldwide incidence of dengue has increased 10-fold over the last two decades, with over 5 million cases reported in 2019 (WHO, 2021). Although the arbovirus burden disproportionately affects poorer populations of tropical and subtropical regions, the territorial expansion of this vector relating to climate change will also be of concern for temperate areas in the world (Ryan et al., 2019). A major challenge in integrated vector management is the lack of agents that can effectively target all mosquito life stages. Furthermore, resistance development, together with environmental damage relating to indiscriminate use of non-targeted pesticides, severely affects the availability of chemical alternatives (Lopes et al., 2019).

Garlic is a food crop extensively cultivated worldwide, with garlic oils extracted on an industrial scale to meet pharmaceutical and food industries demands (FAO, 2018). The United States Environmental Protection Agency classifies garlic oil as a minimum risk pesticide active ingredient, posing little or no risk to human health and the environment (EPA, 2015). Moreover, the multiple complex mechanisms of action already described for garlic oils suggest they have low potential for resistance development (Anwar et al., 2017; Arbach et al., 2019). The studies herein: (i) Evaluate the efficacy of chemically characterized garlic oils against all of the *Ae. aegypti* life stages; (ii) Report field trial simulations, and (iii) Discuss the impact of polysulfide composition on larvae and pupae toxicity.

2. Materials and methods

2.1. Chemicals and analytical instrumental

The organophosphate insecticide temephos, Chinese garlic oil (artificial), diallyl disulfide and diallyl trisulfide were purchased from Sigma Aldrich (Buchs, Switzerland). All other chemicals used in this study were HPLC grade.

^{1}H NMR spectra were recorded on a Bruker 600 MHz spectrometer, with tetramethylsilane used as an internal standard. Gas chromatography coupled to mass spectrometry analysis was performed on a Shimadzu GC-2010 instrument employing the following conditions: DB-5 MS (30 m x 25 mm x 25 µm) column; carrier gas: He (1.3 mL/min, in constant linear velocity mode); injector temperature: 220 ºC, in split mode (1:60); and detector temperature: 250 ºC. The temperature was increased at 3 ºC/min from 60 to 210 ºC. Mass spectra were obtained at electron impact of 70 eV and acquired for the 35 to 400 m/z range. The volume injected was 1 µL. The components were identified using GC retention times, calculated by linear interpolation relative to retention times of their main compounds, and by comparison of mass spectra with the NIST (National Institute of Standards and Technology) database.

2.2. Insect rearing

The larvae (third-instar, L3), pupae (2-24 h), and eggs (stored for 2-4 weeks) used in the assays were collected from an *Ae. aegypti* (Rockefeller strain) colony maintained at the Laboratório de Farmacognosia Insectarium at the Universidade de Brasília. The colony has never been exposed to any insecticide. The mosquitoes were maintained at 28 ± 2 °C, 70 ± 10% relative humidity and a 12-h photoperiod. Egg hatching was conducted in plastic containers with tap water and larvae were fed with protein-based fish food.

Adult insects were fed on filter paper (Whatman, Canterbury, UK) pre-soaked with 10% aqueous sugar solution, which was replaced twice per week. An equine blood meal (Hospital Veterinário of the Universidade de Brasília) was given three times a week to enable egg production.

Adult female mosquitoes used in toxicity and contact irritant assays were collected from the University of Notre Dame (USA) *Ae. aegypti* (Liverpool strain) colony. The mosquitoes were maintained at 27 °C, 80% relative humidity and a 12-h photoperiod. Female groups of 4 to 7 days old were separated in plastic containers and fed the previously described sugar solution until the day prior the tests.

2.3. Larvicidal and pupicidal assays

Larvicidal and pupicidal assays were performed following the WHO guideline recommendations. The samples were dissolved in dimethylsulfoxide (DMSO) and assays performed in 4 replicates, repeated with 3 different larvae batches. The garlic oil LC_{50} and LC_{95} values were determined with six concentrations (4.8; 2.4; 1.8; 1.2; 0.8 and 0.4 ppm) for larvae, and five concentrations (60.0; 50.0; 40.0; 30.0 and 5.0 ppm) for pupae. A total of 1,800 larvae and 1,500 pupae were exposed to garlic oil in 4 replicates of 25 individuals in plastic containers with 200 mL of the testing solutions. The number of dead larvae was recorded after 24, 48 and 72 h exposure. Larvae with no movement after mechanical or luminous stimulation were considered dead. Regarding pupicidal assays, the number of dead pupae and completely emerged mosquitoes were recorded after full mosquito emergence in the negative control replicates.

Mosquitoes with distinguishable head, legs and wings that could move were considered viable, even if they did not completely detach from their exuviae. The containers used in the pupicidal assays were covered with a fine netting to prevent mosquito escape. The positive controls used were: for larvae - temephos (0.0250 ppm), and for pupae - pyriproxyfen (0.0001 ppb to 200 ppm range) and temephos (0.5 ppm). The vehicle negative controls (<1.0% DMSO in tap water) were performed in parallel to ensure test validity.
The assays were performed using the same colony under the same rearing conditions. According to the WHO guidelines, tests with a control mortality > 5% were corrected using Abbott's formula and tests with control mortality > 20% were discarded. The LC_{50} (ppm) values and their respective 95% fiducial limits, together with their lower/upper confidence intervals, were calculated by Probit analysis (Milesi et al. 2013) using the RStudio® software.

2.4. Ovicidal assay

Sections of filter paper containing 120-150 *Ae. aegypti* eggs were placed in 200 mL of garlic oil (100 ppm) solution or the DMSO control (< 1% solution in tap water). The eggs were treated for 48 h. The paper sections with the eggs were subsequently transferred to new plastic containers containing 200 mL of fresh tap water. The containers were then placed in a low-pressure chamber for 30 min to stimulate egg hatching. Newly hatched larvae were fed with fish food and counted after 48 h. Four sections of paper containing eggs from four different batches were used in the assays. Papers were photographed for total egg counting using ImageJ software (Fig. S3). The number of larvae after 48 h of eclosion and the initial number of eggs in the control and treated paper sections were used to calculate the corrected mortality rate using the Henderson-Tilton formula.

2.5. Spatial repellency, contact irritancy and adult toxicity assays

The spatial repellency and contact irritancy assays were performed in the High-Throughput Screening System (HITSS) described by Grieco (Grieco et al., 2015), following the methods described by Achee (Achee et.al, 2019). For the spatial repellency assay, 275 cm^2 nylon netting strips were treated with 1.5 mL of acetone (negative control) or with 7.5, 5.0, 2.5 or 1.0% (v/v) garlic oil solution in acetone and positioned in the HITSS terminal metal chambers. Groups of 20 ± 2 females per replicate were used for each of the 9 replicates.

The total number of mosquitoes, together with the knockdown number, in each part of the HITSS was recorded to calculate the Spatial Activity Index (SAI) and the weighted Spatial Activity Index (wSAI). The SAI value = (Nc - Nt) / (Nc + Nt), where Nc and Nt represent the number of viable mosquitoes in the control and treatment chambers, respectively. SAI ranges from -1 to +1. Negative values indicate an attractant response whereas positive values indicate repellency. The wSAI was calculated by multiplying the SAI by the percentage of responsive mosquitoes in the assay. The PRESP (percentage of response) value = [(Nc + Nt)/ N] *100, where N is the total number of mosquitoes used in the assay. The wSAI represents the magnitude of the repellent or attractant effect, while the SAI concerns the existence (or not) of a directional movement. Data was analyzed using the SAS University Edition software with a non-parametric signed-rank test (PROC UNIVARIATE) to determine if the mean SAI and wSAI were significantly different from zero (SAS, 2018). The mosquitoes were extracted from the chambers and placed in transparent containers to observe 24 h mortality.

For contact irritancy assays, the HITSS is partially disassembled, resulting in one chamber housing either the treated or the control net (Achee et.al, 2019). The same type of netting used in the spatial repellency test was utilized for both the treatment and control samples. The control and treatment chambers were run separately, with 10 females used for each of the 6 replicates. The test was performed similarly to the spatial repellency assay. Garlic oil 1.0% (v/v) solution in ethanol was used as the test solution, while ethanol was the negative control. The number of mosquitoes in the clear (escaping) and metal chambers, together with the knockdown number in both the treated and control devices were recorded. Data was analyzed using a Wilcoxon 2-sample test to evaluate the difference between the number of mosquitoes that escaped the control and treated nets. The mean percentage of treatment escape was corrected considering the escape in the control and knockdown observed in both the treated and control groups. PMD (20% p- menthane-3,8-diol), a biochemical pesticide derived from eucalyptus plants, was used as a positive control for both the spatial repellency and contact irritancy assays.

The adult toxicity assay was performed following the CDC bottle assay protocol (CDC, 2019). In quadruplicate, 250 mL glass bottles (Wheaton Science, Millville, NJ, USA) were coated with 1 mL of 1% (v/v) garlic oil solution, resulting in 10 µL garlic oil/bottle. After bottles dried overnight in a fume hood, 25 female mosquitoes in each replicate (n=100) were inserted and the number of knocked down mosquitoes counted for 120 min. The mosquitoes were then transferred into clean transparent vessels and provided sucrose solution to monitor 24 h mortality. Bottles coated with ethanol were used as control. The diagnostic time was determined as well as the knockdown at 30 min and 24 h mortality.

