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Original Article

# Evaluation of the immunomodulatory effect against splenocytes of Balb/c mice of biflorin obtained from *Capraria biflora* by a new isolation method

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ABSTRACT

Biflorin (6,9-dimethyl-3-(4-methylpent-3-en-1-yl) benzo[de]chromene-7,8-dione) is a promising substance that has been increasingly studied in the past decades due to its diverse pharmacological properties (*i.e.* antitumor, antioxidant, antiinflamatory, antimicrobial activity *etc.*). Aiming the comprehension of its antitumoral activity we investigated the cell proliferation and cytotoxicity habilities of biflorin against mice splenocytes Balb/c. Biflorin was able to stimulate mice splenocytes Balb/c in 48 h of incubation at a concentration of 20.2 μM. Its immunostimulation promoted the production of cytokines such as: TNF-α, IFN-γ, IL-2, IL-6 and IL-17, inducing the immune profile toward a Th1 response. Moreover, an original method which led to an excellent yield with less processing time compared to the methods described in the literature was developed to obtain biflorin, from sawdust of *Capraria biflora* L., Scrophulariaceae. This method shows a great potential of increasing the production of this pharmacological active compound.



## Introduction

Within a great variety of known phytochemicals, naphthoquinones represent an important group of compounds that show antitumor properties, which from the point of view of scientific research, make them good candidates for new drugs prototypes (Moraes et al., 2014; Jardim et al., 2015; Oliveira et al., 2017; Lara et al., 2018).

The biflorin, (6,9-dimethyl-3-(4-methylpent-3-en-1-yl) benzo[de]chromene-7,8-dione) CAS number 5957-32-4;99671-97-3 is a naphthoquinone found in the roots from *Capraria biflora* L. (Lima et al., 1953). This plant belongs to the Schrophulariaceae family, Angiosperm group, is originated at the Antilles and distributed from north to south America (Souza et al., 2012). In Brazil, this species is known as marajó tea, méxico tea, américa tea or river tea and is widely distributed throughout the country (Lemos et al., 2007). In popular medicine, the leaves of this species are used in the treatment of pain and fever, vomiting, diarrhea, hemorrhoids, rheumatism, and swelling. However, pharmacological studies with aqueous extract of the leaves presented analgesic activity, whereas of the roots an antimicrobial activity was reported (Correia, 1984; Lemos et al., 2007). Systematic studies have shown that biflorin induces antitumor activity (Vasconcellos et al., 2007), antioxidant activity (Vasconcellos et al., 2005, 2010), anti-inflammatory, antimicrobial, antibiotic and analgesic action (Souza et al., 2012). Moreover, biflorin is a promising substance due of its pharmacological activity against gram-positive alcohol-acid resistant bacteria (Wisintainer et al., 2014), demonstrating also cytotoxic activity against several tumor cell lines (Vasconcellos et al., 2005, 2007) indicating a potent antitumor therapeutic agent (Vasconcellos et al., 2011, 2015; Wisintainer et al., 2015). Ralph and collaborators (2016), for instance, showed that biflorin reduces the viability of melanoma cell lines by DNA interaction, proving to be a therapeutic option. More recently, Jeon et al. (2017) described the use of biflorin in the treatment of cognitive problems in mice.

Although many studies on the antitumor activity of biflorin have been presented in the literature, the scarcity of data on its

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immunological activation or the description of the cellular stimulation *in vitro*, hinders the discussion on the possible mechanisms involved in interaction between biflorin and immunological cells. Immunostimulant compounds, especially substances that are able to act in antitumor, antiviral and immunological therapies, play a fundamental role in many different research fields being important targets of immune response research (Chacon et al., 2016; Davis and Ballas, 2017).

