Entomopathogenic fungi for the control of larvae and adults of *Aedes aegypti* in Mexico

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Abstract

Objective. To assess larvicide and adulticide activity of different native strains of fungi on *Aedes aegypti*. **Materials and methods.** Third instar larvae were exposed for 72 h at a concentration of $1x10^8$ conidia/ml of 15 fungi; only fungi that significantly affected the larvae were evaluated against the adult phase at a concentration of $2x10^{10}$ conidia/ml. Mortality readings were performed at 24, 48, and 72 h for larvae, and every day to 30 days for adults. **Results.** *Trichoderma longibrachiatum, Aspergillus aculeatus,* and *Metarhizium anisopliae* had the best larvicidal activity at 24 h of exposure ($p<0.05$), causing mortalities of 100, 72, and 62%, respectively. Adult mosquitoes were more affected by *Gliocladium virens* (45% mortality), *M. anisopliae* (30% mortality), and *T. longibrachiatum* (23.33% mortality). **Conclusion.** The larval stage of *Ae. aegypti* was more susceptible than the adult phase to the pathogenic action of native fungi, with *T. longibrachiatum* being with the highest virulence.

Keywords: native fungi; larvicides; mosquito control; *Aedes aegypti*

Cisneros-Vázquez LA, Penilla-Navarro RP, Rodríguez AD, Ordóñez-González JG, Valdez-Delgado KM, Danis-Lozano R, Vázquez-Martínez MG. Hongos entomopatógenos para el control de larvas y adultos de *Aedes aegypti* en México. Salud Publica Mex. 2023;65:144-150.
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Resumen

Objetivo. Evaluar la actividad larvicida y adulticida de 15 cepas nativas de hongos en *Aedes aegypti*. **Material y métodos.** Larvas de tercer instar se expusieron por 72 h a concentraciones de $1x10^8$ conidias/ml del hongo; sólo los que afectaron significativamente a las larvas se evaluaron contra los mosquitos adultos a concentraciones de $2x10^{10}$ conidias/ml. Se documentaron las mortalidades a las 24, 48 y 72 h para larvas, y cada día por 30 días para adultos. **Resultados.** Sólo *Trichoderma longibrachiatum, Aspergillus aculeatus* y *Metarhizium anisopliae* tuvieron la mayor actividad larvicida a las 24 h ($p<0.05$), causando mortalidades de 100, 72 y 62%, respectivamente. Los mosquitos adultos fueron más afectados por *Gliocladium virens* con mortalidades de 45%, *M. anisopliae* (mortalidad de 30%) y *T. longibrachiatum* (mortalidad de 23.33%) ($p<0.05$). **Conclusión.** Las larvas de *Ae. aegypti* fueron más susceptibles a la acción patogénica de los hongos nativos que los adultos, siendo *T. longibrachiatum* el más virulento.

Palabras clave: hongos nativos; larvicidas; control de mosquitos; *Aedes aegypti*

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Dengue, chikungunya, and Zika diseases have suffered large outbreaks worldwide, its viruses are mainly transmitted by *Aedes aegypti*. The Pan American Health Organization reported 2 326 115 people infected with dengue in the Americas region during 2020 and it is estimated that more than half of the world population is at risk of developing this disease.[1,2] On the other hand, chikungunya and Zika diseases were affecting only parts of Asia and Africa, but in the past five years, the strong indigenous transmission was reported in Europe, America, and the islands of Oceania and the Pacific.[3-6]

Control programs are mainly based on the management of mosquito breeding sites and the use of chemical insecticides;[7] unfortunately, these strategies have been insufficient to control mosquito populations. In addition, the development of insecticide resistance by populations of *Ae. aegypti* has been reported in diverse states of Mexico[8,9] and around the world.[10] The global emergence of resistance has led to the search for alternatives to combat the mosquito, such as biological control, which does not present environmental pollution problems.

