

Biocontrol evaluation of extracts and a major component, clusianone, from *Clusia fluminensis* Planch. & Triana against *Aedes aegypti*

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Studies evaluated the effects of hexanic extracts from the fruits and flowers of Clusia fluminensis and the main component of the flower extract, a purified benzophenone (clusianone), against Aedes aegypti. The treatment of larvae with the crude fruit or flower extracts from C. fluminensis did not affect the survival of Ae. aegypti (50 mg/L), however, the flower extracts significantly delayed development of Ae. aegypti. In contrast, the clusianone (50 mg/L) isolate from the flower extract, representing 54.85% of this sample composition, showed a highly significant inhibition of survival, killing 93.3% of the larvae and completely blocking development of Ae. aegypti. The results showed, for the first time, high activity of clusianone against Ae. aegypti that both killed and inhibited mosquito development. Therefore, clusianone has potential for development as a biopesticide for controlling insect vectors of tropical diseases. Future work will elucidate the mode of action of clusianone isolated from C. fluminensis.

Key words: clusianone - larvicidal - *Clusia fluminensis* - *Aedes aegypti*

Many insecticides, including the organochlorines, organophosphates and carbamates, persist in the environment as toxic waste (Miresmailli & Isman 2014) and are neurotoxic not only to humans, but to livestock too (Narahashi 2000, Biondi et al. 2012). Furthermore, the redundant mode of action of insecticides may accelerate the emergence of cross-resistance to other pesticides (Georghiou 1972, Miresmailli & Isman 2014). Therefore, new approaches for insect control need evaluating, including substances from natural sources that may have less of an environmental impact (Isman 2006).

One example of a pest species that is a major vector of human diseases, including urban yellow fever, dengue, Chikungunya and Zika virus is *Aedes aegypti*, for which there are also many reports of populations developing resistance to different insecticides (Braga et al. 2004, Vontas et al. 2012). *Ae. aegypti* has a cosmopolitan distribution, is highly domesticated and anthropophilic (Gubler 2011, Powell & Tabachnick 2013). It is estimated that 3.6 billion people live in areas at risk from dengue infection and 390-500 million cases occur in 124-128 countries worldwide each year (Beatty et al. 2010, Brady et al. 2012,

Bhatt et al. 2013). In Brazil, the current distribution of *Ae. aegypti* extends to almost the whole country (Braga & Valle 2007), causing cyclic outbreaks of dengue fever in various regions, in which there are four main serotypes of the virus in circulation (Bastos et al. 2012, Dick et al. 2012). There is neither a vaccine nor specific treatment for dengue (Bhatt et al. 2013), so that combating the virus is limited to elimination of the mosquito vector (Beatty et al. 2010, Dick et al. 2012). Therefore, the search for new alternative strategies and increased vigilance for vector resistance have become essential for the control of this disease (Maciel-de-Freitas et al. 2014).

Many plant natural products have been tested as insecticides against mosquitoes (Carvalho 2011, Miresmailli & Isman 2014, Reegan et al. 2014) as they are nontoxic to mammals and are promising candidates to replace conventional insecticides to which insects are becoming more resistant (Braga et al. 2004, Shaalan et al. 2005, Cantrell et al. 2012, Vontas et al. 2012). In the majority of these studies, although larvicidal activity has been described for the extracts and the presence of a range of compounds sometimes detected, very few have actually identified the compounds responsible for activity together with their structure (Navarro et al. 2015). Some of the compounds identified have been shown to disrupt the insect endocrine system, acting as agonists or antagonists of insect hormones, killing or even preventing them reaching the adult stage (Bowers et al. 1976, Garcia et al. 1986). One group of such natural plant insecticides includes the terpenes, that are related to terpenoids with insect juvenile hormone (JH) analogue activity and have been commercially used successfully as substitutes for classical insecticides in areas where insect resistance

doi: 10.1590/0074-02760150103

Financial support: PROPI-UFF, CNPq, FAPERJ

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Received 15 March 2015

Accepted 24 June 2015

has been detected (Braga et al. 2005a,b). Apparently, no cross-resistance occurs between JH analogues and the pyrethroids which are generally used for the control of *Ae. aegypti* (Braga et al. 2005a).

