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Article

Extracts and Terpenoids from *Stevia* Species as Potential Anthelmintics for Neglected Tropical Diseases Caused by Cestode Parasites

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Abstract: Cestodes are etiological agents of neglected diseases such as echinococcosis and cysticercosis, which are major public health problems. Antiparasitic treatment relies on a small number of approved drugs, which are often only partially effective, poorly tolerated and require prolonged administration. Thus, the discovery of novel potential treatments is critical. The *Stevia* genus (Asteraceae) includes species that are associated with medicinal uses and are considered a source of bioactive compounds. Here, we analyzed four South American *Stevia* species that have previously demonstrated antiprotozoal properties. The effect of dichloromethane extracts of these species was determined by motility assays on *Mesocestoides vogae*, a laboratory model of cestodes. *Stevia alpina* showed the highest anthelmintic potential followed by *S. maimarensis* and *S. multiaristata*, while *S. aristata* was the least active. The sesquiterpene lactones estafietin and eupatoriopicrin were purified from *S. alpina* and *S. maimarensis*, respectively. Estafietin showed cestocidal activity, inhibiting parasite viability in a dose-dependent manner, even from the first day of incubation. Consistent with the motility effects, the extract of *S. alpina* and estafietin induced major alterations on the morphology of the parasite. The results of this report show that *Stevia* species represent a source of new molecules with potential for the treatment of neglected tropical diseases caused by cestodes.

Keywords: natural bioactive compounds; medicinal plants; *Stevia*; neglected tropical parasitic diseases; *Echinococcus*; anti-parasitic treatment

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1. Introduction

Cestodes, or tapeworms, are an ample and diversified group of obligate endoparasites able to infect a multitude of animal species worldwide. Several members of the class Cestoda are of medical importance since they use humans and other animals as definitive and/or intermediate hosts, causing serious diseases. Among them, *Echinococcus granulosus sensu lato* and *Echinococcus multilocularis* are the etiological agents of cystic and alveolar echinococcosis, respectively; and *Taenia solium* causes cysticercosis. These diseases are among the 20 neglected tropical diseases prioritized by the World Health Organization, that mainly affect people living in poverty principally in tropical and subtropical regions [1]. Currently, the chemotherapeutic options to treat these parasitosis are very limited, relying mainly in the benzimidazoles albendazole and mebendazole, and praziquantel. Benzimidazoles are heterocyclic aromatic compounds formed by the fusion of benzene and imidazole rings. The main mode of action is the selective binding to helminth β-tubulin thereby inhibiting its polymerization and the formation of microtubules, hampering glucose uptake and other cellular functions [2]. Praziquantel is a derivative of pyrazinoquinoline. The mechanism of action of praziquantel is not completely defined. It is known that praziquantel induces high rates of calcium ion influx which results in uncontrolled muscle contraction and paralysis of the worms. The postulated drug targets that trigger this calcium ingress are voltage-gated calcium channels [3], or more recently a transient receptor potential melastatin ion channel (TRPM) [4]. These drugs present some disadvantages in terms of safety, tolerance, efficacy, length of treatment and access for the affected population. For example, 20%-40% inefficacy was reported for benzimidazoles in the treatment of human cystic echinococcosis [5,6], and approximately 60% for alveolar echinococcosis [7]. Albendazole is only parasitostatic and not parasiticide for *E. multilocularis*, implying that a long-term treatment, often life-long, with concomitant adverse effects, is needed to treat alveolar echinococcosis. Currently, the availability of these drugs is low and the cost is high in many endemic countries [8]. This scenario highlights the need for new, effective, safe and affordable drugs to control these cestodiases.

One of the main limitations of research on cestodes is the low availability of parasitic material. Some zoonotic cestodes, such as *E. granulosus s. l.*, cannot be reproduced continuously in experimental models being obtained only from natural infections, which makes the implementation of systematic drug evaluation studies difficult. *Mesocestoides vogae* is a cestode whose tetrathyridium (TTy) larval stage can multiply continuously in laboratory animals, providing a continuous and reproducible source of material for biological assays. Furthermore, it is easily cultivated in vitro and is considered non-zoonotic so it can be handled safely [9,10]. This parasite has been validated as a laboratory model [11], being used for development studies [12–14], and for the identification of new cestocidal compounds in pharmacological studies [15,16].

Natural products have made a significant contribution to the development of medicines against a variety of pathologies. It is estimated that more than 50% of the drugs currently on the market are derived from natural sources or were inspired by nature [17]. In this sense, natural products represent an extensive source of molecules. It is estimated that out of the total of 300,000 plant species inhabiting the Earth, only 6% have been investigated for their pharmacological properties. Furthermore, the molecular complexity and diversity of these compounds, inherent to the biosynthetic machinery of a living organism, are incomparable to the structural possibilities of synthetic molecules. Molecular complexity is often accompanied by highly selective and specific biological activities, as seen in cases such as taxol and artemisinin [18]. These properties make natural products highly attractive for the discovery of new lead molecules in drug design for the development of new medicinal treatments.

The *Stevia* genus (Asteraceae) consists of more than 230 plant species which are mainly distributed from the Southern United States to the South American Andean region. *Stevia rebaudiana* Bertoni is the most popular member of this genus since it is known to produce the diterpene glycoside sweetener, stevioside. Beyond *S. rebaudiana*, numerous other species belonging to the *Stevia* genus are recognized for their medicinal properties [19]. Ethnobotanical records of *Stevia* are described in the book “Natural History of Plants of the New Spain, written between 1570 and 1576 by Francisco Hernandez [20]. *Stevia* species have been popularly employed for the treatment of various ailments

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since the 18th century [19]. Popularly drinks such as decoctions and infusions have been used as febrifuges, to treat inflammation, to cure wounds and for intestinal upsets due to parasites, among other uses [20].

