Cultural importance of *Neurolaena lobata* (L.) Cass. from Nariño (Colombia) and neutralization of the biological activities of *Bothrops asper* venom by its ethanol extract

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Research

Abstract

Background: owing to the difficulties for accessing health centers and the limited availability of antivenoms in rural areas of Colombia, in many rural communities people rely on traditional medicine based on the use of plant extracts as a therapeutic resource for the snakebite envenoming. In the department of Nariño, the potential of these alexiteric plants is unknown. This work determined the cultural importance of *Neurolaena lobata* (L.) Cass., one of the plants most frequently used by the rural

communities of Tumaco, municipality with high incidence of poisonings, and assessed the efficacy of an extract of this plant to inhibit toxic effects of the venom of the snake *Bothrops asper*.

Methods: semi-structured interviews were conducted with 14 randomly sampled individuals and the Cultural Importance index (CI) of the plant was calculated. Phytochemical tests were carried out and a battery of biological assays was applied to determine the neutralizing capacity of the extract on the lethal, coagulant, hemorrhagic, and myotoxic activities of the venom.

Results: the CI of this plant (0.0544) reflects its ethnobotanical value for the rural communities of Tumaco. The ethanolic extract from the leaves contains tannins, flavonoids, alkaloids, saponins, and sesquiterpene lactones. Neutralization assays revealed that the extract inhibited the coagulant activity of *B. asper* venom, but failed to inhibit the myotoxic, and lethal activities, and only partially reduced the hemorrhagic effect. Moreover, the extract showed toxicity when assessed for lethality and myotoxicity.

Conclusions: despite the cultural importance of the plant, our experimental findings do not support the claim that the crude extract of *N. lobata* inhibits the toxicity of the venom of *B. asper* of Colombia.

Keywords: *Bothrops asper*, *Neurolaena lobata*, alexiteric plants.

Background

Snakebite envenoming is a public health problem worldwide, mainly affecting tropical and subtropical countries in Africa, Asia, Latin America, and Oceania (Kasturiratne *et al.* 2008; Gutiérrez *et al.* 2010). In Colombia, the National Public Health Surveillance System (SIVIGILA) reported an annual number of 4778 and 5421 cases in the years 2021 and 2022, respectively. The highest incidence is observed in the departments of Amazonia, Orinoco, and Chocó (Rojas-Bárcenas 2018, 2019; INS, 2021, 2022).

In the department of Nariño, a retrospective work conducted between 2008 and 2017 reported an annual number of 111 snakebite cases, with the Pacific region being the most affected, and the municipality of San Andrés de Tumaco having the highest number of cases (Sevilla-Sánchez *et al.* 2019). Although Nariño is one of the departments with lowest incidence of snakebites, these figures are likely to be underestimations because many cases are not treated in health centers or are transferred to other departments (Ayerbe 2009). Moreover, Nariño is one of the departments with the highest number of deaths caused by snakebites (León 2017; Sevilla-Sánchez *et al.* 2019).

Eco-epidemiologically, species of the genus *Bothrops* are responsible for 90% of bites in Latin America and, in Colombia, the species *B. asper* is responsible for about 50-80% of snakebites (Otero *et al.* 2000; Otero-Patiño *et al.* 2018). Its relative abundance, body size, cryptic coloration, ability to live in human-disturbed environments (Sasa *et al*. 2009), and defensive behavior contribute to the medical impact of this species in Central and South America. In addition, its wide distribution range in the department of Nariño, on the western slopes of the Western Cordillera in the Pacific Region, explains why 78.47% of accidents by snakebite occur in this area (Sevilla-Sánchez *et al*. 2019). The venom of *B. asper* causes a complex clinical picture of local and systemic manifestations, including edema, myonecrosis, hemorrhage, blistering, coagulopathy, cardiovascular shock, and acute kidney injury (Otero-Patiño, 2009). This complex pathophysiology is caused by the direct action of venom toxins and by endogenous mechanisms in affected individuals and may vary depending on the geographical locality of origin of the snake (Alape-Girón *et al*. 2008; Ayerbe 2009; Cañas *et al*. 2020; Mora-Obando *et al*. 2014; Otero-Patiño 2009; Rengifo-Rios *et al*. 2019).