The red-violet substance biflorin was first described in the literature in 1952 (Lima et al., 1953) and, since then, researchers have developed several methods to obtain this compound at a higher yield. For instance, Lima et al. (1958) applied biflorin acetone extracts to water-inactivated silica gel column chromatography using pure benzene and then acetone to yield 0.2% pure biflorin. Later, the same researchers, in order to increase this yield, developed a new method with industrial applicability. Biflorin extracts were obtained with ethyl ether at 50 °C in a Soxhlet apparatus and then treated with 0.5% NaOH solution several times. After recrystallization a 0.4% yield of pure biflorin was reported (Lima et al., 1962). In 2003, Fonseca et al. (2003) used 450 g of the roots of *C. biflora* and obtained 4.6 g of the crude extract applying chloroform. Nevertheless after isolation by silica gel chromatography, only 0.022% yield (100 mg) was observed. Vasconcellos et al. (2007) used 6 kg of the root and isolated 2 g of the crude criteria for two days and after column chromatography, 1.5 g of the pure product was obtained, with an overall yield of 0.025%. All these studies show the inherent difficulty in obtaining higher yields of biflorin, hindering industrial applications of this natural product.

In the present study we used an original method of simultaneous extraction and purification of natural products from herbal materials developed by Góes (2018) and applied to sawdust from roots of *C. biflora*, aiming the scale up production of biflorin. The extraction procedure increases the yield of bilflorin, combining solvent economy and low processing time. The extracted biflorin was applied to study the immunostimulation pathway promoted in spleen Balb/c mice cells.

## Materials and methods

### *Chemicals*

The melting point was determined in a Büchi-510 capillary apparatus. IR spectra were measured on a PerkinElmer® (Spectrum 400) IR spectrophotometer. $^{1}$H NMR $^{13}$C NMR spectra were recorded on a Bruker Avance® DRX-400 spectrometer by using tetramethylsilane as the internal standard. The chemical shifts are reported in $\delta$ units, and coupling constants ($J$) are reported in hertz. High resolution mass spectrometry (HRMS) analysis of the pure compounds was performed with a micrOTOF II apparatus. TLC development was conducted on 0.25 mm silica gel plates ($60F_{254}$, Merck).

### *Extraction and isolation*

First we tested the best elution condition for the biflorin, taking into account a retarding factor ($R_f$) of 0.3–0.45 and the more adequate solvent systems tested were (a) hexane/ethyl acetate 8:2 ($R_f$ 0.39) and (b) hexane/ethyl ether 7:3 (v/v) ($R_f$ 0.30). In order to perform the extraction and isolation of the biflorin we applied the set up described in Fig. 1, were the column was partially filled by 25 g of silica gel and then further filled using 20 g of sawdust from *C. biflora* roots. After adding a quantity of 150 ml of eluent hexane/ethyl acetate 8:2 (v/v) over the sawdust, the system was heated at a temperature sufficient to feed the column (~65 °C).

