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Short communication

Effects of semi-purified fractions from stems of *Clusia hilariana* on the development of *Dysdercus peruvianus*

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ABSTRACT

Plants represent a huge source of substances, with pharmacological potential. Brazil has a diversity of agricultural insect pests and an urgent need for safer methods of insect control. *Dysdercus peruvianus* (Guerin-Meneville, 1831), Pyrrhocoridae, is an economically important species of the Order Hemiptera and a pest of cotton (*Gossypium hirsutum* L., Malvaceae). Secondary metabolites in stems of *Clusia hilariana* Schltdl., Clusiaceae, such as terpenes and benzophenones, have been reported to be insecticidal. The present study investigated the effects of semi-purified fractions of hexane crude extracts from male *C. hilariana* stems on development of *D. peruvianus.* Biological parameters at different stages of development including body malformations, range of molting period and toxicity were evaluated. Most insects died and failed to develop due to attachment of their exuviae to the abdomen. Deformations of wings and defective tarsi also occurred. The secondary metabolites from semi-purified fractions of *C. hilariana* caused mortality, interference in molting and metamorphosis, and body deformations, probably by interacting with the neuroendocrine system. The results demonstrate the potential of *C. hilariana* extracts as an alternative for the control of the phytophagous insect *D. peruvianus* and for the development of environmentally safe and biodegradable bio insecticides.

Introduction

Insecticides are important resources for agriculture, since insect pests are still the main cause of food productivity loss, with a significantly negative impact on agribusiness (Weiss et al., 2004). In 1975, the Brazilian National Program of Agricultural Defense aimed to meet the growing demand for agricultural production through the use of agrochemicals. Indiscriminate use of these products resulted in both ecological imbalances due to disappearance of native insect species and environmental contamination (Silva and Flores, 1993). Unfortunately, common insecticides are highly toxic leaving residues and, in addition, target insects are developing increasing resistance (Weiss et al., 2004).

Research is identifying new alternatives to synthetic pesticides with more selectivity and both lower impacts on the environment and human health. Thus, plant secondary metabolites have been studied as they are phago inhibitors and hormone regulators of insect development with repellent or insecticidal activity (War et al., 2012).

The phytophagous insect genus *Dysdercus*, Hemiptera - Pyrrhocoridae, including more than 300 species, are commonly known as cotton-stained bugs, and associated with fungal spread on cotton (*Gossypium hirsutum* L., Malvaceae) leading to significant economic losses (Gallo, 1998). *D. peruvianus*(Guerin-Meneville, 1831)

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Table 1
Development of *Dysdercus peruvianus* after fourth-instar nymphs topical treatment with semi-purified fractions obtained from *Clusia hilariana* stems hexanic crude extract.

Groups	% Mortality		% Moulting/range days		% Metamorphosis/range		% Deformed insects	
	24 h	28 days	From 4th to 5th instar	Range days	From 5th to adult	Range days	5th instar	Adults
Non-treated insects	0	8,9 ± 0.2 ns	100	3rd–15th	91.1 ± 0.2 ns	10th–27th	0	0
Solvent acetone	2.2 ± 0.1 ns	14.4 ± 0.2 ns	100	1st–17th	85.6 ± 0.3 ns	10th–20th	0	0
Fraction 1/insect:								
0.005 mg	2.2 ± 0.1 ns	36.7 ± 0.3 c	100	1st–26th c	63.3 ± 0.3 c	11th–28th	3 ± 0.2	0
0.010 mg	1.1 ± 0.1 ns	27.7 ± 0.6 a	100	4th–18th	72.2 ± 0,6 a	11th–18th	22. ± 0.6 b	0
0.040 mg	0	18.9 ± 0.3 ns	100	1st–13th	81.1 ± 0.3 ns	10th–17th	5 ± 0.2	2 ± 0,1
Fraction 2/insect:								
0.005 mg	5,6 ± 0.2 ns	14.4 ± 0.1 ns	97.76 ± 0.06 ns	1st–28th b	83.3 ± 0.1 ns	8th–24th b	4 ± 0.2	0
0.010 mg	1.1 ± 0.1 ns	16.7 ± 0.2 ns	100	1st–20th a	83.3 ± 0.2 ns	11th–18th	0	9 ± 0.3
0.040 mg	1.10 ± 0.1 ns	32.2 ± 0.4 b	100	3rd–13th	67.8 ± 0.4 b	10th–22th	18 ± 0.5 b	0
Fraction 3/insect:								
0.005 mg	4.43 ± 0.1 ns	20 ± 0.3 ns	100	1st–24th b	80.0 ± 0.3 ns	8th–26th a	4 ± 0.2	0
0.010 mg	0	34.4 ± 1.16 b	100	1st–20th a	65.6 ± 1.2 b	11th–20th	15 ± 0.4 a	5 ± 0.2
0.040 mg	1.1 ± 0.1 ns	38.9 ± 0.9 c	100	1st–18th b	61.1 ± 0.9 b	8th–25th b	12 ± 0.3 b	0
Starycide®SC:								
0.024 mg	1.10 ± 0.1 ns	68.9 ± 0.2 d	100	1st–13th	31.1 ± 0.2 c	8th–13th c	26 ± 0.7 c	5 ± 0.2
0.048 mg	6.7 ± 0 ns	100	2.23 ± 0.06	1st–15th	0.00	–	36.7 ± 0.4 ns	0

