Revista Brasileira de Parasitologia Veterinária
Brazilian Journal of Veterinary Parasitology
ISSN 1984-2961 (Electronic)
www.cbpv.org.br/rbpv

Original Article

Effects of essential oils on native and recombinant acetylcholinesterases of *Rhipicephalus microplus*

Efeito de óleos essenciais sobre acetilcolinesterases nativa e recombinante de *Rhipicephalus microplus*

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How to cite: Santos EGG, Bezerra WAS, Temeyer KB, Leó AAP, Costa-Junior LM, Soares AMS. Effects of essential oils on native and recombinant acetylcholinesterases of *Rhipicephalus microplus*. *Braz J Vet Parasitol* 2021; 30(2): e002221. https://doi.org/10.1590/S1984-29612021024

Abstract

This study reports the action of essential oils (EO) from five plants on the activity of native and recombinant acetylcholinesterases (AChE) from *Rhipicephalus microplus*. Enzyme activity of native susceptible AChE extract (S.AChE), native resistant AChE extract (R.AChE), and recombinant enzyme (rBmAChE1) was determined. An acetylcholinesterase inhibition test was used to verify the effect of the EO on enzyme activity. EO from *Eucalyptus globulus*, *Citrus aurantifolia*, *Citrus aurantium var.dulcis* inhibited the activity of S.AChE and R.AChE. Oils from the two *Citrus* species inhibited S.AChE and R.AChE in a similar way while showing greater inhibition on R.AChE. The oil from *E. globulus* inhibited native AChE, but no difference was observed between the S.AChE and R.AChE; however, 71% inhibition for the rBmAChE1 was recorded. *Mentha piperita* oil also inhibited S.AChE and R.AChE, but there was significant inhibition at the highest concentration tested. *Cymbopogon winterianus* oil did not inhibit AChE. Further studies are warranted with the oils from the two *Citrus* species that inhibited R.AChE because of the problem with *R. microplus* resistant to organophosphates, which target AChE. *C. winterianus* oil can be used against *R. microplus* populations that are resistant to organophosphates because its acaricidal properties act by mechanism(s) other than AChE inhibition.

Keywords: Cattle tick, *Rhipicephalus microplus*, acetylcholinesterase inhibition, acaricide resistance, essential oils.

Resumo

Este estudo relata a ação de óleos essenciais de cinco plantas na atividade de acetilcolinesterases (AChE) nativas e recombinantes de *Rhipicephalus microplus*. A atividade enzimática do extrato de acetilcolinesterase nativa suscetível (S.AChE) e resistente (R.AChE) e da enzima recombinante (rBmAChE1) foi determinada. Um teste de inibição da AChE foi utilizado, para verificar o efeito dos óleos essenciais sobre a atividade enzimática. Óleos essenciais de *Eucalyptus globulus*, *Citrus aurantifolia*, *Citrus aurantium var. dulcis* inibiram a atividade de S.AChE e R.AChE. Os óleos das duas espécies de *Citrus* inibiram S.AChE e R.AChE de maneira semelhante, mas mostraram maior inibição sobre R.AChE. O óleo de *E. globulus* inibiu a AChE nativa, mas sem diferença entre a S.AChE e a R.AChE; no entanto, 71% de inibição para rBmAChE1 foi observada. O óleo de *Mentha piperita* também inibiu S.AChE e R.AChE, mas houve inibição significativa apenas nas concentrações mais altas testadas. O óleo de *Cymbopogon winterianus* não inibiu a AChE. Estudos adicionais são necessários com os óleos das duas espécies de *Citrus* que inibiram a R.AChE, devido ao problema de *R. microplus* resistente aos organofosforados ter como alvo AChE. O óleo de *C. winterianus* pode ser usado contra populações de *R. microplus,* que são resistentes a organofosforados, porque suas propriedades acaricidas agem por mecanismos diferentes.

Palavras-chave: Carrapato bovino, *Rhipicephalus microplus*, acetilcolinesterase, resistência, óleos essenciais.