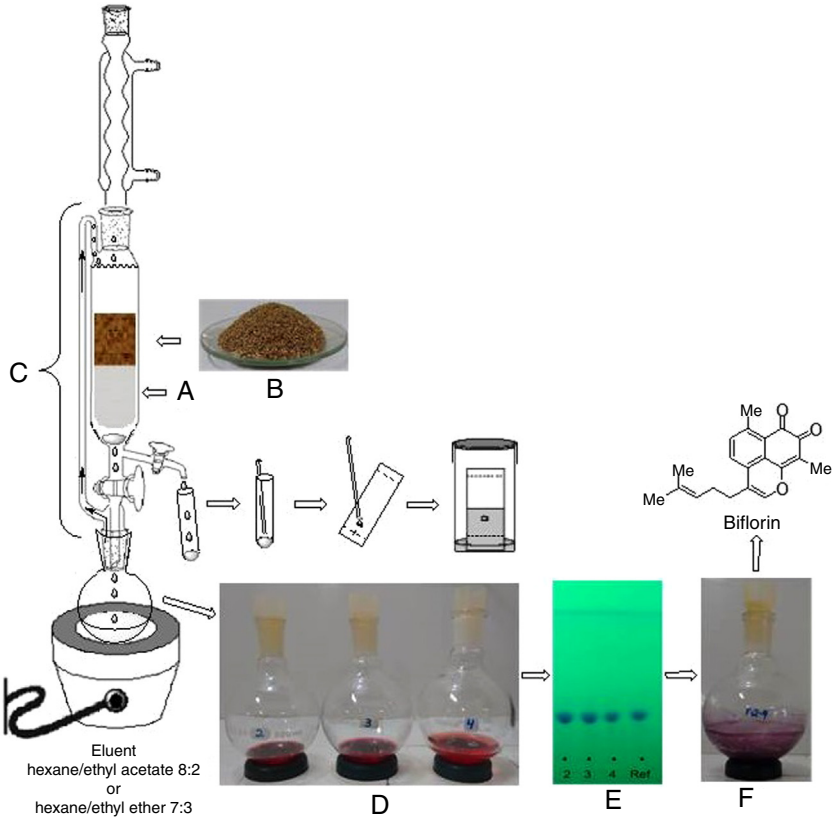


**Fig. 1.** Process for extraction and simultaneous purification of biflorin. (A) Silica gel; (B) root powder of *Capraria biflora*; (C) Góes apparatus; (D) fractions collected containing pure compound; (E) TLC plates hexane/ethyl acetate 8:2 (Rf 0.39); and (F) round-bottom flask containing pure biflorin.

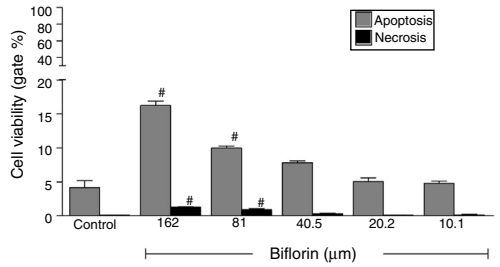


**Fig. 2.** Evaluation of cytotoxicity effect promoted by biflorin in mice spleen cells in 24 h culture. The safety dose to use biflorin is [biflorin] <40.5 μM. Horizontal bars represent the average of two independent experiments performed in duplicate. $^{\#}p = 0.0001$.

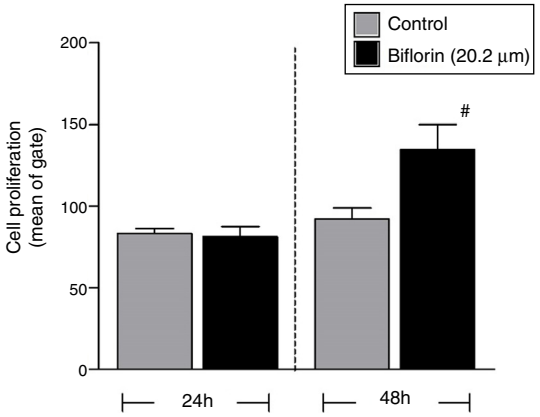


**Fig. 3.** Investigation of cell proliferation in the 24 h and 48 h of incubation with biflorin at 20.2 μM. Biflorin induced higher cell proliferation in 48 h of incubation. Horizontal bars represent the average of two independent experiments performed in duplicate. $^{\#}p = 0.002$.

The extraction/isolation process was performed continuously for 5 h until all biflorin was isolated. We applied the same elution solvent to monitore the fractions by thin layer chromatography (TLC). During the process, four fractions (~10 ml) were collected in the bottom flask. Fractions of 2–4 were added together, rendering a pure violet compound, biflorin.

*Biflorin (6,9-dimethyl-3-(4-methyl-3-pentenyl)naphtha[1,8-bc]-pyran-7,8-diona)*

Violet solid; m.p. 147–148 °C; IR ($cm^{-1}$): 2920; 1682; 1644; 1624; 1596; 1579; 1475; 1437. $^{1}H$ NMR (400 MHz, $CDCl_3$)-$\delta_H$ (multiplicity, *J* in Hz): 1.60 (s, H-14); 1.74 (s, H-15); 2.01 (s, H-17); 2.28 (q, 7.2; H-11); 2.53 (t, 7.2; H-10); 2.74 (s, H-16); 5.18 (s, H-12); 7.10 (s, H-2); 7.42 (d, 8.1; H-5); 7.54 (d, 8.1; H-4). $^{13}C$ NMR (100 MHz, $CDCl_3$)-$\delta_C$: 7.9 (C-17); 18.05 (C-14); 23.3 (C-16) 25.8 (C-15); 27.5 (C-10 and 11); 113.6 (C-9); 116.1 (C-3); 122.7 (C-12); 124.3 (C-9b); 126.8 (C-6); 128.4 (C-4); 129.0 (C-3); 133.5 (C-13); 136.3 (C-5); 140.9 (C-2); 146.7 (C-6); 161.8 (C-9); 178.2 (C-8); 182.2 (C-7). HRMS (EI+): calcd. ($C_{20}H_{20}O_3$): 309.1485. Found: 309.1473. The data corroborate with those described in the literature (Lemos et al., 2007; Souza et al., 2012).

