

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

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12 Abstract

13 Vector control remains the most effective approach to prevent dengue,
14 chikungunya and Zika arboviruses transmission. Conventional insecticides have
15 historically failed to control the *Aedes aegypti* mosquito due to acquired
16 resistance, environmental impact and toxicity. This study evaluated the potential
17 of garlic oil as a biopesticide against the eggs, larvae, pupae and adult forms of
18 *Ae. aegypti* eggs, in accordance with the World Health Organization
19 recommendations. The larvicidal and pupicidal LC₅₀ values were 1.0 ppm and
20 20.3 ppm after 72 h, respectively. The oil maintained its activity in simulated
21 field trials, killing all larvae and pupae at the tested concentrations. At 100 ppm,
22 garlic oil inhibited 59.6 ± 10.6% of egg hatching. Toxicity against the adult form
23 was observed as was its potent spatial repellency. Garlic oils composed of
24 different diallyl polysulfide ratios did not significantly impact insecticidal activity

25 although the garlic oil polysulfide mixtures were more potent than the individual
26 polysulfides. The ovicidal, larvicidal, pupicidal, adulticidal and repellent assays
27 showed the broad activity of garlic oil against *Ae. aegypti*. These results,
28 together with the activity in simulated field trials, support the applicability of
29 garlic oil in integrated mosquito vector control programs.

30 Keywords

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

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35 1. Introduction

36 *Aedes aegypti* L. is the primary mosquito species responsible for the
37 transmission of dengue fever. Worldwide incidence of dengue has increased
38 10-fold over the last two decades, with over 5 million cases reported in 2019
39 (WHO, 2021). Although the arbovirus burden disproportionately affects poorer
40 populations of tropical and subtropical regions, the territorial expansion of this
41 vector relating to climate change will also be of concern for temperate areas in
42 the world (Ryan et al., 2019). A major challenge in integrated vector
43 management is the lack of agents that can effectively target all mosquito life
44 stages. Furthermore, resistance development, together with environmental
45 damage relating to indiscriminate use of non-targeted pesticides, severely
46 affects the availability of chemical alternatives (Lopes et al., 2019).

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

58 2. Materials and methods

59 2.1. Chemicals and analytical instrumental

60 The organophosphate insecticide temephos, Chinese garlic oil (artificial), diallyl
61 disulfide and diallyl trisulfide were purchased from Sigma Aldrich (Buchs,
62 Switzerland). All other chemicals used in this study were HPLC grade.
63 ¹H NMR spectra were recorded on a Bruker 600 MHz spectrometer, with
64 tetramethylsilane used as an internal standard. Gas chromatography coupled to
65 mass spectrometry analysis was performed on a Shimadzu GC-2010
66 instrument employing the following conditions: DB-5 MS (30 m x 25 mm x 25
67 µm) column; carrier gas: He (1.3 mL/min, in constant linear velocity mode);
68 injector temperature: 220 °C, in split mode (1:60); and detector temperature:
69 250 °C. The temperature was increased at 3 °C/min from 60 to 210 °C. Mass
70 spectra were obtained at electron impact of 70 eV and acquired for the 35 to
71 400 m/z range. The volume injected was 1 µL. The components were identified
72 using GC retention times, calculated by linear interpolation relative to retention
73 times of their main compounds, and by comparison of mass spectra with the
74 NIST (National Institute of Standards and Technology) database.

75 2.2. Insect rearing

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

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

87 Adult female mosquitoes used in toxicity and contact irritant assays were
88 collected from the University of Notre Dame (USA) *Ae. aegypti* (Liverpool strain)
89 colony. The mosquitoes were maintained at 27 °C, 80% relative humidity and a
90 12-h photoperiod. Female groups of 4 to 7 days old were separated in plastic
91 containers and fed the previously described sugar solution until the day prior
92 the tests.

93

94 2.3. Larvicidal and pupicidal assays

95 Larvicidal and pupicidal assays were performed following the WHO guideline
96 recommendations. The samples were dissolved in dimethylsulfoxide (DMSO)
97 and assays performed in 4 replicates, repeated with 3 different larvae batches.
98 The garlic oil LC₅₀ and LC₉₅ values were determined with six concentrations
99 (4.8; 2.4; 1.8; 1.2; 0.8 and 0.4 ppm) for larvae, and five concentrations (60.0;
100 50.0; 40.0; 30.0 and 5.0 ppm) for pupae. A total of 1,800 larvae and 1,500
101 pupae were exposed to garlic oil in 4 replicates of 25 individuals in plastic
102 containers with 200 mL of the testing solutions. The number of dead larvae was
103 recorded after 24, 48 and 72 h exposure. Larvae with no movement after
104 mechanical or luminous stimulation were considered dead. Regarding pupicidal
105 assays, the number of dead pupae and completely emerged mosquitoes were
106 recorded after full mosquito emergence in the negative control replicates.

107 Mosquitoes with distinguishable head, legs and wings that could move were
108 considered viable, even if they did not completely detach from their exuviae.
109 The containers used in the pupicidal assays were covered with a fine netting to
110 prevent mosquito escape. The positive controls used were: for larvae -
111 temephos (0.0250 ppm), and for pupae - pyriproxyfen (0.0001 ppb to 200 ppm
112 range) and temephos (0.5 ppm). The vehicle negative controls (<1.0% DMSO in
113 tap water) were performed in parallel to ensure test validity.
114 The assays were performed using the same colony under the same rearing
115 conditions. According to the WHO guidelines, tests with a control mortality > 5%
116 were corrected using Abbott's formula and tests with control mortality > 20%
117 were discarded. The LC₅₀ (ppm) values and their respective 95% fiducial limits,
118 together with their lower/upper confidence intervals, were calculated by Probit
119 analysis (Milesi et al. 2013) using the RStudio® software.

120 2.4. Ovicidal assay

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

131 were used to calculate the corrected mortality rate using the Henderson-Tilton
132 formula.

133 2.5. Spatial repellency, contact irritancy and adult toxicity assays

134 The spatial repellency and contact irritancy assays were performed in the High-
135 Throughput Screening System (HITSS) described by Grieco (Grieco et al.,
136 2015), following the methods described by Achee (Achee et.al, 2019). For the
137 spatial repellency assay, 275 cm² nylon netting strips were treated with 1.5 mL
138 of acetone (negative control) or with 7.5, 5.0, 2.5 or 1.0% (v/v) garlic oil solution
139 in acetone and positioned in the HITSS terminal metal chambers. Groups of 20
140 ± 2 females per replicate were used for each of the 9 replicates.

141 The total number of mosquitoes, together with the knockdown number, in each
142 part of the HITSS was recorded to calculate the Spatial Activity Index (SAI) and
143 the weighted Spatial Activity Index (wSAI). The SAI value = $(N_c - N_t) / (N_c + N_t)$,
144 where N_c and N_t represent the number of viable mosquitoes in the control and
145 treatment chambers, respectively. SAI ranges from -1 to +1. Negative values
146 indicate an attractant response whereas positive values indicate repellency.

147 The wSAI was calculated by multiplying the SAI by the percentage of
148 responsive mosquitoes in the assay. The PRESP (percentage of response)
149 value = $[(N_c + N_t) / N] * 100$, where N is the total number of mosquitoes used in
150 the assay. The wSAI represents the magnitude of the repellent or attractant
151 effect, while the SAI concerns the existence (or not) of a directional movement.
152 Data was analyzed using the SAS University Edition software with a non-
153 parametric signed-rank test (PROC UNIVARIATE) to determine if the mean SAI
154 and wSAI were significantly different from zero (SAS, 2018). The mosquitoes

155 were extracted from the chambers and placed in transparent containers to
156 observe 24 h mortality.