Of the biological control strategies, the use of entomopathogenic fungi presents several advantages compared to other control agents. For example, these agents can be produced through artisanal methods using cheaper substrates[11] and can be disseminated by mosquitoes through copula within the population.[12] Studies about control of malaria vector mosquito have been previously reported, for example, *Beauveria bassiana* induced mortality in *Anopheles stephensi* adult mosquitoes after exposure to surfaces treated with the fungal,[13] while *An. gambiae* adult mosquitoes from Tanzania were susceptible to *Metarhizium anisopliae*.[14] On the other hand, larvae, and adults of *An. albimanus* were susceptible to *M. anisopliae* and *Gliocladium virens* (native fungus) in Mexico.[15]

For *Ae. aegypti* control, significantly high mortality rates were generated in larvae by *M. anisopliae*,[16] *Leptolegnia chapmanii*,[17] and *Aspergillus clavatus*.[18] Meanwhile, *M. anisopliae*, *B. bassiana*,[19,20] *Paecilomyces carneus, Isaria fumosorosea, Lecanicillium muscarium,* and *Lecanicillium psalliotae*[21] infected *Ae. aegypti* adults. On the other hand, *Pa. carneus*, *I. fumosorosea*, *M. anisopliae*, and *B. bassiana*, among others, have induced infection in mosquito eggs.[22]

For these reasons, the goal of this study was to evaluate the pathogenicity of several native strains of fungi in the larval and adult phases of the Mexican *Ae. aegypti* mosquito.

Materials and methods

Aedes aegypti mosquitoes

Mosquitoes from the New Orleans insecticide susceptible strain reared in our laboratory for more than seven years and originally provided by Dr. Karla Saavedra from Colorado State University, were used. Insectary conditions were at 25-27 °C and with a relative humidity of 60-70%. Third-stage larvae and two- to three-day-old adult females (without a blood meal), were used for the bioassays following the World Health Organization (2005) methodology.[23] All experiments were developed from 2010 to 2015.

Fungi

The native fungi strains evaluated consisted of *Fusarium oxysporum* (LBIH-94), *Penicillium citrinum* (LBIH-104), *Talaromyces flavus* (LBIH-111), *Pa. variotii* (LBIH-102), *Aspergillus niger* (LBIH-108), *A. clavatonanicus* (LBIH-105), *A. flavus* (LBIH-107), *A. aculeatus* (LBIH-103), *A. oryzae* (LBIH-100), *A. flavus* (LBIH-106), *Trichoderma longibrachiatum* (LBIH-101), *P. variotii* (LBIH-115) and *P. citrinum* (LBIH-112), which were isolated from *Ae. aegypti* breeding sites in houses from colonies on the outskirts of Tapachula, Chiapas, Mexico.[24] *G. virens* (LBIH-116) was also used and was isolated from *An. albimanus* larval breeding sites on the coast of Chiapas, Mexico.[24] Furthermore, in the present study, we also evaluated *M. anisopliae sensu lato* (LBIH-033, not native to Chiapas) strain, isolated from the beetle *Phyllophaga* spp. and gently provided by *Centro de Investigación y de Estudios Avanzados* (Cinvestav) from Irapuato.

Fungal culture

Fungi were cultivated on Petri dishes containing Sabouraud Dextrose Agar culture media (SDA, Becton Dickinson) and incubated at 27±2 °C inside an environmental chamber (Thermo Electron Corporation Model 818) until sporulation. Conidia suspensions were prepared in the 0.0001% Tween-80 solution. The conidial concentration was estimated by cell counting with a Neübauer chamber (Hausser Scientific) using method B of the technique described by Hansen.[25] The conidial viability was estimated by measuring the conidial germination of samples obtained from the conidia suspension grown

on SDA media.[26] Conidial suspensions with viability higher than 90% were used for the bioassays.

Larvicidal and adulticidal activity bioassays

Larval phase. Bioassays were conducted inside disposable plastic eight oz beakers containing 100 ml of the evaluated conidia suspension at a concentration of $1x10^8$ conidia/ml, with 25 third-stage larvae added. Sixteen treatments were evaluated, including the 15 fungal strains and a 0.0001% Tween-80 control solution without conidia. The bioassays were performed inside an environmental chamber at 27 °C with a 12:12 h light/dark photoperiod. The mortality rates were evaluated at 24, 48, and 72 h, and all treatments were repeated four times.