2.6. Simulated field trials

Larvicide and pupicide simulated field trials were performed in plastic buckets containing 8 L of tap water, distributed in a random manner outside the controlled atmosphere of the laboratory to emulate environmental conditions. The water was left to acclimatize for 24 h, after which 50 third-instar larvae or 50 pupae were added to each container, accordingly. After 2 h adaptation, 10 mL of a garlic oil solution (in DMSO) at different concentrations was added: larvae (4.0, 3.0 and 1.5 ppm) and pupae (60, 45 and 30 ppm). Positive (temephos 0.013 ppm) and negative (<1.0% DMSO) controls were performed in parallel. A thermohygrometer was placed in one of the control containers to monitor temperature and humidity throughout the tests. Each concentration and control were tested in quadruplicate and repeated with 3 different larvae/pupae batches. Containers were covered with a nylon netting to prevent interference. Each container was examined at 24 h intervals, with the number of live and dead individuals used to determine post-treatment mortality. The Chi-Square test was used to determine any statistically significant differences between the proportions of pupae that died in laboratory and simulated field trials at the same concentration.

Spatial repellency assays were conducted in a scaled-up field trial assay similar to the laboratory trial described in Section 2.5. This trial involved two 10 m^3 rooms in the Laboratório de Farmacognosia Insectarium connected by a plastic tube to allow mosquito flux during the experiment (Fig. 4). The treated room had an electric diffuser with 150 µL of garlic oil added to 300 mL water, while the control room housed a humidifier with only water. The opposite-facing rooms were separated by 1.3 m corridor, each with a door. Groups of 20 ± 8 females were used for each of the 9 replicates. The test solution was replaced before the 7^{th} replicate. Five-day old female mosquitoes were placed in the center of the tube connecting the rooms. After 30 sec, the mosquitoes were released and allowed to fly through the apparatus and to the rooms. A cage was assembled inside each room to enable mosquito counting at the end of the test. After 15 min, the number of live and knocked-down mosquitoes in each part of the test (control and treatment rooms, and corridor tubing) was recorded and the Spatial Activity Index (SAI)/ weighted Spatial Activity Index (wSAI) determined as described in Section 2.5.

2.7. Isolated polysulfides and enriched garlic oil assays

Diallyl disulfide (DAS2) was purchased from Sigma Aldrich. Diallyl trisulfide (DAS3), diallyl tetrasulfide (DAS4) and diallyl pentasulfide (DAS5) were isolated from garlic oil using a Varian ProStar preparative HPLC. A Phenomenex Luna C18(2) column (5 µm particle size, 150 x 21.2 mm) was used with an isocratic 90% methanol mobile phase for 30 min at 10 mL/min, monitored at 210 nm. The collected peaks were stored in the freezer prior to hexane extraction. The organic phase was collected and concentrated using a rotary evaporator. Samples were diluted in $CDCl_3$ and analyzed by ^1H NMR. The NMR data was compared to literature data to confirm compound structures (Wang et al., 2013). The isolated compounds were diluted in ethanol (< 2.0% in tap water) and tested in 12-well plates containing 3 mL of tap water and 10 larvae or 5 pupae, accordingly. The LC_{50} values were determined using six polysulfide concentrations for larvae (10.0, 5.0, 2.5, 1.25, 0.63 and 0.31 ppm) and pupae (50.0, 25.0, 12.5, 6.25, 3.13 and 1.56 ppm). A total of 240 larvae and 120 pupae were exposed to garlic oil treatment in quadruplicate.

Individual garlic oils were supplemented with DAS2, DAS3, DAS4 and DAS5 (50% w/w), and tested against larvae and pupae in the aforementioned concentration ranges for the isolated polysulfides. Ethanol, used to dissolve all of different enriched garlic oil samples, was used as a negative control (< 2.0% ethanol in tap water). LC_{50} values were determined using the GraphPad Prism 8 software and the dose-response curves compared by two-way ANOVA followed by Dunnett's test to compare the activity of the different samples with the original garlic oil.

Ethanol stock solutions of the isolated polysulfides and the supplemented garlic oil samples were monitored by HPLC throughout the larvae and pupae assays. A SunFire C18 column (5 μm 4.6 x 250 mm) was used with an isocratic 90% methanol mobile phase for 15 min at 1 mL/ min. Peaks were monitored at 210 nm.

3. Results and discussion

3.1 Garlic oil chemical profile

Garlic oil composition was characterized through the evaluation of diallyl polysulfide ratios using three different methods: GC-MS (Table S1), HPLC-DAD (Fig. S1) and ^{1}H NMR (Fig. S2). The predominant allyl polysulfides detected were: diallyl sulfide (DAS), diallyl disulfide (DAS2), diallyl trisulfide (DAS3), diallyl tetrasulfide (DAS4), diallyl pentasulfide (DAS5) and diallyl hexasulfide

(DAS6) (Table 1). Combined, these diallyl polysulfides (DAPS) account for more than 97% of oil composition. According to its profile, rich in diallyl trisulfide (DAS3), the garlic oil used in this study could be classified as a Cluster #2 garlic oil (Satyal et al., 2017). GC/MS resulted in the identification of 8 peaks, all possessing at least one sulfur atom.

All three analytical techniques revealed DAS3 as the most abundant compound: GC/MS (63.1%), HPLC/UV (53.9%) and ^{1}H NMR (52%). The next most abundant polysulfides were DAS2: GC/MS (26.7%), HPLC (17%), ^{1}H NMR (32%), and DAS4: GC/MS (4.2%), HPLC (19%) and ^{1}H NMR (9.4%).

The diallyl polysulfide ratios observed may differ depending on the technique due to the type of detectors used. Extinction coefficients of polysulfides differ in UV analysis and do not represent a direct relationship between peak areas and concentrations of the different compounds (Lawson et al., 1991). Since peak area directly correlates to the number of hydrogens, NMR analysis could offer a more accurate representation of the proportion of major compounds. However, high detection limits render NMR the least sensitive technique in that it does not detect some minor components. When comparing to data in the literature, it is important to consider the polysulfide composition of the garlic oils in conjunction with the analytical methods employed.

3.2 Larvicidal and pupicidal activity

Larvae (L3 stage) were more susceptible (LC_{50} 1.58 ppm, 24 h) to garlic oil treatment than pupae (LC_{50} 20.34 ppm) (Table 2). A thorough literature search did not identify any previous reports of garlic oil *Ae. aegypti* pupicidal activity, suggesting that this is its first report. In addition, these pupae results support the capacity of garlic oil to interfere in more than one stage of the mosquito life cycle. Unlike water-borne larvae, pupae do not feed, making compound bioavailability considerably more challenging. Larvae could have enhanced garlic oil absorption rates due to filter feeding, while higher pupal endurance could mostly relate to physical barriers rather than biochemical protection. A previous study with ^{35}S-radiolabeled DAS2 reported that mosquito larvae assimilate DAS2 at least 3-fold faster than pupae. At 50 ppm DAS2, 95% of *Culex pipiens* larvae died after 8 h, while only 3% of pupae died after the same exposure time. The ^{35}S activity when all larvae (8 h) and pupae (24 h) died was similar, indicating that the equivalent concentration of DAS2 would kill both life forms (Ramakrishnan et al., 1989). In our experiments, even at the highest concentration (60 ppm), there was almost no pupae mortality after 24 h. Pupae death was mostly observed at adult ecdysis, whereas most larvae mortality was observed after the first 24 h. Owing to the volatile nature of garlic oil, to achieve an adequate concentration to achieve pupae mortality, higher initial concentrations must be used to offset time-dependent losses due to evaporation.

The larvicidal activity of the garlic oil in this study showed 10-fold greater potency than those investigated in previous reports. Sarma et al. determined a larvicidal LC_{50} 16.9 ppm after 24 h, that reached 7.6 ppm after 72 h. However, GC/MS analysis of the garlic oil used in those experiments only annotated 30% of total peak area with major components identified as DAS2 (8.5%) and DAS3 (7.8%) (Sarma et al., 2019). These were significantly lower than those detected in the oil used in the present study (26.7% and 63.1%, respectively) (Table 1). Muturi et al. reported a LC_{50} 7.95 ppm (24 h) for a garlic oil with 49.1% DAS2, 31.1% DAS3 and 11.0% DAS4 (determined by GC/MS) (Muturi et al., 2018; Muturi et al., 2019).

The organophosphate temephos positive control in larvicidal tests showed LC_{50} 0.008 ppm (CI_{95} 0.0076 - 0.0085) after 24 h. In the pupicidal test control, 0.063 ppm temephos had no effect on pupae, while 0.5 ppm (a 62.5-fold higher concentration than its larvicidal LC_{50}) only caused 15% pupae mortality. At present, there is no pupicide available for *Ae. aegypti* mosquito control despite reference to pyriproxyfen pupicidal activity in the literature (Hustedt et al., 2020). Therefore, we investigated pyriproxyfen pupicidal activity in the 0.0001 ppb to 200 ppm range. No pupal mortality or deformations in emerging adults were detected after treatment in this concentration range. Although considered a pupicide, pyriproxyfen only interferes with development into the adult form when administered during the larvae stage at concentrations allowing pupae formation.

