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Original Article

# Laboratory evaluation of *Clusia fluminensis* extracts and their isolated compounds against *Dysdercus peruvianus* and *Oncopeltus fasciatus*

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ABSTRACT

The effects of the hexanic extracts of the fruits and flowers of *Clusia fluminensis* Planch. & Triana, Clusiaceae, as well as their main constituents, the triterpene lanosterol and the benzophenone clusianone, were evaluated on hemipterans *Dysdercus peruvianus* and *Oncopeltus fasciatus*. The topical treatments of insects with the hexanic extracts significantly affected the survival of *O. fasciatus*, but not that of *D. peruvianus*. Concomitantly, extracts delayed the development of both hemipterans. Moreover, isolated lanosterol significantly reduced both the survival and development of *O. fasciatus* and *D. peruvianus*, while clusianone only reduce the survival of *D. peruvianus* and marginally inhibited the development of both insects. The results show the specific activity of lanosterol and clusianone against the two evaluated insect species and indicate the potential of compounds derived from *C. fluminensis* for the development of specific biopesticides for the control of agricultural pests. Subsequent work will examine the mode of action of lanosterol and clusianone isolates from *C. fluminensis*.



## Introduction

Synthetic insecticides were widely used after World War II (McGraw and O'Neill, 2013) and have played important roles in the control of agricultural pests and tropical disease vectors. These compounds, however, are highly toxic and contaminate the environment causing serious human health problems (Longnecker et al., 2001; Weiss et al., 2004).

In addition, the frequent use of pesticides has enhanced insect resistance and resulted in increased dosages and/or subsequent replacement by other chemicals with even higher toxicity (Hemingway and Ranson, 2000). Therefore, new strategies for insect control are under development, especially with natural products of plant origin that are less harmful to the environment (Stoate et al., 2009).

Co-evolution mechanisms between insects and plants have resulted in the selection of plant secondary metabolites, including pyrethrins, alkaloids, rotenoids and terpenoids, with killer, repellent or growth and developmental inhibitory activities against insects (Isman, 2006; Alexenizer and Dorn, 2007; Miresmailli and Isman, 2014).

The vast Brazilian flora is of primary importance in the search for alternative natural insecticides (Mendonça et al., 2005; Giorgi et al., 2013). *Clusia fluminensis* Planch. & Triana, Clusiaceae, is a native plant from Brazil, found in regions of high luminous intensity and water restriction, such as the "restinga" environment (sandy coastal plains) and rocky outcrops (Bittrich, 2010). Moreover in nature, *C. fluminensis* leaves are rarely attacked by insects.

Previous reports showed that the leaves of species from the genus *Clusia* are rich in terpenes, especially triterpenes and sesquiterpenes (Barrios et al., 1990; De Andrade et al., 1998;

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Compagnone et al., 2008; Marín et al., 2008; Guimarães et al., 2013). Nagem et al. (1993) identified the pentacyclic triterpenes, amyrin, friedelin, α-friedelinol and β-friedelinol as well as the tetracyclic triterpene, lupenone, in the leaves of *C. fluminensis*. Recently, lanosterol and clusianone from the fruits and male flowers of *C. fluminensis*, respectively, were isolated and structurally characterized (Silva et al., 2012; Oliveira et al., 2014). Subsequently, clusianone was shown in laboratory assays to induce high mortality and to inhibit development of *Aedes aegypti* larvae (Anholeti et al., 2015).

Clusianone is a benzophenone of which there are many derivatives (Beerhues and Liu, 2009; Li et al., 2014) but only a few studies of their insecticide properties have been made (Middleton and Chadds, 1970; Ranganatha et al., 2013). For example, the benzophenones, cariphenone A and cariphenone B, from *Hypericum carinatum* Griseb., Hypericaceae, have anti-mosquito activity (da Silva et al., 2013), while benzophenones present in commercial UV filters mimic ecdysone activity in *Chironomus riparius* (Meigen, 1804) (Diptera: Chironomidae) and affect development (Ozáez et al., 2014).

Lanosterol is known generally to be the precursor of ergosterol in fungi and cholesterol in animals (Phillips et al., 2006). Although cycloartenol is the main precursor of steroids in vegetable, studies have shown that plants are capable of producing lanosterol directly from 2,3-oxidosqualene cyclase (Suzuki et al., 2006). Studies involving terpenes show that these metabolites are capable of causing changes in the endocrine system of insects, acting as agonists or hormone antagonists, killing them or preventing them from reaching adulthood (Bowers et al., 1976). In a previous study with *C. fluminensis* clusianone showed a significant reduced survival of *Aedes aegypti* larvae. However, no activity was detected with lanosterol on the survival or development of *A. aegypti* larvae (Anholeti et al., 2015).