157 For contact irritancy assays, the HITSS is partially disassembled, resulting in
158 one chamber housing either the treated or the control net (Achee et.al, 2019).

159 The same type of netting used in the spatial repellency test was utilized for both
160 the treatment and control samples. The control and treatment chambers were
161 run separately, with 10 females used for each of the 6 replicates. The test was
162 performed similarly to the spatial repellency assay. Garlic oil 1.0% (v/v) solution
163 in ethanol was used as the test solution, while ethanol was the negative control.

164 The number of mosquitoes in the clear (escaping) and metal chambers,
165 together with the knockdown number in both the treated and control devices
166 were recorded. Data was analyzed using a Wilcoxon 2-sample test to evaluate
167 the difference between the number of mosquitoes that escaped the control and
168 treated nets. The mean percentage of treatment escape was corrected
169 considering the escape in the control and knockdown observed in both the
170 treated and control groups. PMD (20% p- menthane-3,8-diol), a biochemical
171 pesticide derived from eucalyptus plants, was used as a positive control for both
172 the spatial repellency and contact irritancy assays.

173 The adult toxicity assay was performed following the CDC bottle assay protocol
174 (CDC, 2019). In quadruplicate, 250 mL glass bottles (Wheaton Science,
175 Millville, NJ, USA) were coated with 1 mL of 1% (v/v) garlic oil solution, resulting
176 in 10 μ L garlic oil/bottle. After bottles dried overnight in a fume hood, 25 female
177 mosquitoes in each replicate (n=100) were inserted and the number of knocked
178 down mosquitoes counted for 120 min. The mosquitoes were then transferred
179 into clean transparent vessels and provided sucrose solution to monitor 24 h

180 mortality. Bottles coated with ethanol were used as control. The diagnostic time
181 was determined as well as the knockdown at 30 min and 24 h mortality.

182 2.6. Simulated field trials

183 Larvicide and pupicide simulated field trials were performed in plastic buckets
184 containing 8 L of tap water, distributed in a random manner outside the
185 controlled atmosphere of the laboratory to emulate environmental conditions.
186 The water was left to acclimatize for 24 h, after which 50 third-instar larvae or
187 50 pupae were added to each container, accordingly. After 2 h adaptation, 10
188 mL of a garlic oil solution (in DMSO) at different concentrations was added:
189 larvae (4.0, 3.0 and 1.5 ppm) and pupae (60, 45 and 30 ppm). Positive
190 (temephos 0.013 ppm) and negative (<1.0% DMSO) controls were performed in
191 parallel. A thermohygrometer was placed in one of the control containers to
192 monitor temperature and humidity throughout the tests. Each concentration and
193 control were tested in quadruplicate and repeated with 3 different larvae/pupae
194 batches. Containers were covered with a nylon netting to prevent interference.
195 Each container was examined at 24 h intervals, with the number of live and
196 dead individuals used to determine post-treatment mortality. The Chi-Square
197 test was used to determine any statistically significant differences between the
198 proportions of pupae that died in laboratory and simulated field trials at the
199 same concentration.

200 Spatial repellency assays were conducted in a scaled-up field trial assay similar
201 to the laboratory trial described in Section 2.5. This trial involved two 10 m³
202 rooms in the Laboratório de Farmacognosia Insectarium connected by a plastic
203 tube to allow mosquito flux during the experiment (Fig. 4). The treated room had

204 an electric diffuser with 150 μ L of garlic oil added to 300 mL water, while the
205 control room housed a humidifier with only water. The opposite-facing rooms
206 were separated by 1.3 m corridor, each with a door. Groups of 20 ± 8 females
207 were used for each of the 9 replicates. The test solution was replaced before
208 the 7th replicate. Five-day old female mosquitoes were placed in the center of
209 the tube connecting the rooms. After 30 sec, the mosquitoes were released and
210 allowed to fly through the apparatus and to the rooms. A cage was assembled
211 inside each room to enable mosquito counting at the end of the test. After 15
212 min, the number of live and knocked-down mosquitoes in each part of the test
213 (control and treatment rooms, and corridor tubing) was recorded and the Spatial
214 Activity Index (SAI)/ weighted Spatial Activity Index (wSAI) determined as
215 described in Section 2.5.

216 2.7. Isolated polysulfides and enriched garlic oil assays

217 Diallyl disulfide (DAS2) was purchased from Sigma Aldrich. Diallyl trisulfide
218 (DAS3), diallyl tetrasulfide (DAS4) and diallyl pentasulfide (DAS5) were isolated
219 from garlic oil using a Varian ProStar preparative HPLC. A Phenomenex Luna
220 C18(2) column (5 μ m particle size, 150 x 21.2 mm) was used with an isocratic
221 90% methanol mobile phase for 30 min at 10 mL/min, monitored at 210 nm. The
222 collected peaks were stored in the freezer prior to hexane extraction. The
223 organic phase was collected and concentrated using a rotary evaporator.
224 Samples were diluted in CDCl_3 and analyzed by ^1H NMR. The NMR data was
225 compared to literature data to confirm compound structures (Wang et al., 2013).
226 The isolated compounds were diluted in ethanol (< 2.0% in tap water) and
227 tested in 12-well plates containing 3 mL of tap water and 10 larvae or 5 pupae,

228 accordingly. The LC₅₀ values were determined using six polysulfide
229 concentrations for larvae (10.0, 5.0, 2.5, 1.25, 0.63 and 0.31 ppm) and pupae
230 (50.0, 25.0, 12.5, 6.25, 3.13 and 1.56 ppm). A total of 240 larvae and 120
231 pupae were exposed to garlic oil treatment in quadruplicate.
232 Individual garlic oils were supplemented with DAS2, DAS3, DAS4 and DAS5
233 (50% w/w), and tested against larvae and pupae in the aforementioned
234 concentration ranges for the isolated polysulfides. Ethanol, used to dissolve all
235 of different enriched garlic oil samples, was used as a negative control (< 2.0%
236 ethanol in tap water). LC₅₀ values were determined using the GraphPad Prism 8
237 software and the dose-response curves compared by two-way ANOVA followed
238 by Dunnett's test to compare the activity of the different samples with the
239 original garlic oil.
240 Ethanol stock solutions of the isolated polysulfides and the supplemented garlic
241 oil samples were monitored by HPLC throughout the larvae and pupae assays.
242 A SunFire C18 column (5 µm 4.6 x 250 mm) was used with an isocratic 90%
243 methanol mobile phase for 15 min at 1 mL/ min. Peaks were monitored at 210
244 nm.

245 3. Results and discussion

246 3.1 Garlic oil chemical profile

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

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

257 All three analytical techniques revealed DAS3 as the most abundant compound:
258 GC/MS (63.1%), HPLC/UV (53.9%) and ¹H NMR (52%). The next most
259 abundant polysulfides were DAS2: GC/MS (26.7%), HPLC (17%), ¹H NMR
260 (32%), and DAS4: GC/MS (4.2%), HPLC (19%) and ¹H NMR (9.4%).