Stevia genus is characterized by the presence of sesquiterpene lactones, diterpenes, longipinanes, and flavonoids as main phytochemical constituents. Extracts and compounds isolated from species of this genus have demonstrated diverse pharmacological activities, being antioxidant, antiviral, anti-inflammatory, and antiproliferative, the most frequently reported [21]. Within the genus, several species have shown antiparasitic activity, including phytochemicals isolated from *S. satureiifolia* var. satureiifolia, *S. alpina* and *S. maimarensis* that have demonstrated trypanocidal properties [22–25] and the extracts of *S. satureiifolia* var. satureiifolia, *S. entreriensis*, *S. multiaristata* and *S. aristata* which have exerted trypanocidal, leishmanicidal, and/or anti-*Echinococcus granulosus* activities [25–27].

Considering the potential of natural products in the drug discovery process and the antiparasitic activity demonstrated by some species of *Stevia* genus, the aim of this work was to analyze the effect of *Stevia* extracts and isolated compounds on *M. vogae* in the search for selective and effective drugs to treat neglected tropical diseases caused by cestodes.

2. Results

2.1. Stevia Extracts

Before the evaluation of the anti-DENV-2 activity, the pure natural compounds were

The dichloromethane extracts of *S. alpina*, *S. multiaristata*, *S. maimarensis* and *S. aristata* showed yields from the dried plant material of 5.0, 16.6, 5.3 and 2.5%, respectively. The analysis of the chemical composition of the extracts was performed by HPLC coupled with a UV-diode-array detector (DAD). The chromatograms obtained can be seen in Figure 1.

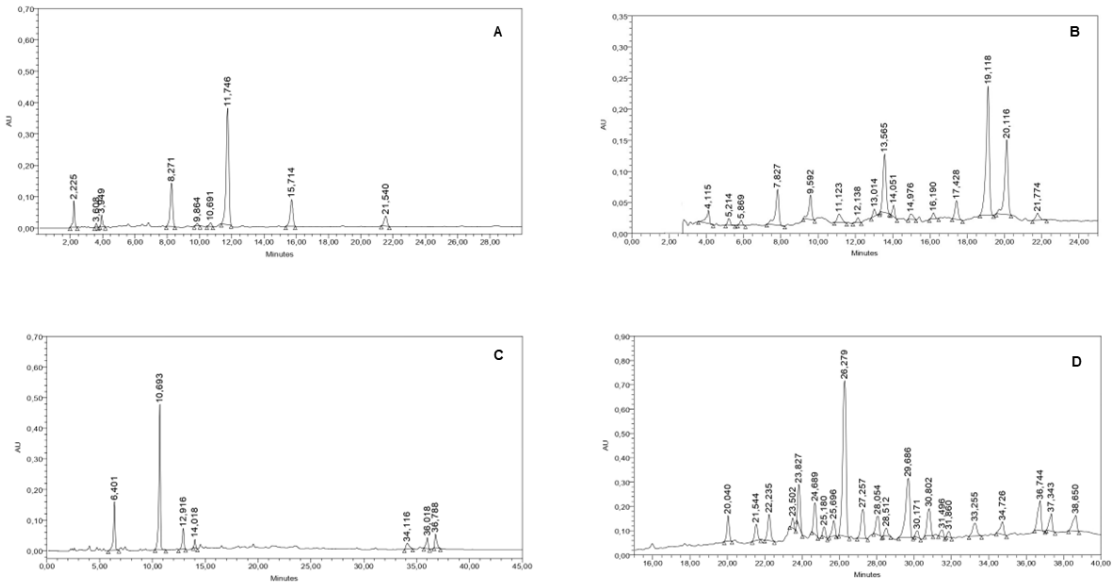

Figure 1. DAD-HPLC chromatograms of the organic crude extract of *Stevia alpina* (A), *Stevia multiaristata* (B), *Stevia maimarensis* (C) and *Stevia aristata* (D).

The dichloromethane crude extract of *S. alpina* showed nine peaks. A major peak of 52.43% of the total peaks area was observed at a Rt (Retention time) of 11.746 min (Figure 1A). This compound showed an UV spectrum with a maximum absorption (λmax) at 210 nm. After processing the extract as described in subsection 2.4, the major component of *S. alpina* was purified and identified as compound 1. The chromatogram of *S. multiaristata* organic crude extract showed sixteen peaks. Three

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major peaks at Rts of 19.119, 20.116 and 13.565 min, representing 32.39, 18.76 and 13.56 % of the total peaks area, were detected (Figure 1B). The dichloromethane crude extract of *S. maimarensis* showed seven peaks with one major peak that represented 53.35% of the total peaks area at a Rt of 10.693 min (Figure 1C). The UV spectrum of this peak did not show a specific pattern with a λmax at 212 nm. This phytochemical was later purified as explained in subsection 4.4 and identified as compound 2. The *S. aristata* organic crude extract presented 22 peaks. A major peak which represented 28.25 % of the total peak area was observed at a Rt of 26.279 min. This compound showed an UV spectrum with maximum absorptions at 276 and 340 nm (Figure 1D).

2.2. Pure Phytochemicals Isolated from Stevia Alpina and S. maimarensis

The sesquiterpene lactones estafietin (compound 1) and eupatoriopicrin (compound 2) were isolated from the aerial parts of *S. alpina* and *S. maimarensis*, respectively. These compounds were identified by spectroscopic methods comparing the experimental spectra with those found in the literature [28,29]. The chemical structures of 1 and 2 can be seen in Figure 2. The purity levels of these phytochemicals were 93.3 and 94.6 % for compounds 1 and 2, respectively.