*Dysdercus peruvianus* (Guérin-Menéville) (Hemiptera: Pyrrhocoridae) and *Oncopeltus fasciatus* (Dallas) (Hemiptera: Lygaeidae) are often used as models for drug testing in basic research and applied entomology (Fernandes et al., 2013; Tietbohl et al., 2014). The cotton stainer bug, *D. peruvianus*, damages cotton seeds and spoils the cotton fibers. It is also a vector for phytopathogenic microorganisms and may result in great losses in cotton production (Gallo, 1988). In Brazil, *Dysdercus* spp. are targets for insecticides in cotton plantations, however, there are few specific products for the control of these pests (Gallo, 1988). The milkweed bug, *O. fasciatus*, is also a hemipteran insect, but unlike *Dysdercus* spp., is not usually regarded as a pest species but has been used extensively as a model in biological research (Liu and Kaufman, 2009).

The present paper investigates the activity of *C. fluminensis* hexanic extracts, and their major isolated components, lanosterol and clusianone, against two pest insect species, *D. peruvianus* and *O. fasciatus*. The results show that compounds from *C. fluminensis* have selective activities with lanosterol killing *O. fasciatus* with greater efficiency than *D. peruvianus* while clusianone killed *D. peruvianus* but not *O. fasciatus*.

## Material and methods

### *Insect colonies*

Colonies of *Oncopeltus fasciatus* and *Dysdercus peruvianus* were established in the Laboratory of Insect Biology of the Universidade Federal Fluminense, and kept at constant temperature (26 ± 1 °C), photoperiod (16L:8D) and relative humidity (60% ± 5) (Milano et al., 1999). The insects were housed in transparent glass pots, covered in netting and water provided *ad libitum*. *O. fasciatus*, were reared under similar conditions to *D. peruvianus* but fed, respectively, on sunflower seeds (*Helianthus annuus* L., Asteraceae) and cotton seeds (*Gossypium hirsutum* L., Malvaceae) (Feir and Beck, 1963; Feir, 1974). The seeds were placed inside the pots during the mating and laying period and up to the first instar. From the second instar on, the insects were transferred to clean pots each week, in order to avoid seed contamination with insect feces and facilitate cleaning, the seeds were placed on top of the netting closing the pots. All the insecticidal activity experiments were conducted at a constant temperature of 26 ± 1 °C.

### *Plant material and extract preparation*

Flowers from male individual and fruits from female individual of *Clusia fluminensis* Planch. & Triana, Clusiaceae, were collected in the summer and autumn, respectively, in Niterói (Rio de Janeiro State, Brazil). The identification of plants and the preparation of extracts were as described previously (Silva et al., 2012; Anholeti et al., 2015).

### *Isolation of substances and chemical analysis of crude extracts*

The polyisoprenylated benzophenone, clusianone, and the triterpene, lanosterol, were isolated and analyzed from the flowers and fruits, respectively, of *C. fluminensis* using various chromatographic and mass spectrometric techniques as described by Silva et al. (2012), Oliveira et al. (2014) and as modified in Anholeti et al. (2015).

### *Insect bioassays*

Randomly selected, 4$^{th}$ instar nymphs of *O. fasciatus* and *D. peruvianus*, were treated with sample solutions, which were applied topically to the dorsal cuticle of each insect. The crude extracts from *C. fluminensis* were dissolved in ethanol at a concentration of 1 mg/ml, and 1 μl of each sample was applied. The isolated substances (clusianone and lanosterol) were applied similarly, except that they were dissolved in acetone to a concentration of 0.7 mg/ml (0.7 μg/insect).

The control groups were untreated (C) or treated solely with the solvents (SC) used to dissolve the samples. Biological evaluation of the results of the different treatments was performed daily from the beginning of the 4$^{th}$ instar up to the adult stage. Observations were made of survival (mortality), the intermolt and metamorphosis periods, and the presence of premature adult characteristics and body deformities. The experiments were terminated at the death or emergence to adults of all insects from the control groups.

All experiments were repeated at least twice with batches of ten fully engorged insects with replicates of six for each of the three groups (experimental, C and SC). The results are derived from the media of the percentage of each replicate from the day after topical application on 4$^{th}$ instar nymphs (1$^{st}$ day) to the last day of observation.

### *Data and statistical analysis*

All graphs were created with GraphPad Prism 6.05 software (GraphPad Software, San Diego, CA, USA), showing the survival and the developmental course of the juvenile stage and adults.