Adult phase. The fungal strains with the highest pathogenicity against the larval were evaluated against adult females. A suspension of 2x10 conidia/ml was prepared from each fungus. Subsequently, sterile filter paper (16x14cm) was impregnated with 3.5 ml of each solution and allowed to dry at room temperature for 24 h. Treated filter paper was placed inside plastic beakers (16oz) similar to reported by Garcia-Munguia and colleagues.[27] Filter papers impregnated with a 0.01% Tween-80 solution without conidia, were used as controls. Groups of 20 female mosquitoes were exposed to filter papers for 30 days and kept inside an environmental chamber at 27 °C with 70% relative humidity and a photoperiod (12:12). The bioassays were repeated three times. All mosquitoes were fed a 5% sucrose solution impregnated in cotton, which was placed on top of the lid mesh. Mortality rates were recorded every day to 30 days after exposure. In order to determine the sporulation rate, all dead mosquitoes were washed with 1% sodium hypochlorite solution for 20 sec and rinsed twice in sterile distilled water. Finally, to stimulate the growth of the fungus, the mosquitoes were placed in Petri dishes containing filter paper and surgical cotton moistened with sterile tri-distilled water. The observation was carried out ten days after the death of the insect.

Doses-response bioassays

Dose-response bioassay. The strain with the highest pathogenicity potential against mosquito larvae was selected to determine CL_{50} and CL_{90} using $2x10^7$ $1x10^7$ $7x10^6$ $6x10^6$ and $5x10^6$ conidia/ml concentrations, and 0.0001% Tween 80 without conidia, as a control. All experiments consisted of four replicates, and the mortality rates were quantified at 24 h.

Statistical analysis

The larval activity of the fungi against *Ae. aegypti* was performed using the Kruskal Wallis test, and to identify statistically significant among treatments the Dunn test. For the adulticidal activity, mortalities caused by fungi were compared using a one-way ANOVA, and multiple comparisons were made with the Tukey test. In addition, sporulation percentages were analyzed using one-way ANOVA. All the statistical analyses mentioned above were performed using GraphPad Prism 6 package. Finally, lethal concentrations (CL_{50} and CL_{90}) were determined using a Probit regression applied to treatments and the mortality rate, which was determined using the Probit software (EPA, version 1.5).

Results

Larvicidal and adulticidal activity bioassays

All of the evaluated fungi caused mortality in larvae with significant differences among them in its larvicide capacity ($p<0.05$) (table I). At 24 h of exposure, *T. longibrachiatum, A. aculeatus,* and *M. anisopliae* were the most effective agents against larvae, with mortality rates of 100, 72, and 62%, respectively. However, at 72 h of exposure, some fungi increased their mortality effectivity as was shown by *F. oxysporum* (from 38 to 100%), *A. aculeatus* (from 72 to 96%), *A. niger* (from 8 to 88%), and *M. anisopliae* (from 62 to 78%). In contrast, *Pa. variotii* (LBIH-115) and *Ta. flavus* were the least virulent fungi and showed no significant differences compared to the control ($p>0.05$).

During a 30-day interval, the mortality rate of *Ae. aegypti* adults by *G. virens* was 45% (SE=5), *M. anisopliae* 30% (SE=5), and *T. longibrachiatum* 23.3% (SE=2.8) ($F=23.12$; $df=3$; $p<0.001$) (figure 1). In the sporulation study, infection rates between 83 and 85% were observed among the three fungi showing no statistical differences ($F=0.043$; $df=2$; $p=0.958$) (figure 2). While the mosquitoes of the control group did not develop any fungus in their cuticle (figure 2).

Doses-response bioassays

As *Ae. aegypti* larval phase was more susceptible to entomopathogenic fungi than the adult phase in all the cases tested, and *T. longibrachiatum* strain-induced 100% larvae mortality at a faster rate than the other strains, a calculation of its lethal concentrations at 50 and 90% (LC_{50} and LC_{90}) was performed. At 48 h of exposure

Table I

PERCENT OF MORTALITY (MEDIAN) OF THIRD-STAGE *AE. AEGYPTI* LARVAE EXPOSED 24 AND 72 H TO 15 NATIVE ENTOMOPATHOGENIC FUNGI. CONTROL REPRESENTS MOSQUITO LARVAE WITHOUT FUNGAL EXPOSURE. ALL EXPERIMENTS WERE CARRIED OUT BETWEEN 2010 AND 2015 UNDER LABORATORY CONDITIONS. CHIAPAS, MEXICO