The only scientifically validated treatment for snakebite envenoming is the intravenous administration of antivenoms, which are antibody preparations from the plasma of animals immunized with snake venoms (León *et al*. 2018). However, factors such as the insufficient availability of antivenom in the country (Gómez-Cardona *et al.* 2017; Otero-Patiño, 2009), as well as the limitations in points of care, geographical conditions and deterioration of road infrastructure in some areas, which prevent rapid access to public health centers, limit the distribution and administration of this essential medicine, and increase the likelihood of mortality and other complications of envenomings (Vásquez *et al*. 2013).

In this context, in the face of limited medical care and accessibility of antivenom, many rural populations have empirically and traditionally used natural alternatives as antidotes against snake venoms, such as the application or ingestion of plant extracts (also known as alexiteric plants) (Otero *et al*. 2000; Giovannini and Howes, 2017; Rengifo-Rios *et al*. 2019). Although

the use as anti-inflammatory, tranquilizing, antitussive, anticarcinogenic, muscle relaxant, antidiabetic, antihypertensive, and analgesic effects of many medicinal plant species has been demonstrated (Süntar 2020), their use for snakebite envenoming has been less explored (Soares *et al.* 2005).

Phytochemical studies, based on ethnobotanical knowledge, have found that different plant families can inhibit several toxic effects (local and systemic) induced by snake venoms due to the presence of active principles such as 4-nerolidylcatechol and edunol (Núñez *et al*. 2004), or secondary metabolites such as polyphenols (e.g. tannins, flavonoids), which act as chelating agents for zinc or calcium (Castro et al. 1999; Patiño *et al*. 2012). These ions are necessary for the enzymatic activities of metalloproteases and phospholipases A_2, proteins generally associated with the hemorrhagic and myotoxic effects induced by the venom of some snake species (Gutiérrez *et al*. 2010).

Neurolaena lobata (L.) Cass., commonly called "Gavilana", is a plant of the Asteraceae family, with medicinal properties described against hemorrhage, ulcers, fever, and inflammation (Gracioso *et al*. 1998; McKinnon *et al*. 2014) and with bibliographic records of use in snakebite envenomings (Suárez, 2015), being therefore considered by some authors to be an alexiteric plant (Carbonó-Delahoz and Dib-Diazgranados, 2013). This plant is frequently used by indigenous, Afro-descendant, and peasant communities in the department of Nariño, such as the village of El Candelo, Río Rosario-Tumaco (Nariño), as a low-cost treatment option due to the difficulty of accessing antivenom treatment (Suárez 2015). However, to date, the cultural importance of the species for the rural community has not been systematically characterized.

Previous works report that the ethanolic extract of *N. lobata* (L.) Cass. has a low or moderate capacity to neutralize the coagulant and hemorrhagic effects induced by *B. asper* venom from Costa Rica, Guatemala, and northwestern Colombia, being ineffective against other venom activities, such as lethality and *in vitro* phospholipase A_2 enzymatic activity (Otero et al. 2000; Saravia et *al*. 2001). However, the effectiveness of extracts of specimens from southwestern Colombia against different biological effects of *B. asper* venom from the same region is unknown.

This research combines an ethnobotanical analysis of *N. lobata* (L.) Cass. and a phytochemical analysis of the extract, with a panel of *in vitro* and *in vivo* biological assays in murine models to have a comprehensive overview of the cultural importance of the species, to carry out a phytochemical analysis of an extract of the plant, and to determine the neutralizing capacity of the extract against toxic effects induced by *B. asper* venom.