*Plant material*

The botanical material of the species *Capraria biflora* L., Scrophulariaceae, specifically the roots, was collected in May/2017, in an area located in the Itamaracá Island (Pernambuco, Brazil: coordinates 7°44′43.8″ S 34°49′32.1″ W). The exsicata were identified (by Dr. Rita de Cássia Pereira, herbarium curator) and deposited in the herbarium of the Agronomic Institute of Pernambuco, registered under the Nr. 91544. For simultaneous extraction and purification process of the biflorin, roots of *C. biflora* were collected and kept at *ca.* 28 °C for one week to remove plant humidity. After this, the roots were pulverized to reach fine particles.

*Animals*

Female BALB/c mice (6–8 weeks old) were raised and maintained at the animal facilities of the Keizo Asami Immunopathology Laboratory located in Federal University of Pernambuco, Brazil. Mice were kept under standard laboratory conditions (20–22 °C and 12 h day and night cycle) with free access to a standard diet (Labina/Purina, Campinas, Brazil) and water. All experimental procedures were performed with accordance of Ethics Committee of Animal Use of Federal University of Pernambuco (protocol number 0048/2016).

*Preparation of splenocytes*

This procedure was performed in accordance with a previous protocol (Melo et al., 2011). After euthanasia of animals with hydrochloride of xylazine 2% and hydrochloride of ketamine 10% followed by cervical dislocation, the spleen of each mouse was aseptically removed and placed in a Falcon tube containing RPMI 1640 with fetal calf serum (complete medium). In a vertical flow, each spleen was transferred to a Petri dish where they were soaked. The cell suspensions obtained from each spleen were transferred to Falcon tubes containing approximately 10 ml of incomplete medium. Spleen homogenates were overlaid onto a Ficoll-PaqueTM PLUS layer, with the density adjusted to 1.076 g/ml, and centrifuged at 1000 × *g* at room temperature for 25 min. The interface cell layer containing immune cells was recovered by Pasteur pipette, washed twice in phosphate-buffered saline (PBS) and centrifuged twice at 500 × *g* for 10 min. Cells were counted in a Neubauer chamber, and cell viability was determined by the trypan blue exclusion method. Cells were only used when viability was >98%.

*Analysis of cell viability*

Splenocytes from BALB/c mice treated with 10.1–162 μM of biflorn for 24 h of incubation as well as untreated cells were centrifuged at 450 × *g* at 22 °C for 10 min. After discarding the supernatant, 1 ml of PBS 1× was added to the precipitate and after ressuspension, the cells were centrifuged again (450 × *g*, 22 °C, 10 min). The pellet was resuspended in 300 μl of binding buffer, transferred to a labeled cytometer tube, and Annexin V conjugated with fluorescein isothiocyanate (1:500) and propidium iodide (20 μg $ml^{-1}$) were added. Flow cytometry was performed on a FACSCalibur platform (BD Biosciences, San Jose, USA) and the results were analyzed using CellQuest Pro software (BD Biosciences).

*Proliferation assay using CFSE staining*

Same protocol to obtain splenocytes was used for proliferation assay. After acquiring splenocytes, cell solution was centrifuged at 300 × *g* at room temperature for 5 min with sterile PBS 1× containin SFB 5% (pH 7.2). The cell solution was adjusted then to $10^7$ cells/ml and received 5 mmol $l^{-1}$ of 5(6)-carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE). Cells were incubated for 10 min at 28 °C in the dark and then centrifuged twice at 300 × *g*/5 min with sterile PBS 1×. Stained cells were cultured for 24 h with either 20.2 μM

