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Activity against *Leishmania sp.* (Trypanosomatidae) of 8β-isovalerianyloxy-9α-hydroxy-calyculatolide isolated from *Neurolaena lobata* (Astereceae) in Costa Rica

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***Neurolaena lobata* (Astereceae) has a variety of uses in traditional medicine, including the treatment of protozoan infections, ulcers, diabetes and inflammatory skin disorders. The therapeutic properties are mainly attributed to the sesquiterpene lactones produced by *N. lobata* (Astereceae). In this study, 8β-isovalerianyloxy-9α-hydroxy-calyculatolide, a sesquiterpene lactone present in the mature leaves of *N. lobata*, was isolated. The compound was characterized by spectroscopic techniques with an IC_{50} value of 3.75 ± 1.63 µg/mL against *Leishmania* sp in *in vitro* tests. This study emphasizes the importance of identifying the components responsible for the biological activities of plant-based treatments. Such knowledge is vital for the standardization and development of phytopharmaceuticals.**

Key words: *Neurolaena lobata*, sesquiterpene lactones, *in vitro*, *Leishmania sp.*, phytochemical screening, Costa Rica.

INTRODUCTION

Leishmaniasis is a disease caused by a parasitic protozoan of the genus *Leishmania*, which is transmitted to humans through the bite of infected female sandflies. The disease mainly affects populations with limited economic resources, who are vulnerable due to problems such as malnutrition, weak immune systems, poor housing conditions and their location in areas affected by climate change, leading to an increase in the number of biological vectors involved in the transmission of the parasite, as well as an increase in the multiplication of the parasite within the biological transmitter (Alvar et al., 2012; WHO, 2020).

According to data reported by the World Health Organisation (WHO), it is estimated that leishmaniasis affects approximately 700,000 to 1,000,000 individuals annually, of which 26,000 to 65,000 die, a figure surpassed only by malaria (WHO, 2017; WHO, 2020). The countries reporting the highest global incidences of leishmaniasis are Syria, Afghanistan, Pakistan, Brazil, Iran, Algeria, Iraq, and Colombia (WHO, 2020).

There are three main forms of leishmaniasis: cutaneous (CL), visceral (LV) and mucocutaneous (CML). It is estimated that approximately 0.2–0.4 million new cases of VL and 0.7–1.2 million new cases of CL occur worldwide each year (WHO, 2017). In 2018, more than 5000 endemic cases of CL were reported in 10 countries, accounting for 85% of the total world incidence of CL leishmaniasis. For VL leishmaniasis, 90% of reported cases globally occur in seven countries: Brazil, Ethiopia, India, Kenya, Somalia, South Sudan, and Sudan (WHO, 2020). CL leishmaniasis manifests as the formation of skin ulcers on the face and extremities, which after healing (even several years later), can reappear in mucocutaneous form, a condition

considered to be severe and can lead to destruction of the nose and soft palate of the patient if not treated early and properly. The LV form of leishmaniasis is the most severe and can lead to prolonged fever, anaemia and haemorrhage, with a mortality rate of 10%. Approximately 400,000 people worldwide are diagnosed with LV leishmaniasis each year (Handler et al., 2015).

In Costa Rica, the average incidence of leishmaniasis in the 2005-07 triennium was 35.5 cases per 100,000 population, rising to 30.5 cases in the 2014–16 triennium. The most affected group was those under 15 years of age, with an incidence rate of 44.42 cases per 100,000 population. In San Ramón, Guatuso, Turrialba and Talamanca, the incidence rates exceeded the average incidence rate (Jaramillo-Antillón et al., 2018). However, according to the Pan-American Health Organization (PAHO) (2019), there was a considerable reduction in the incidence rate in Costa Rica in 2019 compared to 2017, with a 48% decrease in the cutaneous form of leishmaniasis, which is popularly known in Costa Rica as "papalomoyo". The visceral form of leishmaniasis has only been reported once in Costa Rica, in a minor from the province of Guanacaste (Carrillo et al., 1999; Guerrero, 2015).

The most common treatment for leishmaniasis is an injection containing pentavalent antimony (meglumine antimoniate), which can cause cardiotoxicity, hepatotoxicity, pancreatitis, and other side effects (Shahian and Alborzi, 2009; Baiocco et al., 2009). Another drug commonly used as an alternative treatment, but with high toxicity to the liver and kidneys, is amphotericin B, which must be administered very slowly intravenously (Freitas-Junior et al., 2012). Miltefosine is an antileishmanial drug that, according to Freitas-Junior et al. (2012), was the first effective oral treatment; however, despite having low toxicity, miltefosine is classified as teratogenic (Sundar, 2007).