Scientists, however, need to increase the number of alternative natural insecticides available for the control of *Ae. aegypti* in order to build up an arsenal of natural products able to counter the resilience of these mosquitoes to develop resistance. Thus, another group of natural pesticides derived from plants with potential as insect control agents are the benzophenones. Many derivatives of these compounds with different structures and a multiplicity of functions have been described (Beerhues & Liu 2009, Li et al. 2014). Very few of these compounds have been investigated as potential insect control agents, although the insecticidal activity of benzophenones has been known for many years (Middleton & Chadds 1973). Only recently has their mosquito larvicidal activity been realised with synthetic benzophenone indole analogues (Ranganatha et al. 2013) and with the benzophenones, cariphenone A and cariphenone B, isolated from a *Hypericum carinatum* (da Silva et al. 2013a). In addition, a commercial preparation of benzophenones used in ultraviolet (UV) filters has been shown to disrupt the endocrine system, activating hormonal genes and altering embryonic development of *Chironomus riparius* (Ozáez et al. 2014).

One source of benzophenones is *Clusia fluminensis* (Planch. & Triana) (Clusiaceae), a native species from the Brazilian coast, commonly used as an ornamental plant because of its evergreen leaves and high tolerance to dehydration. Significantly, these leaves rarely show signs of herbivory, which are always undamaged and bright (Bittrich 2010). In 2000, Porto et al. identified and quantified, by high performance liquid chromatography, polyisoprenylated benzophenones, including clusianone and spiritone, in resins obtained from the male flowers of *C. fluminensis*. Recently, clusianone from male flowers of *C. fluminensis* was also isolated and structurally characterised (Silva et al. 2012).

In the quest for alternative natural biological control agents against mosquito larvae, the present paper reports on the larvicidal activity of hexanic extracts from *C. fluminensis* against *Ae. aegypti* (Linnaeus) (Diptera: Culicidae). Subsequently, a benzophenone (clusianone) was isolated, structurally elucidated and investigated as the source of the larvicidal activity.

MATERIALS AND METHODS

Insect colonies - Eggs of *Ae. aegypti*, Rockefeller strain, were provided by Dr José Bento Pereira Lima [Brazilian Army Biology Institute, Oswaldo Cruz Foundation (Fiocruz), state of Rio de Janeiro (RJ), Brazil]. They hatched in the rearing water after about 1 h and groups of 500 newly hatched larvae were placed in plastic bowls (25 x 15 x 7 cm) containing 500 mL of dechlorinated water and 500 mg of fish feed flakes (Tetramin® Tropical Flakes). The larvae were kept in incubators BOD at 26 ± 1°C until third instars emerged after approximately 72 h.

Plant material - Flowers from male and fruits from female *C. fluminensis* were collected, with the permis-

sion of the Brazilian Army, in a rocky outcrop at Forte Barão do Imbuhy, in the city of Niterói, RJ. Flowers and fruits were available in the summer of 2006 and autumn of 2007, respectively. The plant material was identified by Dr Marcelo Guerra Santos and a voucher specimen was deposited at the herbarium of the Faculty of Teacher Education, Rio de Janeiro State University (licence 9213).

Preparation of plant extracts - Fruits of *C. fluminensis* were dried in an oven at 40°C and subsequently fragmented while whole flowers were used while still partially fresh. The crude extracts were prepared by macerating plant organs with analytical grade hexane as a solvent at room temperature. The solvent was renewed every seven days and then evaporated under reduced pressure.