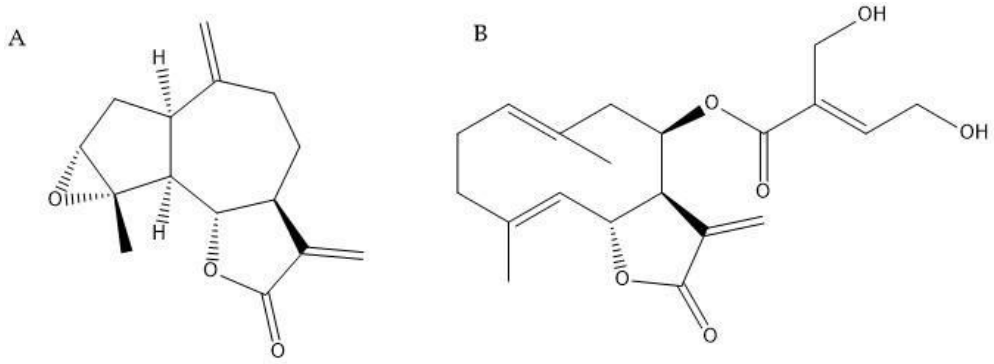

Figure 2. Chemical structures of estafietin (A) and eupatoriopicrin (B), isolated from *Stevia alpina* and *Stevia maimarensis*, respectively.

2.3. Effect of Stevia Extracts and Compounds on Cestode Viability

As detailed in subsection 4.9, the cestocidal activity of *Stevia* species extracts and compounds was evaluated with a worm tracker device that quantifies *M. vogae* TTy motility. Additionally, the alterations on TTy structures were recorded daily with a camera attached to an inverted optical microscope. The morphology of *M. vogae* TTy larvae, when obtained from the mouse peritoneal cavity, consists of an anterior scolex with four suckers as attachment organs; an internal region with a compact, acoelomate and non-segmented parenchyma; and a well-defined external syncytial (multinucleate) tegument that covers the body [30].

Stevia alpina extract showed the highest cestocidal activity of the four species analyzed here. This extract showed almost complete reduction of viability from day 1 at all concentrations tested (96-100%, $p<0.0001$ days 1-9 for all concentrations) (Figure 3A). The other three extracts showed a dose-dependent response. At high concentration (1000 µg/mL) they killed TTy from day 1 (96-100%, $p<0.001$-$P<0.0001$, days 1-9) (Figures 3B, 4A and 4B). *S. multiaristata* showed a high cestocidal activity also at 500 µg/mL (84-97%, $p<0.001$-$p<0.0001$, days 1-9) (Figure 3B). *S. maimarensis* and *S. aristata*, showed a similar cestocidal activity. At 500 µg/mL it was observed an initial reduction of viability (*S. maimarensis*: 59%, $p<0.05$, day 1; 78%, $p<0.01$, day 2; *S. aristata*: 78%, $p<0.0001$, day 2) and a delayed high cestocidal action (days 3-9: *S. maimarensis*: 84-96%, $p<0.01$-$p<0.001$; *S. aristata*: 96-100%, $p<0.001$-$p<0.0001$). An unexpected stimulatory effect was displayed at 100 µg/mL (*S. maimarensis*: 26-37% of increase, $p<0.01$-$p<0.001$, days 2-9) (Figure 4A) (S. aristata: 8% of increase on days 6 and 8, $p<0.05$) (Figure 4B).

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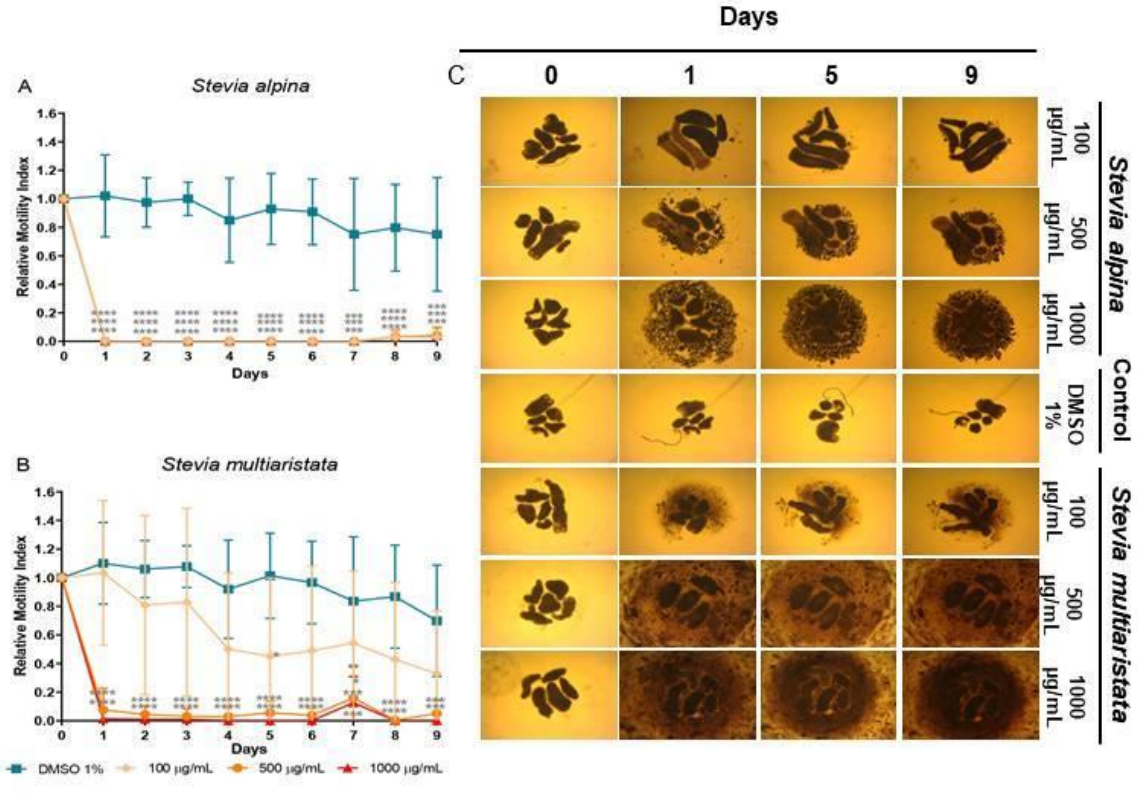