Control 1: non-treated insects; Control 2: insects treated with acetone only. Fraction 1: insects treated with Fraction 1 (hexane 100%). Fraction 2: insects treated with Fraction 2 (hexane and ethyl acetate 25%). Fraction 3: insects treated with Fraction 3 (hexane and ethyl acetate 50%). Control 3: insects treated with positive control (Starycide®SC 4,8 p/p triflumuron). Significance: a – $p < 0.05$; b – $p < 0.01$; c – $p < 0.001$; d – $p < 0.0001$. ns: not significant ($p > 0.05$). All results are expressed as ± SEM.

is important for field research and laboratory experimentation since they are widespread, have short developmental cycles and are easily maintained (Fernandes et al., 2013).

The plant family Clusiaceae is primarily tropical with fifteen genera and 800 species and is a source of many pharmaceuticals. Clusia L., with 300–400 species, is one of the largest genera of this family (Stevens, 2001 *onwards*). The *Clusia* species produce secondary metabolites, including terpenes, and benzophenones with potential insecticide activities although testing against insects is limited (*e.g.* Anholeti et al., 2015). In Brazil, 126 Clusiaceae species belonging to eleven genera, and 67 *Clusia* species has been recorded (Clusiaceae in Flora do Brasil 2020 em construção).

Previously, Duprat et al. (2017) studied the effects of extracts of the fruits and flowers of *Clusia fluminensis* Planch. & Triana on the hemipterans *Dysdercus peruvianus* and *Oncopeltus fasciatus* (Dallas, 1852) with only the mortality of the latter insects significantly affected. The present study, with the extracts of stems from *Clusia hilariana* Schltdl., a Brazilian endemic species, reports significant mortality of treated *D. peruvianus*. It also provides more details of the deformations induced in *D. peruvianus* and of the analysis of the components involved with potential for development in biological control programs

Materials and methods

Plant material

Stems of *Clusia hilariana* Schltdl., Clusiaceae, staminate individuals were collected in Rio de Janeiro State, Brazil (location: S22°12′59.9″ WO41°35′51.6″). The identification of plants and the preparation of extracts were as described previously (Duprat et al., 2017). Stems were dried at 40 °C and reduced to small fragments with an industrial blender.

Crude extracts

The plant material (29,989 g) was extracted by static maceration with hexane (1:7, plant:solvent). The solvent was renewed six times and then filtered and reduced in rotary evaporator, resulting in the crude hexanic extract of stems (CHMSH, 1243 g). Column chromatography and gas chromatography are as detailed in the supplementary material.

Insect colony and treatments

The experiments were carried out on *D. peruvianus* fourth instar nymphs as described by Fernandes et al (2013) with modifications. Four groups of thirty insects each were used in triplicate and tests were performed by topical application. Control 1 group received no treatment. Control 2 group received treatment with acetone. The positive control Starycide®SC 4.8 w/w triflumuron was used on Control Group 3 and insects were treated with 0.5 μl (0.024 mg/insect) or 1 μl (0.048 mg/insect) of the pure product on their dorsal cuticle. The fourth group was treated with semi-purified fractions of *C. hilariana.* solubilized in acetone. For testing purposes, 5, 10 or 40 mg of semi-purified fractions 1, 2 and 3 were dissolved in 1 ml of acetone, resulting in solutions in final concentrations of 5, 10 and 40 mg/ml. The following biological parameters were evaluated: different stages of development; body malformations; range of molting period and toxicity (death within 24 h and at different developmental stages).