Chemical analysis of crude extracts - Gas chromatography coupled with mass spectrometry (GC-MS) - The crude extracts obtained from flowers of *C. fluminensis* were analysed by GC-MS performed on an Agilent model 6890N gas chromatograph equipped with a mass selective detector, model 5973N. A volume of 1 µL of the solution with a concentration 1 mg/mL was injected. The following conditions were utilised: carrier gas, helium at 2.0 mLmin⁻¹, split ratio 5:1, injector temperature 300°C and ion source temperature 230°C. The oven temperature was programmed from 150-300°C at 10°C min⁻¹ and held at 300°C for 15 min. Sample injection volume was 1 µL. The mass spectra of the major components of *C. fluminensis* flowers were analysed and compared with the Wiley mass spectral databases and the literature data.

Nuclear magnetic resonance (NMR) analysis - The NMR spectra were acquired using a BRUKER DRX 400 spectrometer operating at 400 MHz for ¹H and 100 MHz for ¹³C. Chemical shifts were measured relatively to an internal tetramethylsilane reference. Spectral data of clusianone was published for our group previously in Silva et al. (2012) and the purification and identification of lanosterol in Oliveira et al. (2014). The structure of lanosterol was confirmed by MS compared to the equipment's database (Wiley Database) and NMR.

Isolation of clusianone - The polyisoprenylated benzophenone, clusianone, was isolated as a tautomeric pair from the crude hexanic extract of flowers of *C. fluminensis* by countercurrent chromatography using the solvent system n-hexane-acetonitrile-methanol (2:1.25:0.5, v/v/v), as described by Silva et al. (2012). The material under study is endotoxin free.

Insect bioassays - *Ae. aegypti* larvae were used three days after hatching and were formed into groups of 10 larvae in the third instar with six replicates. The groups were placed in 300 mL disposable plastic cups containing 50 mL of dechlorinated water. The cups were then randomly allocated to groups, with the aid of the pwgen (Windows Phone) program. The dried extracts and isolates were weighed and solubilised with 0.1% dimethyl sulfoxide (DMSO) to make up to 50 µL, with a final concentration of 50 mg/L in the water test. These substances were thoroughly mixed in the water test to minimise the possibility of adhesion of particles to the hydrophobic

plastic surfaces. The control groups were untreated and the solvent controls (SC) were treated solely with 0.1% DMSO used to dissolve the samples. During the tests, larvae were fed with 30 mg per cup of hamster food (Alcon®, Brazil), immediately after completion of the tests and three days later. The results are derived from the media of the percentage of each replicate.

All experiments were followed until the death or emergence of all insects in control group. All insects remained in the BOD incubators, at 26 ± 1 °C. Counts were made daily to evaluate dead insects and developmental stages. All experiments were repeated at least twice.

Data and statistical analysis - All graphs were created with GraphPad Prism 6.01 software (GraphPad Software, USA). The graphics analysed the survival (and mortality) and the developmental course of juvenile forms and adults. The Gehan-Breslow-Wilcoxon test compared the whole survival curves and larval and adult development (GraphPad Prism 6.01 software). No corrections for multiple comparisons were made (Rothman 1990). The Barnard's test (Barnard package v.1.3) (Erguler 2012, R Development Core Team 2014) was used to analyse the development of *Ae. aegypti* pupae, comparing the mean values only in a representative day indicated by an arrow in graphs. In all experiments, only p values < 0.05 were considered statistically significant.

Ethics - Eggs of *Ae. aegypti* were provided by Dr José Bento Pereira Lima from insects treated according to the

Ethical Principles in Animal Experimentation approved by the Ethical Committee in Animal Experimentation (Fiocruz). The protocol is from National Council for the Control of Animal Experimentation/Ministry of Science, Technology and Innovation (cobea.org.br), which is associated with the American Association for Animal Science, Federation of European Laboratory Animal Science Associations, International Council for Animal Science and Association for Assessment and Accreditation of Laboratory Animal Care International. The authors declare that they have no competing interests. The funders, from Brazilian Govern, had no role in study design, data collection and analysis, decision to publish or preparation of the paper.

RESULTS

The bioassays with the hexanic extract of *C. fluminensis* fruit showed that in the treated groups there were no effects on the survival rates or development of *Ae. aegypti* (Fig. 1 A-D).