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

271 3.2 Larvicidal and pupicidal activity

272 Larvae (L3 stage) were more susceptible (LC₅₀ 1.58 ppm, 24 h) to garlic oil
273 treatment than pupae (LC₅₀ 20.34 ppm) (Table 2). A thorough literature search
274 did not identify any previous reports of garlic oil *Ae. aegypti* pupicidal activity,
275 suggesting that this is its first report. In addition, these pupae results support

276 the capacity of garlic oil to interfere in more than one stage of the mosquito life
277 cycle. Unlike water-borne larvae, pupae do not feed, making compound
278 bioavailability considerably more challenging. Larvae could have enhanced
279 garlic oil absorption rates due to filter feeding, while higher pupal endurance
280 could mostly relate to physical barriers rather than biochemical protection. A
281 previous study with ³⁵S-radiolabeled DAS2 reported that mosquito larvae
282 assimilate DAS2 at least 3-fold faster than pupae. At 50 ppm DAS2, 95% of
283 *Culex pipiens* larvae died after 8 h, while only 3% of pupae died after the same
284 exposure time. The ³⁵S activity when all larvae (8 h) and pupae (24 h) died was
285 similar, indicating that the equivalent concentration of DAS2 would kill both life
286 forms (Ramakrishnan et al., 1989). In our experiments, even at the highest
287 concentration (60 ppm), there was almost no pupae mortality after 24 h. Pupae
288 death was mostly observed at adult ecdysis, whereas most larvae mortality was
289 observed after the first 24 h. Owing to the volatile nature of garlic oil, to achieve
290 an adequate concentration to achieve pupae mortality, higher initial
291 concentrations must be used to offset time-dependent losses due to
292 evaporation.

293 The larvicidal activity of the garlic oil in this study showed 10-fold greater
294 potency than those investigated in previous reports. Sarma et al. determined a
295 larvicidal LC₅₀ 16.9 ppm after 24 h, that reached 7.6 ppm after 72 h. However,
296 GC/MS analysis of the garlic oil used in those experiments only annotated 30%
297 of total peak area with major components identified as DAS2 (8.5%) and DAS3
298 (7.8%) (Sarma et al., 2019). These were significantly lower than those detected
299 in the oil used in the present study (26.7% and 63.1%, respectively) (Table 1).
300 Muturi et al. reported a LC₅₀ 7.95 ppm (24 h) for a garlic oil with 49.1% DAS2,

301 31.1% DAS3 and 11.0% DAS4 (determined by GC/MS) (Muturi et al., 2018;
302 Muturi et al., 2019).

303 The organophosphate temephos positive control in larvicidal tests showed LC₅₀
304 0.008 ppm (CI₉₅ 0.0076 - 0.0085) after 24 h. In the pupicidal test control, 0.063
305 ppm temephos had no effect on pupae, while 0.5 ppm (a 62.5-fold higher
306 concentration than its larvicidal LC₅₀) only caused 15% pupae mortality. At
307 present, there is no pupicide available for *Ae. aegypti* mosquito control despite
308 reference to pyriproxyfen pupicidal activity in the literature (Hustedt et al., 2020).
309 Therefore, we investigated pyriproxyfen pupicidal activity in the 0.0001 ppb to
310 200 ppm range. No pupal mortality or deformations in emerging adults were
311 detected after treatment in this concentration range. Although considered a
312 pupicide, pyriproxyfen only interferes with development into the adult form when
313 administered during the larvae stage at concentrations allowing pupae
314 formation.

315 Visual inspection of pupae that died after garlic oil treatment (LC₅₀ 20.34 ppm)
316 evidenced a dark coloration, probably due to tissue necrosis after detoxification
317 attempts (Fig 1A). Sublethal garlic oil concentrations triggered pupae
318 abnormalities impeding the emergence of healthy mosquitoes often causing
319 malformations incompatible with mosquito survival (Fig. 1B-1E). In some cases,
320 the mosquito was completely formed but could not detach from the exuviae
321 during the molting process (Fig 1C-D). In the 5-15 ppm range, some
322 mosquitoes were completely formed, however with leg and wing malformations
323 that interfered with their fitness and survival (Fig 1D-F). Incomplete mosquito
324 detachment from the exuviae prevented flight and generally caused death after
325 a few hours, probably due to exhaustion. Some completely formed and

326 detached mosquitoes exhibited wing defects and were therefore unable to close
327 their wings during rest (Fig 1F). We observed direct correlation between the
328 concentration used and the impact on adult formation, with lower concentrations
329 relating to a lower degree of abnormality. In the wild, such abnormalities in
330 emerging mosquitoes compromise their survival and capacity to transmit
331 arboviral diseases. In summary, not only do garlic oils demonstrate direct
332 activity against pupae, they also indirectly control the adult form by interfering in
333 the life cycle at sublethal concentrations.

334

335 3.3. Isolated polysulfides and enriched garlic oil assays

336 Unveiling the potency of different polysulfides could potentially inform the
337 development of more potent insecticides by modifying the polysulfide
338 compositions of garlic oils to optimize their efficacy. DAS2-DAS5 were purified
339 from garlic oil by preparative scale HPLC and their individual structures
340 confirmed by ¹H NMR (Fig. S2) (Wang et al., 2013). These polysulfides were
341 individually tested as well as being used to supplement, and subsequently
342 measure the effect native garlic oils whose DAPS ratios have been altered.
343 When individually tested, DAS3 and DAS4 were most active polysulfides after
344 24 h against larvae, but less active than the original garlic oil (Fig. 2A and Table
345 S2). However, larvicidal activity of all of DAS2-DAS5 appeared to be more
346 equipotent after 72 h (Table S2). Interestingly, all the isolated polysulfides were
347 less active than the garlic oils supplemented with each of them individually in
348 the first 24 h for larvae. Sarma et al. reported that DAS2 and DAS3 were less
349 active than garlic oil after 72 h against *Ae. aegypti* larvae, although DAS3 was
350 more active in the first 24 h (Sarma et al., 2019).

351 The original garlic oil together with the DAS4 supplemented garlic oil were the
352 most active samples against larvae after 24 h (Fig. 2A and Table S2). All the
353 other samples tested in larvae had statistically different potencies when
354 compared to the original oil at 24 h. Interestingly, the LC₅₀ of a DAS2-rich garlic
355 oil (49.1%) was 7.95 ppm (CI₉₅ 7.19 - 8.66) (Muturi et al., 2018; Muturi et al.,
356 2019). The polysulfide ratio of the aforementioned oil was similar to our DAS2-
357 enriched garlic oil (Table S3), however, the LC₅₀ herein was lower (LC₅₀ 5.6
358 ppm, CI₉₅ 4.9 - 6.4 ppm). Another DAS2-supplemented garlic oil with 4.3% DAS,
359 37.4% DAS2, 10.9% DAS3 and 0.4% DAS4 showed an LC₅₀ of 7.05 ppm (CI₉₅
360 6.12 – 7.82) against *Culex pipiens* larvae after 48 h. The original garlic oil with
361 7.2% DAS2, 16.3% DAS3 and 0.7% DAS4 had a slightly higher LC₅₀ of 8.01
362 ppm (CI₉₅ 7.64 – 8.36) (Kimbaris et al., 2009).

363 After 72 h, the garlic oil supplemented with DAS5 was the most active with an
364 LC₅₀ of 0.5 ppm against larvae (Fig. 2B and Table S2). The pupicidal assays of
365 garlic oil individually supplemented with DAS4 or DAS2 determined significantly
366 more potent LC₅₀ values (4.0 and 5.5 ppm, respectively), than the original garlic
367 oil (10.2 ppm) (Fig. 2C and Table S2).

368 The garlic oil activities described in this section differ from those reported in the
369 previous section due to the different test methods used. The WHO protocol,
370 with 25 larvae/ pupae in 200 mL of test solution, resulted in: LC₅₀ 1.6 ppm
371 (larvae) and 20.3 ppm (pupae), while in this section, with 12-well plates with 10
372 larvae/ 5 pupae in 3 mL of test solution, resulted in: LC₅₀ 2.3 ppm (larvae) and
373 10.2 ppm (pupae). Therefore, the LC₅₀ values are not only impacted by the
374 garlic oil DAPS profile, but also by the testing method employed.