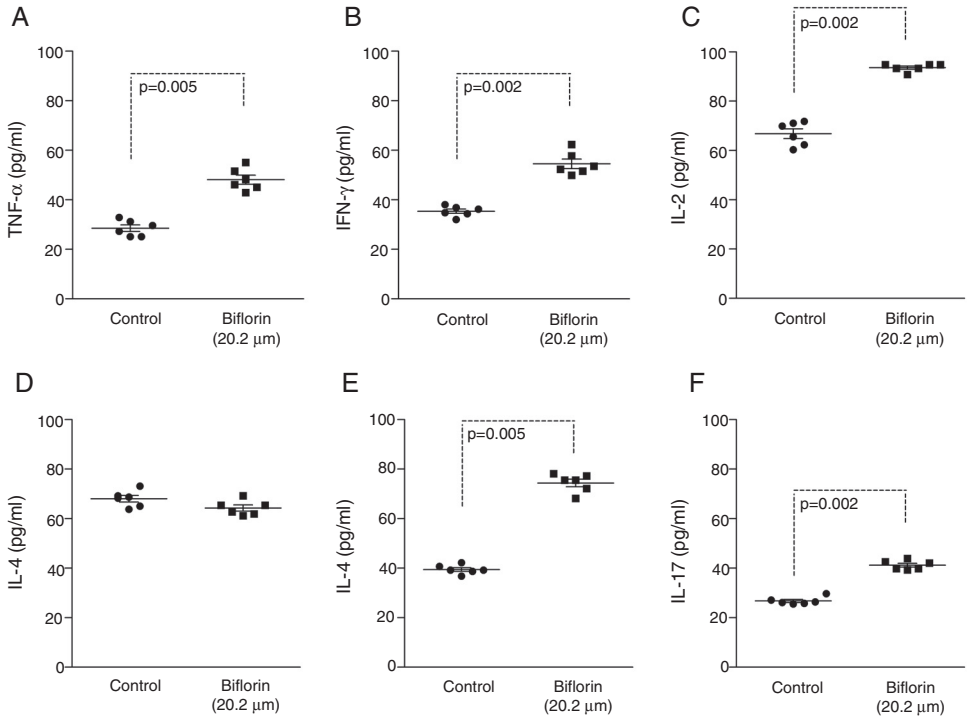


**Fig. 4.** Study about immunostimulatory effect promoted by biflorin (20.2 μM) in mice splenocytes cultures. Biflorin stimulated higher production of TNF-α (A), IFN-γ (B), IL-2 (C), IL-6 (E) and IL-17 (F). Similar values between control and biflorin treated cells were observed to IL-4 production (D).

of biflorin or with culture medium only (negative control). After culture time, cells were centrifuged (300 × *g*/5 min), and analyzed on a FACSCalibur platform (Becton Dickinson Biosciences) coupled with a cell Quest Pro software (Becton Dickinson).

*Measurement of cytokine production in splenocyte cultures*

Supernatants of cultures treated or not with biflorin (20.2 μM) for 24 h were collected for quantification of cytokines using the CBA (Cytometric Bead Array) Mice Th1/Th2 Cytokine Kit (Becton Dickinson Biosciences, USA) for simultaneous detection of interleukins (IL-2, IL-4, IL-6, IL-10, IL-17), tumor necrosis factor-alpha (TNF-α), and interferon-gamma (IFN-γ). The assays were performed according to manufacturer's instructions and data were acquired on a FACSCalibur platform. Six individual cytokine standard curves (0–5000 pg/ml) were run in each assay. The range of detection was set as 3000–5000 pg/ml. The cells were analyzed using the FACSCalibur platform according to manufacturer's instructions.

*Statistical analysis*

Data were analyzed using non-parametric tests. To detect the differences between groups, the Wilcoxon test was used. Student's *t*-test was used to analyze the results from cell viability assay. All results were expressed as mean ± SD and a value of $p < 0.05$ was considered as statistically significant.

## Results and discussion

*Simultaneous extraction and purification*

For the separation and the purification of the chemical compounds of plant origin, mainly, they are often subjected to extraction. Then, chromatographic procedures for purification are done, being commonly applied to column chromatography or followed by recrystallization.

In order to improve the yield of biflorin, as well as to save material and time, we used a new method of simultaneous extraction and purification (Góes, 2018). The results demonstrated that this process (Fig. 1) showed to be more efficient and with excellent yield compared to the methods described in the literature. Applying 5 h and using 20 g sawdust, 25 g of silica gel and 150 ml of eluent, we obtained 126.6 mg of the pure compound, yielding 0.6%. As far as we are aware, this is the highest yield obtained compared to those reported in the literature for biflorin. In this process it is also possible to increase the amount of sawdust and to maintain the same amounts of the eluent and silica. For the process of simultaneous extraction and purification it is necessary a Góes apparatus (c) (patent registration: BR 10 2017 021099 5) (Góes, 2018), a condenser; a round bottom flask; a heating blanket; sample test tubes; capillary tube for preparation of TLC chromatography plates; glass chromatographic vessel; silica gel chromatographic plates.