The bioassays with the hexanic extract of *C. fluminensis* flowers (FL) showed no significant effect on *Ae. aegypti* survival in comparison with the SC ($p = 0.1797$) (Fig. 2A). In contrast, the analyses of the effects of FL on the development of larvae to pupae and pupae to adults showed significant delays in molting ($p \leq 0.0001$) between the FL-treated and SC groups (Fig. 2B-D). Thus, on the fifth day, 88.3% of the SC group had molted to pupae, while 93.3% of the FL group were still in the larval

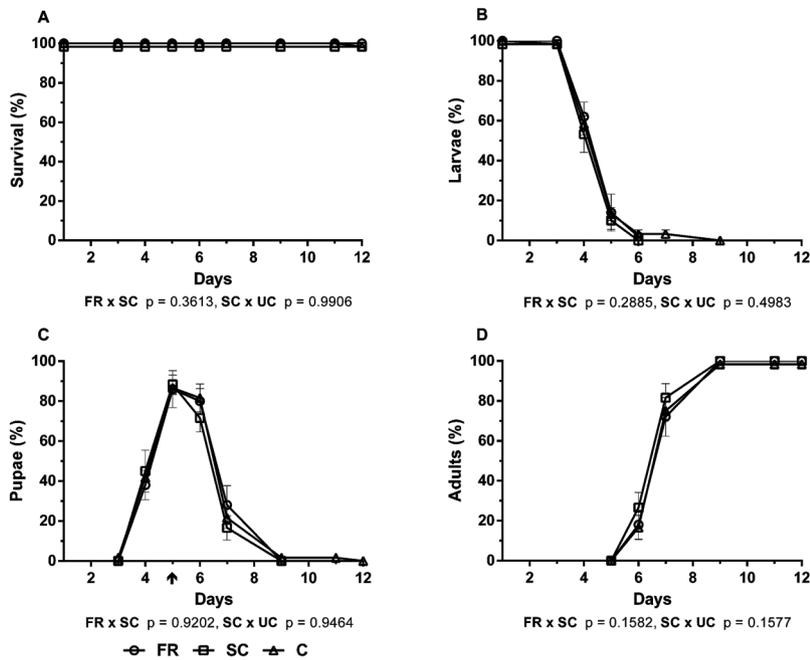


Fig. 1: effects of extracts of fruit from *Clusia fluminensis* (FR) on survival (A) and development of larvae (B), pupae (C) and adult (D) of *Aedes aegypti* at different days after experimental treatment (FR) compared with the solvent control (SC) that was compared with untreated control (UC). Each point represents the mean of at least six replicates with 10 insects and bars show standard error. Third instar *Ae. aegypti* larvae received 50 mg/L extract in the test water. Statistical analyses (under the graphs) with the Barnard's test were used (C) on a representative day indicated by an arrow and the Gehan-Breslow-Wilcoxon test was used (A, B, D) to compare the entire curve between the groups (FR x SC and SC x C). Significant differences (p value < 0.05) are in bold.