375 While some of the differences in activity are significant, the overall trend is a
376 higher activity of the enriched garlic oils in comparison to the isolated
377 polysulfides. Potency variations of the different garlic oils are significant, but
378 may be irrelevant due to the low level (low ppm range) required to achieve
379 larvicidal and pupicidal activities. In addition, some garlic oil compositions were
380 more active against pupae, and less active against larvae. For instance, DAS4-
381 supplemented garlic oil was significantly less active on larvae (after 72 h) but
382 was significantly more active against pupae than the original garlic oil (Fig. 2).
383 Collectively, these results indicate that alterations in individual DAPS
384 proportions (DAS2-DAS5) would not significantly impact global mosquito
385 control, providing the concentration of the DAPS mixture is maintained. The
386 literature suggests that DAS supplementation may negatively affect the
387 larvicidal activity of garlic oils by a reduction in the proportion of the other
388 polysulfides. A DAS-enriched garlic oil showed significantly lower larvicidal
389 activity (LC₅₀ 24.3 ppm) than the original garlic oil (LC₅₀ 8.0 ppm) for *Culex*
390 *pipiens* larvae after 48 h (Kimbaris et al., 2009).

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

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

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

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

440 3.4 Simulated field trials: larvicidal and pupicidal assays

441 The trials involved exposing the *Ae. aegypti* Rockefeller strain to garlic oil
442 treatment conditions simulating their natural breeding sites. Larvae and pupae
443 were added to plastic buckets containing tap water and placed outside the
444 laboratory conditions. During these trials, the water temperature ranged from
445 9.1 to 34.8 °C, with the lowest relative humidity of 24%. The three
446 concentrations tested against pupae (60.0, 45.0 and 30.0 ppm) caused 100%
447 mortality. These concentrations were approximately the LC₁₀₀, LC₉₀ and LC₈₀ in
448 the laboratory experiments. The concentrations of 60.0 and 30.0 ppm were also

449 tested under laboratory conditions (28 ± 2 °C, $70 \pm 10\%$ relative humidity and
450 12 h photoperiod). The latter caused significantly less mortality in the laboratory
451 ($70.7 \pm 10.4\%$ in 200 mL cups) compared to the field setting ($99.8 \pm 0.3\%$ in 8 L
452 buckets) ($p < 0.0001$). The different volume to surface area ratio of the test
453 vessels may account for the difference observed. A higher volume to surface
454 area ratio may retard garlic oil evaporation and cause higher mortality due to
455 prolongation of the initial concentration.

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

461 3.5 Ovicidal activity

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

469 Another study reported 100% egg mortality with a 100 ppm garlic oil solution
470 (Sarma et al., 2020), in which only the larvae that spontaneously hatched after
471 72 h were accounted. In the present study, no larvae hatched spontaneously
472 after 72 h exposure to garlic oil solution, while up to 85% hatched in the
473 controls. Since the garlic oils are larvicidal, the treated water could be killing

474 newly hatched larvae instead of affecting the eggs. To verify the ovicidal activity
475 of our test solution, we transferred the 72 h treated eggs to clean tap water and
476 stimulated hatching in a low-pressure chamber.

477 The egg stage is recognized as the most resilient of the *Ae. aegypti* life cycle,
478 with eggs remaining viable for several months under dry conditions (Kliwer,
479 1961). Mosquito populations are therefore able to endure long dry seasons in
480 some tropical areas. For instance, central regions in Brazil experience 4 to 5
481 months of intense drought every year. The subsequent wet season results in
482 exponential mosquito population growth. Transovarial virus transmission has
483 been reported, suggesting it could be one of the mechanisms for maintaining
484 virus circulation in interepidemic seasons (Joshi et al., 2002). *Ae. aegypti* eggs
485 are a crucial, and perhaps the most challenging target, for the development of a
486 multifaceted control strategy.

487 3.6 Spatial repellency, contact irritancy and adult toxicity assays

488 Mosquito repellency is recognized as a promising tool to control arbovirus
489 transmission. The main objective of this technology is to prevent mosquitoes
490 approaching areas where they could find a human host. An ideal repellent
491 would not cause mortality at the applied repellency concentration, resulting in
492 lower pressure for resistance development (Achee et al., 2012). Garlic oil was
493 tested at 7.5, 5.0, 2.5 and 1.0% (v/v) using the HITSS (High-Throughput
494 Screening System) to assess its spatial repellency activity. The SAI (spatial
495 activity index) values of the three higher concentrations were statistically
496 different from the negative control, reaching 0.67 ± 0.17 (2.5%), 0.71 ± 0.1
497 (5.0%) and 0.56 ± 0.24 (7.5%). The highest wSAI (weighted spatial activity
498 index) obtained was 15.2 ± 6.8 (7.5%), which is considered low. The wSAI

499 levels were a result of the low PRESP (percentage of response) values, all
500 under 20%. A low PRESP was also observed for the 20% PMD positive control,
501 for which only 21.6% ($\pm 4.9\%$) mosquitoes responded. The wSAI 17.2 ± 5.7 and
502 SAI 0.64 ± 0.23 of the PMD positive control were comparable to the garlic oil
503 treatments at lower doses (2.5%, 5.0% and 7.5%). The SAI and wSAI for the
504 garlic oil 1.0% concentration were not significant.

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

515 Since we noted absence of knocked down mosquitoes and potential repellent
516 activity, we conducted the same assay in a scaled-up system to simulate a field
517 setting. Two rooms of the insectarium were connected with a plastic tubing that
518 allowed mosquitoes to fly freely from one room to the other. A cage was
519 assembled inside each room to enable mosquito counting at the end of the test
520 (Fig. 4). At the concentration tested (0.015 mL/m^3), the mean wSAI was 31.9,
521 with 0.5 SAI (± 0.17) and 61.1% PRESP. Raw data analysis indicated an
522 oriented movement of the mosquitoes escaping from the garlic oil-treated room.
523 More than half of the mosquitoes flew to the control room. The repellency

524 observed in the scaled-up system was more prominent than in the HITSS
525 apparatus. Collectively, these results warrant broader discussion and
526 consideration regarding the validity of HITSS spatial repellency data generated
527 for highly volatile scented compounds, beyond the crude SAI and wSAI values.
528 The second repellent strategy tested contact irritancy. This test differs from the
529 aforementioned spatial repellency assay in that mosquitoes directly contact the
530 surface treated with the sample. At 1.0%, garlic oil caused significant contact
531 irritancy, with $66.0 \pm 7.86\%$ ($p = 0.004$) of the mosquitoes escaping from the
532 treated chamber. No significant knockdown was observed, suggesting that the
533 activity, at this concentration, is non-toxic to *Ae. aegypti*. The PMD control
534 caused $42.4\% \pm 14.13\%$ ($p=0.002$) contact irritancy at 20% concentration. By
535 direct comparison, garlic oil is more irritant to mosquitoes than the commercial
536 pesticide.