Treatments	*Median(IQR) 24 h*	*Median(IQR) 72 h*
Trichoderma longibrachiatum	100a	----------
Aspergillus aculeatus	72(65-80)ab	96(92-100)a
Metarhizium anisopliae	62(57-78)ab	78(72-92)ab
Fusarium oxysporum	38(33-43)bcd	100(100-100)a
Gliocladium Virens	22(17-31)de	60(52-67)bc
Penicillium citrinum (LBIH-104)	22(16-32)def	58(52-66)bcd
Paecilomyces variotii (LBIH-102)	22(14-31)def	52(48-60)cd
Aspergillus oryzae	18(12-24)ef	32(24-39)ef
Aspergillus flavus (LBIH-106)	14(9-19)fg	50(45-59)de
Aspergillus niger	8(5-12)fgh	88(81-96)a
Talaromyces flavus	2(0-4)ghi	4(4-8)ghi
Aspergillus clavatonanicus	0(0-4)ghi	52(45-56)cde
Paecilomyces variotii (LBIH-115)	0(0-4)gi	4(0-4)hi
Penicillium citrinum (LBIH-112)	0i	12(12-15)g
Aspergillus flavus (LBIH-106)	0i	12(9-19)g
Control	0i	0hi

Treatments with the same letter showed not significant differences between them, and with different letters showed significant differences between them with a $p<0.05$. N=300 mosquitoes larvae per treatment.

using the highest concentration of $2x10^7$ conidia/ml, *T. longibrachiatum* induced a 100% larval mortality rate, this exposure time was used to perform a Probit analysis, and an LC_{50} of $5.7x10^6$ conidia/ml and an LC_{90} of $9.6x10^6$ conidia/ml (table II) were obtained.

Discussion

The 15 native fungal strains evaluated in *Ae. aegypti* from México presented different pathogenicity levels, being *T. longibrachiatum* the most virulent fungi to its larvae phase. No study has reported the pathogenicity of the *Trichoderma* genus fungi in larval or adult *Ae. aegypti*; nonetheless in this study, *T. longibrachiatum* at $1.05x10^8$ conidia/ml induced mortality rates of 100% in larvae, as *M. anisopliae* from Brazil at $5x10^6$ conidia/ml, and *A. clavatus* from Senegal at $21x10^7$ conidia/ml caused

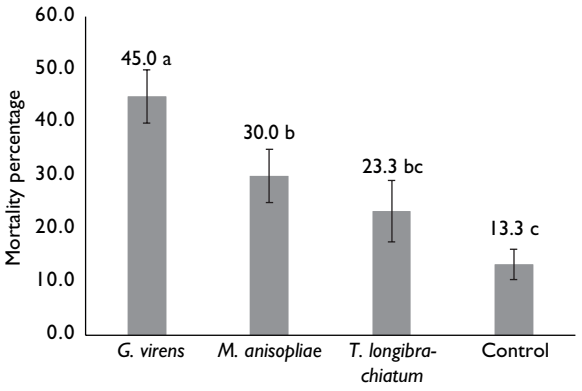

FIGURE 1. MORTALITY PERCENT OF *AEDES AEGYPTI* ADULT (2 TO 3 DAY-OLD) EXPOSED DURING 30 DAYS TO *GLIOCLADIUM VIRENS*, *METHARIZIUM ANISOPLIAE* AND *TRICHODERMA LONGIBRACHIATUM*. CONTROL REPRESENTS MOSQUITOES WITHOUT FUNGAL EXPOSURE. TREATMENTS WITH THE SAME LETTER SHOWED NOT SIGNIFICANT DIFFERENCES BETWEEN THEM, AND WITH DIFFERENT LETTERS SHOWED SIGNIFICANT DIFFERENCES BETWEEN THEM WITH A $p<0.05$. ALL EXPERIMENTS WERE CARRIED OUT BETWEEN 2010 AND 2015 UNDER LABORATORY CONDITIONS. CHIAPAS, MEXICO