In addition to the problems with side effects, some patients do not complete their treatment, which increases the risk of the parasite developing resistance to the drugs (Freitas-Junior et al., 2012).

In recent years, alternative active compounds have been investigated to control leishmaniasis with higher efficacy and fewer side effects. Current approaches include the use of a mixture of anti-parasitic compounds (Abamor and Allahverdiyev, 2016) and the combination of an antileishmanial drug with other natural active compounds, such as plant extracts or oils, to enhance the effect and reduce the toxicity of the drug (Van Griensven et al., 2010; Oryan, 2015).

A large number of plant extracts have shown activity against leishmaniasis, ranging from crude extracts and semi-purified fractions to pure compounds, which include sesquiterpene lactones, diterpenoids and alkaloids (Iqbal et al., 2016).

Neurolaena lobata is a plant of the Asteraceae family, which is widely distributed throughout Central America and the Caribbean, and can also be found from southern Mexico to the north-western region of South America. This plant has a great variety of uses in traditional medicine and has been identified as being active against protozoa, such as *Leishmania* sp., *Plasmodium falciparum* and *Plasmodium berghei* (Berger et al., 2001; Chinchilla et al., 2011; Chinchilla et al., 2014), as well as against different types of cancer, ulcers, inflammatory skin disorders, diabetes and pain of different origins (Lajter et al., 2014). The vast majority of these medicinal properties are attributed to the sesquiterpene lactones produced by this species as these compounds are the secondary metabolites present mainly in these plants. Sesquiterpene lactones have been used to reverse the resistance of some *Leishmania* species to certain drugs (Pérez-Victoria et al., 1999) and have also shown bioactivity against malaria and a powerful anti-inflammatory activity (Mckinnon et al., 2014).

In Costa Rica, a study carried out on plants collected at the Alberto Manuel Brenes Biological Reserve (ReBAMB) to detect bioactivity against parasites identified antileishmanical properties of *N. lobata*, along with other promising plants (Chinchilla et al., 2014). The present work reports the activity against *Leishmania* sp. of the compound 8β-isovalerianyloxy-9α-hydroxy-calyculatolide, a sesquiterpene lactone isolated from the mature leaves of *N. lobata*.

MATERIALS AND METHODS

Collection of plant material

Mature leaves of *N. lobata* (known as Gavilana in Costa Rica) (number 23 in the UCIMED herbarium) were collected in the REBAMB. This reserve is located 42 km northwest of the city of San Ramón, in the province of Alajuela, Costa Rica (10°13′49"N, 84°36′10"W). The reserve is located at an altitude of between 600 and 1640 m, with an average temperature of 21°C and a relative humidity of 98% (Sánchez, 2000). The plant material was collected and packed in plastic bags and transported to the laboratory according to protocols previously described by Chinchilla et al. (2011).

Preparation of crude extract

The plant material (500 g) was washed and dried in an oven at 40°C for 1 week, then ground in a Trapp® brand chopper/grinder and stored in plastic bags in a fridge (4°C). The material was macerated with 70% v/v ethanol and stored in an amber bottle for 1 week with occasional stirring. After this period, extracts were vacuum filtered using Whatman 1 paper and concentrated at 40°C in a rotary evaporator (Büchi R-114, Switzerland) to obtain the crude extract.

Phytochemical screening

The methodology described by Sharapin et al. (2000) was used with the following modifications: liquid-liquid extractions were carried out with 40 mL of crude extract and 15 mL of ethyl ether (in triplicate) to obtain an aqueous phase (AQ1) and an ether extract. The obtained ether extract was concentrated to dryness to give fraction (E). Half of the volume of the aqueous phase (AQ1) was hydrolysed with 15 mL of 3 mol/L HCl and subsequently extracted with ethyl ether to give an ether phase (AQ2) and a hydrolysed aqueous phase.

Each extract was subjected to qualitative tests, as described by Sharapin et al. (2000), to determine the secondary metabolites present. Different methods of chemical analysis indicated the presence of terpenes (vanillin), alkaloids (Dragendorff), flavonoids (Shinoda), coumarins (KOH), triterpenes and sterols (Liebermann-Burchard), and quinones (Bornträger-Kraus) in the ether phases (E and AQ_2). Phenols and tannins ($FeCl_3$), polysaccharides (Lugol), reducing sugars (Fehling), saponins (foam) and alkaloids (Dragendorff) were determined to be present in the aqueous sample (AQ1). The hydrolysed aqueous sample also contained anthocyanins, as determined by an acid-base test. Antiparasitic tests were carried out on each of the samples obtained after the liquid-liquid extraction to determine the extracts that had activity.