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

547 Previous papers reported the potential use of garlic oil as a skin repellent
548 (Rajan et al., 2005; Campbell et al., 2011). The antennae of female *Ae. aegypti*

549 mosquitoes responded to garlic oil and its isolated polysulfides, that were also
550 active in contact repellent assays (Campbell et al., 2011). An experiment with a
551 Y-tube olfactometer showed that the garlic oil repellency lasted for less than 30
552 min, which would be insufficient for effective repellent activity (Mitra et al.,
553 2020). From an epidemiological perspective, the impact of skin contact
554 repellents in preventing disease transmission remains controversial as it
555 depends on individual compliance for a regular successful outcome (Norris and
556 Coats, 2017). In opposition, spatial repellents have been recognized as an
557 interesting innovative alternative to contact repellents and may constitute an
558 important tool in integrated pest control management (Norris and Coats, 2017;
559 Achee et al., 2019). In fact, garlic products have been commercially explored as
560 mosquito repellent agents in some products, such as Mosquito Barrier[®] and
561 Mosquito-less[®] marketed in the United States and Canada. Their availability in
562 the market is not proof of quality, since these products qualify for registration
563 exemption. As such, they are not required to have proven efficacy or safety
564 assessment prior to commercialization. The lack of robust scientific evidence of
565 garlic repellency potential means authors inadvertently declare garlic as an
566 ineffective repellent (Maia and Moore, 2011). To our knowledge, this is the first
567 scientific report of garlic oil trials as spatial repellents and insecticides against
568 adult mosquitoes.

569

570 4. Conclusions

571 Ovicidal, larvicidal, pupicidal, adulticidal and spatial repellency assays showed
572 the broad activity of garlic oil against all *Aedes aegypti* life stages. Garlic oils
573 are unique as insecticides in that they affect eggs, larvae and pupae, which

574 commonly coexist at mosquito breeding sites. These results, together with its
575 endurance in simulated field trials and the industrial production of garlic oil,
576 highlight its suitability for integrated mosquito vector control programs. Evidence
577 shown in this study suggests that alterations in garlic oil composition may not
578 significantly affect its broad activity providing the final concentration of diallyl
579 polysulfides remains unchanged. The activity and stability data of isolated diallyl
580 polysulfides and garlic oil polysulfide mixtures suggests isolation efforts may not
581 result in higher potency. Lack of persistency in the environment, mainly due to
582 high volatility, demands technological development to explore garlic oil
583 commercial use against *Ae. aegypti*. Mixing garlic oil with other essential oils or
584 scented volatile compounds to address the characteristic aroma would enable
585 indoor and outdoor garlic oil applications. The results shown here suggest the
586 promising application of garlic oils to control mosquito approach to humans,
587 either as spatial repellents, immature stage control or mosquitocidal agents,
588 providing low persistency and aroma can be technologically addressed.

589

590 CRediT authorship contribution statement

591

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

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

600

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609

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 711 [dengue](https://www.who.int/en/news-room/fact-sheets/detail/dengue-and-severe-dengue)

712

713 **Table and Figure captions**

714

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

717

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

718 *DAPS: diallyl polysulfides

719

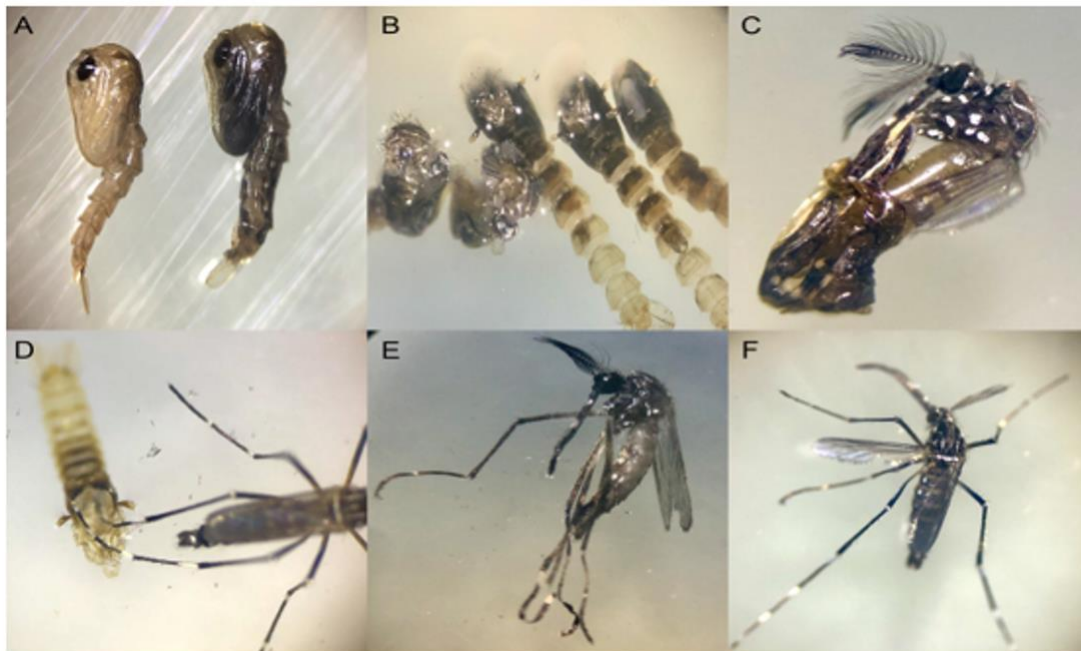
720

721 **Table 2.** Garlic oil LC₅₀/LC₉₅ values (ppm) against *Ae. aegypti* larvae and
 722 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

723 ^aCI: confidence interval. ^bp: p value for chi-square.

724
725



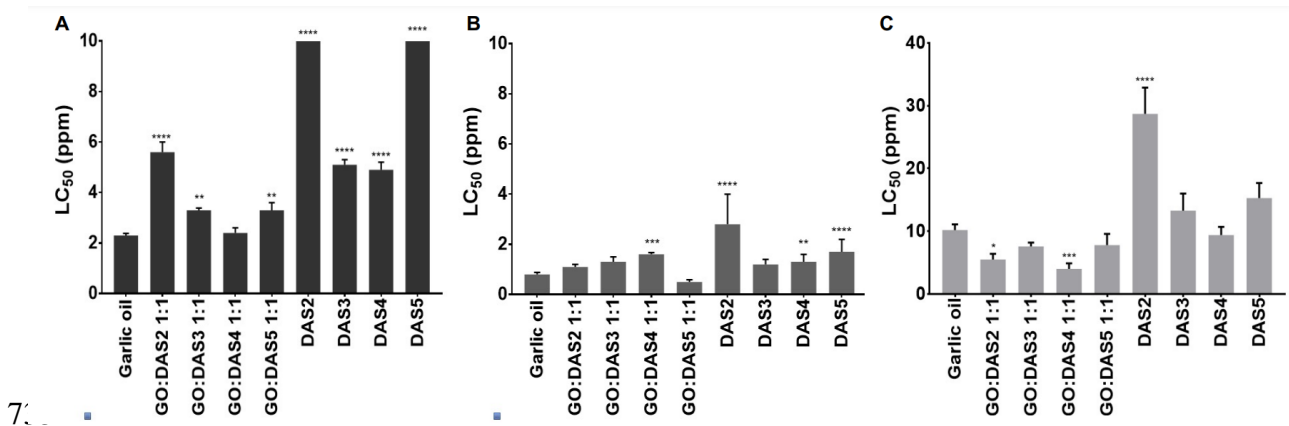
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727 **Fig. 1.** Mortality and abnormalities in pupae treated with garlic oil: 1A. a dead
728 pupa (right) compared to a healthy pupa (left); 1B. dead pupae during adult
729 emergence; 1C. a partially-formed mosquito, died during ecdysis; 1D. a
730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