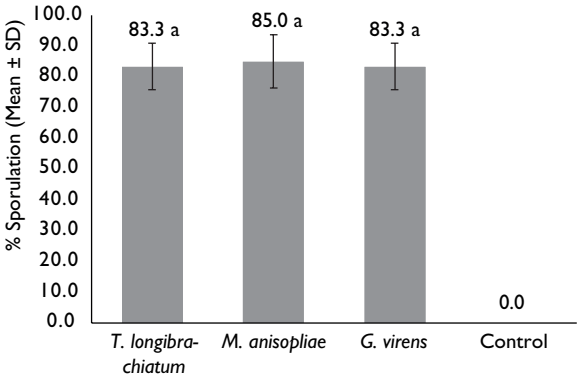

FIGURE 2. SPORULATION OF *TRICHODERMA LONGIBRACHIATUM*, *METHARIZIUM ANISOPLIAE* AND *GLIOCLADIUM VIRENS* ON *AEDES AEGYPTI* ADULT AFTER 10 DAYS OF DEATH. THE CONTROL REPRESENTS DEAD MOSQUITOES WITH NO PRIOR EXPOSURE TO ANY OF THE THREE FUNGUS. TREATMENTS WITH THE SAME LETTER SHOWED NOT SIGNIFICANT DIFFERENCES BETWEEN THEM, AND WITH DIFFERENT LETTERS SHOWED SIGNIFICANT DIFFERENCES BETWEEN THEM WITH A $p<0.05$. ALL EXPERIMENTS WERE CARRIED OUT BETWEEN 2010 AND 2015 UNDER LABORATORY CONDITIONS. CHIAPAS, MEXICO

Table II
Lethal concentrations for third-stage *Aedes aegypti* larvae after 48 h of exposure to *Trichoderma longibrachiatum*. All experiments were carried out between 2010 and 2015 under laboratory conditions. Chiapas, Mexico

Point	Concentration	95% confidence limits	
		Lower	Upper
LC1	$2.2x10^6$ conidia/ml	$1.6x10^6$ conidia/ml	$2.7x10^6$ conidia/ml
LC5	$2.9x10^6$ conidia/ml	$2.3x10^6$ conidia/ml	$3.4x10^6$ conidia/ml
LC10	$3.3x10^6$ conidia/ml	$2.7x10^6$ conidia/ml	$3.8x10^6$ conidia/ml
LC15	$3.7x10^6$ conidia/ml	$3.1x10^6$ conidia/ml	$4.1x10^6$ conidia/ml
LC50	$5.7x10^6$ conidia/ml	$5.3x10^6$ conidia/ml	$6x10^6$ conidia/ml
LC85	$8.7x10^6$ conidia/ml	$8x10^6$ conidia/ml	$9.7x10^6$ conidia/ml
LC90	$9.6x10^6$ conidia/ml	$8.7x10^6$ conidia/ml	$1x10^7$ conidia/ml
LC95	$1.1x10^7$ conidia/ml	$9.9x10^6$ conidia/ml	$1.3x10^7$ conidia/ml
LC99	$1.4x10^7$ conidia/ml	$1.2x10^7$ conidia/ml	$1.8x10^7$ conidia/ml

X^2 calculated=5.299; X^2 tabular 0.05=7.815.

in the *Ae. aegypti* larvae,[16,18] and as *A. sulphureus* from Brazil at $2.68x10^8$ conidia/ml in *Ae. fluviatilis* larvae.[28] Therefore, these results show that *T. longibrachiatum* is a potential entomopathogen fungus to control larvae of *Ae. aegypti* from Mexico.

Conversely, *M. anisopliae* fungus is well known for its pathogenic success against crop plagues,[29] and commercial products based on this fungus are available on the market. In this study, *M. anisopliae* only induced a 65% mortality rate in *Ae. aegypti* larvae, which is lower than the rate induced by the *M. anisopliae* strain reported by Silva and collaboratos[16] (100% in *Ae. aegypti* larvae). Moreover, Vázquez-Martínez and collaborators[24] reported a 100% mortality rate when evaluating this same strain of *M. anisopliae* in third-stage *An. albimanus* larvae. This finding suggests that *Ae. aegypti* is less susceptible to *M. anisopliae* infection than *An. albimanus*, being both mosquito species from the North of the American Continent.