The ether extract was found to have antiparasitic activity; therefore, one or more of the metabolites present in this extract must be responsible for the antiparasitic activity, and coumarins, terpenes and sterols are the most likely candidates.

Compound purification

Antiparasitic tests revealed that the ether extract had activity against *Leishmania* sp. All stored material was macerated for one week using ethyl ether in an amber bottle with occasional stirring at room temperature. The ether extract was then vacuum filtered using Whatman 1 paper and the solvent was evaporated at 30°C using a Büchi R-114 brand rotary evaporator.

The separation of the ether extract components was carried out by column chromatography using a glass column with a Teflon key and silica gel (230–400 mesh) as stationary phase. The mobile phase consisted of a concentration gradient from hexane to ethyl acetate, starting with 100% hexane and ending with 100% ethyl acetate. Eighteen fractions were obtained. The obtained fractions were analysed by thin layer chromatography (TLC), using F_{254} silica gel plates of 20 × 20 cm and 0.2 mm thickness, to identify fractions with the same behaviour, which were then tested for their anti-parasitic activity. Of the 18 fractions, fractions 6 and 7 showed the highest activity against *Leishmania* sp. Fractions 6 and 7 were purified by preparative layer chromatography using J.T. Baker analytical plates (SiF_{254} TLC silica gel) and a mobile phase of hexane:ethyl acetate (3:7).

Identification of 8β-isovalerianyloxy-9α-hydroxy-calyculatolide

Gas chromatography analyses were carried out with a Shimadzu instrument, model GC-17ª, with a mass spectrometer detector in split mode; model QP-5000, coupled to an AT-5 column (5% diphenylpolysiloxane), length 30 m, 0.25 mm ID and ionisation detector (electron impact 70 eV) and the databases Wiley139, NIST107 and SZTERP. The sample was also analysed by ^{13}C Nuclear Magnetic Resonance (NMR) and ^{1}H NMR using a Bruker Ascend instrument operated by an Advance III 600 console with a 5-mm BBO 600 MHz probe (frequencies of 600.1324005 MHz for ^{1}H and 150.9178981 MHz for ^{13}C).

Parasites

The promastigotes of the *Leishmania mexicana* (OCR with known characteristics) strain, which have well-known characteristics, were cultured in Rugai medium for maintenance of the strain and in RPMI (Roswell Park Memorial Institute) medium supplemented with 10% foetal bovine serum for the experiments.

In vitro tests

The extracts were treated according to the methodology described by Chinchilla et al. (2014). Briefly, in a first presumptive test, extracts and controls were placed in the presence of 5×10^5 to 5×10^6 promastigotes and the antiparasitic activity was determined according to the parameters described previously. In a second trial, the IC_{50} value of promising extracts was determined; serial dilutions of the extracts were performed according to the dry weight previously established for each sample, followed by refrigeration for 24 h and determination of parasite viability. Each process was performed in triplicate, in no less than three successive experiments, and readings were carried out by the same person in all cases. Different concentrations of extracts and purified products were used to determine the concentration that inhibited 50% of the organisms (IC_{50}). The Probit method (Díaz et al., 2004) was used to calculate the IC_{50} values of the antiparasitic activity. This method uses the data obtained for each dilution of the extract, with the known weight of the component under study, in terms of the antiparasitic activity, less than and greater than 50%. With this information, a graph of effect vs. dose can be established, which is a fundamental aspect in the calculation of IC_{50} values. All experiments were performed using negative controls, parasites in MEM medium only, and positive controls, parasites killed by heating.

Table 1. Phytochemical screening of the dried mature leaves of *N. lobata.*

Metabolite	Ether extract (E)	Aqueous extract (AQ_1)	Hydrolysed aqueous extract (AQ_2)
Alkaloids	+	+	+
Flavonoids	+	-	+
Coumarins	+	-	+
Triterpenes and sterols	+	-	+
Quinones	+	-	+
Tannins	+	+	-
Reducing compounds	-	+	-
Terpenes	+	-	-
Saponins	-	+	-

+, Presence; - Absence

We graded the IC_{50} values as follows: < 10 µg/mL: very active; 10–50 µg/mL: active; 50–100 µg/mL: slightly active; and >100 µg/mL: inactive (Rasoanaivo et al., 1999).

Cytotoxicity tests

The toxicity of the extracts towards the blood of white laboratory mice (strain Swiss Hds: CD-1) was investigated according to the procedure described by Luize et al. (2005) and our previous study (Chinchilla et al., 2014). Cytotoxicity was determined by observation of globular lysis or agglutination, with 1:80 dilution being established as the limit of toxicity; all tests were performed in triplicate. All handling of the animals was carried out according to the appropriate ethical guidelines and permits (CICUA-07-10).