The Gehan-Breslow-Wilcoxon test was performed employing Graph Prism software version 6.05 in order to compare the whole curves of survival and adult development. Moreover, the Suissa and Shuster test employing Z-pooled as statistic, using “Exact” package version 1.6 for R program version 3.3.1 (Suissa and Shuster, 1985; Calhoun, 2015; R Core Team, 2016), was used to compare proportions of immature individuals between independent groups in representative days of each experiment indicated by an arrow in

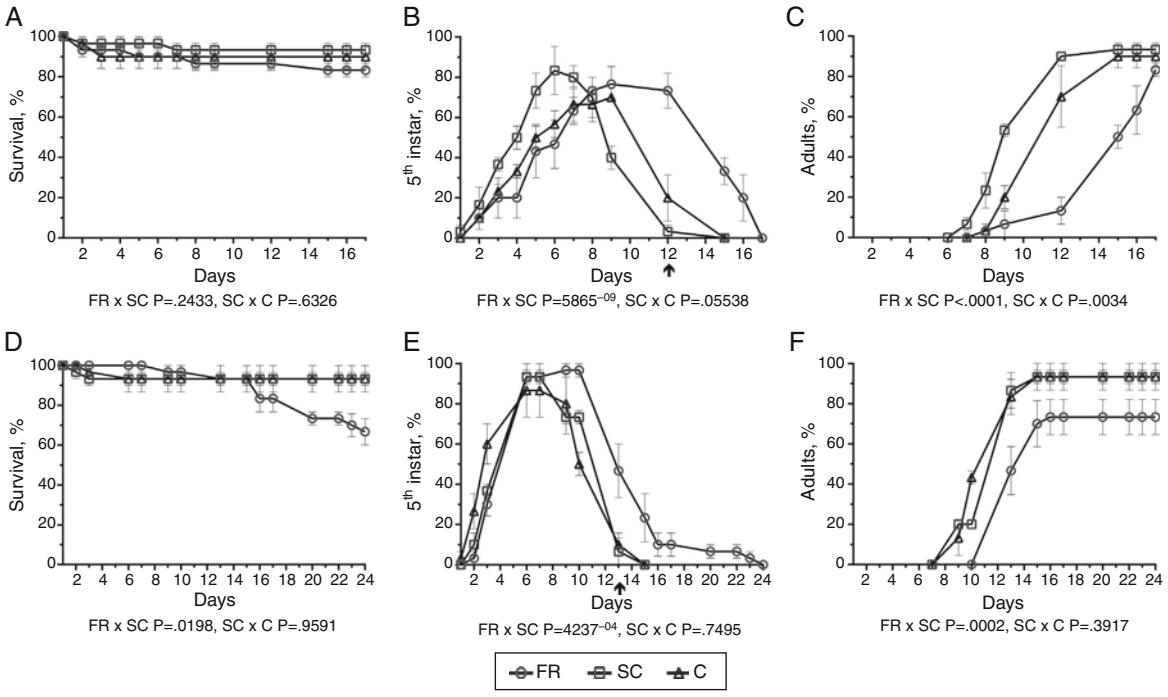


**Fig. 1.** Effects of extracts of fruit (FR) from *Clusia fluminensis* on survival (A, D) and development of nymphs (B, E), adults (C, F) of *Dysdercus peruvianus*, (A, B, C) and *Oncopeltus fasciatus* (D, E, F), at different days after experimental treatment. (FR, ○) compared with the solvent control (SC, □), that was compared with the untreated control (C, Δ). Fourth instar hemipteran nymph were topically treated with 1 μg of extract in 1 μl of solvent. Statistical analyses (under the graphs) with the Barnard's test were used (B, E) on a representative day indicated by an arrow and the Gehan-Breslow-Wilcoxon test was used (A, C, D, F) to compare the entire curve between the groups (FR × SC and SC × C). Each point represents the medium of at least six replicates with ten insects and bars show SE. Significant differences ($p$ value < 0.05) are in bold.

graphs. The $p$-values generated by Exact package were confirmed by Fortran program XUN2X2 version 2.0 (Berger, 1996) and Barnard package version 1.6 for R program (Erguler, 2015). In all experiments, only $p$-values < 0.05 were considered statistically significant and no corrections for multiple comparisons were made (Rothman, 1990).

## Results

The bioassays with the hexanic extract of *C. fluminensis* fruit (FR) showed that in the treated groups there was no effect on survival rates of *D. peruvianus* (Fig. 1A). In contrast, in experiments with *O. fasciatus*, the survival curve showed a significant ($p$ < 0.02, in bold)