Visual inspection of pupae that died after garlic oil treatment (LC_{50} 20.34 ppm) evidenced a dark coloration, probably due to tissue necrosis after detoxification attempts (Fig 1A). Sublethal garlic oil concentrations triggered pupae abnormalities impeding the emergence of healthy mosquitoes often causing malformations incompatible with mosquito survival (Fig. 1B-1E). In some cases, the mosquito was completely formed but could not detach from the exuviae during the molting process (Fig 1C-D). In the 5-15 ppm range, some mosquitoes were completely formed, however with leg and wing malformations that interfered with their fitness and survival (Fig 1D-F). Incomplete mosquito detachment from the exuviae prevented flight and generally caused death after a few hours, probably due to exhaustion. Some completely formed and detached mosquitoes exhibited wing defects and were therefore unable to close their wings during rest (Fig 1F). We observed direct correlation between the concentration used and the impact on adult formation, with lower concentrations relating to a lower degree of abnormality. In the wild, such abnormalities in emerging mosquitoes compromise their survival and capacity to transmit arboviral diseases. In summary, not only do garlic oils demonstrate direct activity against pupae, they also indirectly control the adult form by interfering in the life cycle at sublethal concentrations.

3.3. Isolated polysulfides and enriched garlic oil assays

Unveiling the potency of different polysulfides could potentially inform the development of more potent insecticides by modifying the polysulfide compositions of garlic oils to optimize their efficacy. DAS2-DAS5 were purified from garlic oil by preparative scale HPLC and their individual structures confirmed by ^{1}H NMR (Fig. S2) (Wang et al., 2013). These polysulfides were individually tested as well as being used to supplement, and subsequently measure the effect native garlic oils whose DAPS ratios have been altered. When individually tested, DAS3 and DAS4 were most active polysulfides after 24 h against larvae, but less active than the original garlic oil (Fig. 2A and Table S2). However, larvicidal activity of all of DAS2-DAS5 appeared to be more equipotent after 72 h (Table S2). Interestingly, all the isolated polysulfides were less active than the garlic oils supplemented with each of them individually in the first 24 h for larvae. Sarma et al. reported that DAS2 and DAS3 were less active than garlic oil after 72 h against *Ae. aegypti* larvae, although DAS3 was more active in the first 24 h (Sarma et al., 2019).

The original garlic oil together with the DAS4 supplemented garlic oil were the most active samples against larvae after 24 h (Fig. 2A and Table S2). All the other samples tested in larvae had statistically different potencies when compared to the original oil at 24 h. Interestingly, the LC_{50} of a DAS2-rich garlic oil (49.1%) was 7.95 ppm (CI_{95} 7.19 - 8.66) (Muturi et al., 2018; Muturi et al., 2019). The polysulfide ratio of the aforementioned oil was similar to our DAS2-enriched garlic oil (Table S3), however, the LC_{50} herein was lower (LC_{50} 5.6 ppm, CI_{95} 4.9 - 6.4 ppm). Another DAS2-supplemented garlic oil with 4.3% DAS, 37.4% DAS2, 10.9% DAS3 and 0.4% DAS4 showed an LC_{50} of 7.05 ppm (CI_{95} 6.12 – 7.82) against *Culex pipiens* larvae after 48 h. The original garlic oil with 7.2% DAS2, 16.3% DAS3 and 0.7% DAS4 had a slightly higher LC_{50} of 8.01 ppm (CI_{95} 7.64 – 8.36) (Kimbaris et al., 2009).

After 72 h, the garlic oil supplemented with DAS5 was the most active with an LC_{50} of 0.5 ppm against larvae (Fig. 2B and Table S2). The pupicidal assays of garlic oil individually supplemented with DAS4 or DAS2 determined significantly more potent LC_{50} values (4.0 and 5.5 ppm, respectively), than the original garlic oil (10.2 ppm) (Fig. 2C and Table S2).

The garlic oil activities described in this section differ from those reported in the previous section due to the different test methods used. The WHO protocol, with 25 larvae/ pupae in 200 mL of test solution, resulted in: LC_{50} 1.6 ppm (larvae) and 20.3 ppm (pupae), while in this section, with 12-well plates with 10 larvae/ 5 pupae in 3 mL of test solution, resulted in: LC_{50} 2.3 ppm (larvae) and 10.2 ppm (pupae). Therefore, the LC_{50} values are not only impacted by the garlic oil DAPS profile, but also by the testing method employed.

While some of the differences in activity are significant, the overall trend is a higher activity of the enriched garlic oils in comparison to the isolated polysulfides. Potency variations of the different garlic oils are significant, but may be irrelevant due to the low level (low ppm range) required to achieve larvicidal and pupicidal activities. In addition, some garlic oil compositions were more active against pupae, and less active against larvae. For instance, DAS4-supplemented garlic oil was significantly less active on larvae (after 72 h) but was significantly more active against pupae than the original garlic oil (Fig. 2). Collectively, these results indicate that alterations in individual DAPS proportions (DAS2-DAS5) would not significantly impact global mosquito control, providing the concentration of the DAPS mixture is maintained. The literature suggests that DAS supplementation may negatively affect the larvicidal activity of garlic oils by a reduction in the proportion of the other polysulfides. A DAS-enriched garlic oil showed significantly lower larvicidal activity (LC_{50} 24.3 ppm) than the original garlic oil (LC_{50} 8.0 ppm) for *Culex pipiens* larvae after 48 h (Kimbaris et al., 2009).

Natural garlic oils often contain low levels of other organosulfur compounds that may also contribute to their potency. The garlic oils investigated herein are artificial and mainly composed of diallyl polysulfides, being the only enriched compounds. Based on the chemical analysis of the original garlic oil involving three different techniques: GC-MS, HPLC-DAD and ^{1}H NMR (Table 1, Table S1, Fig. S1-S2), it is unlikely that components other than the polysulfides were responsible for the insecticidal activities detected. The higher activities of the combined oils, when compared to the isolated polysulfides, could be a result of an interesting synergistic insecticidal combination between these compounds.

Contrarily, Sarma et al. suggested a DAS2 and DAS3 mixture presented an antagonistic effect against both larvae and adult *Ae. aegypti* mosquitoes (Sarma et al., 2019). However, natural products, specifically essential oils are recognized as more effective larvicides than their isolated compounds (Silvério et al., 2020). A combination of different compounds found in the oils not only impacts activity, but could also impair the development of resistance due to the different mechanisms of action of the distinct compounds (Anwar et al., 2017; Arbach et al., 2019).

Once purified, individual diallyl polysulfides are prone to disproportionation back into garlic oil-like polysulfide mixtures, especially those possessing longer sulfur chains (Arbach et al., 2019). Samples of DAS2, DAS3, DAS4, DAS5, garlic oil and all 1:1 garlic oil combinations (prepared in ethanol for the biological assays) were stored at room temperature and reanalyzed periodically by HPLC. As previously reported, the stability of longer polysulfide chains is inversely proportional to sulfur chain length, as observed for DAS4 and DAS5 in Fig. 3A. In a previous study, 50% of DAS5 was lost in the first 4 h after HPLC recovery (Arbach et al., 2019), while the present study showed that only 25% of DAS5 remained intact after 24 h. DAS2 and DAS3 remained stable during the entire experiment. The chemical instability of longer chain polysulfides may directly impact their biological activity. The reduction of DAS4 and DAS5 was accompanied by the formation of the other polysulfides until they reached equilibrium (Fig. 3A). Originally purified DAS4 and DAS5 exhibited similar HPLC profiles after 72 h storage in solution at room temperature, with DAS3 and DAS4 comprising approximately 82% of the samples.

The original garlic oil, a steady state equilibrium mixture of diallyl polysulfides, did not show any alterations in composition after 72 h when compared to Time 0 (Fig. 3B-3C; Fig. S4E). Garlic oil supplemented with DAS2 or DAS3 also remained stable throughout the experiment (Fig. S4A-B). As observed for the isolated DAS4 and DAS5, the garlic oil supplemented with these compounds reached equilibrium after 24 h, richer in DAS3 and DAS4 when compared to the original oil (Fig. 3C; Fig. S4C-D). As the HPLC samples were stored in sealed vials prior to analysis, they were probably not identical to those in the biological assays. The HPLC analyses suggested compound instability. In addition, other bioassay variables included the use of tap water, incompletely sealed plates and a higher temperature in the insectarium. Given the instability of long chain polysulfides, together with their fast volatilization, the toxicity to larvae and pupae observed after 72 h may be the delayed result of the initial DAPS exposure. In brief, the lower activity of the isolated polysulfides, together with the instability of the higher chain ones, indicate that efforts to purify and utilize individual polysulfides as single entities is not worthwhile.

3.4 Simulated field trials: larvicidal and pupicidal assays

The trials involved exposing the *Ae. aegypti* Rockefeller strain to garlic oil treatment conditions simulating their natural breeding sites. Larvae and pupae were added to plastic buckets containing tap water and placed outside the laboratory conditions. During these trials, the water temperature ranged from 9.1 to 34.8 ºC, with the lowest relative humidity of 24%. The three concentrations tested against pupae (60.0, 45.0 and 30.0 ppm) caused 100% mortality. These concentrations were approximately the LC_{100}, LC_{90} and LC_{80} in the laboratory experiments. The concentrations of 60.0 and 30.0 ppm were also tested under laboratory conditions (28 ± 2 °C, 70 ± 10% relative humidity and 12 h photoperiod). The latter caused significantly less mortality in the laboratory (70.7 ± 10.4% in 200 mL cups) compared to the field setting (99.8 ± 0.3% in 8 L buckets) ($p < 0.0001$). The different volume to surface area ratio of the test vessels may account for the difference observed. A higher volume to surface area ratio may retard garlic oil evaporation and cause higher mortality due to prolongation of the initial concentration.