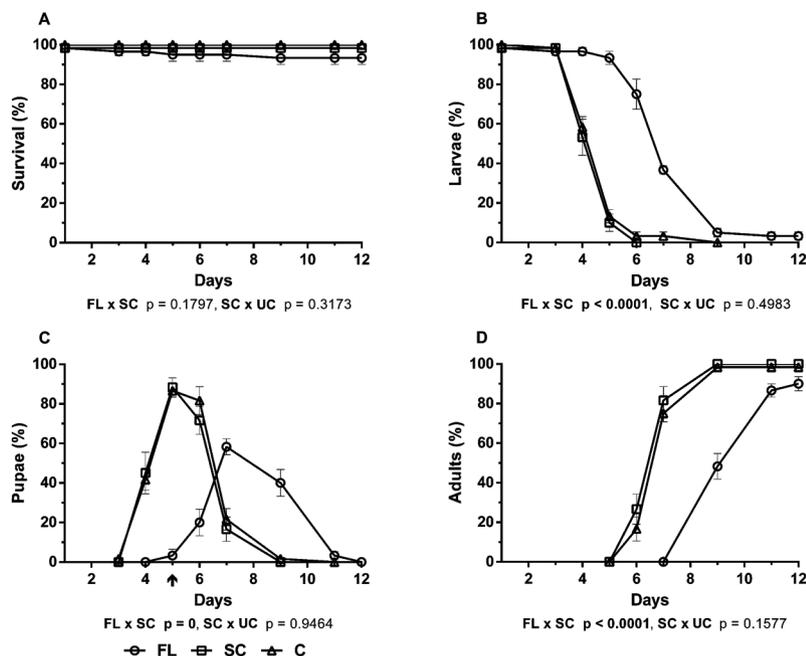


Fig. 2: effects of extracts of flower from *Clusia fluminensis* (FL) on survival (A) and development of larvae (B), pupae (C) and adult (D) of *Aedes aegypti* at different days after experimental treatment (FL) compared with the solvent control (SC) that was compared with untreated control (UC). The assays were executed and analysed as in the Fig. 1 legend and Materials and Methods.

stage (Fig. 2B, C). Furthermore, on day 7, 81.7% of the SC had reached adults, while none of the FL group had reached this stage (Fig. 2D).

Since only the flower extract affected the development of *Ae. aegypti*, this alone was submitted to GC-MS. The chromatogram obtained for the hexanic extract of flowers of *C. fluminensis* showed that the major component (retention time: 18.424 min) was the benzophenone, clusianone. This substance was identified by comparison of the mass fragmentation pattern with the data provided by Delle Monache et al. (1991) and represented 54.85% of the hexane extract (Fig. 3). In addition, lanosterol was also detected (retention time: 19.155 min) in the same extract and contributed to 9.74% of the sample composition (Fig. 3).

The purification protocols and chemical structures of clusianone from the flowers of *C. fluminensis* have been described previously (Silva et al. 2012). Subsequently, bioassays with this purified substance were performed against *Ae. aegypti*.

Bioassays with clusianone showed a significant reduced survival of the *Ae. aegypti* ($p < 0.0001$) (Fig. 4A), so that by the first day of treatment 20% of larvae had already died compared with the SC group (Fig. 4A). In the clusianone-treated group, by the last day (12th) of treatment, 93.3% of insects were dead, while more than 93% were still alive in the SC group (Fig. 4A). The few specimens of *Ae. aegypti* that survived the treatment with clusianone remained as larval instars (Fig. 4B), with none molting to pupae or adults (Fig. 4C, D).

In contrast with the effect of clusianone, no activity was detected by the isolated lanosterol on the survival or development of *Ae. aegypti* larvae in the bioassays (results not showed).

DISCUSSION

In this present study, clusianone was further identified and purified from FL, following the original protocols described by Silva et al. (2012). Clusianone is the major component from the flowers of *C. fluminensis*, representing almost 55% of the crude extract composition.

Crude fruit extracts of *C. fluminensis* had no activity on the survival or development of *Ae. aegypti* and preliminary results showed that clusianone is not present in these extracts, in which lanosterol is the main compound. FL also had no effects on survival, but delayed the development of *Ae. aegypti*. In addition, the purified clusianone not only caused high larval mortality and delayed development, but also completely blocked the molting of surviving treated-insects. These results show four important facts. Firstly, the value of analysing not only mortality, but also the effect on development in the evaluation of plant extracts activities against insects. Secondly, that fractionated and purified components may show enhanced activities in bioassays in comparison with those of crude extracts. Thirdly, the necessity of assaying different regions of the plant tissue for activity, when testing plant extracts for insecticide properties. Fourthly, they report the first description of clusianone's larvicidal activity against *Ae. aegypti* larvae.