Figure 3. Effect of *Stevia* extracts in *Mesocestoides vogae* tetrathyridia. (A-B) *In vitro* cestocidal activity determined by worm motility for 9 days using a worm tracker device for the extracts: (A) *Stevia alpina* and (B) *Stevia multiaristata*. Relative motility indices were measured from three independent biological replicates, each one in quadruplicate. Error bars represent the standard deviation and the asterisks indicate those values that showed differences with statistical significance compared with the negative control, according to two-way ANOVA test and Dunnett's post-tests (*$p < 0.05$, **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$). (C) Inverted optical microscope images (40X) of *S. alpina* and *S. multiaristata* extracts in *M. vogae* tetrathyridia at different days of treatment. The extracts were evaluated at concentrations of 100, 500 and 1000 µg/mL. Parasites incubated with the drug vehicle (DMSO 1%) were used as a negative control. Morphology alterations and extensive damage on the tegument are observed.

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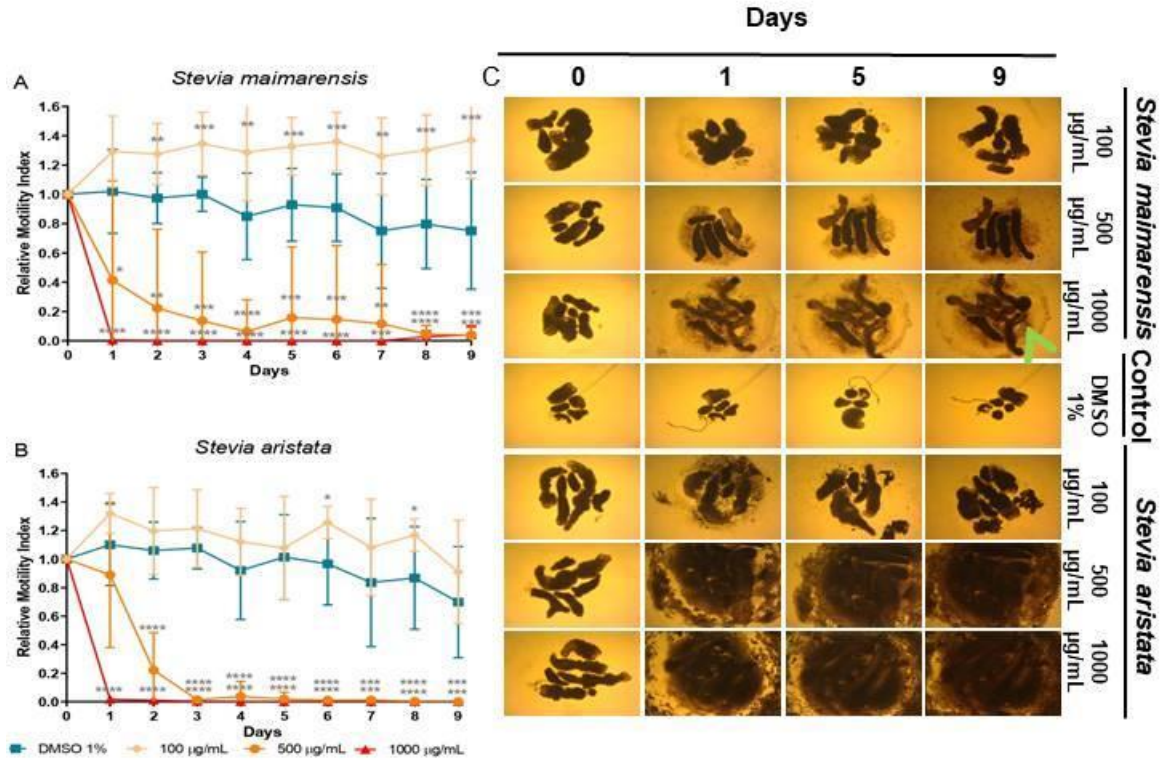

Figure 4. Effect of *Stevia* extracts in *Mesocestoides vogae* tetrathyridia. (A-B) *In vitro* cestocidal activity determined by worm motility for 9 days using a worm tracker device for the extracts: (A) *Stevia maimarensis* and (B) *Stevia aristata*. Relative motility indices were measured from three independent biological replicates, each one in quadruplicate. Error bars represent the standard deviation and the asterisks indicate those values that showed differences with statistical significance compared with the negative control, according to two-way ANOVA test and Dunnett's post-tests (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001). (C) Inverted optical microscope images (40X) of *S. maimarensis* and *S. aristata* extracts in *M. vogae* tetrathyridia at different days of treatment. The extracts were evaluated at concentrations of 100, 500 and 1000 µg/mL. Parasites incubated with the drug vehicle (DMSO 1%) were used as a negative control. Morphology alterations, extensive damage on the tegument with the presence of influx (green arrows) of medium culture into the worm are observed.