Fungi of the *Aspergillus sp*. genus have been previously reported as pathogens for *Aedes sp*. larvae. Seye and collaborators[18] showed that *A. clavatus* fungi ($17x10^7$ conidia/ml), a native fungus from Senegal, induced a mortality rate of 100% after 24 h. Meanwhile, in the present study, *A. aculeatus* and *A. niger* induced mortality rates of 96 and 88%, respectively, after 72 h of exposure. These rates are similar to those induced by *A. kanagawaensis* ($5.95x10^5$ conidia/ml) and *A. ochraceus* ($6.3x10^5$ conidia/ml), native fungi from Brazil[28] in *Ae. fluviatilis* after 72 h (80 and 97%, respectively), which indicates that our native *Aspergillus* have the same ability to control larval population of *Ae. aegypti* in Mexico.

Using a similar method to that employed in the present study, previous experiments have exposed adult *Ae. aegypti* mosquitoes to *M. anisopliae,* natives fungi from Kenya and Brazil,[19,20] and *B. bassiana*[27] (a native fungus from Northern Mexico, $6x10^8$ conidia/ml) for 24 to 48 h and reported a median lethal time (LT_{50}) of three to five days. The fungi *G. virens*, *T. longibrachiatum,* and *M. anisopliae,* which were used in this study, did not induce a mortality rate of 50% in 30 days and were unable to compete with the pathogenicity of other fungi described in the literature. In contrast, Farenhorts and Knols[30] showed that fungal activity depends on several factors, including the application method, conidial substrate, and formulation used to homogenize the conidia when preparing the conidial suspension. Because these factors could mask the action of *T. longibrachiatum*, *G. virens*, and *M. anisopliae* against the adult phase of *Ae. aegypti*, they should be evaluated to investigate a possible increase in the pathogenicity of the aforementioned strains.

The native fungus *T. longibrachiatum* dramatically affected the larval phase of *Ae. aegypti* (with a 100% mortality rate), but it showed a reduced infectious capacity against the adult phase. This difference could be explained by two known factors. One factor may be the potential metabolite production of *T. longibrachiatum,* as previously described for the use of *T. viride* against *Cx. quinquefasciatus*,[31] in which filtered fungal cultures were toxic to the larvae. The second factor may be the ingestion of conidia by the larvae, as in the case of *Culicinomyces clavisporu*.[32] This fungus produces conidia that, after being ingested by the larvae, adhere to and penetrate the larvae's intestines, causing their death. In addition to ingestion, conidia can reach the larvae through natural orifices, such as the spiracle and the anus, causing the larvae to suffocate through mechanical blockage of the tracheal tract.[33]

On the other hand, the sporulation rate generated by our native fungi remained in a range of 83 to 85%, while Scholte and collaboratos[19] reported 93.5% of *M. anisopliae* sporulation in female *Ae. aegypti* from Kenya in a period of three days of observation. Our infection rates are relatively similar to that reported in the literature; however, our native strains needed more time to generate mycelium in the cuticle of *Ae. aegypti*.

Toxicity is the ability of a substance to produce harmful effects on a living being, when in contact with it, and can be expressed by means of the average lethal concentration (LC_{50}). In the present study, the LC_{50} ($5.7x10^6$ conidia/ml) and LC_{90} ($9.6x10^6$ conidia/ml)

values were obtained when *T. longibrachiatum* was used against *Ae. aegypti*, and are similar to those obtained for *G. virens*, another native strain from the Chiapas state, which has an LC_{50} value of $3.55x10^6$ and an LC_{90} value of $8.674x10^7$ conidia / ml against third-stage larvae of *An. albimanus*.[15] Meanwhile, the values reported for the LC_{50} of *M. anisopliae* against *Cx. quinquefasciatus* larvae from Brazil (LC_{50} of $1.97x10^4$ conidia / ml),[34] and *Ae. albopictus* mosquito larvae from Pakistan ($LC_{50}= 1.09x10^5$ conidia / ml)[35] were lower than those obtained in the present study, indicating that *T. longibrachiatum* is less toxic to mosquitoes because it needs more conidia to generate mortalities similar to those reported in the literature.

In conclusion, in the present study, the larval phase of *Ae. aegypti* mosquitoes were more susceptible to entomopathogenic fungal infection than the adult phase. *Trichoderma longibrachiatum* fungus presented the highest virulence, which makes it a good candidate for biological *Ae. aegypti* larval control strategies. However, it is necessary to carry out future studies to determine if there are secondary metabolites active against *Ae. aegypti* and if they have an impact on beneficial organisms and human health.

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Declaration of conflict of interests. The authors declare that they have no conflict of interests.

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