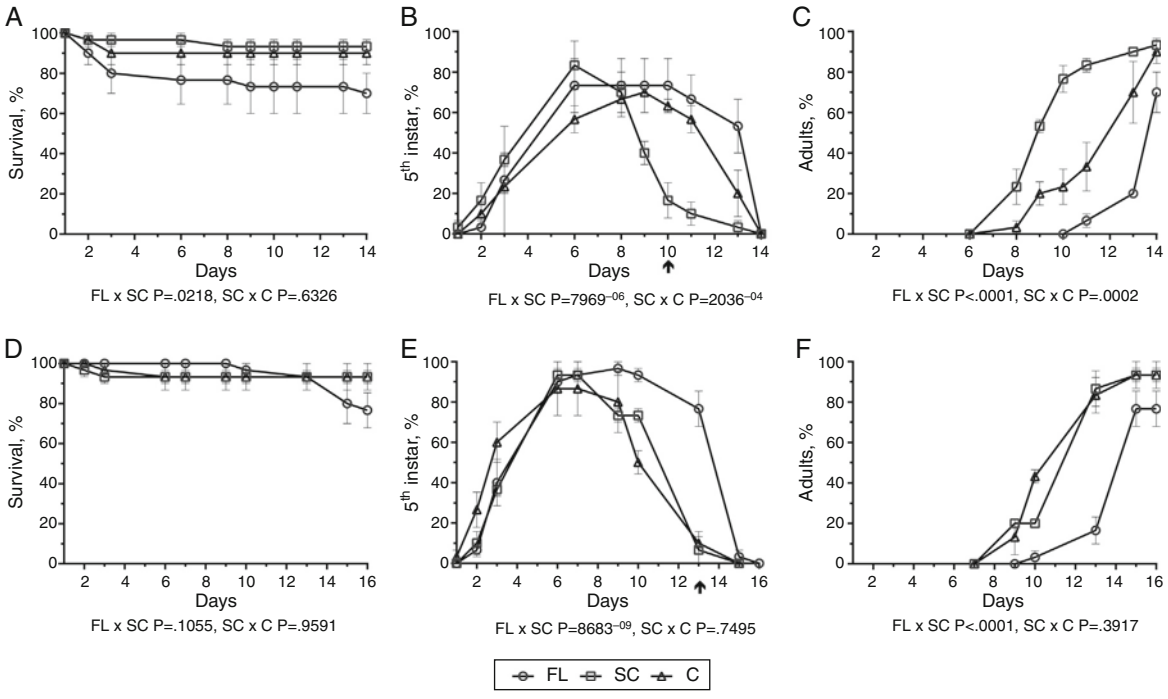


**Fig. 2.** Effects of extracts of flower (FL) from *Clusia fluminensis* on survival (A, D) and development of nymphs (B, E), adults (C, F) of *Dysdercus peruvianus*, (A, B, C) and *Oncopeltus fasciatus* (D, E, F), at different days after experimental treatment. (FL, ○) compared with the solvent control (SC, □), that was compared with the untreated control (C, Δ). The assays were executed and analyzed as in Fig. 1 legend and "Materials and methods" section.