Estafietin, the main compound of *S. alpina*, induced a strong dose-dependent reduction of viability at 500 (97-100%, p<0.001-p<0.0001) and 100 µM (79-97%, p<0.001-p<0.0001), with no effect at 50 µM (Figure 5A). Eupatoriopicrin was less effective than estafietin. This compound, obtained from *S. maimarensis*, showed at 500 µM a delayed reduction of viability (53%, day 1 60%, day 2; 71-76%, days 3-9; p<0.05-p<0.01). Unlike the parent extract, that produced an 8% increase of viability, TTy treated with lower concentrations of this terpene displayed similar values as negative control parasites (Figure 5B).

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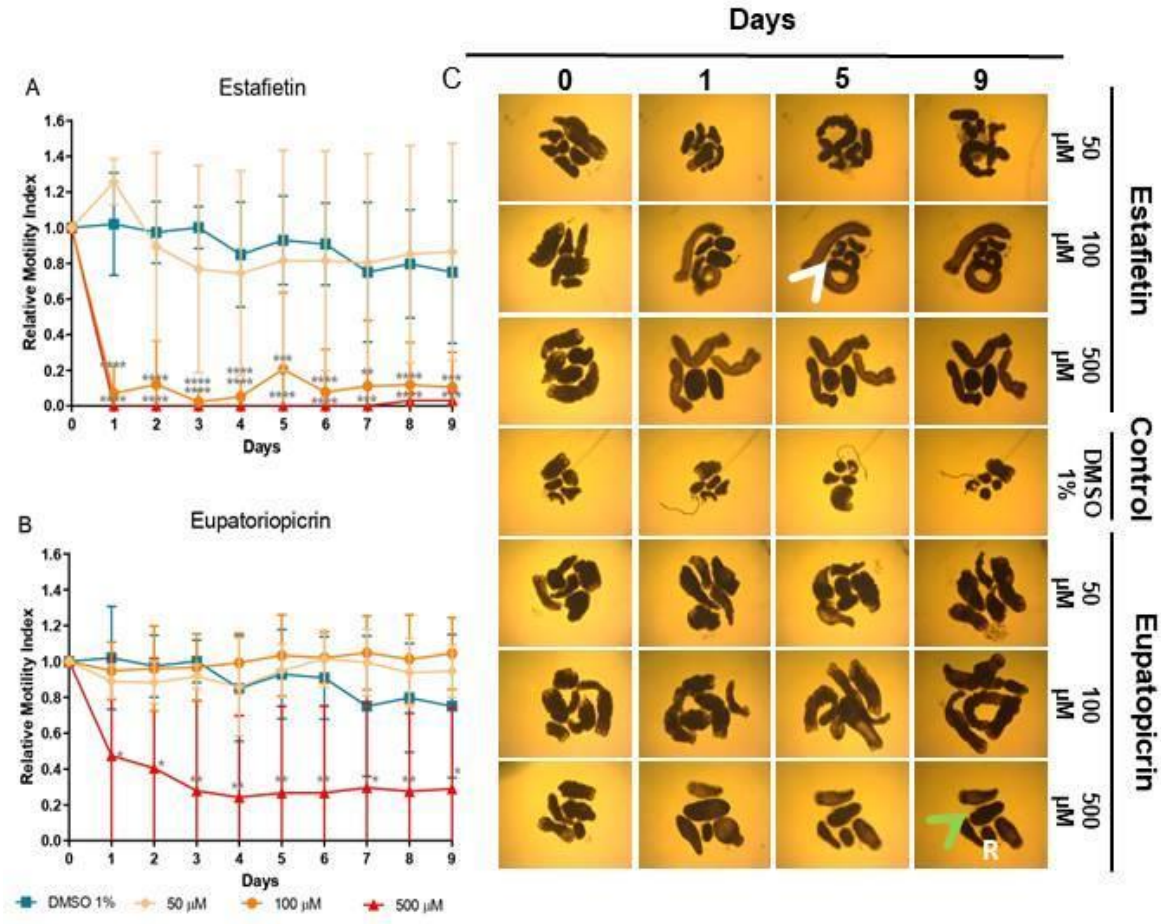

Figure 5. Effect of estafietin and eupatoriopicrin in *Mesocestoides vogae* tetrathyridia. (A-B) *In vitro* cestocidal activity determined by worm motility for 9 days using a worm tracker device for the compounds: (A) Estafietin and (B) Eupatoriopicrin. Relative motility indices were measured from three independent biological replicates, each one in quadruplicate. Error bars represent the standard deviation and the asterisks indicate those values that showed differences with statistical significance compared with the negative control, according to two-way ANOVA test and Dunnett's post-tests (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001). (C) Inverted optical microscope images (40X) of estafietin and eupatoriopicrin in *M. vogae* tetrathyridia at different days of treatment. The compounds were evaluated at concentrations of 50, 100 and 500 µM. Parasites incubated with the drug vehicle (DMSO 1%) were used as a negative control. Morphology alterations, extensive damage on the tegument with the presence of influx (green arrows) of medium culture into the worm and blebs (white arrows), as well as cellular debris (R) are observed.

The results of microscopical observations confirmed those obtained with the WMicrotracker. At concentrations of extracts or compounds that reduced viability, we observed a lack of motility with an evident alteration of general morphology, compared with untreated parasites (Figures 3C, 4C and 5C). The main change observed in *S. alpina*, *S. maimarensis* and *S. aristata* was an elongation of the body, in addition to a loss of tegumental definition. Also, cellular debris was observed in the culture medium. TTys treated with *S. multiaristata* looked more compact (tegumental debris couldn't be observed because of the extract precipitation), and some alterations of morphology and extensive damage in the tegument that lines the worms were observed.