Received February 4, 2021. Accepted February 18, 2021.
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Introduction

The tick *Rhipicephalus (Boophilus) microplus* (Canestrini, 1888) (Acari, Ixodidae) is an economically important ectoparasite of cattle that impairs livestock production systems in tropical and subtropical parts of the world (Pérez de León et al., 2020). The cattle tick *R. microplus* causes direct host damage through its obligate blood feeding habit and is a vector of pathogens, including species of *Babesia* and *Anaplasma* that cause bovine babesiosis and anaplasmosis, respectively (Roy et al., 2018). Synthetic chemicals with acaricidal properties are used to treat livestock infestation with *R. microplus*, which is associated with high expenses to farmers (Reginato et al., 2017; Ferreira et al., 2018). In Brazil, the annual economic losses of *R. microplus* is at least $3.2 billion (Grisi et al., 2014).

The indiscriminate use of acaricides has made several classes of these chemical agents ineffective due to the development and selection of resistant *R. microplus* populations (Reck et al., 2014; Rodriguez-Vivas et al., 2018). Commercially available classes of acaricides that are used extensively include the organophosphates (OP) and carbamates (CB). Synthetic chemicals in these classes of acaricides exert their inhibitory action on acetylcholinesterase (AChE) (Anderson & Coats, 2012), which is a hydrolase enzyme that plays a vital role in cholinergic neurotransmission. Inhibition of AChE activity results in hyperexcitability of neurons that leads to seizures, nervous system collapse, and death of the organism (Sharifi et al., 2017; Temeyer, 2018).

Invertebrates have different AChE isoforms (Baxter & Barker, 2002). In *R. microplus* three paralogous genes encoding AChEs (rBmAChE1, rBmAChE2 and rBmAChE3) were confirmed and expressed in neural and non-neural tissues (Temeyer et al., 2010). The different biochemical properties of these isoforms and the variation in enzymatic activity between tissues indicates the physiological plasticity of AChE in *R. microplus* (Temeyer et al., 2020). For example, AChE1 is expressed in the salivary glands, ovaries and synganglion whereas AChE2 is only expressed in the synganglion (Baxter & Barker, 2002; Temeyer et al., 2013). However, it is known that AChE insensitivity is related to resistance to OPs and CBs, and that in *R. microplus* this is a primary mechanism of resistance to compounds belonging to those classes of acaricides (Temeyer et al., 2010).

Essential oils are among the repertoire of natural products that can be used as alternative treatment against tick infestations because they offer advantages over synthetic acaricides (Hüe et al., 2015; Valente et al., 2017). These include the slow development of resistance by pests, low toxicity to mammals, low environmental impact and reduction of residues in products of animal origin (Abdelgaleil et al., 2009; Salman et al., 2020). As compared to conventional synthetic acaricides, essential oils that are efficacious can enhance the safety of treatment for livestock infested with *R. microplus* (Gross et al., 2017; Wang et al. 2019). The composition of essential oils includes volatile secondary metabolites known for their significant role in plant defense mechanisms (Silva Lima et al., 2018). Essential oils are a complex mixture of substances from various chemical families, however the most common compound found are terpenes (mono and sesquiterpenes) and phenylpropanoids (Dhifi et al., 2016; Salman et al., 2020).

Essential oils are known to have pesticidal and repellent properties (Pinto et al., 2015; Soares et al., 2016; Carroll et al., 2017) and some essential oils have also been investigated for their inhibition capacity of AChE (Salleh & Khamis, 2020). As example, essential oil of *Origanum syriacum* inhibited AChE of *Culex quinquefasciatus* (López et al., 2019), and *Eucalyptus globulus* essential oil inhibited AChE of *Rhipicephalus annulatus* (Arafa et al., 2020). However, there are no scientific reports on the ability of essential oils from *Eucalyptus globulus*, *Citrus aurantifolia*, *Citrus aurantium var. dulcis*, *Mentha piperita*, and *Cymbopogon winterianus* to inhibit AChE from *R. microplus*. In this study we investigated the action of essential oils from those plants on AChE activity in larvae of acaricide susceptible and resistant strains of *R. microplus*, and on rBmAChE1.

Materials and Methods

Tick populations

Ticks from two populations of *R. microplus*, a susceptible (Porto Alegre strain) and other resistant (resistant to organophosphate, synthetic pyrethroids, phenylpyrazole, amidinic, macrocyclic lactone, and benzoylphenyl urea derivatives - Jaguar strain) (Reck et al., 2014) were obtained by artificial infestations of cattle, which were not recently exposed to acaricide. The experimental procedures were approved by the animal research ethics committee of the Federal University of Maranhão (UFMA) under protocol number 23115.008186/2017-18.