Regarding larvae, 4.0 and 3.0 ppm caused 100% mortality after 24 h. The 1.5 ppm sample caused > 95% mortality after 48 h, a concentration that affects almost 50% of the larvae under laboratory conditions. These results in the field environment, demonstrated that variable weather conditions (temperature, humidity and light parameters) did not impact garlic oil efficacy.

3.5 Ovicidal activity

At 100 ppm, the garlic oil solution inhibited 59.6 ± 10.6% egg hatching after the Henderson-Tilton correction. On average, eclosion was 28.3 ± 5.9% for the garlic oil treatment and 70.7 ± 9.4% for the DMSO control. Egg viability ranged from 19.4 to 33.6% in the treated samples and from 54.7 to 87.7% in the control. Hatching rates may vary according to storage time and conditions (Soares-Pinheiro et al., 2016). Water temperature can also significantly impact viability and delay larvae emergence (Byttebier et al., 2014).

Another study reported 100% egg mortality with a 100 ppm garlic oil solution (Sarma et al., 2020), in which only the larvae that spontaneously hatched after 72 h were accounted. In the present study, no larvae hatched spontaneously after 72 h exposure to garlic oil solution, while up to 85% hatched in the controls. Since the garlic oils are larvicidal, the treated water could be killing newly hatched larvae instead of affecting the eggs. To verify the ovicidal activity of our test solution, we transferred the 72 h treated eggs to clean tap water and stimulated hatching in a low-pressure chamber.

The egg stage is recognized as the most resilient of the *Ae. aegypti* life cycle, with eggs remaining viable for several months under dry conditions (Kliewer, 1961). Mosquito populations are therefore able to endure long dry seasons in some tropical areas. For instance, central regions in Brazil experience 4 to 5 months of intense drought every year. The subsequent wet season results in exponential mosquito population growth. Transovarial virus transmission has been reported, suggesting it could be one of the mechanisms for maintaining virus circulation in interepidemic seasons (Joshi et al., 2002). *Ae. aegypti* eggs are a crucial, and perhaps the most challenging target, for the development of a multifaceted control strategy.

3.6 Spatial repellency, contact irritancy and adult toxicity assays

Mosquito repellency is recognized as a promising tool to control arbovirus transmission. The main objective of this technology is to prevent mosquitoes approaching areas where they could find a human host. An ideal repellent would not cause mortality at the applied repellency concentration, resulting in lower pressure for resistance development (Achee et al., 2012). Garlic oil was tested at 7.5, 5.0, 2.5 and 1.0% (v/v) using the HITSS (High-Throughput Screening System) to assess its spatial repellency activity. The SAI (spatial activity index) values of the three higher concentrations were statistically different from the negative control, reaching 0.67 ± 0.17 (2.5%), 0.71 ± 0.1 (5.0%) and 0.56 ± 0.24 (7.5%). The highest wSAI (weighted spatial activity index) obtained was 15.2 ± 6.8 (7.5%), which is considered low. The wSAI levels were a result of the low PRESP (percentage of response) values, all under 20%. A low PRESP was also observed for the 20% PMD positive control, for which only 21.6% (± 4.9%) mosquitoes responded. The wSAI 17.2 ± 5.7 and SAI 0.64 ± 0.23 of the PMD positive control were comparable to the garlic oil treatments at lower doses (2.5%, 5.0% and 7.5%). The SAI and wSAI for the garlic oil 1.0% concentration were not significant.

Although a trend in repellency could be observed regarding increasing concentration, the SAI and wSAI of the higher concentrations did not significantly alter. The percentages of mosquitoes escaping (PRESP) from the central chamber for all concentrations were considered low (< 20%), indicating that the majority of mosquitoes (> 80%) did not attempt to escape to either the control or the treated chamber. However, mosquitoes were agitated during the experiment, exhibiting disturbed behavior with nondirectional flight. As such it seems plausible that, due to the strong smell and high volatility of the garlic oil, the apparatus became completely saturated during the experiments thereby preventing the mosquitoes from navigating the escape route.

Since we noted absence of knocked down mosquitoes and potential repellent activity, we conducted the same assay in a scaled-up system to simulate a field setting. Two rooms of the insectarium were connected with a plastic tubing that allowed mosquitoes to fly freely from one room to the other. A cage was assembled inside each room to enable mosquito counting at the end of the test (Fig. 4). At the concentration tested (0.015 mL/m^3), the mean wSAI was 31.9, with 0.5 SAI (±0.17) and 61.1% PRESP. Raw data analysis indicated an oriented movement of the mosquitoes escaping from the garlic oil-treated room. More than half of the mosquitoes flew to the control room. The repellency observed in the scaled-up system was more prominent than in the HITSS apparatus. Collectively, these results warrant broader discussion and consideration regarding the validity of HITSS spatial repellency data generated for highly volatile scented compounds, beyond the crude SAI and wSAI values.

The second repellent strategy tested contact irritancy. This test differs from the aforementioned spatial repellency assay in that mosquitoes directly contact the surface treated with the sample. At 1.0%, garlic oil caused significant contact irritancy, with 66.0 ± 7.86% ($p = 0.004$) of the mosquitoes escaping from the treated chamber. No significant knockdown was observed, suggesting that the activity, at this concentration, is non-toxic to *Ae. aegypti*. The PMD control caused 42.4% ± 14.13% ($p=0.002$) contact irritancy at 20% concentration. By direct comparison, garlic oil is more irritant to mosquitoes than the commercial pesticide.

To determine the potential toxicity to adult mosquitoes, glass bottles were coated with 10 μL (approximately 10 mg) garlic oil. The diagnostic time for this concentration was 25 min, with 100% knockdown. After 24 h, all mosquitoes remained motionless, confirming their death. The diagnostic time of 25 min is comparable to the diagnostic time of commonly used pesticides such as malathion and permethrin (CDC, 2020). On the other hand, a higher amount of garlic oil is needed to reach this diagnostic time. For instance, for 15 and 10 min diagnostic times, 0.4 mg of malathion and 0.043 mg permethrin are required. Garlic oil could be tested at lower amounts to determine the lowest acceptable diagnostic dose.

Previous papers reported the potential use of garlic oil as a skin repellent (Rajan et al., 2005; Campbell et al., 2011). The antennae of female *Ae. aegypti* mosquitoes responded to garlic oil and its isolated polysulfides, that were also active in contact repellent assays (Campbell et al., 2011). An experiment with a Y-tube olfactometer showed that the garlic oil repellency lasted for less than 30 min, which would be insufficient for effective repellent activity (Mitra et al., 2020). From an epidemiological perspective, the impact of skin contact repellents in preventing disease transmission remains controversial as it depends on individual compliance for a regular successful outcome (Norris and Coats, 2017). In opposition, spatial repellents have been recognized as an interesting innovative alternative to contact repellents and may constitute an important tool in integrated pest control management (Norris and Coats, 2017; Achee et al., 2019). In fact, garlic products have been commercially explored as mosquito repellent agents in some products, such as Mosquito Barrier ® and Mosquito-less® marketed in the United States and Canada. Their availability in the market is not proof of quality, since these products qualify for registration exemption. As such, they are not required to have proven efficacy or safety assessment prior to commercialization. The lack of robust scientific evidence of garlic repellency potential means authors inadvertently declare garlic as an ineffective repellent (Maia and Moore, 2011). To our knowledge, this is the first scientific report of garlic oil trials as spatial repellents and insecticides against adult mosquitoes.

4. Conclusions

Ovicidal, larvicidal, pupicidal, adulticidal and spatial repellency assays showed the broad activity of garlic oil against all *Aedes aegypti* life stages. Garlic oils are unique as insecticides in that they affect eggs, larvae and pupae, which commonly coexist at mosquito breeding sites. These results, together with its endurance in simulated field trials and the industrial production of garlic oil, highlight its suitability for integrated mosquito vector control programs. Evidence shown in this study suggests that alterations in garlic oil composition may not significantly affect its broad activity providing the final concentration of diallyl polysulfides remains unchanged. The activity and stability data of isolated diallyl polysulfides and garlic oil polysulfide mixtures suggests isolation efforts may not result in higher potency. Lack of persistency in the environment, mainly due to high volatility, demands technological development to explore garlic oil commercial use against *Ae. aegypti*. Mixing garlic oil with other essential oils or scented volatile compounds to address the characteristic aroma would enable indoor and outdoor garlic oil applications. The results shown here suggest the promising application of garlic oils to control mosquito approach to humans, either as spatial repellents, immature stage control or mosquitocidal agents, providing low persistency and aroma can be technologically addressed.

CRediT authorship contribution statement

Renata Garcia Dusi: conceptualization, investigation, formal analysis, writing - original draft, writing - review & editing; Laís da Silva Morais: methodology, writing - review & editing; Natália Mendes Gomes Magalhães: conceptualization, methodology, writing - review & editing; Lorena Carneiro Albernaz: formal analysis, methodology, writing - review & editing; Chris J. Hamilton: formal analysis, methodology, supervision, writing - review & editing;

Laila Salmen Espindola: supervision, funding acquisition, resources, writing - review & editing.