3. Discussion

The scarcity of safe, effective and affordable drugs to treat diseases caused by cestodes such as echinococcosis and cysticercosis, which mainly affect vulnerable populations, highlights the importance of finding new treatment alternatives. In previous works, we assessed the effect of extract

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of plants from the Asteraceae family on *E. granulosus sensu stricto* (s.s.) protoscoleces, and cysts obtained from the murine experimental model and observed that the extracts from *S. aristata* [26] and *S. multiaristata* [27] showed *in vitro* protoscolicidal potential. Both *Stevia* extracts also caused damage to the germinal layer of murine cysts and produced a significant reduction in the parasitic mass obtained from mice infected with *E. granulosus* s.s. protoscoleces. Based on these findings, we hypothesized that major compounds of *Stevia* extracts could have cestocidal potential. To test this hypothesis, we evaluated the potential of the mentioned *Stevia* extracts and two additional ones from *S. alpina* and *S. maimarensis*, to reduce the viability of *M. vogae* TTy. Then, we isolated the major compounds of two of these extracts and evaluated their cestocidal potential on these parasites. The cestocidal effect was measured by the quantification of worm motility using the worm microtracker device [31] adapted to cestodes [32–35]. This method allows the simultaneous evaluation of a high number of compounds, objectively and quantitatively. It also enables continuous, real-time, and non-invasive measurements, facilitating the assessment of parasitic viability on the same plate throughout the entire testing period. For the assay, we used *M. vogae* TTy, since this is a cestode laboratory model that allows the implementation of systematic drug evaluation studies. The results obtained showed that all the extracts tested produced a high and significant reduction of *M. vogae* TTy viability at the highest concentration tested (1000 μg/mL). At lower concentrations *S. alpina* was the most potent, reducing parasite viability by more than 95%, from the first day of incubation.

Estafietin, the major compound present in *S. alpina* extract, showed a high cestocidal potential being able to kill 100% of parasites at the highest concentration tested -500 μM- and more than 76% of parasites at 100 μM, even from the first day of incubation. On Vero cells, this compound presented a 50% cytotoxicity concentration (CC_{50}) value of 800.8 μM [36], thus indicating it does not show overt toxicity against this human cell line. The early effect of this compound suggests that it could help shorten cestocidal treatments. This is of major importance taking into consideration the need for prolonged treatment in echinococcosis treatments with the currently used drug albendazole. Using the same parasite model and motility measure method as in this work, we have previously shown a delayed effect for albendazole at 20 μM which induced 50% viability reduction only from day 4 of incubation [28]. Eupatoriopicrin, isolated from *S. maimarensis* extract, showed cestocidal capacity although it was less potent and selective than estafietin (CC_{50}=257.7 μM) [36]. This compound reduced parasite motility or did not change it, according to the concentration used. It did not increase parasite motility, suggesting that the low increase in motility observed with *S. maimarensis* could be due to other/s compounds present in the extract. Both estafietin and eupatoriopicrin are sesquiterpene lactones (STLs), which are a class of terpenoid compounds, mainly found in species of the Asteraceae family. This group of phytochemicals presents a fifteen-carbon backbone (C15) with a ɣ-lactone ring closed toward C6 or C8 and a methylene group conjugated to the carbonyl group, forming an α,β-unsaturated ɣ-lactone moiety [37]. According to their skeletal arrangement estafietin and eupatoriopicrin are classified as guaianolide and germacranolide type, respectively. STLs have been thoroughly studied due to their wide range of biological activities. These activities include antimicrobial, antitumor, anti-inflammatory, molluscicidal, antihelminthic, antiprotozoal, among others [29]. Moreover, STLs play a crucial role in plant-insect interactions, serving as attractants, deterrents, and antifeedants. Initially considered highly cytotoxic, chemical modifications have enhanced their biological activities while reducing their cytotoxicity, renewing interest in them as lead compounds in the drug discovery process.

Several studies investigated the anthelmintic properties of plant extracts or essential oils [38]. However, only a few of them have focused on the effect of isolated compounds from plants. Concerning STLs, some studies of their effect on *E. multilocularis* were reported. Since dihydroartemisinin and artesunate were effective against *E. multilocularis* metacestodes *in vitro* but not *in vivo* (mouse model), the *in vitro* effects of synthetic ozonides (1,2,4-trioxolanes) were investigated. These compounds were shown to induce structural alterations in the parasites [39]. As far as we know there are no further reports of the effects of STLs on cestodes.

Herein, we showed that estafietin, as well as its parent extract of *S. alpina*, produced an early and potent *in vitro* cestocidal effect. Experiments to assess their *in vivo* effect in animal models will be

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conducted in the future. The results of this work suggest that sesquiterpene lactones, terpenoid compounds present in Asteraceae, such as *Stevia* species, alone or in combination with currently used drugs, could be considered potential candidates for the development of new medicines to treat neglected diseases caused by cestode parasites.

4. Materials and Methods

4.1. Plant Materials

The aerial parts of the *Stevia* species used in this work were collected from different locations within Argentine territory. In order to preserve the genetic resource and the natural ecosystem, these plant materials were harvested conservatively, cutting 10-15% of the aerial parts with scissors [24]. *Stevia maimarensis* (Hieron.) Cabrera was collected in Jujuy Province, Tilcara Department: Perchel, in March 2017. *Stevia alpina* Griseb. was collected in Catamarca Province, Provincial Route 307, Km 49, in April 2015. *Stevia aristata* D. Don ex Hook. & Arn. was collected in Entre Ríos Province, La Paz Department, Provincial Route 1, Esquivel stream, in December 2012. *Stevia multiaristata* Spreng. was collected in Entre Ríos Province, Paraná Department, Pueblo Brugo, Paraná River in December 2012. The identification of these wild species was conducted by the renowned taxonomists Dr. Gustavo Giberti and Hernan Bach. Voucher specimens are available at the Museo de Farmacobotánica "Juan A. Domínguez", Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Argentina, under the identification numbers BAF12264, BAF12266, BAF797 and BAF798, respectively.