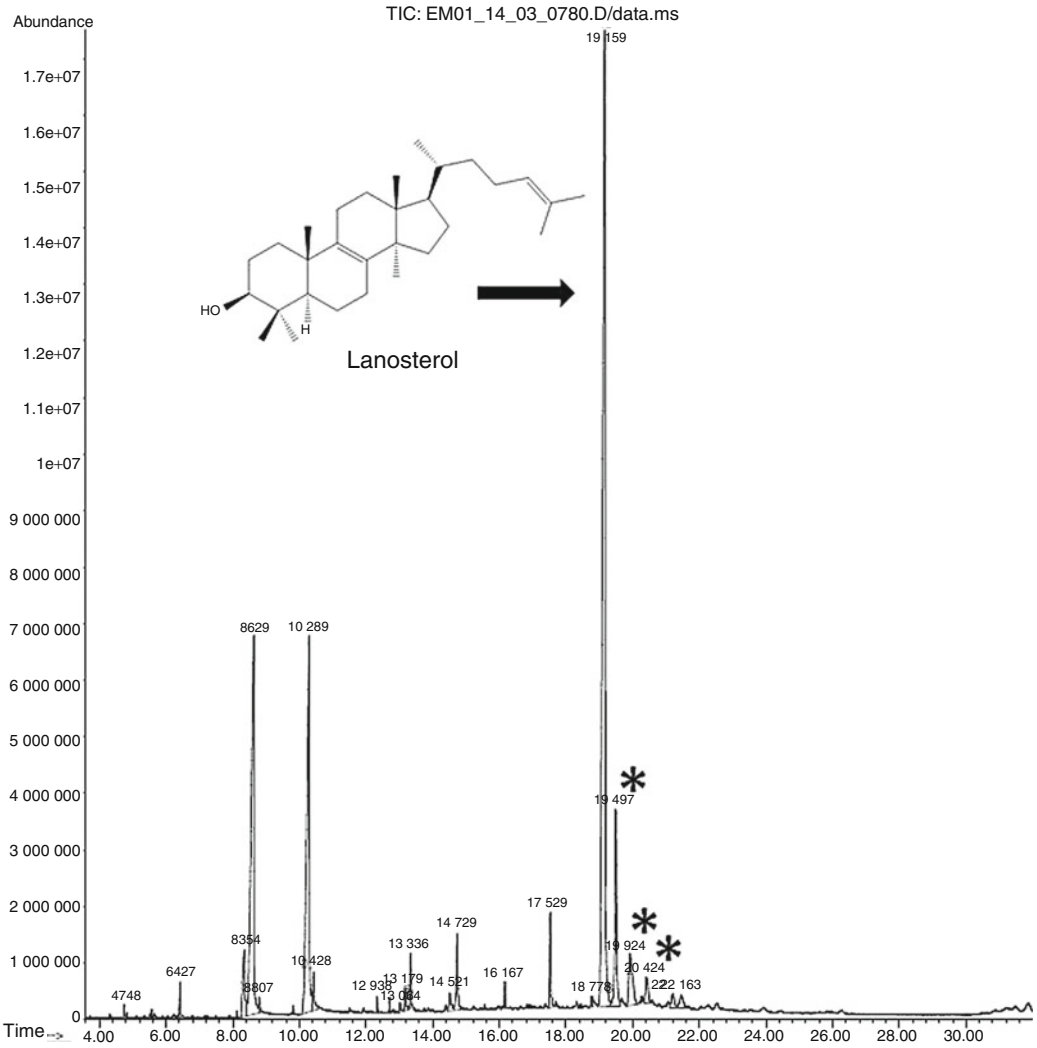


**Fig. 3.** Chromatogram obtained for the hexanic extract of fruits of *Clusia fluminensis* by GC–MS showing the triterpene lanosterol as the major component. * Lanosterol isomers.

mortality rate in FR group compared with the solvent control (SC) (Fig. 1D), so that by the last day of testing ($24^{th}$), 33.3% of the treated insects were dead compared to 6.7% of the control groups (Fig. 1D).

In addition, the development of nymphs and adults in FR treated *O. fasciatus* and *D. peruvianus* showed significant delays ($p \leq 0.0002$) in comparison with the controls (Fig. 1B, C and E, F). For example, with *D. peruvianus* at day 12, 90% of the insects from the SC group had metamorphosed (Fig. 1C), while 73.3% of the insects of the FR group were still juveniles (Fig. 1B). Likewise, at day 13 with *O. fasciatus* 86.7% of the insects from the SC group were adults (Fig. 1F) while 46.7% of the FR group were still nymphs (Fig. 1E).

In contrast with the FR, the bioassays with the hexanic extract of *C. fluminensis* flowers (FL), resulted in significant mortality in the *D. peruvianus* ($p = 0.0218$) experimental group compared with the SC (day 14. 3% *versus* 6.7%, respectively, Fig. 2A) as well as no significant effect on the *O. fasciatus* survival in comparison with the SC ($p = 0.1055$, Fig. 2D).

Similar to the effects of FR on development to adults, both insect species showed significant delays in molting ($p \leq 0.0001$) between the FL-treated and the SC groups (Fig. 2C and F). For example, on day 10 with *D. peruvianus*, 76.7% of the insects in the SC group had molted to adults, while none of them had metamorphosed in the FL group (Fig. 2B and C). Likewise, on the day 13 with *O. fasciatus*, 86.7% of the insects had become adults in the SC group, while only 16.7% of the FL group had reached this stage (Fig. 2E and F).

The hexanic extracts of the fruits and flowers were submitted to GC–MS. The chromatogram obtained for the hexanic extract of fruits of *C. fluminensis* showed a peak at 19.16 min, corresponding to a substance that represents 40.6% of the sample composition. The mass spectrum of this substance is similar to that provided by the equipment's database for the triterpene, lanosterol. The mass fragmentation pattern observed is also consistent with the data provided by Shin et al. (2000) for this substance. The triterpene is accompanied by three isomers (retention times: 19.50, 19.92, 20.42 min) with the same mass fragmentation pattern of lanosterol, and that together correspond to 10.73% of sample composition (Fig. 3). Other substances present in appreciable amount in this extract, with retention times of 8.63 min and 10.29 min (Fig. 3), showed mass fragmentation patterns consistent with fatty acids, and were identified, respectively, as palmitic and oleic acids.

Moreover, the GC–MS data obtained for the hexanic extract of flowers of *C. fluminensis* suggests the presence of lanosterol (9.7%) and clusianone (54.8%), as shown in a previous paper (Anholeti et al., 2015).