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References

Achee NL, Bangs MJ, Farlow R, Killeen GF, Lindsay S, Logan JG, et al. Spatial repellents: from discovery and development to evidence-based validation. Malar J. 11(1):164, 2012.

Achee NL, Sardelis MR, Dusfour I, Chauhan KR, Grieco JP. Characterization of Spatial Repellent, Contact Irritant, and Toxicant Chemical Actions of Standard Vector Control Compounds. J Am Mosq Control Assoc. 25(2):156–67, 2019.

Anwar A, Gould E, Tinson R, Groom M, Hamilton CJ. Think Yellow and Keep Green—Role of Sulfanes from Garlic in Agriculture. Antioxidants. 6(1), 2017.

Arbach M, Santana TM, Moxham H, Tinson R, Anwar A, Groom M, et al. Antimicrobial garlic-derived diallyl polysulfanes: Interactions with biological thiols in Bacillus subtilis. Biochim Biophys Acta Gen Subj. 1863(6):1050–8, 2019.

Byttebier B, De Majo MS, Fischer S. Hatching Response of Aedes aegypti (Diptera: Culicidae) Eggs at Low Temperatures: Effects of Hatching Media and Storage Conditions. J Med Entomol. 51(1):97–103, 2014.

Campbell C, Gries R, Khaskin G, Gries G. Organosulphur constituents in garlic oil elicit antennal and behavioural responses from the yellow fever mosquito. J Appl Entomol. 135(5):374–81, 2011.

CDC. Centers for Disease Control and Prevention. Guideline for Evaluating Insecticide Resistance in Vectors Using the CDC Bottle Bioassay. Atlanta, GA: Centers for Disease Control and Prevention; p. 1–2; 2019.

CDC. CONUS Manual for evaluating insecticide resistance in mosquitoes using the CDC bottle bioassay kit. 2020. Available at: https://www.cdc.gov/zika/pdfs/CONUS-508.pdf.

EPA. Environmental Protection Agency. Active Ingredients Eligible for Minimum Risk Pesticide Products [Internet]. Available at: https://www.epa.gov/sites/production/files/2015-12/documents/minrisk-active-ingredients-tolerances-2015-12-15.pdf

FAO. Food and Agriculture Organization of the United Nations. FAOSTAT. Crops. 2018.

Grieco JP, Achee NL, Sardelis MR, Chauhan KR, Roberts DR. A novel high-throughput screening system to evaluate the behavioral response of adult mosquitoes to chemicals. J Am Mosq Control Assoc. 21(4):404–11, 2005.

Hustedt JC, Boyce R, Bradley J, Hii J, Alexander N. Use of pyriproxyfen in control of Aedes mosquitoes: A systematic review. PLoS Negl Trop Dis. 14(6), 2020.

Joshi V, Mourya DT, Sharma RC. Persistence of dengue-3 virus through transovarial transmission passage in successive generations of Aedes aegypti mosquitoes. Am J Trop Med Hyg. 67(2):158–61, 2002.

Kimbaris AC, Kioulos E, Koliopoulos G, Polissiou MG, Michaelakis A. Coactivity of sulfide ingredients: a new perspective of the larvicidal activity of garlic essential oil against mosquitoes. Pest Manag Sci. 65(3):249-254, 2009.

Kliewer JW. Weight and Hatchability of Aedes aegypti Eggs (Diptera: Culicidae)1. Ann Entomol Soc Am. 54(6):912–7, 1961.

Lawson L, Wang Z-Y, Hughes B. Identification and HPLC Quantitation of the Sulfides and Dialk(en)yl Thiosulfinates in Commercial Garlic Products. Planta Med. 57(04):363–70, 1991.

Lopes RP, Lima JBP, Martins AJ. Insecticide resistance in Culex quinquefasciatus Say, 1823 in Brazil: a review. Parasit Vectors. 12(1):591–591, 2019.

Maia, MF, Moore, SJ. Plant-based insect repellents: a review of their efficacy, development and testing. Malar J .10, S11, 2011.

Milesi P, Pocquet N, Labbé P. BioRssay: a R script for bioassay analyses.

2013.

Mitra S, Rodriguez SD, Vulcan J, Cordova J, Chung H-N, Moore E, et al. Efficacy of Active Ingredients From the EPA 25(B) List in Reducing Attraction of Aedes aegypti (Diptera: Culicidae) to Humans. J Med Entomol. 57(2): 477-484, 2020.

Muturi EJ, Ramirez JL, Zilkowski B, Flor-Weiler LB, Rooney AP. Ovicidal and Larvicidal Effects of Garlic and Asafoetida Essential Oils Against West Nile Virus Vectors. J Insect Sci Online. 18(2):43, 2018.

Muturi EJ, Hay WT, Behle RW, Selling GW. Amylose Inclusion Complexes as Emulsifiers for Garlic and Asafoetida Essential Oils for Mosquito Control. Insects. 10 (10): 337, 2019.

Norris EJ, Coats JR. Current and Future Repellent Technologies: The Potential of Spatial Repellents and Their Place in Mosquito-Borne Disease Control. Int J Environ Res Public Health. 14(2):124, 2017.

Ramakrishnan V, Chintalwar GJ, Banerji A. Environmental persistence of diallyl disulfide, an insecticidal principle of garlic and its metabolism in mosquito, Culex pipiens quinquifasciatus Say. Chemosphere. 18(7):1525–9, 1989.

Rajan TV, Hein M, Porte P, Wikel S. A double-blinded, placebo-controlled trial of garlic as a mosquito repellant: a preliminary study. Med Vet Entomol. 19(1):84–9, 2005.

Ryan SJ, Carlson CJ, Mordecai EA, Johnson LR. Global expansion and redistribution of Aedes-borne virus transmission risk with climate change. PLoS

Negl Trop Dis. 13(3), 2019.

Sarma R, Adhikari K, Mahanta S, Khanikor B. Combinations of Plant Essential Oil Based Terpene Compounds as Larvicidal and Adulticidal Agent against Aedes aegypti (Diptera: Culicidae). Sci Rep. 9(1):9471, 2019.

Sarma R, Adhikari K, Mahanta S, Khanikor B. Twenty Essential Oils as Ovicidal Agent Against Aedes aegypti (Diptera: Culicidae). Natl Acad Sci Lett. 43 (6): 497-500, 2020.

SAS Institute Inc. SAS Studio. Cary, NC, USA: SAS Institute Inc.; 2018.

Satyal P, Craft JD, Dosoky NS, Setzer WN. The Chemical Compositions of the Volatile Oils of Garlic (Allium sativum) and Wild Garlic (Allium vineale). Foods. 6(8), 2017.

Silvério MR, Espindola LS, Lopes NP, Vieira PC. Plant Natural Products for the Control of Aedes aegypti: The Main Vector of Important Arboviruses. Molecules. 25(15), 2020.

Soares-Pinheiro VC, Dasso-Pinheiro W, Trindade-Bezerra JM, Tadei WP. Eggs viability of Aedes aegypti Linnaeus (Diptera, Culicidae) under different environmental and storage conditions in Manaus, Amazonas, Brazil. Braz J Biol. 77(2):396–401, 2016.

Wang K, Groom M, Sheridan R, Zhang S, Block E. Liquid sulfur as a reagent: synthesis of polysulfanes with 20 or more sulfur atoms with characterization by UPLC-(Ag)-coordination ion spray-MS. J Sulfur Chem. 34(1/2):55–66, 2013.

WHO. World Health Organization. Dengue and severe dengue [Internet]. Geneva: World Health Organization; 2021. Available at: https://www.who.int/en/news-room/fact-sheets/detail/dengue-and-severe-dengue

Table and Figure captions

Table 1. Percentage composition of garlic oil diallyl polysulfides (DAS) determined by GC-MS, HPLC-UV and ^{1}H NMR.

Method	DAS	DAS2	DAS3	DAS4	DAS5	DAS6	DAPS*
GC-MS	4.6	26.7	63.1	4.2	ND	ND	98.6
HPLC-DAD	1.7	16.6	53.9	19.6	4.2	1.9	97.9
^{1}H NMR	7.0	29.2	52.0	9.4	1.8	0.6	100.0

*DAPS: diallyl polysulfides

Table 2. Garlic oil LC_{50}/LC_{95} values (ppm) against *Ae. aegypti* larvae and pupae.

	Exposure time (h)	LC_{50} (CI_{95})[a]	LC_{95} (CI_{95})	Chi (p)[b]	Slope	Intercept
Larvae	24	1.58 (1.49 - 1.67)	2.99 (2.71 - 3.38)	0.17	5.93	-1.17
	48	1.06 (1.02 - 1.10)	1.91 (1.81 - 2.04)	1.0	6.47	-0.17
	72	0.92 (0.87 - 0.97)	1.77 (1.66 - 1.92)	0.96	5.77	0.21
Pupae		20.34 (18.03 - 22.44)	56.95 (51.93 - 63.66)	0.46	3.21	-4.81

[a]CI: confidence interval. [b]p: p value for chi-square.