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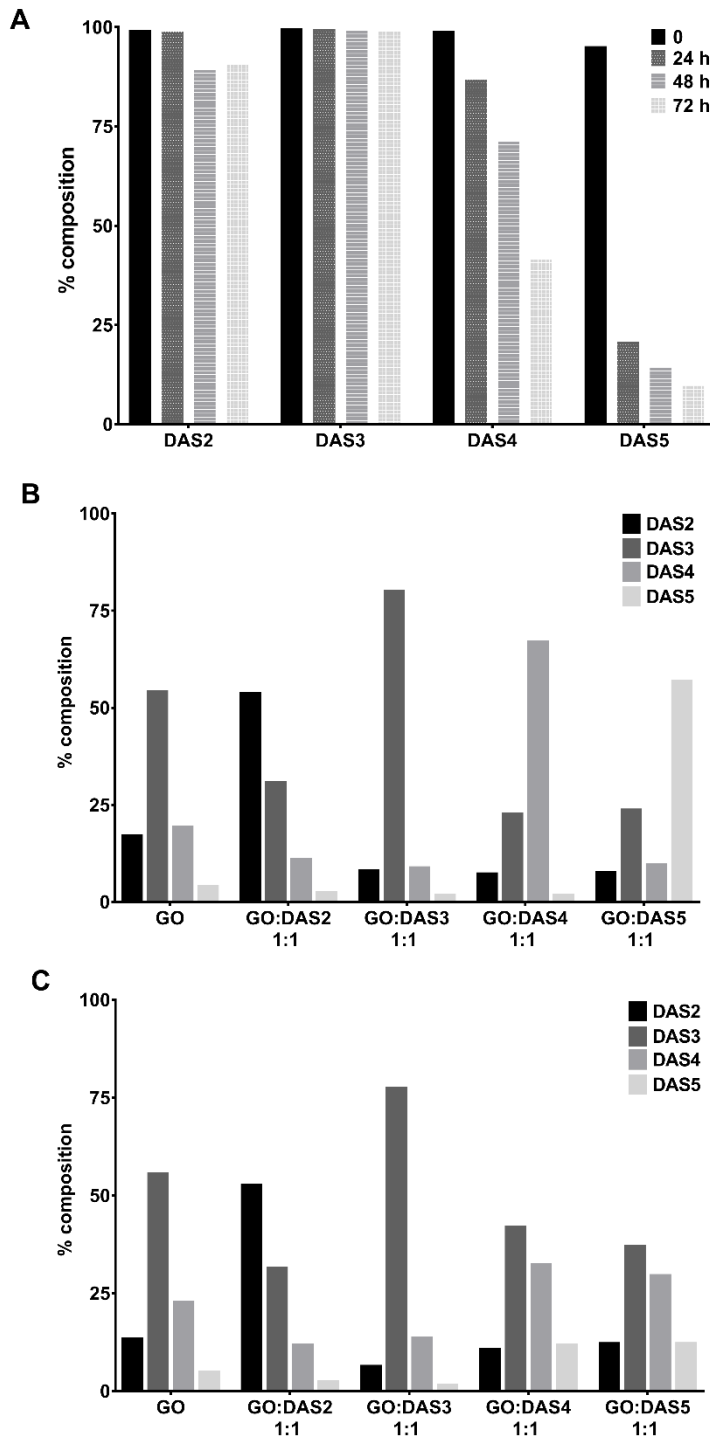
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736 **Fig. 2.** LC₅₀ values of isolated polysulfides, garlic oil and garlic oil supplemented
 737 with polysulfides against *Ae. aegypti* larvae after 24 h (2A), larvae after 72 h
 738 (2B) and pupae (2C). Statistical significance (p values) related to the original
 739 garlic oil potency. *: p ≤ 0.05; **: p ≤ 0.01; ***: p ≤ 0.001; ****: p ≤ 0.0001.

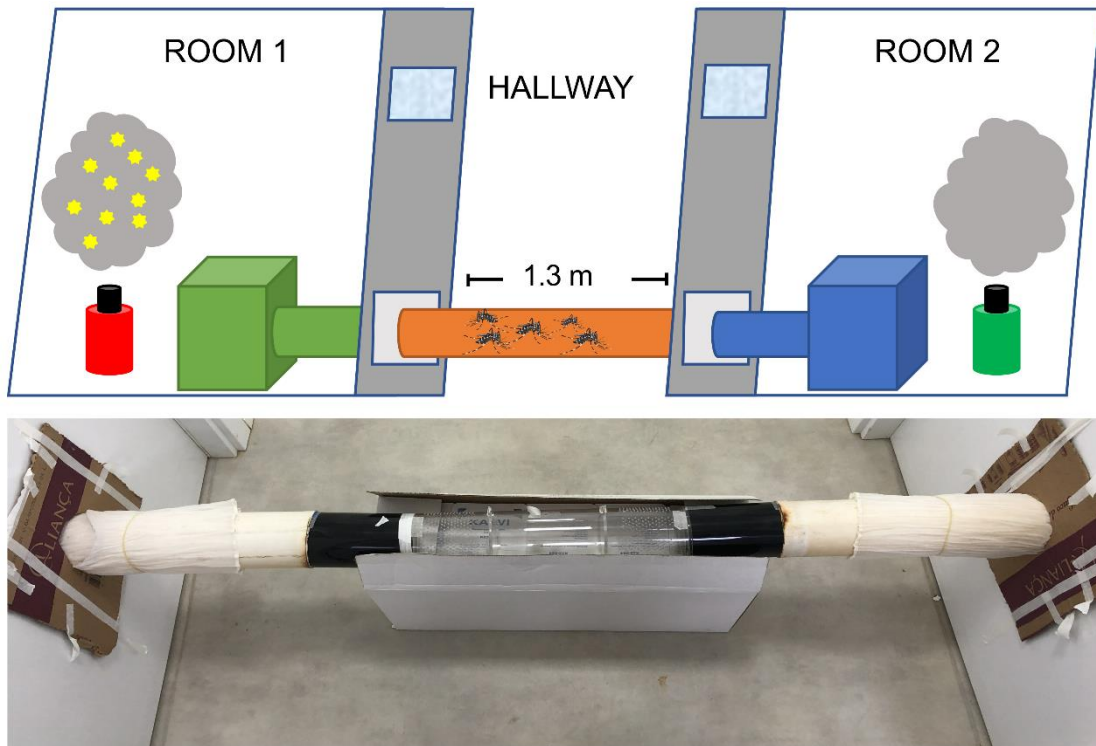
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742

743 **Fig. 3.** Peak area percentages (%) of diallyl polysulfides (DAPS) determined by
 744 HPLC-UV detection (210 nm) at different time points: 3A. individual polysulfides;
 745 3B. garlic oil composition at time 0; 3C. garlic oil composition after 72 h.



746

747 **Fig. 4.** Schematic representation of the spatial repellency simulated field trial.

748 The distance between the rooms is 1.3 m. Room 1: Test room with vaporized

749 garlic oil solution and mosquito cage (green). Room 2: Control room with

750 vaporized water and mosquito cage (blue).

751

752

753 **Appendix A. Supplementary data**

754

755 **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

756 ^aRI = Retention index determined with respect to a homologous series of n-alkanes on a
757 DB-5 MS column.