The clusianone effects, however, seem to have similar characteristics to those found with synthesised benzophenone tagged indole derivatives that resulted in mortality in *Culex quinquefasciatus* larvae (Ranganatha et al. 2013), as well as those reported for extracts from species of *Hypericum* against culicids (Cetin et al. 2011). A fraction of *H. carinatum* enriched with the benzophenones,

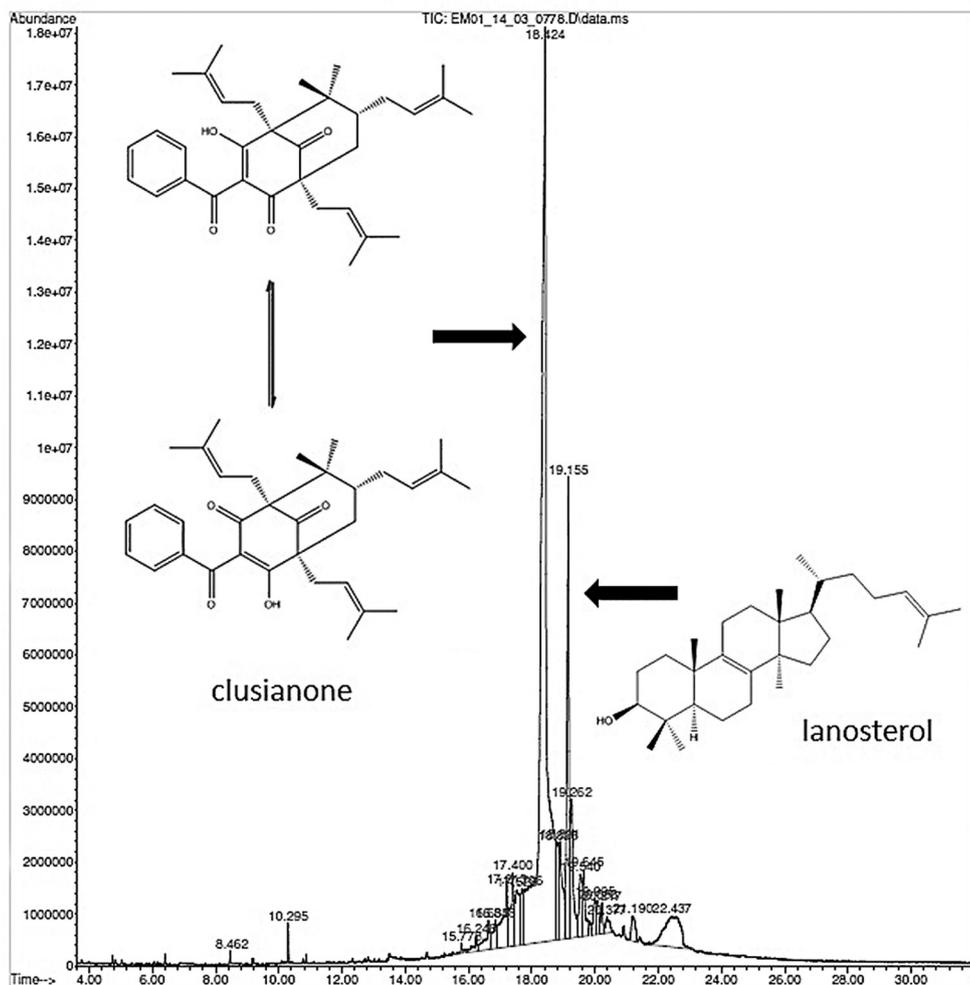


Fig. 3: chromatogram obtained for the hexanic extract of male flowers of *Clusia fluminensis* by gas chromatography coupled with mass spectrometry showing the benzophenone/clusianone and the triterpene lanosterol as major components.

cariphenone A and cariphenone B has also shown to have larvicidal activity against *Ae. aegypti*, but at a lower concentration than that used in our experiments with clusianone (da Silva et al. 2013a). The genus *Hypericum* is often considered to be a member of the family Clusiaceae.