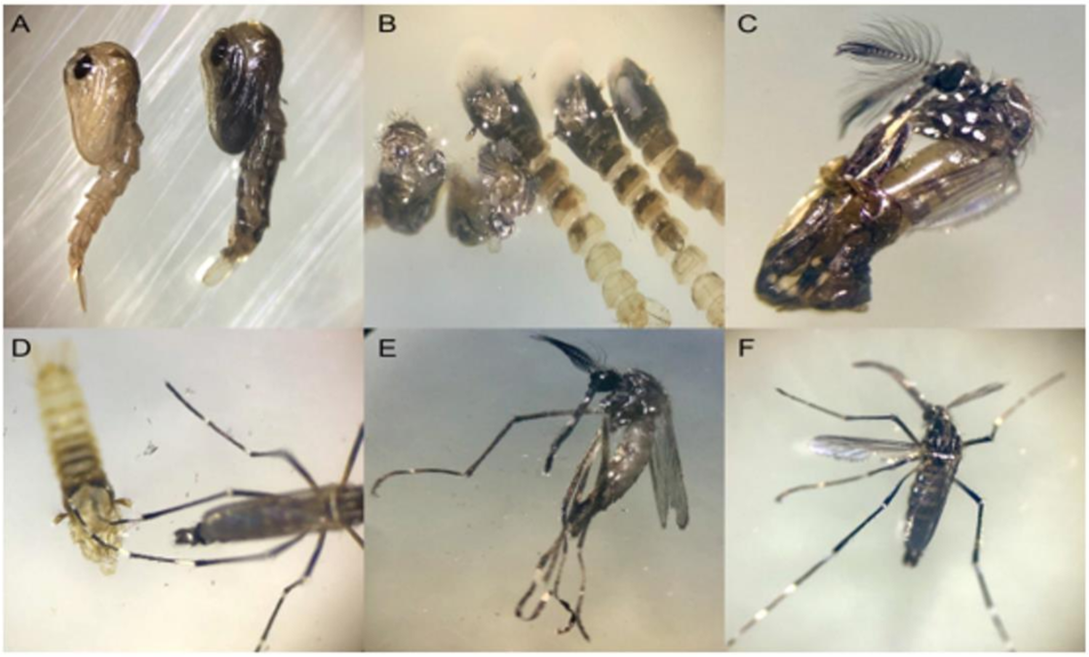

Fig. 1. Mortality and abnormalities in pupae treated with garlic oil: 1A. a dead pupa (right) compared to a healthy pupa (left); 1B. dead pupae during adult emergence; 1C. a partially-formed mosquito, died during ecdysis; 1D. a completely formed mosquito still attached to the exuviae; 1E. a mosquito with malformed legs; 1F. a mosquito with wing and proboscis malformation.

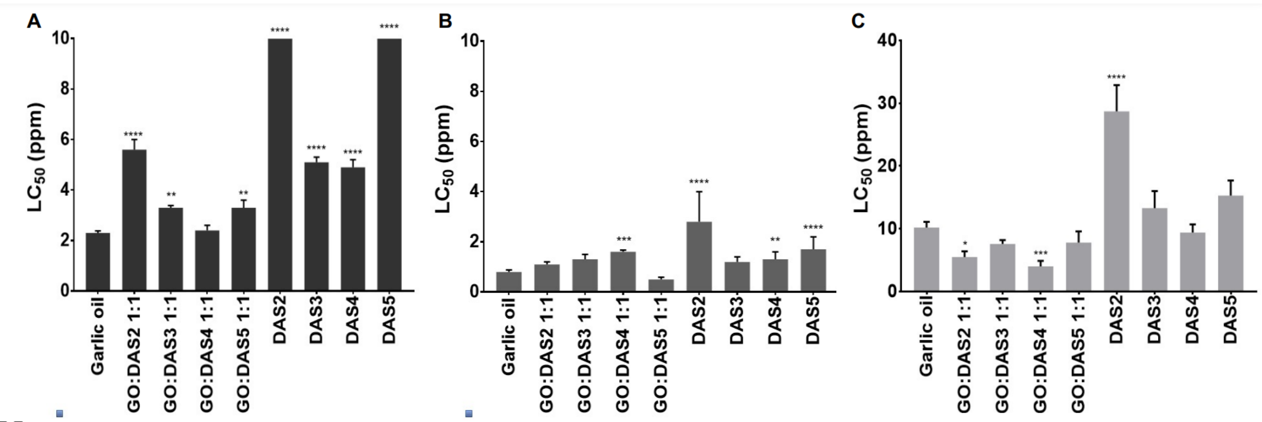

Fig. 2. LC_{50} values of isolated polysulfides, garlic oil and garlic oil supplemented with polysulfides against *Ae. aegypti* larvae after 24 h (2A), larvae after 72 h (2B) and pupae (2C). Statistical significance (p values) related to the original garlic oil potency. *: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$; ****: $p \leq 0.0001$.

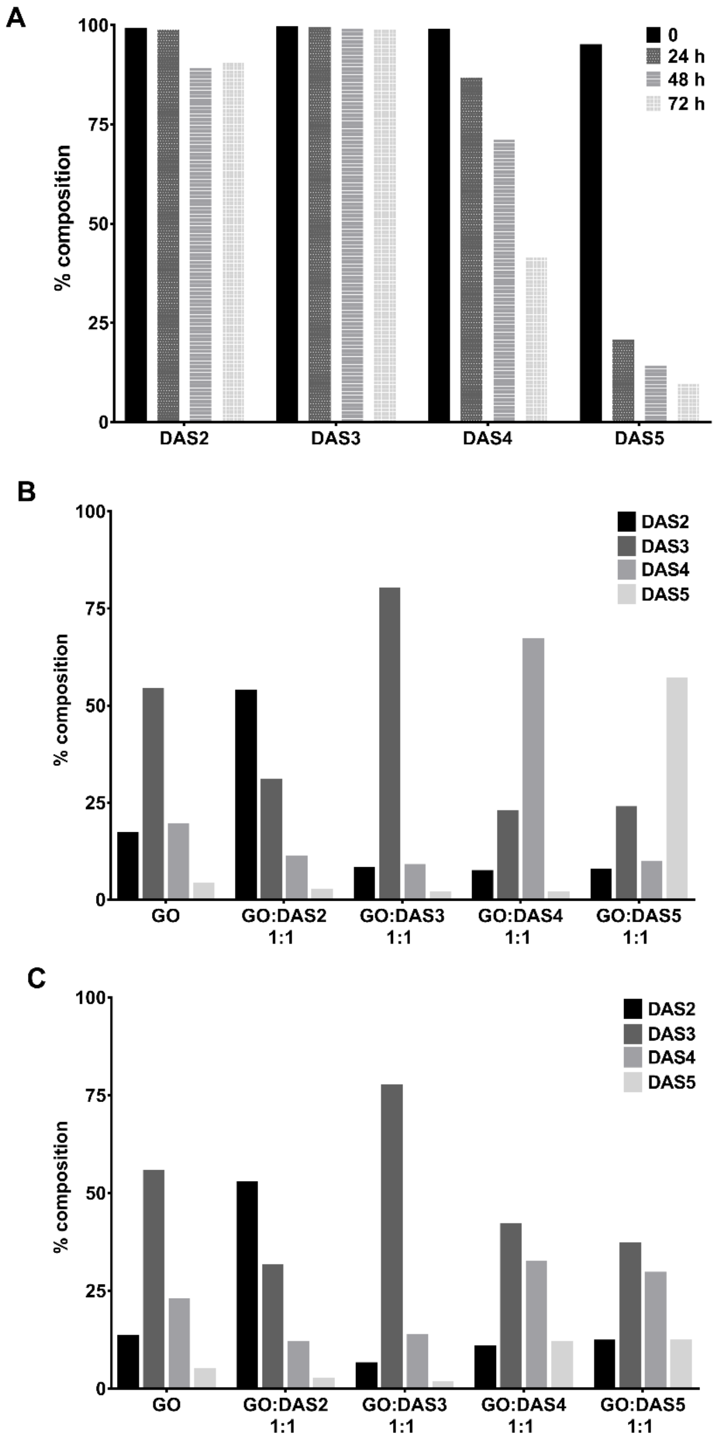

Fig. 3. Peak area percentages (%) of diallyl polysulfides (DAPS) determined by HPLC-UV detection (210 nm) at different time points: 3A. individual polysulfides; 3B. garlic oil composition at time 0; 3C. garlic oil composition after 72 h.