Scanning electronic microscopy analysis (SEM)

SEM was performed on insects of control groups 1 and 2, on insects that died during the experiment, and on insects with deformations after treatment with fractions 1, 2 and 3 at concentrations of 5, 10 or 40 mg/ml. Insects were fixed in 2.5% glutaraldehyde (GA). The treatment of insects for SEM followed that of Alencar et al. (2019). The statistical tests for group comparisons were performed using Excel 2010 and Graphpad Prism Program 6. The significance of results was analyzed using PAST (Paleontological Statistics) Program, version 2.16, subjected to Probit. Differences between treated and control groups were considered not significant once $p > 0.05$.

Results and discussion

Topical treatment of *D. peruvianus* fourth instar nymphs with semi-purified fractions from *C. hilariana* at different doses significantly increased mortality of *D. peruvianus*, with a time of exposure-dependent response (Table 1). The most significant results were with Fraction 1 at 0.005 mg/insect (36.7 ± 0.3%), Fraction 2 at 0.040 mg/insect (32.2 ± 0.4%) and Fraction 3 both 0.010 mg/insect and also at 0.040 mg/insect (34.4 ± 1.2% and 38.9 ± 0.9%). In contrast, control Group 1 only showed mortality, at 8.90 ± 0.2%, after 28 days treatment while control Group 2

Fig. 1. Abnormalities after treatment with semi purified fractions of *Clusia hilariana* hexanic crude extracts stems at varying concentrations on 4th instar nymphs of *Dysdercus peruvianus*. Deformation from 4th to 5th molt nymph with Fraction 1 (hexane 100%) at doses of 0.010 mg/insect (A); 0.020 mg/insect (B); 0.040 mg/insect (C); Deformation from 5th to adult molt nymph at dose 0.040 mg/insect (D); Deformation from 5th to adult nymph with Fraction 2 (hexane and ethyl acetate 25%) at doses of 0.005 mg/insect (E); 0.020 mg/insect (F); Deformation from 4th to 5th molt nymph at dose of 0.040 mg/insect (G); Deformation from 5th to adult molt nymph with Fraction 3 (hexane and ethyl acetate 50%) at dose of 0.010 mg/insect (H–I); Deformation on 4th instar nymph after treatment with Fraction 3 (hexane and ethyl acetate 50%) at dose of 0.040 mg/insect (J). 5th and 4th instar nymphs of *D. peruvianus* (K); Adult *D. peruvianus* insect (L). Bars = 0.5 cm.