Fully engorged females were naturally detached, and then were collected, washed with distilled water, dried on filter paper, weighed and separated into groups containing ten specimens each (maximum weight difference

was ± 0.5 g). The ticks were incubated (27 °C and relative humidity ≥ 80%), for 14-21 days, for oviposition (Silva Lima et al., 2018) and subsequent hatching to produce larvae used in preparation of crude larval extracts.

Obtaining the native and recombinant AChEs

In order to extract the multiple AChEs present in the larval extracts, *R. microplus* larvae were macerated using a mortar and pestle for 5 min in 100 mM sodium phosphate buffer, pH 7.0, containing 5 mM EDTA, 0.5% (v/v) Triton X-100, and 5 µL.mL^{-1} protease Inhibitor mix (Sigma-Aldrich, St. Louis, MO, USA), at a 1:25 ratio (larva weight/ buffer volume). The extract was left standing for 25 min at 4 °C and centrifuged at 4 °C for 30 min at 15.000 x *g*. The supernatant was recovered, stored at 4 °C, and used as a source of AChEs of susceptible and resistant strain named respectively, native susceptible AChE extract (S.AChE) and native resistant AChE extract (R.AChE).

The recombinant enzyme (rBmAChE1) was obtained according to Temeyer et al. (2010). Briefly, total RNA was isolated from pooled larvae of susceptible strain. Gene-specific primers were utilized to direct synthesis of first-strand cDNA from RNA template using Reverse Transcriptase, and complete BmAChE1 coding regions were amplified by high fidelity PCR from cDNA, sequenced, and expressed in baculovirus vectors. Recombinant expression clones were assembled, sequenced, and expressed in baculovirus infected Sf21 cell cultures.

Protein concentration was determined using bovine serum albumin (BSA) as standard (Bradford, 1976). Results were expressed in milligrams of proteins per milliliter (mg.mL^{-1}).

Determination of AChE activity

The S.AChE, R.AChE and rBmAChE1 activity was determined according to Ellman et al. (1961), modified as described by Li et al. (2005). The reaction mixture consisted of 10 µL of the S.AChE or R.AChE (1.5 mg protein mL^{-1} final concentration) or rBmAChE1 diluted 30 X with buffer (50mM sodium phosphate, pH 7.5), 100 µL 50 mM sodium phosphate buffer, pH 7.5, and 100 µL of the reaction solution. The reaction solution contained 0.24 mM acetylthiocholine iodide (Sigma-Aldrich) and 0.64 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) (Sigma-Aldrich) prepared in the above sodium phosphate buffer. In the blank sample, the AChE aliquot was replaced by the buffer. Reaction was conducted in a 96 well microplate for 30 min and monitored every 5 min by recording the absorbance (Abs) at 405 nm (Microplate Reader, Biochrom). Activity was calculated using the equation: Activity (abs/mL/min) = $[(T_{30}-T_0)/30] \times 100$, where T_0 and T_{30} = sample absorbance - blank absorbance measured at zero and 30 min reaction. Only linear reactions throughout the monitoring period were considered.

Inhibition of AChE activity

Essential oils from *E. globulus* (chemical composition - 83% 1.8-cineol, 9% limonene, 4% alfa-pinene, 3% p-cimene), *C. aurantifolia* (chemical composition - 57% limomene, 14% γ-terpinene, 12% β-pinene, 2% α-pinene, 1.5% mircene, 1.5% geranial), *C. aurantium var. dulcis* (chemical composition - 96% limonene, 1.8% mircene, 0.5% α-pinene, 0.3% sabinene), *M. piperita* (35% menthol, 26% menthone, 6.0% 1.8-cineol, 5.0% isomenthone, 5.0% methyl acetate, 4.0% neomenthol) and *C. winterianus* (chemical analysis was not performed) were commercially purchased (Ferquima). The chemical analysis were performed by company and informed to the authors. The essential oils were individually diluted in ethanol to 20 mg.mL^{-1} (stock solution). From the stock solution, an essential oil solution at 2 mg.mL^{-1} was prepared in 50 mM sodium phosphate buffer, pH 7.5. The final essential oil concentrations tested were 1.00, 0.67, 0.44, 0.30, 0.20, 0.13, 0.088, 0.058, 0.039, 0.026, 0.017 and 0.012 mg.mL^{-1}. These concentrations were selected by a preliminary pilot test performed.