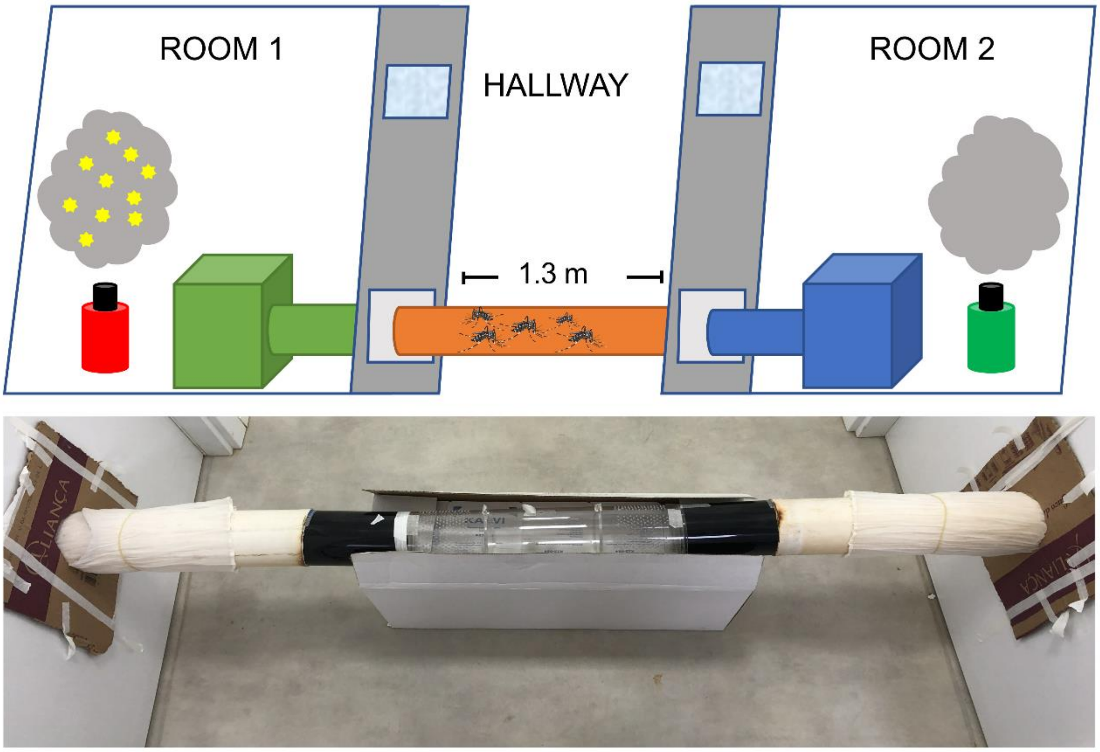

Fig. 4. Schematic representation of the spatial repellency simulated field trial. The distance between the rooms is 1.3 m. Room 1: Test room with vaporized garlic oil solution and mosquito cage (green). Room 2: Control room with vaporized water and mosquito cage (blue).

Appendix A. Supplementary data

Table S1. Garlic oil composition by GC-MS.

RI[a]	Compound	Molecular Weight	Peak Area (%)
840	1,2-dithiolane	106	0.63
896	diallyl sulfide	114	4.62
1078	diallyl disulfide	146	26.67
1212	2-vinyl-4H-1,3-dithiin	144	0.30
1300	diallyl trisulfide	178	63.06
1537	diallyl tetrasulfide	210	4.23
1808	4,5,9-trithia-1,11-dodeca-diene	220	0.32
-	6-methyl-4,5,8,9,10-penta-thio-trideca-1	284	0.16
	Total identified		100.0

[a]RI = Retention index determined with respect to a homologous series of n-alkanes on a DB-5 MS column.

Table S2. *LC_{50} (ppm) of isolated polysulfides, garlic oil and garlic oil supplemented with polysulfides against Ae. aegypti larvae (L3 stage) and pupae.*

Sample	**LC_{50} (CI_{95})***		
	Larvae 24 h	Larvae 72 h	Pupae
DAS2	>10	2.8 (1.2 - 12.6)	28.7 (19.9 - 37.4)
DAS3	5.1 (4.6 - 5.6)	1.2 (0.8 - 1.7)	13.3 (8.3 - 20.2)
DAS4	4.9 (4.3 - 5.6)	1.3 (0.8 - 2.1)	9.4 (7.0 - 12.5)
DAS5	>10	1.7 (0.8 - 3.4)	15.3 (11.0 - 21.5)
Garlic oil (GO)	2.3 (2.1 - 2.5)	0.8 (0.6 - 1.0)	10.2 (8.5 - 12.3)
1:1 GO + DAS2	5.6 (4.9 - 6.4)	1.1 (0.8 - 1.5)	5.5 (3.8 - 7.6)
1:1 GO + DAS3	3.3 (3.1 - 3.5)	1.3 (0.9 - 1.8)	7.6 (6.1 - 9.0)
1:1 GO + DAS4	2.4 (2.0 - 2.8)	1.6 (1.4 - 1.7)	4.0 (2.0 - 6.3)
1:1 GO + DAS5	3.3 (2.7 - 4.1)	0.5 (0.3 - 0.7)	7.8 (4.4 - 12.5)

[a]CI_{95} : 95% confidence interval.

Table S3. Peak area percentages (%) of diallyl polysulfides (DAPS) determined by HPLC-UV detection (210 nm) at different time points in the original and DAPS supplemented garlic oils.

Sample	Time (h)	%				
		DAS2	DAS3	DAS4	DAS5	DAS2-DAS5
Original GO	0	16.9	53.9	19.0	3.8	**93.6**
	24	15.6	54.9	20.8	4.1	**95.3**
	48	14.4	55.0	21.5	4.3	**95.2**
	72	13.1	55.3	22.6	4.7	**95.7**
GO : DAS2 1 : 1	0	53.4	30.5	10.8	2.2	**96.9**
	24	52.9	30.3	10.9	2.1	**96.2**
	48	52.6	30.9	11.6	2.2	**97.3**
	72	52.5	31.3	11.6	2.1	**97.5**
GO : DAS3 1 : 1	0	7.9	79.8	8.6	1.5	**97.8**
	24	7.3	77.8	11.6	1.2	**97.9**
	48	6.7	77.1	12.9	1.5	**98.1**
	72	6.1	77.3	13.4	1.3	**98.1**
GO : DAS4 1 : 1	0	7.0	22.5	66.8	1.6	**97.9**
	24	7.3	77.8	11.6	1.2	**97.9**
	48	7.1	40.2	37.2	11.3	**95.8**
	72	8.2	43.5	32.8	11.4	**96.0**
GO : DAS5 1 : 1	0	7.5	23.5	9.3	56.6	**96.8**
	24	8.0	39.8	31.5	13.5	**92.8**

48	10.3	38.7	30.6	12.6	**92.2**
72	12.0	36.8	29.3	12.0	**90.0**

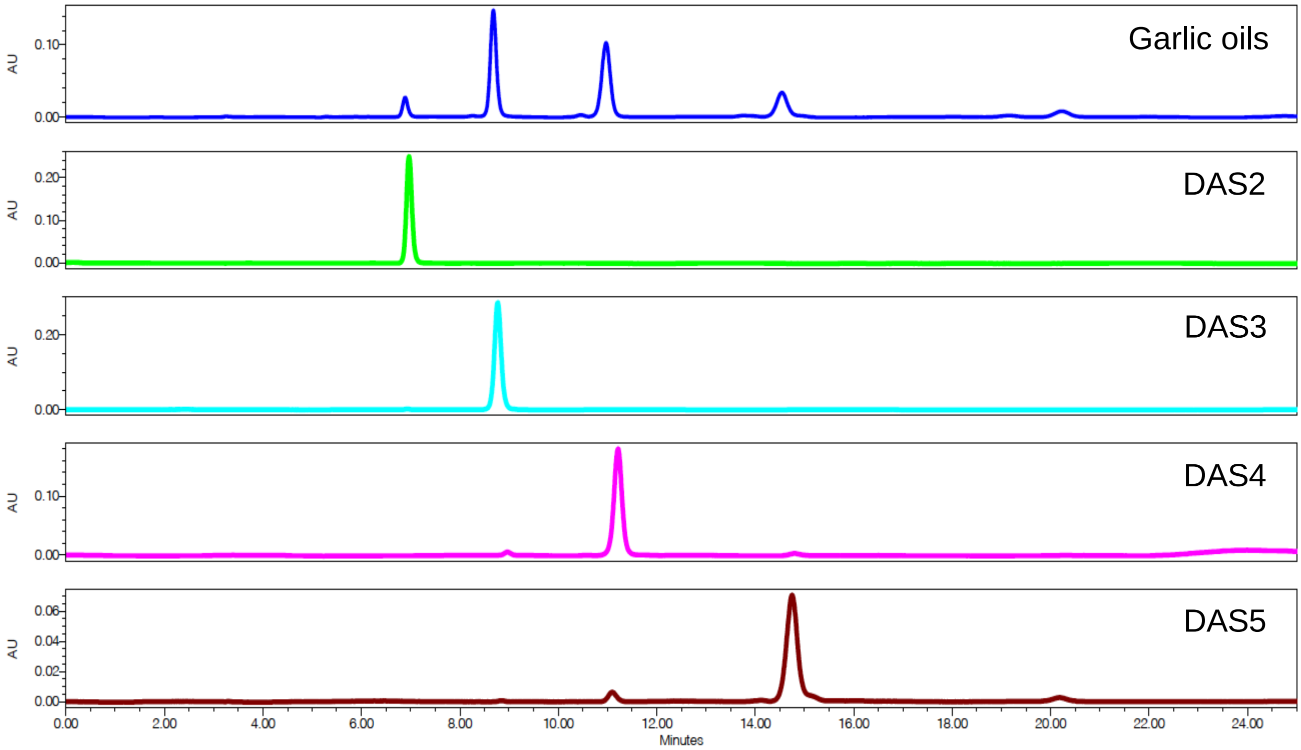

Fig. S1. HPLC-DAD chromatograms (at 210 nm) of the enriched garlic oils and respective isolated polysulfides.