Materials and Methods

Study area

The work was conducted in the municipality of San Andrés de Tumaco, located in the department of Nariño in southwestern Colombia, at 1º48'24'' north latitude and 78º45'53'' longitude, on the Pacific coast (Supplementary Fig. S1). It spreads over 3,778 km^2 and the urban area is about 38 km^2. It has a Tropical humid weather, with annual rainfall of 2292 mm, which is high even during the driest month; the least amount of rain is recorded in August with an average of 81 mm and the highest rainfall occurs in June, with an average of 348 mm. It has a relative humidity between 80-85% and the average annual temperature is 25.8 °C; the highest average temperatures are recorded in April (25.3-26.2 °C) and November is the coldest month of the year (Dirección General Marítima - Centro de Investigaciones Oceanográficas e Hidrográficas del Pacífico, 2012; Cámara de Comercio de Tumaco, 2019).

Collection of plant material

For the search and collection of *Neurolaena lobata* (L.) Cass. specimens, two field trips were made to the municipality of San Andrés de Tumaco. Supplementary Figure S1 shows the exact location and collection site of the plant corresponding to the Mar Agrícola farm at 1°40'49.7'' north latitude and 78°44'55.5.5'' west longitude in the vicinity of Inguapí del Carmen at an altitude of 60 m above sea level, area characterized by presenting a tropical humid forest type ecosystem (Alcaldía Municipal de Tumaco, 2008).

A total of 17 botanical samples were collected and taken to the PSO herbarium of the University of Nariño, where their taxonomy was corroborated, and their identification certified. A specimen was deposited in the herbarium and its name was checked (https://wfoplantlist.org/, accessed July 16, 2023).

Ethnobotanical data collection and analysis

Ethnobotanical information on *N. lobata* (L.) Cass. related to its use in snakebites by the community was obtained through semi-structured interviews applied to the people of two sampling areas, one rural and one urban, in the municipal center of

Tumaco (Supplementary Fig. S2), after obtaining the informed consent of the participants approved by the ethics committee of the University of Nariño (approval record number 038 of October 2020). Because 88.70% of the neighborhoods in the municipality of Tumaco correspond to slums, they do not have a total census of its population (Rivera-Cortes and Colorado-Marquinez 2019). Therefore, the interviews were conducted with a statistically representative sample, defined as 10% of the total number of inhabited houses in each of the sampling areas (Geilfus 2002; López 2004).

In the rural area represented by the village of Inguapí del Carmen (Supplementary Fig. S2A), in the vicinity of the Mar Agrícola station of the Universidad de Nariño, 14 individuals were interviewed, chosen at random from the total number of inhabited houses. In the urban area (Supplementary Fig. S2B), a total of 320 houses were included corresponding to the neighborhoods Esfuerzo I, Esfuerzo II, and Villa Carolay, located in the sector of Ciudadela Universitaria, whose inhabitants are mainly dedicated to agriculture and fieldwork, of which 32 individuals were randomly selected and interviewed following the acceptance of each participant.

The interview included questions related to the use of traditional medicine in snakebite victims, with emphasis on the plant species *N. lobata* (L.) Cass., the parts of the plant used in the treatment, the quantity applied, the mode of preparation (maceration or decoction), the application techniques (drink, bath, poultice, vapors), the duration of the treatment, and the sites where they usually collect the plant material. The information was recorded in a data matrix. Based on the analysis of the ethnobotanical data obtained, the plant part (e.g. root, stem, or leaves) to be used for the preparation of the extract was defined.

Calculation of the Cultural Importance index

The cultural importance of *N. lobata* (L.) Cass. was estimated using the Cultural Importance index (CI) developed by Pieroni (2001) and modified by González-Insuasti *et al.* (2008). For this purpose, variables related to the tradition of use and the form of consumption of the plant in the treatment of snakebites were analyzed (Table 1). The Cultural Importance index of *N. lobata* (L.) Cass. was calculated using the following formula:

$$CI = \frac{Cp \times \mathrm{Up} \times \mathrm{Nc} \times Ap \times Faq \times Pum \times Ua \times \mathrm{EBa} \times \mathrm{Otu} \times Equ \times Qp \times Fap}{10000}$$