Benzophenyl and benzopyran compounds, although not benzophenones, have also recently been reported to have similar effects to clusianone against mosquitoes. For example, benzophenyl urea compounds may disrupt chitin, causing changes in development or even mortality in *Culex* and *Aedes* mosquitoes in low doses (Fontoura et al. 2012, Belinato et al. 2013). Additionally, benzopyrans, present mostly in a *Hypericum polyanthemum* lipophilic extract, showed larvicidal activity against *Ae. aegypti* in high concentrations, while in sublethal levels exhibited growth-inhibiting activity to prevent pupae formation and adult emergence (da Silva et al. 2013b). This last effect was also detected with clusianone against *Ae. aegypti* in the present study, where the few survivors remained as larvae. This result is very important for the evaluation of the potential use of these compounds as insecticides, because *Ae. aegypti* treated with a successful commercial analogue of

JH (methoprene) also remained as larvae or died as pupae, without emerging as adults (Braga et al. 2005a,b).

Finally, the tetranortriterpenoid, azadirachtin, purified from *Azadirachta indica* A. (Juss.) (Meliaceae) acts on the insect neurosecretory cells. Juvenile forms after contact with this product remain irreversibly in the larval stage (Garcia et al. 1986, Mulla & Su 1999), as similarly observed after *Ae. aegypti* treatment with clusianone isolated from *C. fluminensis*. Likewise, benzophenone-3 used in UV filters affects embryogenesis by delaying hatching and mimicking the action of ecdysone, thereby disrupting the insect endocrinological equilibrium (Ozáez et al. 2014). Therefore, evidence from previous studies and for clusianone indicates that many naturally-occurring plant insect control agents affect the pest endocrine homeostasis to kill, delay or interrupt the development of their target species.

In conclusion, the present research shows, for the first time, the insecticidal activity of the benzophenone, clusianone, against mosquitoes and represents a promising start for the development of natural plant products for the control of insect vectors of tropical diseases.

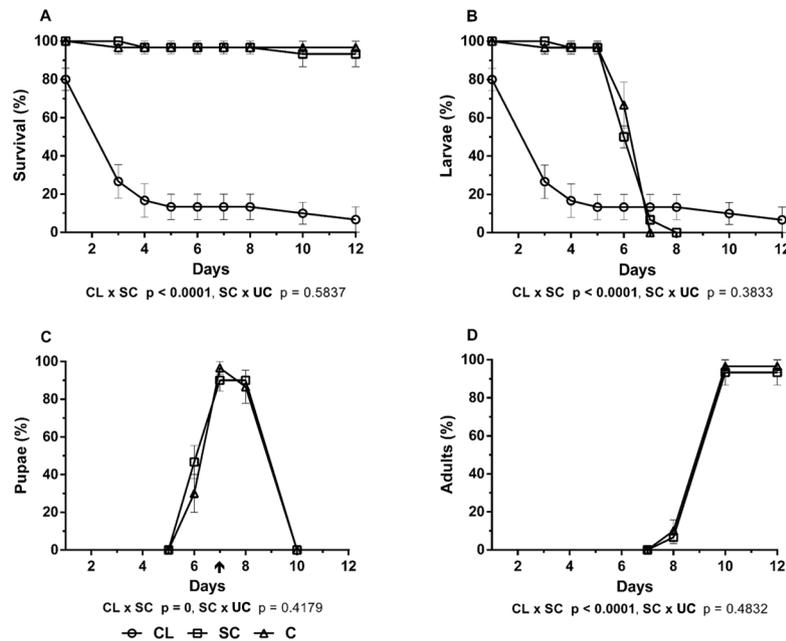


Fig. 4: effects of clusianone (CL) isolated from *Clusia fluminensis* on survival (A) and development of larvae (B), pupae (C) and adult (D) of *Aedes aegypti* at different days after experimental treatment (CL) compared with the solvent control (SC) that was compared with untreated control (UC). The assays were executed and analysed as in the Fig. 1 legend and Materials and Methods.

ACKNOWLEDGEMENTS

To Felipe Leite and Jefferson OC de Oliveira (UFF), for technical assistance, and to the command of Forte Barão do Imbuhy (21st Group of Field Artillery), for permission to collect *C. fluminensis*.

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