758

759

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

Sample	<i>LC</i> 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)

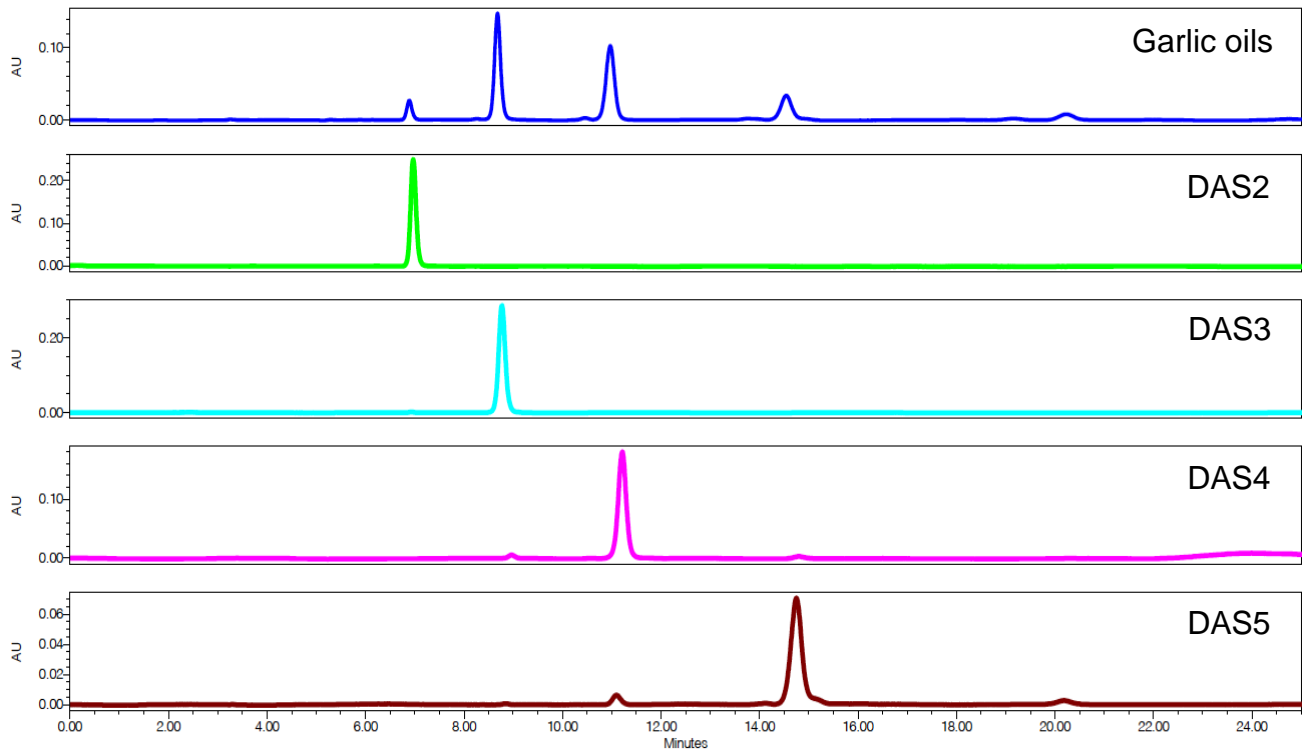
762 ^aCI₉₅ : 95% confidence interval.

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

Sample	Time (h)	%				DAS2- DAS5
		DAS2	DAS3	DAS4	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

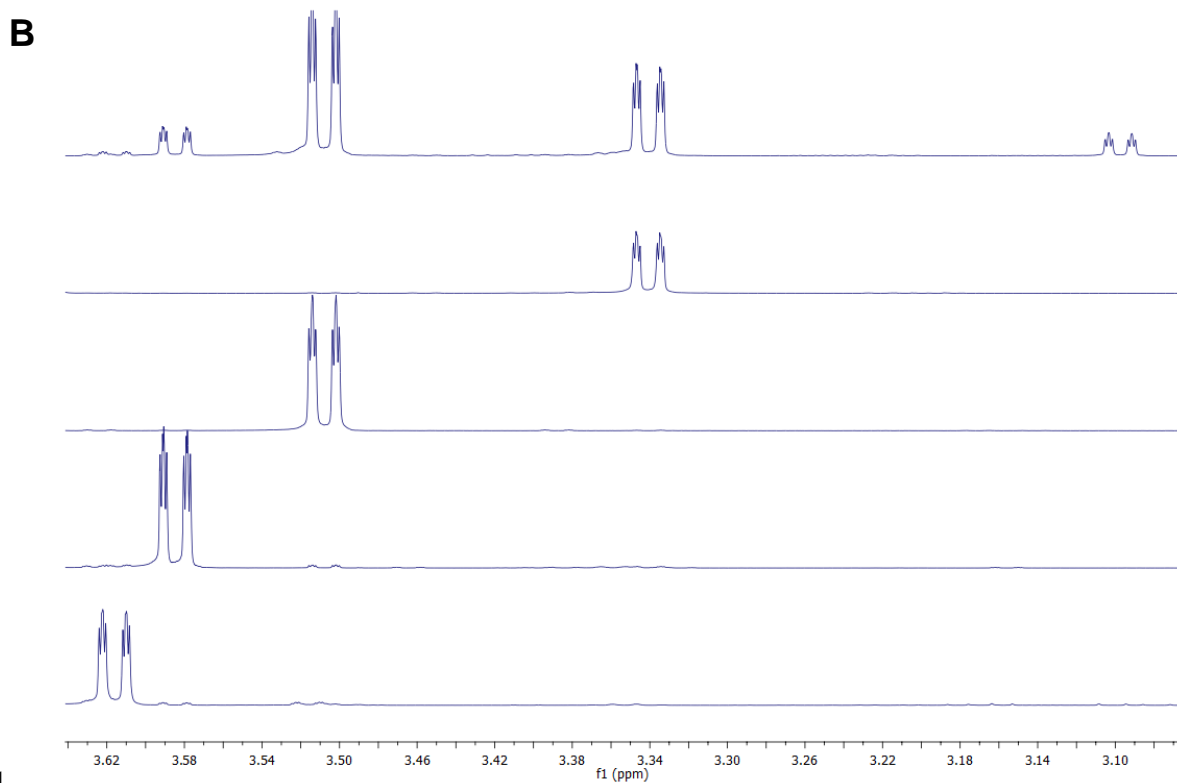
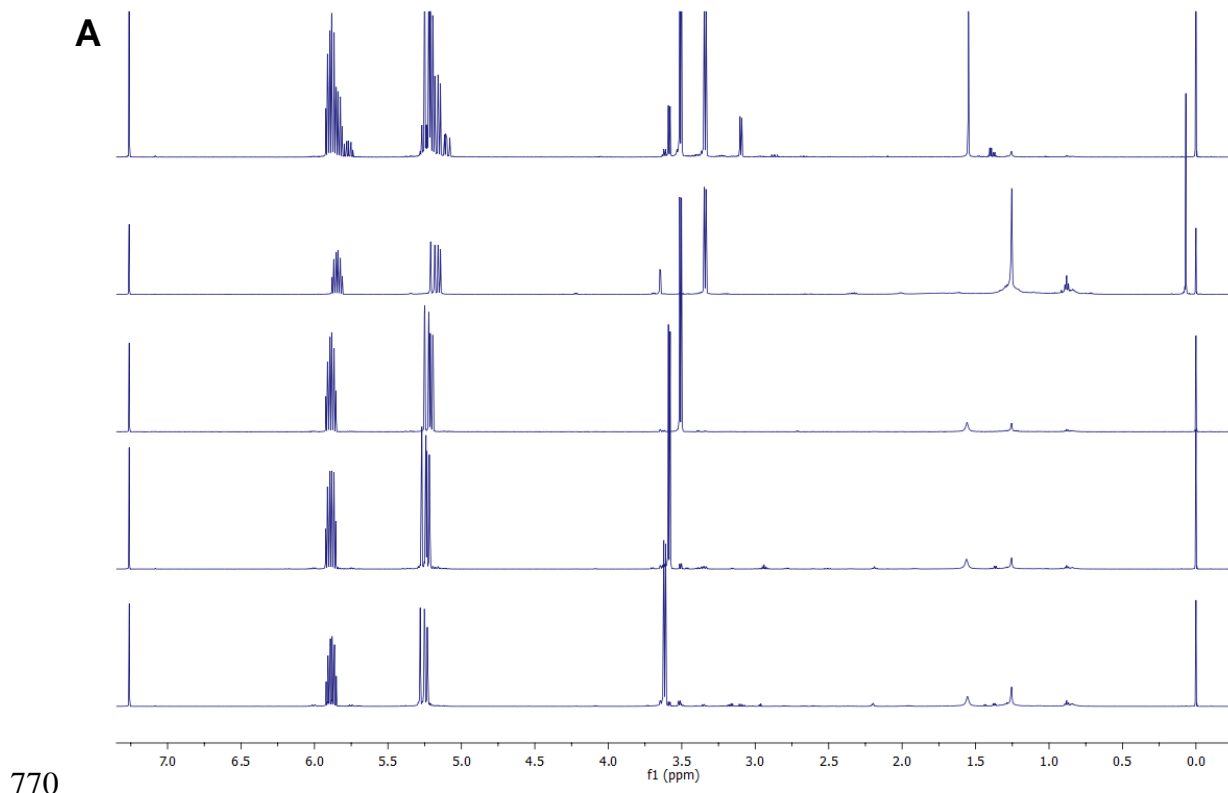
48	10.3	38.7	30.6	12.6	92.2
72	12.0	36.8	29.3	12.0	90.0