Extraction by maceration

A total of 5 kg of fresh leaf material was collected to prepare the extract. All the fresh material was dried in the shade at room temperature for 15 days and pulverized. It was subjected to extraction by chemical maceration according to the methodology used by Castro *et al.* (1999), with some modifications. For this, 200 g of dried leaf material was macerated for 72 hours at room temperature with absolute ethanol and stored in a dark place. The crude extract was filtered and concentrated in vacuum under reduced pressure in a rotary evaporator at 38°C and then dried in a desiccator with silica for 36 hours (Rengifo-Rios *et al.* 2019; Saravia *et al.* 2015). The green-brown residue was stored at -20°C until biological tests and phytochemical analysis.

Phytochemical characterization

A qualitative analysis of the metabolites was carried out: tannins, flavonoids, alkaloids, saponins and sesquiterpene lactones were tested. Tannic acid, quercetin, atropine solution (1%), and ginseng extract, were used as positive controls and one tube of extract only was prepared as negative control (Domínguez 1973; Santa Cruz 1986; Lima-Ortiz and Morales-Coromac 2014).

Determination of tannins

A 1% extract solution (50 mg in 5 mL EtOH) was prepared, and 1 mL of the extract was added to each of three tubes. Then 5 drops of 1% w/v gelatine solution (50 mg gel in 5 mL) were added to tube 1, 4 drops of gel-salt were added to tube 2 with the following concentrations: 1% gelatine and 10% NaCl, and 4 drops of 10% w/v $FeCl_3$ were added to tube 3. For each tube, two additional tubes were prepared with 1mL of tannic acid as a positive control, any change in staining was observed and recorded (Lima-Ortiz and Morales-Coromac 2014; Medinilla 2010; Santa Cruz 1986).

Determination of flavonoids

A sample of the extract (80 mg) was dissolved in 4 mL of 80% methanol and distributed in 7 test tubes as follows: tube 1: 0.5 mL of the extract plus 0.5 mL of concentrated sulfuric acid; tube 2: 0.5 mL of the extract plus 3 to 5 drops of 10% w/v ferric chloride; tube 3: 0.5 mL of the extract plus 0. 5 mL of concentrated hydrochloric acid and subsequent water bath for 5 minutes; tube 4: 0.5 mL of the extract plus 10 mm magnesium metal and 0.5 mL of concentrated hydrochloric acid (Shinoda Test); tube 5: 0.5 mL of the extract plus 5 drops of sodium hydroxide; tube 6: 0.5 mL of the extract plus 5 drops of boric acid

solution in acetic anhydride. For each of the tubes, an additional tube was prepared with quercetin as a positive control, the formation of precipitates or changes in coloring were observed and the presence/absence of flavonoids was determined. Orange to red colors indicates the presence of flavones; red to dark red color indicates the presence of flavanols; dark red to reddish-purple color indicates the presence of flavanone in Shinoda Test (Mg + HCl); yellow color indicates presence of flavones and flavanols; orange indicates the presence of flavanones; red or red-blue chalcones or aurones with concentrated sulfuric acid; orange or red colors indicate the presence of 5-hydroxyflavones with boric acid/ $(Ac)_2O$ test; 3,4-flavanandiols give red color and catechins give brown-yellow colors with HCl 2N; and yellow color indicates the presence of flavonoids with NaOH solution (Berenguer-Rivas et al. 2018; Janmin et al. 2022; Godlewska *et al.* 2023).

Determination of alkaloids

A sample of the plant extract (100 mg) was weighed and 2 drops of 10% w/v ammonium hydroxide were added. Then, 2.5 mL of methanol was added at 60°C, then filtered and the filtrate was acidified with 2N hydrochloric acid. Three tubes were prepared from this initial solution: 5 drops of Mayer's reagent were added to tube 1, 5 drops of Dragendorff's reagent to tube 2, and 5 drops of Wagner's reagent to tube 3. For each tube, positive controls were prepared with 1% atropine and, finally, the presence of turbidity or precipitates was observed and recorded (Lima-Ortiz and Morales-Coromac 2014; Medinilla 2010; Santa Cruz 1986).