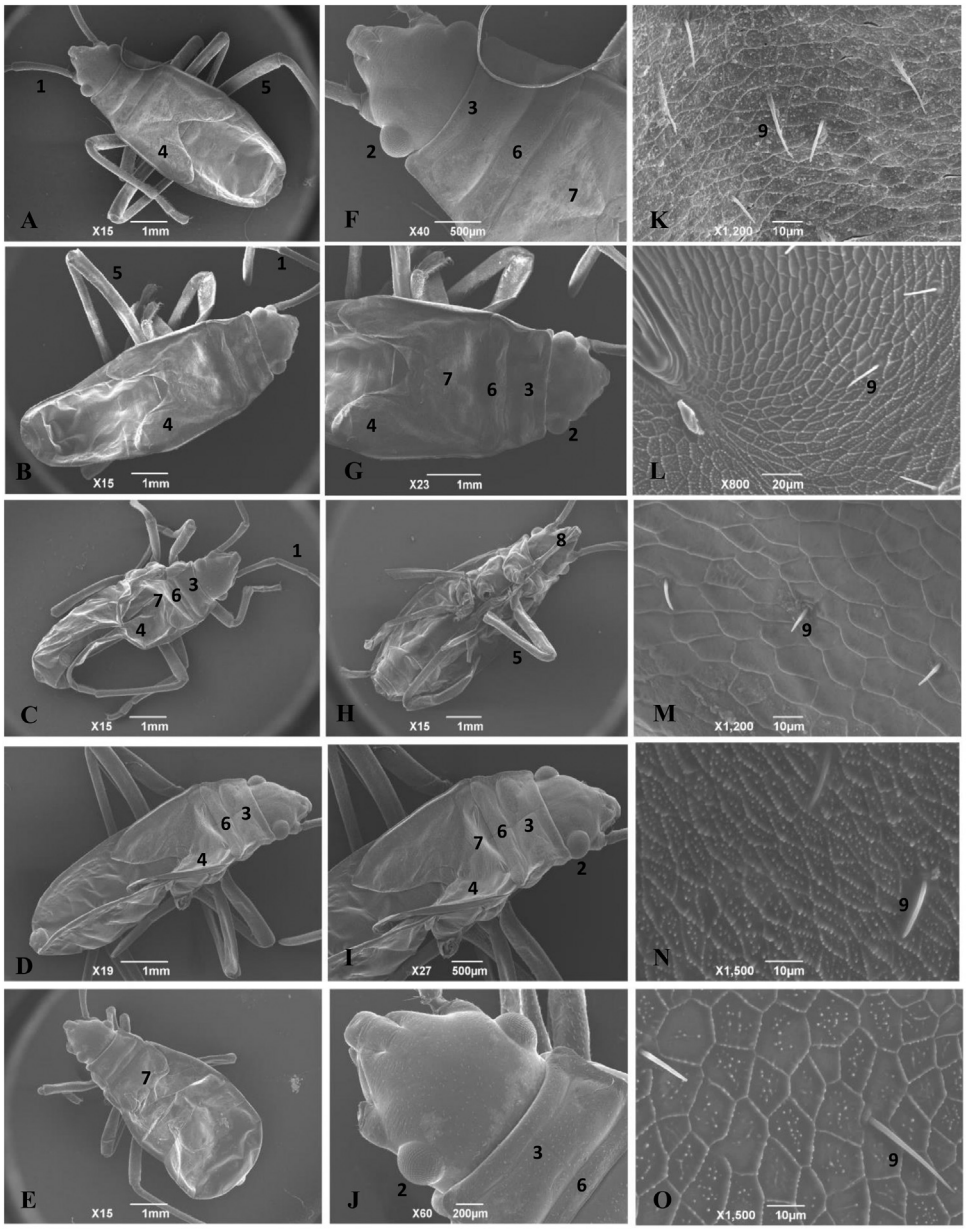

Fig. 2. Electronmicrography obtained with scanning electron microscopy of *Dysdercus peruvianus* insect fourth instar nymphs after topical treatment with Control Group 1 and Control Group 2, and semi purified fractions obtained from hexanic crude extract of *C. hiariana* stems. Dorsal image of fourth instar nymphs: untreated-Control 1 (A); treated with acetone solvent-Control 2 (B); treated with Fraction 1 (hexane 100%) at 0.005 mg/insect dose. Deformities in legs and abdomen, alteration in texture of alar bristle and wrinkled cuticle (C); treated with Fraction 2 (hexane and ethyl acetate 25%) at 0.010 mg/insect dose. Deformed and elongated alar bristle and wrinkled cuticle (D); treated with Fraction 3 (hexane and ethyl acetate 50%) at 0.040 mg/insect dose. Alar bristle was not formed and wrinkled cuticle (E); detailed dorsal image of fourth instar nymph head and thorax: untreated-Control 1 (F); treated with acetone solvent-Control 2 (G); ventral image of fourth instar nymph treated with Fraction 1 (hexane 100%) at 0.005 mg/insect dose (H); treated with Fraction 2 (hexane and ethyl acetate 25%) at 0.010 mg/insect dose and deformed alar bristle (I); treated with Fraction 3 (hexane and ethyl acetate 50%) at 0.040 mg/insect dose (J). Scanning electron microscopy of fourth instar nymph epidermis: untreated-Control 1 (K); treated with acetone solvent-Control 2 (L); treated with Fraction 1 (hexane 100%) at 0.005 mg/insect dose. Smooth tegument and absence of dots in integument responsible for aeration (M); treated with Fraction 2 (hexane and ethyl acetate 25%) at 0.010 mg/insect dose (N); treated with Fraction 3 (hexane and ethyl acetate 50%) at 0.040 mg/insect dose (O). Delimited cells composing epidermis and integrated in integument for all treatments and controls. No changes were observed in eyes, in antennas and in hairs for all treatments and controls. 1: antennae; 2: eye; 3: pronotum; 4: wing pad; 5: leg; 6: mesonotum; 7: scutellum; 8: rostrum; 9: integument setae.