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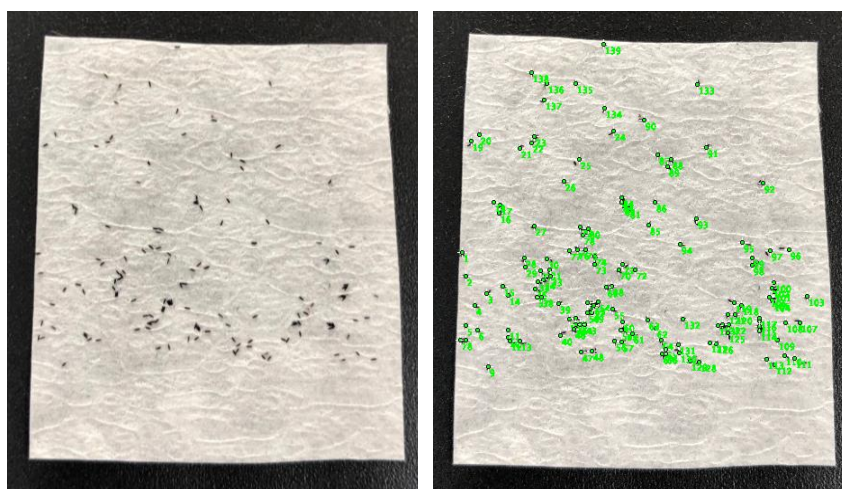


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768 **Fig. S1.** HPLC-DAD chromatograms (at 210 nm) of the enriched garlic oils and respective isolated
 769 polysulfides.

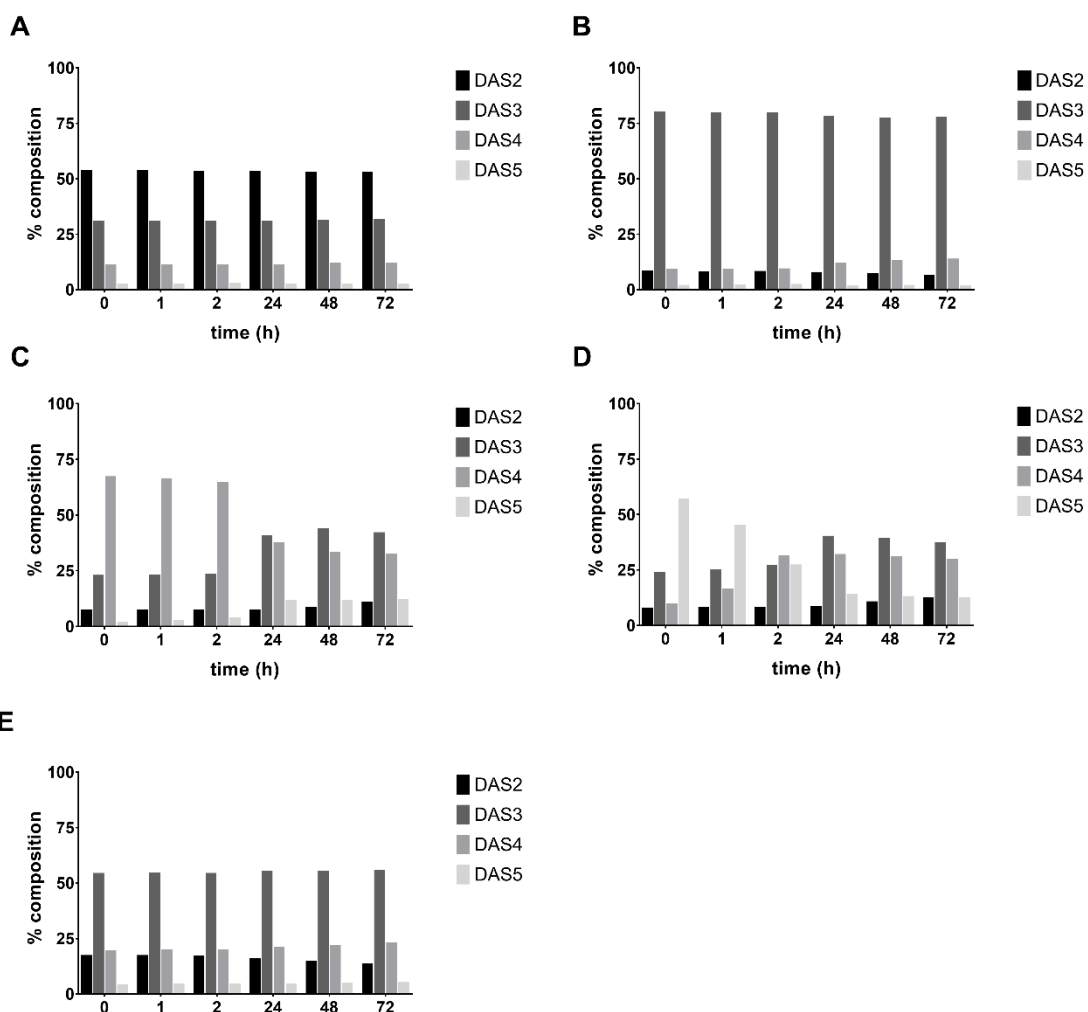


772 **Fig. S2A:** Garlic oil and garlic polysulfides in CDCl_3 from top to bottom: garlic oil, diallyl disulfide
 773 (DAS2); diallyl trisulfide (DAS3); diallyl tetrasulfide (DAS4); diallyl pentasulfide (DAS5). **S2B:**
 774 Stacked ^1H NMR spectra of garlic oil and polysulfides in the 3.30 to 3.70 ppm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



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Fig. S3. Egg counting using the ImageJ software.



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Fig. S4. Peak area percentages (%) of diallyl polysulfides (DAPS) determined by

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HPLC-UV detection (210 nm) at different time points: **3A.** garlic oil + DAS2

783 (1:1); **3B.** garlic oil + DAS3 (1:1); **3C.** garlic oil + DAS4 (1:1); **3D.** garlic oil +

784 DAS4 (1:1); **3E.** original garlic oil.

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