Table 1. Variables used for estimating the medicinal Cultural Importance index

Variable	Symbol	Scale
Know the plant	Cp	No=1 Yes=2
Use the Plant	Up	No=1 Yes=2
Common name	Nc	No = 0.5; yes = 1
Acquisition of the plant	Ap	Occasional = 1; occasional and exclusive= 2; exclusive= 3
Method of acquisition	Faq	Through harvesting = 1; through the purchase = 2; through purchasing and harvesting =3
Perception of medicinal use	Pum	Not medicinal = 0.5 Don't know = 1 Low medicinal importance in treatment =2 Medium medicinal importance in the treatment =3. High medicinal importance in the treatment =4. Very high medicinal importance in the treatment =5
Use in snakebites	Ua	Don't know =1; No=5; Yes=10
Other uses	Otu	Don't know = 0.5 One use = 1 Two uses = 2 Three uses = 3
Used against envenoming by *Bothrops asper*	EBa	No= 0.5 Don't know = 1 Yes= 2
Part of the plant used	Equ	Don't know = 0.25 Roots = 0.54 Immature plant = 0.75 Floral buttons = 0.75 Stems = 1 Seeds = 1 Young leaf whorls = 1.5 Adult leaves = 2 Flowers = 2.5 Fruits = 3 Leaves and stems = 3.5
Preparation process	Qp	Mixed cooked =1 Cooked and alone =2

		Macerated in alcohol =3 Macerated in water =4 Raw and mixed = 5 Raw and alone =6
Form of application	Fap	Don't know = 0.5 Compress =1 Drink =2 Poultice =3

Determination of saponins

They were determined by using the foam test. Briefly, three tubes were labeled as follows: tube 1, to which 10 mg of the plant extract was added in 1 mL of ethanol; tube 2, to which 1 mL of ginseng extract (ginsenosides) dissolved in 4 mL of water (positive control) was added, and tube 3, in which 2 mL of water were added (negative control). Subsequently, 10 mL of distilled water was added to all tubes and placed in a water bath at 60°C for 30 min. After this time, the tubes were allowed to cool, then sealed and shaken, left to stand for 30 min, and finally the formation of a foam layer was observed as a positive indication of the presence of saponins (Lima-Ortiz and Morales-Coromac 2014; Medinilla 2010; Santa Cruz 1986).

Identification of sesquiterpene lactones

A qualitative analysis of sesquiterpene lactones present in the *N. lobata* (L.) Cass. extract was performed according to the methodology used by Domínguez et al. (1973) and Villacorta *et al*. (2017).

Legal test for α, β-unsaturated lactones

A sample of the extract (5 mg) was dissolved in 3 drops of pyridine, 1 drop of 0.5% sodium nitroprusside solution was added and then 4 drops of 2N KOH were added dropwise. Finally, the presence/absence of a pink coloration was observed as an indication of test positivity (Domínguez *et al*. 1973; Villacorta *et al.* 2017).

Ferric hydroxamate test for lactones

A sample of the extract (10 mg) was weighed and placed in a tube. Then, 2 drops of 2N methanolic solution of hydroxylamine hydrochloride and 1 drop of 2N methanolic solution of KOH were added. Subsequently, the mixture was heated to boiling for 2 min, allowed to cool, and brought to acid pH with 0.5N HCl. Finally, 1 drop of 1% ferric chloride was added and the presence or absence of a violet, red, or pink coloring was observed, indicating the positivity of the test (Domínguez *et al*. 1973; Villacorta *et al.* 2017).

Detection by thin-layer chromatography (TLC)

Two samples of the extract were run on a silica gel plate using 9:1 chloroform-methanol as the elution system. The plates were UV spotted and then each sample was developed with either 50% H_2SO_4 in water or Vanillin - H_2SO_4. Finally, it was heated on iron and observed for the presence/absence of a red, green, or black coloration indicating test positivity for the first sample, and a blue coloration indicating test positivity for the second sample (Domínguez *et al*. 1973; Villacorta *et al*. 2017).