caused 2.2 ± 0.1% and 14.4 ± 0.2% mortality after 24 h and 28 days, treatment, respectively. Control groups 1 and 2 insects molted to 5th instar nymphs achieving the adult stage — metamorphosis respectively (91.1 ± 0.2% and 85.6 ± 0.3%, Table 1). Control 3 treated with Starycide®SC 4.8 w/w Triflumuron, a reference insecticide and inhibitor of chitin synthesis, was highly toxic at 0.048 mg/insect, since nymphs did not metamorphose, and only 2.2 ± 0.1% of nymphs reached the 5th instar and recorded 6.7 ± 0.0% and 100% mortality at 24 h and 28 days, respectively. A dose of 0.024 mg/insect caused 1.1 ± 0.1% mortality at 24 h and 68.9 ± 0.2% ($p < 0.0001$) within 28 days, with 31.1 ± 0.2% ($p < 0.001$) of 5th instar nymphs becoming adults after treatment (Table 1).

Treatments with semi-purified fractions also caused deformations (Fig. 1), preventing most insects reaching adults. This was triggered even at the lowest doses, suggesting that these fractions contain high concentration of active components.

Previous studies with *C. fluminensis* isolated the triterpene, lanosterol, from hexanic extracts of flowers and fruits, which after topical treatment in *D. peruvianus* at low doses (0.001 mg/insect), produced adult malformations during the metamorphosis of some

5th instar insects *i.e.* wing deformations and wrinkled cuticle. While hexane crude extracts of flowers and fruits of *C. fluminensis* caused representative mortality (Duprat et al., 2017). As shown in Table 2 (supplementary material), Fractions 2 and 3 contain triterpenes, which may contribute to the effects in *D. peruvianus*.

Metamorphosis inhibition rates were more pronounced for Fraction 3 at 0.010 mg/insect (65.6 ± 1.2% ($p < 0.01$), and 0.040 mg/insect (61.1 ± 0.9%, $p < 0.01$), for Fractions 1 at 0.005 (63.3 ± 0.3 $p < 0.001$) and Fraction 2 at 0.04 mg (67.8 ± 0.4%, $p < 0.01$) compared with control groups ± 91.1 ± 0.2% and 85.6 ± 0.3%) (Table 1).

Additionally, doses of 0.010 mg/insect (Fraction 1), 0.040 mg/insect (Fraction 2), 0.010 mg/insect and 0.040 mg/insect (Fraction 3), respectively, induced the appearing of 22 ± 0.6% ($p < 0.01$), 18 ± 0.5% ($p < 0.01$), 15 ± 0.4% ($p < 0.05$) and 12 ± 0.3% ($p < 0.01$) of 5th instar insects with deformities (Table 1) such as: wing or antennae malformations; defective tarsi and wrinkled cuticle (Fig. 1). Insects that were confined in exuviae died (Fig. 1G). The Starycide®SC treated insects (Control 3) also developed similar deformations in 26 ± 0.7% ($p < 0.001$) (0.024 mg/insect) and 36.7 ± 0.4% (0.048 mg/insect) of 5th instars.

SEM (Fig. 2) showed that *C. hilariana* extracts wrinkled the cuticle but did not affect the integument. Fraction 3 had the most potent cuticular effects. altering the hydrocarbon, protein or enzyme conformation in some way The results also suggest that *C. hilariana* metabolites act as juvenile hormone analogues, since when treated with the Fractions at 0.005 mg/insect and 0.01 mg/insect the intermolt period was significantly extending, for example, with Fraction 2, at 0.005 mg/insect, from 3rd to 15th day to 1st to 28 th (Table 1). The fractions could be interfering in neuroendocrine system by blocking the production of protoracicotropic or ecdysone hormones, or affecting cuticle sclerotization, making it more resistant to degradation. Another possibility would be interference with IGR (Insect Growth Regulator) activity, specifically as inhibitors of the chitin synthesis (Klowden, 2013).