The purification protocols and chemical structures of lanosterol and clusianone, respectively, from fruits and flowers of *C. fluminensis*, have been described previously (Silva et al., 2012; Oliveira et al.,

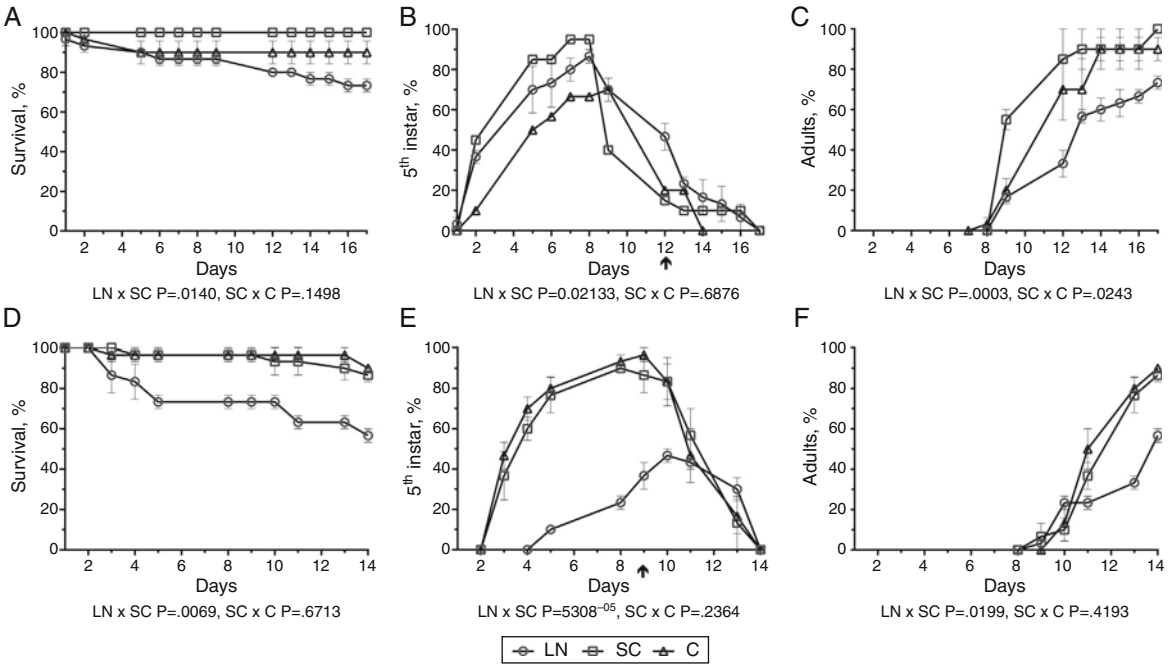


**Fig. 4.** Effects of extracts of lanosterol (LN) isolated from *Clusia fluminensis* on survival (A, D) and development of nymphs (B, E), adults (C, F) of *Dysdercus peruvianus*, (A, B, C) and *Oncopeltus fasciatus* (D, E, F), at different days after experimental treatment. (LN, ○) compared with the solvent control (SC, □), that was compared with the untreated control (C, Δ). The assays were executed and analyzed as in Fig. 1 legend and "Materials and methods" section.

2014). Subsequently, bioassays with these two purified substances were performed against *D. peruvianus* and *O. fasciatus*.

In the bioassays with lanosterol-treated *D. peruvianus* and *O. fasciatus*, the survival rates were significantly reduced in comparison with the SC groups ($p = 0.014$, Fig. 4A and $p = 0.0069$, Fig. 4D, respectively). Regarding the development of the nymphs to adults, significant delays occurred with lanosterol treatment although variations were observed between the *D. peruvianus* and *O. fasciatus* groups. With *D. peruvianus*, there was a significant difference in the adult development between the lanosterol and SC curves ($p = 0.0003$, Fig. 4C). Thus, by day 12, 33.3% of the nymphs from the lanosterol group had molted compared with 85% in the SC group (Fig. 4B and C). Similarly, with *O. fasciatus*, at day 9 of lanosterol treatment, only 36.7% of the nymphs of *O. fasciatus* had molted to 5th instar whereas in the control groups ($p = 5.308^{-05}$) at least 93.9% of insects had already reached this same nymphal stage (Fig. 4E). The differences between the curves of development to adult were also statistically significant for the lanosterol-treated insects compared to the SC group ($p = 0.0199$, Fig. 4F).

In experiments with lanosterol-treated *O. fasciatus* and *D. peruvianus*, 3.3% (SE ± 3.3) malformed insects with deformed wings and ruffled cuticle were observed (Fig. 5). These abnormal forms appeared after 4–6 days testing with 4th to 5th instar nymphs that should have molted to 5th instars. These malformed specimens died after or during molting, often confined within the old cuticle.

The bioassays with clusianone showed a significant reduction in survival of *D. peruvianus* ($p < 0.0048$, Fig. 6A) but not of *O. fasciatus* ($p > 0.6685$, Fig. 6D) in comparison with the SC groups. On the last day with *D. peruvianus* (17th day), only 66.7% of insects survived compared with 100% of the SC group (Fig. 6A). Regarding development, significant delays ($p = 0.01037$ at 7 days, Fig. 6B and C) were recorded with *D. peruvianus* but, surprisingly, clusianone accelerated significantly ($p = 4.077^{-04}$ at 11 days) the development of *O. fasciatus* (Fig. 6E and F).