Venom

A venom pool of 7 adult individuals of *Bothrops asper*, from the Pacific basin in the municipality of El Tambo (department of Cauca), kept in captivity at the Centro de Investigaciones Biomédicas de la Universidad del Cauca (CIBUC), was used. For venom extraction, the snakes were immobilized and after they bit into a glass funnel covered with Parafilm®, the venom was obtained, freeze-dried, and stored at 20°C until use.

Experimental animals and ethical considerations

The biological activities were carried out on white mice (*Mus musculus*) Swiss Webster strain (CFW) from the LABBIO laboratory of the Universidad del Valle - Sede San Fernando, without distinction of sex, and weights between 16 and 20 grams. Mice were weighed and distributed in cages in groups of 4 animals. They were kept in appropriate environmental conditions of temperature and relative humidity (18-22ºC and 70-80% respectively) with the availability of LabDiet 5010 autoclavable food and water ad libitum throughout the experiment (Cubillos et al. 1999; Mora-Obando *et al*. 2014). The tests carried out in mice were approved by the Research Ethics Committee of the University of Nariño (approval record number 038 of October 2020).

Neutralization tests on the biological activities of B. asper venom

For the neutralization assays of the lethal, coagulant, and hemorrhagic activities of *B. asper* venom by *N. lobata* (L.) Cass. extract, the venom challenge doses were determined according to standardized protocols recommended by the World Health Organization (WHO 2017). They were calculated from the minimum doses of each biological activity, previously reported for this species by Mora-Obando *et al*. (2014) and Rengifo-Rios *et al*. (2019). Because the minimum myotoxic dose for *B. asper* from southwestern Colombia has not been established, a fixed dose of venom of 50 µg was used, which is a dose capable of producing a prominent myotoxic effect by the venom of this same population of the species (Mora-Obando *et al*. 2014) (Table 2). The tests were carried out by preincubating the venom with the extract for 30 min at 37°C.

Table 2. Minimum and challenge doses of *B. asper* venom were used in the experimental design.

Biological activity	Reference dose	Challenge dose [µg/mouse](2)
Lethal Dose $_{50}$ (LD_{50}) [µg/mouse]	100.9 (83.2-122.8)(1)	201.8
Minimum Coagulant Dose (MCD) [µg]	0.37 ± 0.05(1)	0.74
Minimum Hemorrhagic Dose (MHD) [µg]	1.44 ± 0.20(1)	14.4

(1) Mora-Obando et al. (2014); (2) The challenge doses used correspond to 2 LD_{50}s (lethality), 2 MCD (coagulant activity) and 10 MHD (hemorrhagic activity).

The plant ethanolic extract was dissolved in phosphate buffer saline solution (PBS), pH 7.2, using Tween-80 (2.5-10% v/v) as an emulsifier, according to the protocol of Saravia-Otten *et al*. (2015). The experimental design of the extract/venom ratios (w/w) evaluated in each of the neutralizing activities was carried out based on the studies of Saravia-Otten *et al*. (2001, 2015, 2017a, 2017b, 2021; 2022) and Rengifo-Rios *et al*. (2019). All biological assays included a venom control, an extract control (highest amount) to determine the intrinsic activity of the extract, and a diluent control (PBS-Tween 80) (Saravia-Otten *et al*., 2021). The neutralizing efficacy of the extracts for lethal, hemorrhagic and myotoxic activities, when there was neutralization, was expressed as Median Effective Dose (ED_{50}), corresponding to the mg extract/mg venom ratio in which 50% of the effect was neutralized. In the case of coagulant activity, neutralization was expressed as Effective Dose (ED), i.e., the ratio mg extract/mg venom at which the clotting time is prolonged three times as compared to venom controls.