Statistical analysis

Excel 2016 from Microsoft office was used for statistical analysis to calculate the mean, standard deviation and confidence interval (1-α= 99%) of the concentrations with antiparasitic activity.

RESULTS

Phytochemical screening of dried mature leaves of *N. lobata,* shown in Table 1, indicated the presence of alkaloids in all extracts, while ether extracts (obtained before and after the hydrolysis process) contained flavonoids, coumarins, quinones, triterpenes, and sterols. Other metabolites detected were terpenes and tannins in the ether extract and reducing compounds, tannins and saponins in the aqueous extract. The hydrolysed aqueous extract was tested only for the presence of anthocyanins.

The ether extract obtained by phytochemical screening showed the highest activity against *Leishmania* sp.

Therefore, this extract was chosen for purification by column chromatography. The sesquiterpene lactone, 8β-isovalerianyloxy-9α-hydroxy-calyculatolide, was isolated with an IC_{50} value of 3.75 ± 1.63 µg/mL. None of the extracts, fractions and isolated product showed red blood cell toxicity at active concentrations.

The molecular mass of the isolated compound was determined by HRESIMS, yielding a molecular ion at 379 m/z $[M+H]^+$. The isolated compound was identified by analysis of ^{13}C and ^{1}H NMR data and compared with the data previously reported for the compound by Passreiter et al. (1995), which are shown in Tables 2 and 3, respectively.

Figure 1 shows the chemical structure of the sesquiterpene isolated from the dried mature leaves of *N. lobata.* Since the sample was not analysed by NOESY-2D, the stereochemistry of the compound could not be determined; however, the structure is considered to have the same absolute stereochemistry as that reported by Passreiter et al. (1995).

DISCUSSION

Although leishmaniasis is a treatable and curable disease, treatment of leishmaniasis remains a public health challenge in certain developing countries. Therefore, the search is ongoing for promising active compounds in plant extracts, identified through i*n vitro* and *in vivo* tests, which could be developed as alternatives for the treatment of the disease.

Moraes-Neto et al. (2019) highlight plants of the Asteraceae family as having the largest variety of species with interesting components that are likely to be leishmanicidal treatments, including *Ambrosia tenuifolia*, *Artemisia annua*, *Baccharis uncinella*, *Mikania micrantha*, *Neurolaena lobata*, *Tithonia diversifolia,* and *Vernonia polyanthes*.

In the preliminary study by Chinchilla et al. (2014), *N. lobata* was one of the 67 species analysed that showed promising activity against *Leishmania* sp. One type of compound that has shown good activity against *Leishmania* is sesquiterpene lactones. The possible

Table 2. ^{13}C-NMR data for 8β-isovalerianyloxy-9α-hydroxy-calyculatolide (150 MHz, CD_3OD, TFA).

Carbon	δ obtained(ppm)	δ reported* (ppm)
1	206.0	204.3
2	103.4	103.7
3	194.2	193.1
4	31.2	31.4
5	40.3	40.9
6	72.6	72.9
7	45.6	45.8
8	78.3	77.4
9	75.0	74.6
10	91.6	91.0
11	140.9	139.9
12	169.5	168.8
13	122.3	123.9
14	17.7	18.8
15	15.0	16.2
1'	171.4	171.6
2'	42.4	42.8
3'	25.2	25.2
4'	21.1	22.3
5'	21.2	22.4

*Source: (Passreiter et al., 1995).

Table 3. ^{1}H-NMR data (600 MHz, CD_3OD, TFA) for 8β-isovalerianyloxy-9α-hydroxy-calyculatolide.

Hydrogen	δ (ppm)	
	Obtained	*Previously reported
2	5.74	5.58
4	3.14 (7.2 Hz)	3.04 (7.0 Hz)
5a	2.00	2.06
5b	2.65	2.60
6	4.43	4.49
7	3.74 (3.0; 3.6 Hz)	3.83 (2.5; 3.0 Hz)
8	4.98 (5.0 Hz)	5.08 (5.0 Hz)
9	3.96 (5.0 Hz)	4.12 (5.0 Hz)
13a	5.79 (3.6 Hz)	5.74 (3.0 Hz)
13b	6.26 (3.0 Hz)	6.34 (2.5 Hz)
14	1.46	1.49
15	1.42 (7.2 Hz)	1.40 (7.0 Hz)
2'a	2.14	2.11
2'b	2.10	2.09
3'	1.98 (7.0 Hz)	1.98 (6.6 Hz)
4'	0.92 (7.0 Hz)	0.91 (6.6 Hz)
5'	0.92 (7.0 Hz)	0.90 (6.6 Hz)