The Insect Growth Regulators (IGR) are third generation insecticides, including the use of exogenous hormones or substances analogous to these hormones (Staal, 1975). IGR cause similar effects on insects, as described in the present study, including incomplete molting, adults confined in exuviae, deformed nymphs and inhibition of wing growth (Mondal and Parween, 2000). The bioassays performed with Control 3 Starycide® SC 4.8% w/w (Triflumuron) addressed research on the neuroendocrine complex that supposedly could be involved with the results obtained with phytophagous insects. The deformations after treatment with triflumuron, were similar to those after treatment with semi-purified fractions of *C. hilariana* and described above for IGR. Treatment of *R. prolixus* 5th instar nymphs with triflumuron also induces enhanced metamorphosis inhibition and high death rates in 24 h (Mello et al., 2008).

Chemical analysis of semi-purified fractions from *C. hilariana* showed that Fraction 1 consists of sesquiterpenes and hydrocarbons. Fraction 2 is mainly composed of triterpenes, while in Fraction 3 the major compounds are fatty acids (Table 2–supplementary material). In a previous work with essential oils from staminate flowers of *C. hilariana*, terpenes were observed as major components (Nogueira et al., 2001). Oleic, stearic and palmitic acids were also identified as major components of *C. hilariana* floral resins (Porto et al., 2000). These acids were also observed in fractions 2 and 3 of *C. hilariana*. (Table 2–supplementary material).

As shown in Table 2 (supplementary material), the fractions obtained from *C. hilariana* are mainly constituted by fatty acids and triterpenes, and substances of lipophilic character, including sesquiterpenes, which may cross the cuticle lipid barrier through the insect tegument and act on insects causing conformational changes in the wax molecules, or specifically in hydrocarbons, interfering with the biochemical processes and causing physiological disorders (Scalerandi et al., 2018). They can also interfere with the octopaminergic receptors of insect's nervous system, which are responsible for the neuromodulation of juvenile hormone, and for the regulation of its ion channels and sensory system (Farooqui, 2007).

Fernandes et al. (2013) showed that α and β-amyrin from the ethanolic extract of fruits of *Manilkara subsericea* Mart., Sapotaceae, caused increasing mortality rates in *D. peruvianus* 4th instar nymphs, high inhibition rates of molting to the 5th instar and adults as well as wing deformations and wrinkled cuticle. The triterpene β-amyrin was also identified in the present work in Fraction 2 and therefore probably contributed to the mortalities and deformations recorded here.

There are few studies of *C. hilariana* and its biological effects against insects. One such study, however, confirms the role of triterpenes in delaying molting and insect mortalities using hexanic and methanolic extracts of the flowers, both staminate and pistilate, and led to the isolation of the triterpene oleanolic acid and the benzophenones, nemorosone. These were tested at 100 μg/ml of blood in the hematophagous insect, *Rhodnius prolixus* and resulted in delay of molting and toxicity with oleanolic acid and lack of toxicity with benzophenones (Kelecom et al., 2002).

In conclusion, semi-purified fractions from *C. hilariana* stems demonstrated median insecticidal activity against *D. peruvianus*, probably interfering in the adequate functioning of insect's neuroendocrine system, increasing mortality rates, interfering in molting and in metamorphosis. *C. hilariana* has active substances, such as terpenes, that may act alone or synergistically in insect physiology and interfere in their growth and development. Information on the effects of fractions of *C. hilariana* on *D. peruvianus* may contribute to development of biodegradable and more target selective substances for agricultural use with the purpose of controlling pests by sustainable pest management programs.

Integrated pest management (IPM) or integrated pest control (IPC) is a broad-based approach that integrates different methods to control pests employing various techniques with the use of synthetic insecticides alternately with green insecticides with could be derived from secondary insect metabolites. Green pesticides can also be used to produce organic and agricultural foods without using commercial pesticides, fertilizers or genetically modified organisms. (Koul et al., 2008).

Authors' contributions

HCR undertook the biological tests and ran laboratory studies with semi-purified fractions; MCA and SRP analyzed chemical data from plant material; MGS collected plant material and performed taxonomic identification; SRP and DF planned experiments; HCR, MCA, SRP and DF drafted the manuscript and interpreted data; MRF interpreted data; MCA, SRP and DF supervised laboratory work; HCR undertook statistical analysis and shoot scientific photography; CBM and MSG helped with the manuscript critical reading, and JRSM helped with Scanning Electron Microscopy. All the authors have read the final manuscript and approved the submission.

Conflict of interest

All authors have none to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.bjp.2019.07.005.

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