The AChE inhibitory activity was evaluated by mixing 10 µL of the S.AChE, R.AChE or rBmAChE1 with 100 µL of the essential oil and 100 µL of the reaction solution described above. Propoxur (Sigma-Aldrich) was used as a positive control. It was similarly prepared as the essential oil, but at 0.25, 0.05, 0.025, 0.005, 0.0025 and 0.0005 mM final concentrations (Prado-Ochoa et al., 2014). In the negative control, essential oils were replaced by the phosphate buffer and ethanol. Reactions were conducted in a 96 well microplate for 30 min and monitored every 5 min by recording the absorbance (Abs) at 405 nm (Microplate Reader, Biochrom).The percentage of enzyme inhibition was calculated by comparison with the negative control as follows: AChE inhibition (%) = 100 - [(As / Ac) x 100], where: As = AChE activity for each concentration; Ac = negative control (AChE activity without essential oil). Only linear regression reactions throughout the monitoring period were considered.

Statistical analyses

The data were obtained from the triplicate inhibition assays of two independent experiments for each essential oil on the S.AChE and R.AChE and rBmAChE1. The data were initially transformed to log (X) and normalized; subsequently, nonlinear regression was performed to obtain the IC50 (50% inhibition concentration) and the F test was used by pair to compare the curves. All analysis were performed using the GraphPad Prism 7.0 software (GraphPad Inc., San Diego, CA, USA).

Results and Discussion

Acaricidal and repellent activities, and egg hatch inhibition are among the biological properties against ticks reported for essential oils of plant species in the genus *Citrus* (Pazinato et al., 2016; Stefanidesova et al., 2017; Vinturelle et al., 2017). In our experiments *C. aurantium var. dulcis* and *C. aurantifolia* inhibited S.AChE and R.AChE in varying degrees. Stronger inhibition against R.AChE was exhibited by *C. aurantifolia* oil (64.0 ± 13.1%) at 0.44 mg.mL^{-1} and *C. aurantium var. dulcis* (49.8 ± 8.5%) at 0.67 mg.mL^{-1}. However, the inhibition decreased at highest concentrations (Figures 1A and 1B). There was statistically significant difference among IC50 of S.AChE, R.AChE and rBmAChE1 after treatment with *C. aurantium var. dulcis*, and *C. aurantifolia*.