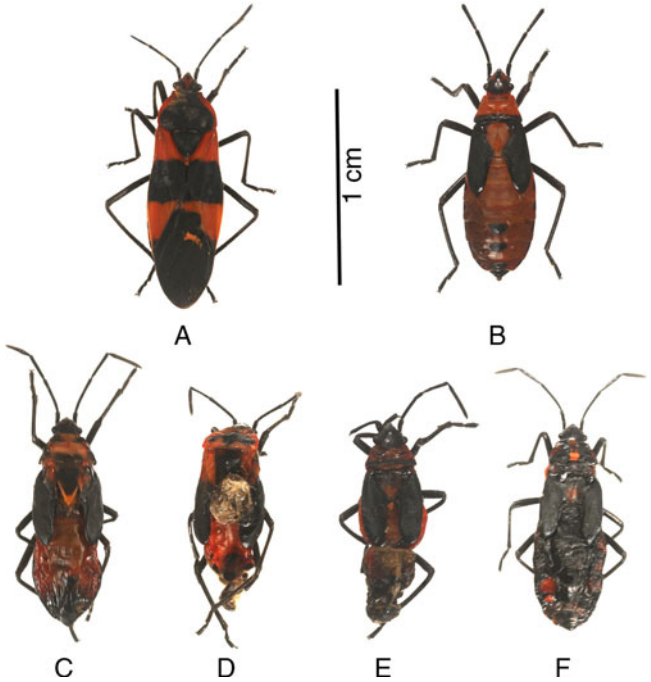


**Fig. 5.** Normal 5th instar (A) and adult (B) of *Oncopeltus fasciatus* from control group (PC). Deformed specimens of *O. fasciatus* from 4th instar abnormal molt after treatment with lanosterol (C to F). Bar = 1 cm.

Interestingly, only in assays with *D. peruvianus* were significant differences detected between C and SC groups. Control groups containing the solvent (SC) had the effect of advancing the development of this insect species, compared with the C group (Figs. 1C, 2B and C, 4C, 6B and C).

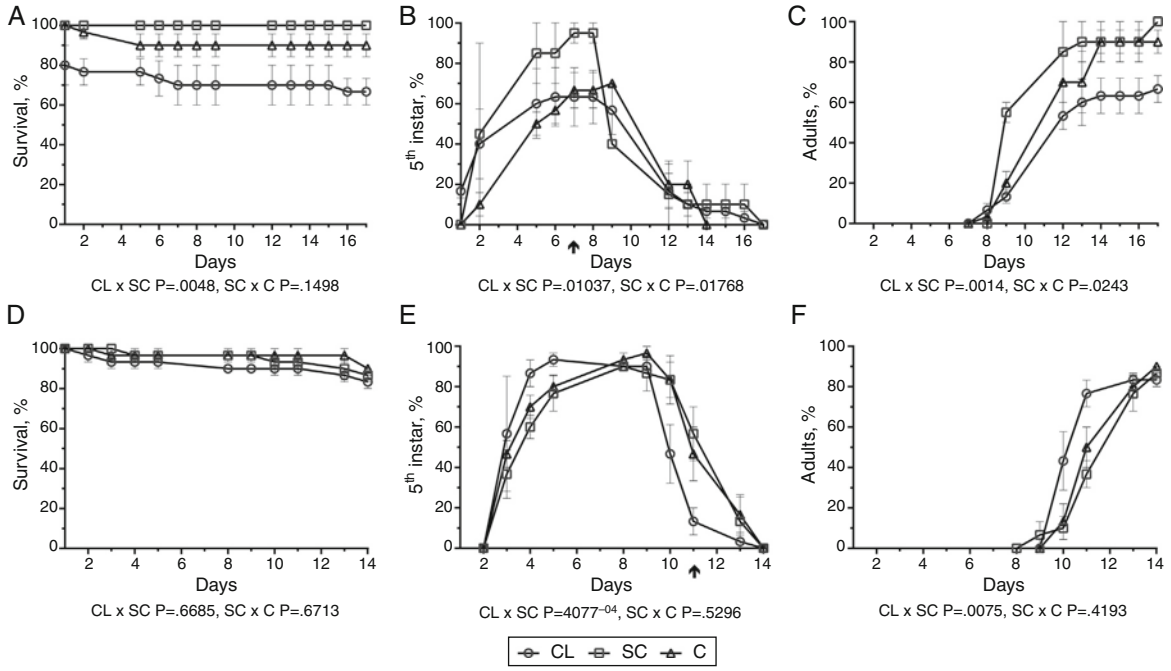


**Fig. 6.** Effects of extracts of clusianone (CL) isolated from *Clusia fluminensis* on survival (A, D) and development of nymphs (B, E), adults (C, F) of *Dysdercus peruvianus*, (A, B, C) and *Oncopeltus fasciatus* (D, E, F), at different days after experimental treatment (CL, ○) compared with the solvent control (SC, □), that was compared with the untreated control (C, Δ). The assays were executed and analyzed as in Fig. 1 legend and "Materials and methods" section.