The plant materials collected from each species were dried at room temperature protected from sunlight and humidity and afterwards manually grounded to be stored until used.

4.2. Extract Preparation

Once dried and grounded, the plant materials were extracted to obtain the crude organic extract from each species. Consequently, the material was extracted twice by maceration with dichloromethane (CH_2Cl_2) (10% w/v) for 5 minutes. The resulting extracts were filtered (Schleicher and Schuell-Whatman, grade 0859, medium, smooth, 90 mm) and taken to dryness at 40°C under vacuum in a rotary evaporator (Buchi R-134). The yield of the extraction process was calculated as: yield (%) = (weight of the obtained extract (g) × 100)/weight of initial plant material (g).

4.3. Chromatographic Analysis of the Stevia Crude Extracts

The chromatographic analysis of the crude extracts of *Stevia* species was performed by high-performance liquid chromatography (HPLC) with a Waters equipment coupled to a photodiode array detector (Waters 2996), a Rheodyne injection valve (20 µL), pump (Waters Delta 600), Waters 600 controller, and in-line degasser. A reversed-phase column (Agilent Eclipse Plus C-18, 4.6 × 250 mm, 5 µm particle size) was used, and the photodiode array detector was set at 210 nm. The extracts were dissolved in water: acetonitrile (1:1) at 10 mg/mL concentration. The solutions were filtered with a nylon filter (0.45 µM, Agilent) and eluted with a gradient of water (A) and acetonitrile (B) in 40 min. The specific gradients used were 0-98% B for the extract *S. aristata*, 35-95% B for *S. multiaristata*, *S. maimarensis* and *S. alpina.* The flow rate was 1.0 mL/min, and the elution was performed at room temperature. Chromatograms were recorded and processed using the Empower Pro 3 software. The water employed to prepare the mobile phase was of ultrapure quality (Milliq). Acetonitrile (HPLC) J. T. Baker and methanol (HPLC) J. T. Baker were used.

4.4. Isolation and Purification of Phytochemicals

The crude extract of *S. alpina* (1.5 g) was fractionated by Silicagel 60 column chromatography (40 × 3 cm, 80 g, 230–400 mesh) eluted with CH_2Cl_2: ethyl acetate (EtOAc) (9.5:0.5). Fractions of 75 mL each were collected and tested by TLC (SP: Silicagel F_{254}; MP: CH_2Cl_2:EtOAc (9.5:0.5); SR: sulfuric anisaldehyde). From fractions 3-6 a major compound precipitated in the form of white sharp needles. This precipitate was dissolved in the minimum volume of a heated mixture of heptane: EtOAc (2:1)

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to be cooled to 4°C for 24 h in order to facilitate the crystallization process. Afterwards, the crystals were separated from the solution and taken to dryness under vacuum to afford compound **1**.

The crude extract of *Stevia maimarensis* was submitted to a dewaxing process to eliminate sterols and lipids. The extract obtained as described in subsection 2.2 was suspended in 200 mL of ethanol: water (70:30) to be partitioned three times with 60 mL of hexane (Hx). The remaining hydroalcoholic suspension was extracted thrice with 60 mL of CH_2Cl_2. The resulting dichloromethane sub-extracts were gathered, dried with anhydrous sodium sulfate (Biopack), filtered, and taken to dryness under reduced pressure. The dewaxed extract of *S. maimarensis* (10 g) was fractionated by column chromatography (50 × 5 cm) using Silicagel 60 (180 g, 230–400 mesh) as stationary phase (SP) and CH_2Cl_2:EtOAc (1:2) as mobile phase (MP). 40 fractions of 50 mL each were collected. The fractions were tested by Thin Layer Chromatography (TLC) [SP: Silicagel F_{254}; MP: Hx:EtOAc (5:5); SR (spraying reagent): sulfuric anisaldehyde]. Between fractions 17 and 29 one pure compound was detected. These fractions were gathered and taken to dryness under reduced pressure to afford yellow crystals corresponding to compound **2**.

4.5. Purity Assessment and Identification of the Isolated Compounds

The purity analysis of compounds **1** and **2** was carried out using HPLC. A Waters chromatograph equipped with a UV-visible diode array detector (Waters 2996) and a pump (Waters Delta 600) was utilized. An Agilent Eclipse Plus C-18 analytical column of 4.6 × 250 mm and 5 µm particle size was employed. Gradients of water:acetonitrile (A:B) were used as MP (45-95% B and 35-95% B, for compounds **1** and **2**, respectively.) The compounds were dissolved in a mixture of A:B (1:1) using an ultrasonic bath. Subsequently, the solutions were filtered using 0.45 µm nylon filters. In all cases, a 20 µL loop and a flow rate of 1 mL/min were used. The runtime was set to 30 min. A run for the solvent was performed by injecting the solvent mixture used to dissolve the compounds. The purity was calculated as: purity (%) = (area of the major peak × 100) / ∑ area of every peak.

The identities of the isolated compounds were determined by spectroscopic methods: proton nuclear magnetic resonance (^{1}H-NMR) and carbon nuclear magnetic resonance (^{13}C-NMR), heteronuclear single quantum correlation (HSQC), heteronuclear multiple bond correlation (HMBC), correlated spectroscopy (COSY) (Bruker Avance 600) (600 MHz in CDCl3), electron impact mass spectrometry (EI-MS), and spectrophotometry (UV), comparing experimental spectra with literature data [11,12].