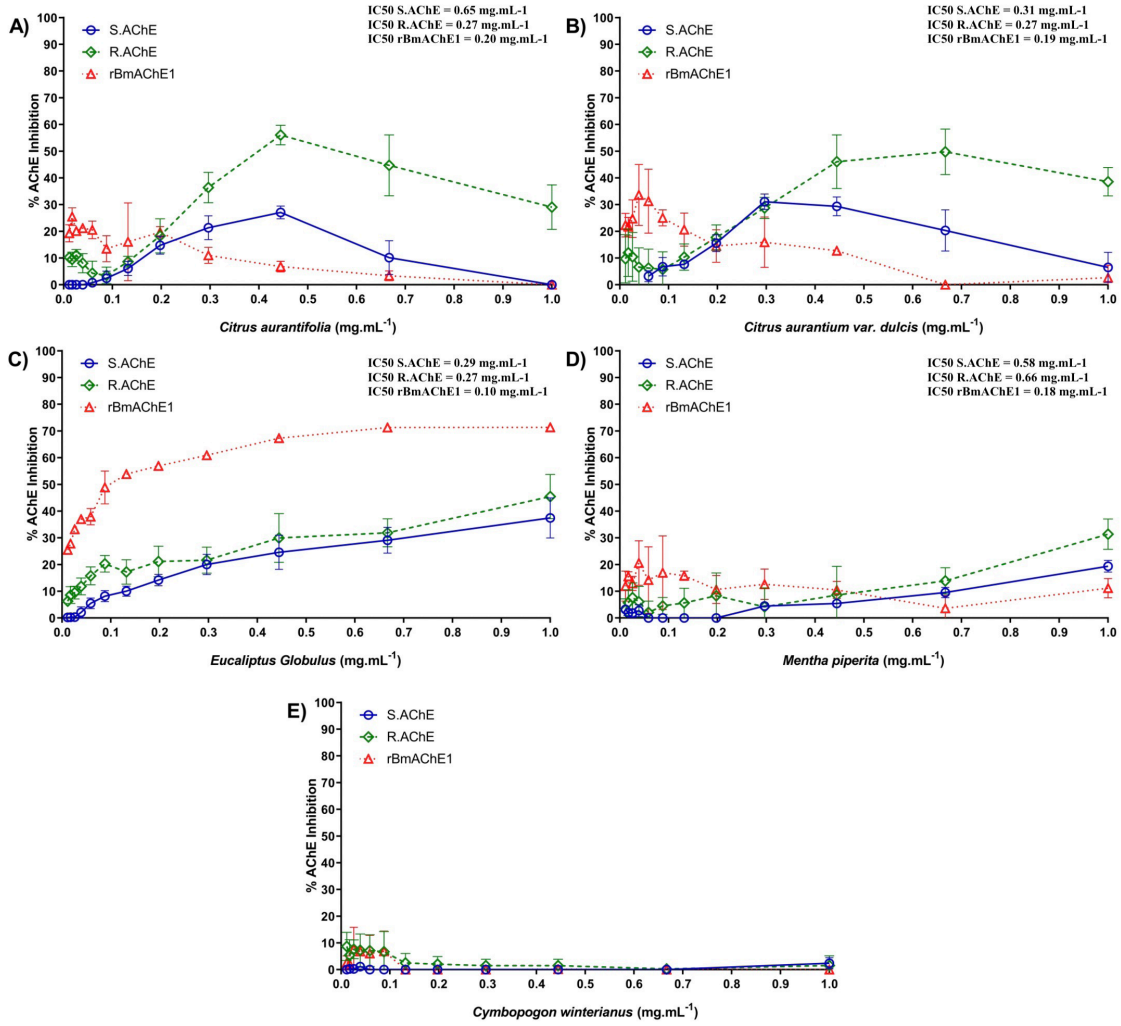

Figure 1. Effect of different concentrations of essential oils (mg.mL^{-1}) on the acetylcholinesterase activity of *R. microplus* expressed as percentage of inhibition (%) and its . The essential oils used (A) *Citrus aurantifolia*; (B) *Citrus aurantium var. dulcis*; (C) *Eucalyptus globulus*; (D) *Mentha piperita*, and (E) *Cymbopogon winterianus.* S.AChE: Native susceptible AChE extract; R.AChE: Native resistant AChE extract; rBmAChE1: Recombinant enzyme; IC50: 50 percent inhibitory concentration. Each point represents the mean of the values obtained from two independent experiments performed in triplicate.

The *Citrus* oil inhibition profiles for S.AChE and R.AChE reported here suggest that structural difference between the AChE of susceptible and resistant tick larvae result in different sensitivities to *Citrus* oils, which is consistent with the known mechanism of OP resistance in *R. microplus* associated with AChE insensitivity (Temeyer, 2018). Bioassays are warranted to determine if our *in vitro* results translate into acaricidal and/or repellent activity against *R. microplus* by the oils of *C. aurantifolia* and *C. aurantium var. dulcis*. By comparison, monoterpenes in oils of other *Citrus* plants have been shown to be active against *R. microplus* and *Dermacentor reticulatus* (Pazinato et al., 2016; Stefanidesova et al., 2017). In this regard, the monoterpene limonene has been shown to be acaricidal against *R. microplus* (Ferrarini et al., 2008; Vinturelle et al., 2017).