## Discussion and conclusion

In the present study, the triterpene, lanosterol, was further identified and purified from the hexanic extracts of the fruits (FR) and flowers (FL) of *C. fluminensis*, following the original protocols described by Oliveira et al. (2014). In crude extracts of the flowers, lanosterol represents only 10% of the contents (Anholeti et al., 2015) while in extracts of the fruits, this compound makes up 40.46% of the extract. The high lanosterol content in the FR extract probably accounts for some similarities in survival and development obtained with the two insect species tested with the FR extract and purified lanosterol (compare Figs. 1 and 4). *O. fasciatus* was more sensitive to the FR extract and lanosterol, in terms of mortality, than *D. peruvianus*, and no activity was detected against *Ae. aegypti* with either treatment (Anholeti et al., 2015). Molting aberration in last stage *Oncopeltus* nymphs, treated with IGR's pesticides have been described (Redfern et al., 1982). However, the appearance of some precocious adults with deformations after the 4$^{th}$ instar hemipterans were treated (Fig. 5) could indicate the anti-juvenile hormone activity of lanosterol (Bowers et al., 1976).

The effect of plant metabolites with anti juvenile hormone activity has been described against many insects, especially after the identification of the precocenes purified from plants of the genus *Ageratum* (Bowers et al., 1976; Pratt et al., 1980). These substances have marked effects on hemipterans and induce the appearance of early adults (adultoids) with characteristics similar to those found in the presumptive adultoid bugs resulting from the treatment with lanosterol in the present study (Masner et al., 1979; Unnithan et al., 1977; Jurberg et al., 1984).

The lanosterol effect is similar to the precocenes, have less effects in holometabolous insects (Kelly and Fuchs, 1978; Staal, 1986; Erezyilmaz et al., 2006), which could explain why *A. Aegypyi* was not susceptible to lanosterol (Anholeti et al., 2015) even up to concentrations of 100 μg (unpublished result).

Apparently, the precocenes inactivate the corpora allata and consequently interrupt the production of juvenile hormone (Bowers and Aldrich, 1980). However, anti-JH activity can also occur with substances that compete with JH for the recognition receptors in the cells of target tissues (Staal, 1986). In addition, some substances, such as lanosterol, can act as precursors in the biosynthesis of JH, producing a hormone with altered specificity (Staal, 1986). Lanosterol can be a precursor of cholesterol and the biosynthesis of juvenile hormone is similar to that of cholesterol (Miao et al., 2002).

Following application, the uptake of lanosterol into the hemolymph may not only modify the synthesis of JH but also the JH receptors on the target cells by substituting for cholesterol in the cell membrane and significantly altering the structural and functional integrity of the cell (Miao et al., 2002) to bind or respond appropriately to JH. Cholesterol substitution by lanosterol may also interfere/modify the entry of small molecules and ions into and out of epithelial cells of the exoskeleton and compromise the chitin synthase secretory pathway producing the cuticle (Merzendorfer and Zimoch, 2003) and result in insect deformations.

In the present study, and in contrast to their effects on *Aedes aegypti* (Diptera: Culicidae) (Anholeti et al., 2015), FL and clusianone had no or limited effects on killing the hemipterans and delaying their development. Moreover, clusianone was further identified and purified from the hexanic extracts of flowers (FL) of *C. fluminensis*. Clusianone at 55% is the major component of *C. fluminensis* flowers extract (Anholeti et al., 2015) with lanosterol also present in FL but making up less than 10% of the composition.

In *O. fasciatus*, clusianone actually accelerated development, as well the solvent controls in *D. peruvianus*. We have observed in our laboratory that some compounds that stress the nymphs can accelerate their development to the adults stage (unpublished observation), as if attempting to escape an unfavorable environment.

Further studies must be carried out to clarify the mode of action of lanosterol and clusianone isolated from *C. fluminensis* since they appear to affect hemimetabolous and holometabolous insects differentially. The present work shows the potential of these substances for the development of biopesticides with more specificity of action against agricultural pests.

## Ethical disclosures

**Protection of human and animal subjects.** The authors declare that no experiments were performed on humans or animals for this study.

**Confidentiality of data.** The authors declare that no patient data appear in this article.

**Right to privacy and informed consent.** The authors declare that no patient data appear in this article.

## Authors contributions

MCA, MRF, MACK, SRP prepared extracts, isolated substances and analyzed chemical data from the plant material. MGS collected the plant material and identified the plant. RCD, MSG, DF, CBM, NAR conceived, designed research and analyzed data of insect bioassays. MCA, RCD, DF, NAR and CBM wrote the manuscript. RCD, BPS, JPFP, MCA conducted insect bioassays. All authors read and approved the manuscript.

## Conflicts of interest

The authors declare no conflicts of interest.

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