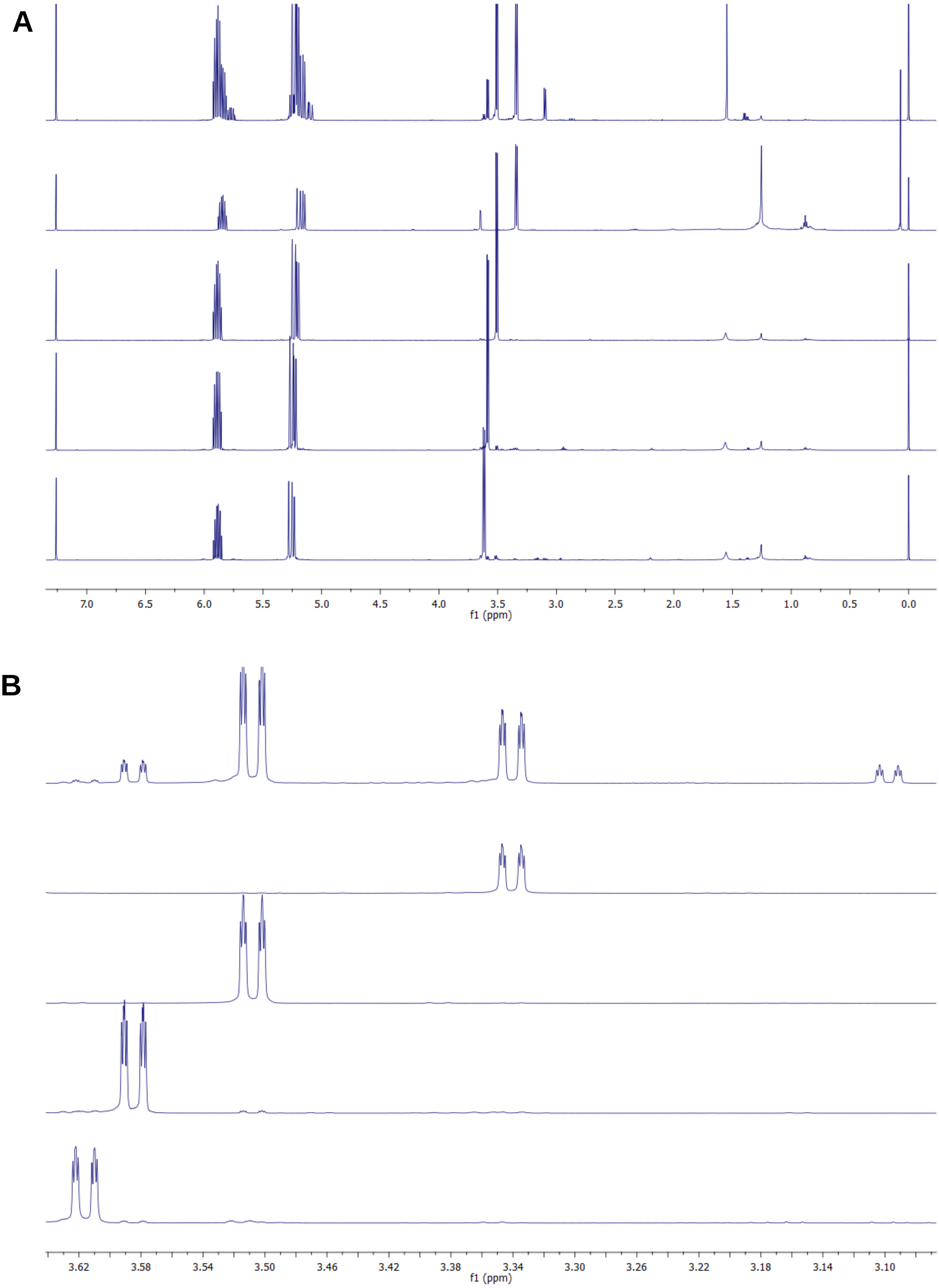

Fig. S2A: Garlic oil and garlic polysulfides in $CDCl_3$ from top to bottom: garlic oil, diallyl disulfide (DAS2); diallyl trisulfide (DAS3); diallyl tetrasulfide (DAS4); diallyl pentasulfide (DAS5). **S2B**: Stacked ^{1}H NMR spectra of garlic oil and polysulfides in the 3.30 to 3.70 ppm region.

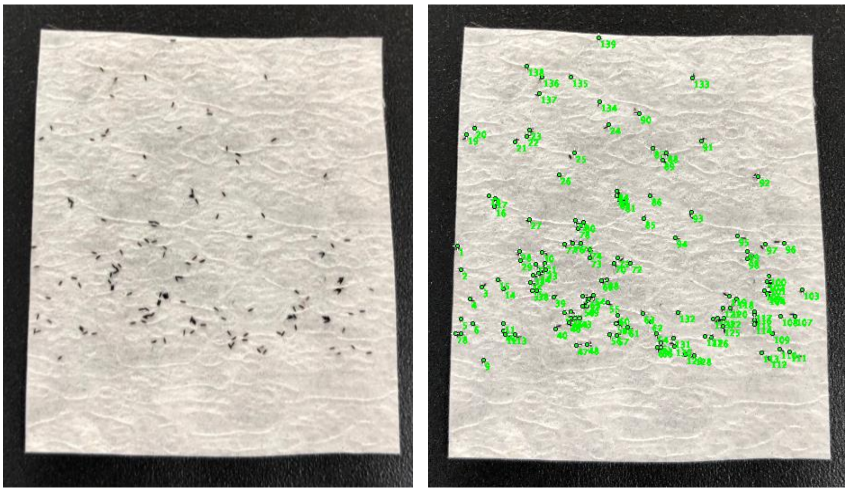

Fig. S3. Egg counting using the ImageJ software.

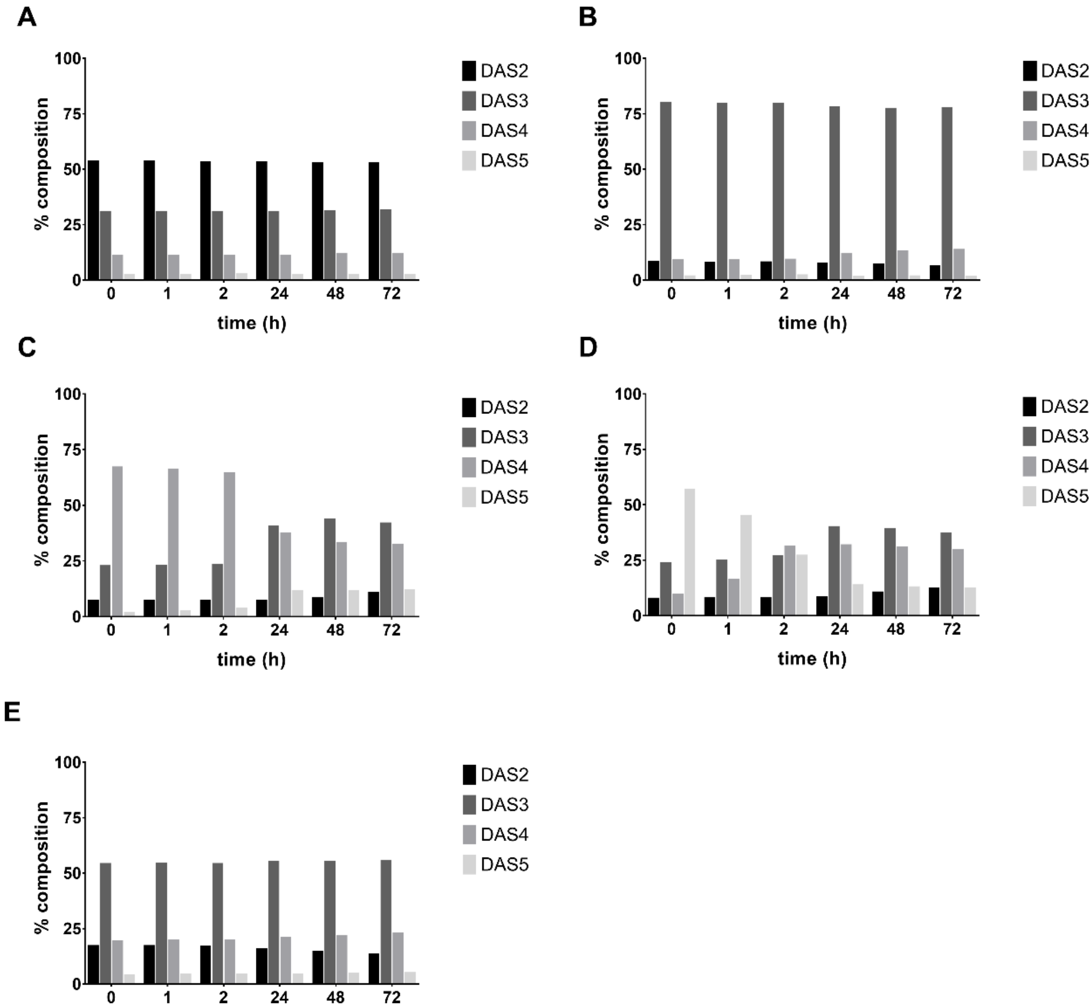

Fig. S4. Peak area percentages (%) of diallyl polysulfides (DAPS) determined by HPLC-UV detection (210 nm) at different time points: **3A**. garlic oil + DAS2

(1:1); **3B**. garlic oil + DAS3 (1:1); **3C**. garlic oil + DAS4 (1:1); **3D**. garlic oil + DAS4 (1:1); **3E**. original garlic oil.