Eucalyptus globulus oil inhibited 37.5 ± 7.5 and 45.5 ± 8.3% of S.AChE and R.AChE at 1 mg.mL^{-1}, respectively. Significant difference in inhibitory activity was observed between the S.AChE (IC50 = 0.29 mg.mL^{-1}) and R.AChE (IC50 = 0.27 mg.mL^{-1}). However, the oil from *E. globulus* strongest inhibited the rBmAChE1 with IC50 of 0.10 mg.mL^{-1} (Figure 1C). Because the other oils tested did not inhibit rBmAChE1, we hypothesize that differences between their components afford them various levels of inhibitory activity against the AChE isoforms considering that *R. microplus* has at least three functional AChEs (Temeyer et al., 2010), and that the larval extracts obtained in this study likely contained the three native AChEs. The pesticidal activity of this oil was associated with a high content of 1.8-cineole, also known as eucalyptol (Miresmailli et al., 2006; George et al., 2009). In a previous study with *R. microplus*, eucalyptol showed greater AChE inhibition against the resistant strain (IC_{50} 0.36 mg.mL^{-1}) than the susceptible strain (IC_{50} 3.41 mg.mL^{-1}) (Cardoso et al., 2020). Furthermore, 0.01 M of eucalyptol inhibited AChE 64.9% in larvae of the beetle *Tribolium castaneum* (Abdelgaleil et al., 2009). The observed differences in rBmAChE1 inhibition by essential oils are strongly suggestive as to their role in the total activity of AChE present in tick larvae, indicating that rBmAChE1 is only partially responsible for the total AChE pool activity, since *C. aurantifolia* and *C. aurantium var. dulcis* oils predominantly target other tick AChEs, in contrast, *E. globulus* oil which inhibited rBmAChE1.

Mentha piperita (popularly known as peppermint) essential oil also showed AChE inhibition. The inhibition rates recorded at the highest concentration (1 mg.mL^{-1}) tested were 31.4 ± 5.7%, 19.4 ± 2.2%, and 11.2 ± 3.6% for the R.AChE (IC50 = 0.58 mg.mL^{-1}), S.AChE (IC50 = 0.66 mg.mL^{-1}), and rBmAChE1 (IC50 = 0.18 mg.mL^{-1}), respectively (Figure 1D). These results suggest that AChE inhibition was caused by a relatively minor component of the peppermint oil or the majors component had low activity. Peppermint oil is acaricidal, repellent, and known to contain menthol and menthone as major compounds (Chagas et al., 2016). *M. piperita* oil also has fumigating action and this activity is promoted by the rapid volatilization of 1.8-cineole (Mkolo et al., 2011).

Cymbopogon winterianus (popularly known as Citronella) essential oil is widely used and commercialized for its repellent and acaricidal activity. These properties of citronella oil are attributed to the presence of volatile substances such as citronellal, eugenol, geraniol, which are major components that act synergistically (Olivo et al., 2008; Singh et al., 2014a, b). Citronella oil did not inhibit the *R. microplus* AChEs in our experiments (Figure 1E) and the IC50 were not obtained. Thus, *C. winterianus* oil can be used against *R. microplus* populations that are resistant to carbamates and organophosphates because its acaricidal properties act by mechanism(s) other than AChE inhibition.

Although the oils tested in this study had shown to be active against ticks of different species including *R. microplus* (George et al., 2009; Singh et al., 2014b; Chagas et al., 2016), questions remained on their mode of action. The pesticidal effect of essential oils results from the synergistic interactions between their bioactive components (Isman, 2015). Essential oil components can act simultaneously on different molecular targets (Politi et al., 2019). Based on our experience (Costa-Júnior et al., 2016; Cardoso et al., 2020), the *in vitro* assays reported here focused on the inhibition of AChE in *R. microplus* to investigate the mode of action of essential oils from the five plants selected for this study.

Conclusion

The oils of *E. globulus*, *C. aurantifolia*, *C. aurantium var. dulcis* and *M. piperita* showed various degrees of inhibition on S.AChE and R.AChE, but only *E. globulus* oil inhibited rBmAChE1. The profiles of AChE inhibition for the five essential oils tested provided useful information to understand their mode of acaricidal activity, corroborating for further studies on the use of essential oils as candidates for new acaricides. Further studies are needed to determine the utility of these essential oils under field conditions to manage populations of *R. microplus* that are resistant to commercially available synthetic acaricidal chemicals.

Acknowledgements

The U.S. Department of Agriculture is an equal opportunity employer and provider. We thank the FAPEMA (Maranhão State Research Foundation) for financial support under process UNIVERSAL-00739/17 and for awarding a fellowship to Soares, A.M.S., under process BEPP-02011/18); Santos, E.G.G and Bezerra, W.A.S. We thank CNPq (Brazilian National Council for Scientific and Technological Development) for awarding a fellowship to L.M. Costa-Junior. We also thank the FINEP (Funding Authority for Studies and Projects) and FAPEMA for supporting the IECT (Science and Technology Institute of Maranhão) Biotechnology.

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