* Source: (Passreiter et al., 1995).

targets of sesquiterpene lactones are associated with key enzymes and metabolites of the parasite, such as ornithine decarboxylase, an enzyme involved in the biosynthesis of polyamides in *Leishmania sp.*, mitogen-activated protein kinase 3 and pteridine reductase 1 (Herrera-Acevedo et al., 2020). Sesquiterpene lactones can also cause oxidative stress in the parasite by inhibiting key enzymes that maintain redox balance in *Leishmania* (Barrera et al., 2013).

In a previous study, Berger et al. (2001) reported two sesquiterpene lactones (neurolenin B and neurolenin C/D) from *N. lobata* with activity against *L. mexicana*. This report led us in an attempt to identify which of the various metabolites obtained in the phytochemical study of *N. lobata* in Costa Rica were responsible for the antiparasitic activity. Although a sesquiterpene lactone was also found in our study, its chemical structure is different from those already reported and it also has activity against

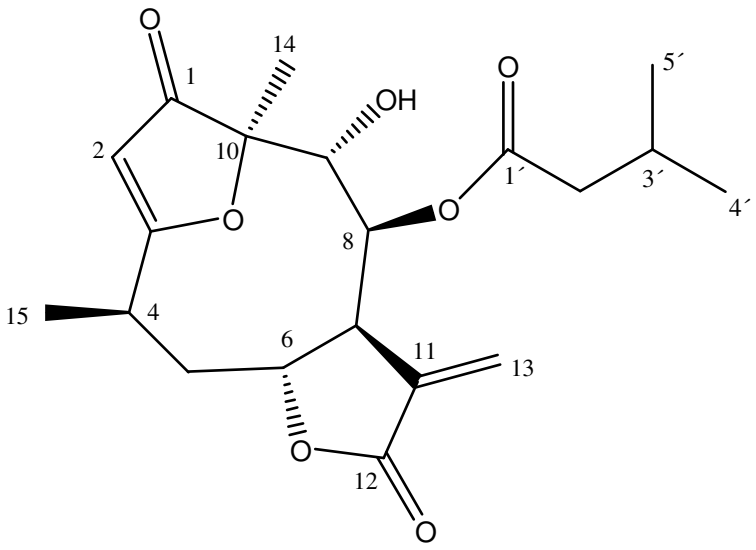

Figure 1. Chemical structure of the compound 8β-isovalerianyloxy-9α-hydroxy-calyculatolide.

Leishmania.

Passreiter et al. (1995) determined the presence of 8β-isovalerianyloxy-9α-hydroxy-calyculatolide via NMR spectroscopic techniques; in the same way, the presence of the said molecule was confirmed in our study using those techniques (NMR), which allowed us to compare our results with those previously reported by these authors as well as confirming in our case that it is not only the same molecule, but that the compound 8β-isovalerianyloxy-9α-hydroxy-calyculatolide is the metabolite in *N. lobata* responsible for the activity against *Leishmania* sp. in the present study.

In the present study, 8β-isovalerianyloxy-9α-hydroxy-calyculatolide showed the highest resistance against *Leishmania* at 3.75 ± 1.63 µg/mL for all the fractions. It should be noted that the antileishmanical activity of this isolated component has not been reported previously. However, this sesquiterpene lactone has shown positive activity against other parasites, including malaria (François et al., 1995; Passreiter and Isman, 1997), and a 5β-hydroxy-8β-isovalerianyloxy-9α-hydroxy-calyculatolide derivative with anti-inflammatory and anti-proliferative activity against human tumour lines has been described by Lajter et al. (2014).

One limitation of this type of study is the extrapolation of *in vitro* results to *in vivo* tests on intracellular parasites such as *Leishmania*. There are no data on the possible toxicity of the compound in experimental animals, which could lead to its eventual inability to be used in *in vivo* models.

On the other hand, work is being done to develop research protocols in an *in vivo* model that can confirm not only the antiparasitic activity but also the innocuousness of the molecule.

Conclusion

Natural products continue to be an interesting source for generating new antiparasitic drugs, as they provide a natural resources with almost unlimited chemical diversity. The possibility of developing new drugs that can contribute to the control of parasitic diseases makes it desirable to search for compounds that have promising characteristics, such as low toxicity, good safety profile, good efficacy, and are affordable to the poorest and most vulnerable people. This finding opens the possibility of further studies to show if this activity can also be standardized *in vivo* systems, an aspect that we are already examining.

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