*Study of the immunostimulatory profile of biflorin*

Several *in vivo* and *in vitro* pharmacologic assays showed that even at lower concentrations biflorin is a potent pharmacological agent showing good biological activities (Vasconcellos et al., 2007; Montenegro et al., 2013; Carvalho et al., 2013; Ralph et al., 2016). Aiming to convert biflorin in a pharmacological compound, we investigated the immunostimulatory profile promoted by this compound on spleen mice cells. For this, we first evaluated if biflorin is cytotoxic this cell line.

Results showed that biflorin can be safely used for concentration values lower to 40.5 μM in 24 h cultures (Fig. 2). In this context, we chose the concentration of 20.2 μM to evaluate the immunological stimulation promoted by biflorin.

Our assays on immunostimulation were performed applying immune cell proliferation and cytokines production. Results about cell proliferation showed that biflorin was able to stimulate mice splenocytes in 48 h of incubation (Fig. 3). Moreover, this compound induced higher cytokines production and led the immunological

profile to Th1 response. In fact, biflorin stimulated the higher production of TNF-α, IFN-γ, IL-2, IL-6 and IL-17 cytokines (Fig. 4).

However, naphthoquinones, especially β-lapachone, had similar effects to our study, where this substance represses the induction of nitric oxide synthase and tumor necrosis factor (TNF) (Tzeng et al., 2003), which may be applied in anti-inflammatory therapies (Choi et al., 2012; Tseng et al., 2013), as well as in the therapy of neuroinflammatory disorders (Lee et al., 2015) due to its function in modulating cytokines with both Th1 and Th2 profiles.

As mentioned, biflorin, a naphthoquinone, induced the Th1 immunological profile, being a relevant response for antitumor and antiviral activity, corroborating with the studies carried out by Vasconcellos et al. (2007). These authors showed that biflorin increased the efficacy of fluoracil (5-FU), caused morphological changes in mice spleens and reduced the rate of tumor proliferation. Moreover, the results obtained in the present study demonstrate that biflorin induced the production of cytokines, such as IFN-γ and IL-2. These cytokines have primordial function, mainly in tumor rejection and inhibition of viral infections, as well as the role of instigating the transcription of various proteins, inflammatory genes, such as the synthesis of nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) (Vila-de-Sol et al., 2008; Tanaka et al., 2012). Studies have reported that interleukin-6 (IL-6) is essential in inflammatory, chronic and autoimmune diseases, as well as in the differentiation of Th17 (Leech et al., 2012; Kimura and Kishimoto, 2010; Hunter and Jones, 2015) which is important in inflammatory responses and in defense against infections caused by extracellular microorganisms (Miossec and Kolls, 2012; Misiak et al., 2017).

## Conclusion

The results demonstrate that the new extraction and purification proves to be rentable process. In fact, we obtained 0.6% of pure biflorin, a percentage higher than the findings in the literature. This procedure can also be used on an industrial scale, not only due to the good yields but also due to the green process applying a reduced amount of solvent and time. We observed that biflorin stimulated the production of cytokines such as TNF-α, IFN-γ, IL-2, IL-6, IL-17. Finally, biflorin presents as a promising molecule because this molecule induced a Th1 immunological profile, being a relevant compound for future antitumor and antiviral assays.

## Ethical Disclosures

**Protection of human and animal subjects.** The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

**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.

## Author contributions

AJSG designed the study and the figures composed and edited; MSLS elaborated the manuscript and performed the experiment *in vitro*. AJA collected and registered the botanical material; VMBL and CMLM performed *in vitro* experiment; JAS and GGO performed the spectrometric analysis and characterized the isolated compound; RMFS and BSS critically revised the manuscript for clarity and intellectual content. All authors read and approved the final manuscript.

## Conflicts of interest

The authors declare no conflicts of interest.

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