4.6. Drugs Preparation for Biological Assays

Stock solutions of the isolated *Stevia* compounds and the crude extracts were prepared using dimethyl sulfoxide as the vehicle (DMSO) at concentrations of 30 mg/mL and 100 mg/mL, respectively. The solutions were afterward fractionated and stored at -18ºC and thawed in an ultrasonic bath at 40ºC to later be diluted as necessary for each biological assay.

4.7. Ethics Statement Assays

Experiments involving the use of experimental animals were conducted strictly in accordance with the protocols approved by the Comité Institucional para el Cuidado y Uso de Animales de Laboratorio (CICUAL), Facultad de Medicina, Universidad de Buenos Aires (UBA), Argentina (protocols: ''*In vivo* passages of cestode parasites from *Mesocestoides vogae*" CD Nº 1127/2015 and ''Histone modifying enzymes in flatworms: study of their potential as new drug targets in diseases of importance in veterinary medicine and human health" CD Nº 187/2020).

4.8. Parasite Material

The *M. vogae* tetrathyridia (TTy) used in this work were maintained in the laboratory by alternate intraperitoneal infection in Wistar rats and BALB/c mice, as described previously [40]. The experimental animals were bred and housed in a temperature-controlled light cycle room with food and water *ad libitum* at the animal facilities of Instituto de Investigaciones en Microbiología y

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Parasitología Médica (IMPaM), Universidad de Buenos Aires (UBA) and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Ciudad Autónoma de Buenos Aires, Argentina. After 3 months of infection, mice were euthanized by CO_2 inhalation. TTy were collected from the peritoneal cavity, using standard aseptic techniques, and washed three times with sterile PBS solution, pH 7.2. Finally, before being employed in experiments, TTy were size-selected using monofilament polyester meshes to a final size of 150 – 250 μm and incubated for 24 h in 5 mL of MvRPMI medium –a modified RPMI 1640 medium without phenol red (Sigma-Aldrich, USA) complemented with 10% v/v inactivated fetal bovine serum (INTERNEGOCIOS SA, Argentina), 2.4 g/L of HEPES Free acid (JT Baker, USA), 2.5 g/L of glucose (4.5 g/L final concentration, Britania, Argentina), 2 g/L of Sodium Bicarbonate (Anedra, Argentina) and 1% v/v Pen/Strep (Penicillin-Streptomycin 10,000 U/mL, Gibco, USA) at 37ºC under 5% CO_2 atmosphere.

4.9. In Vitro Anthelmintic Assays

The *in vitro* effect of each extract and compound on parasite viability was studied as described before by our group [32–35] with minimum modifications. Viability was evaluated with a motility assay employing a worm tracker device (WMicrotracker MINI, Designplus SRL, Argentina) [31], that was previously adapted to measure the movement of *M. vogae* TTy [33]. Briefly, the 24 h incubated TTy, as described in 4.8, were distributed in U-shaped 96-well microplates (Greiner Bio-One, Germany) (five TTy per well) with 150 μL of MvRPMI medium at 37ºC under 5% CO_2 atmosphere and incubated an additional 24h. Incubations with extracts or compounds were performed the following day. Extracts were tested at concentrations of 100, 500 and 1000 μg/mL and pure compounds at concentrations of 50, 100 and 500 μM. As positive controls, pre-treated parasites with ethanol 70% for 30 min and the antiparasitic drug praziquantel at 20 μM were used. All motility assays were performed using an equal amount of the drug vehicle (1% DMSO final concentration) and the corresponding negative control (1% DMSO). To determine the effects of the treatments, TTy were incubated for nine days without changing the medium in the same conditions as described above. Measurements of motility with the WMicrotracker and microscopical observations were performed before adding the compounds (day 0) and daily afterward. The data was collected from three independent biological replicates, each corresponding to TTy obtained from a different mouse, in quadruplicate for each tested condition. Relative motility indices (RMI) of each well respective to its motility before adding the compounds were calculated as described previously [32–35]. Statistical analyses were carried out using GraphPad Prism 8.0.2. Repeated two-way ANOVA tests were used to analyze the effects of the compounds on TTy motility. Significant differences ($P<0.05$) were determined by Dunnett's comparisons post-tests, comparing each treatment concentration with the negative control group (each run on each day). The percentages of reduction (or increase) of viability, as described in the Results section, were taken from the relative motility index by using the formula % reduction = (1-RMI)*100 (for example, an RMI of 0.2 corresponds to 80% of viability reduction. Microscopical observation of motility and morphological changes of the TTy were also employed in all assays. The observation was performed with an inverted microscope (Primo Vert, Carl Zeiss, Germany) and images were taken using a digital video camera (AxioCam ERc5c, Carl Zeiss, Germany).

5. Conclusions

This study provides laboratory evidence of the *in vitro* effect of the STL estafietin, as well as its parent extract of *S. alpina* on cestodes. The early and potent cestocidal effects observed suggest that *Stevia* extracts and compounds can be explored as new therapeutic alternatives, to be applied alone or in combination with classical medicines such as albendazole to treat tropical neglected diseases caused by these types of parasites.

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M.C.R.,V.P.S.; data curation, A.M.C., M.P.C.C., J.B.; writing—original draft preparation, M.P.C.C., J.B., M.C.R.; writing—review and editing, M.P.C.C., J.B., A.M.C., H.R.V., A.EB, V.P.S..; visualization, M.P.C.C., A.M.C., M.C.R.; supervision, V.P.S.. M.C.R.; project administration, V.P.S., M.C.R.; funding acquisition, V.P.S., M.C.R. All authors have read and agreed to the published version of the manuscript.

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