Neutralization of lethal activity

From a stock solution of venom, aliquots were prepared and incubated at 37ºC for 30 min with variable amounts of the extract in the following mg extract/mg venom ratios: 25, 50, and 100. The extract and venom controls were performed in parallel and, additionally, 2 mice were injected with PBS-Tween 80 (2.5%) as control. After the incubation time, 0.5 mL of each of the mixtures, containing 2 LD_{50}s of venom, was injected intraperitoneally into groups of 4 mice. Animals were monitored for 48 h and the number of mice killed at each level was recorded.

Neutralization of the coagulant activity

A fixed concentration of venom was mixed with various dilutions of the extract in a volume of 0.1mL in the following ratios: 6.25, 12.5, 25, 50, 100, 200, 400, 800, 800, 1600 mg extract/mg venom. Mixtures were incubated at 37°C for 30 min. Subsequently, 0.1 mL of each mixture, containing 2 MCD of venom, was added to 0.2 mL of citrated human plasma, previously incubated for 3-5 min in a water bath at 37°C. This assay was performed in triplicate and the clotting time was recorded with a stopwatch. Controls of venom alone and extract alone were included.

Neutralization of hemorrhagic activity

A fixed concentration of venom was incubated (37°C, 30 min) with various dilutions of the extract in the following ratios: 25, 50, and 100 mg extract/mg venom. Controls included samples of venom alone and extract alone. Then, 0.1 mL of each venom-extract mixture, containing a challenge dose of venom (14.4 µg), was injected intradermally into the abdominal region of groups of 5 mice. Two hours later, mice were sacrificed by cervical dislocation, the skin was removed, and the hemorrhagic lesion was photographed. The hemorrhagic area of the internal surface of the skin was quantified with Inkscape software (version 0.92) using the method described by Jenkins *et al*. (2017).

Neutralization of myotoxic activity

A fixed concentration of venom was prepared and mixed with various extract dilutions to obtain the following ratios: 12.5, 25, and 50 mg extract/mg venom in a volume of 0.1 mL. Controls of venom and extract were included. The mixtures were incubated for 30 min at 37°C and then groups of 5 mice received 0.1 mL of each mixture intramuscularly into the right gastrocnemius, containing 50 µg venom. After three hours, mice were euthanized by cervical dislocation and a 0.5 mL blood

sample was withdrawn by cardiac puncture into a microtainer tube with anticoagulant (EDTA). After centrifugation at 1800 *g* at 4°C for 10 min, plasma was collected, and the creatine kinase (CK) activity was determined using a commercial kit (Biosystems) and expressed in international units per liter (U/L).

Data Analysis

A descriptive statistical analysis of the demographic data obtained from the interviewed individuals was carried out in Excel (Microsoft Office®, 2010). Data were entered into a basic data matrix (BDM), standardized by linear transformation and similarity/difference was calculated using average taxonomic distance. Subsequently, a cluster analysis was performed using the UPGMA method of unweighted arithmetic averages to observe clusters of informants regarding the cultural importance of *N. lobata* (L.) Cass. considering the socio-economic status, age, origin, and general use of the plant. The results obtained with UPGMA were complemented with a principal component analysis (PCA), which allowed us to determine the variables that contribute to the cultural importance of *N. lobata* (L.) Cass. in the study area. Statistical analyses were carried out with NTSYS version 2.1.

The results of the *in vitro* neutralization experiments of the coagulant activity were represented in dose-response plots. On the X-axis, the extract/venom ratios and on the Y-axis, the response variable (time) were plotted. For the coagulation assay, a regression analysis was applied using Excel (Microsoft Office®, 2010) and from the best-fit curves, the corresponding equations were obtained to calculate ED. Results were presented as mean and standard deviation. Normality and significance tests were performed in GraphPad Prism 5 using the Kolmogorov-Smirnov test and Dunnett's method for multiple comparisons respectively.

Results and Discussion

Macroscopic characteristics and botanical description

N. lobata (L.) Cass. is known by interviewees by the common name of "Gavilana" and "Tres dedos". It is characterized as an erect herb with striated and pubescent stems; it is commonly much branched and reaches 3 m in height (Fig. 1A). It has simple, lanceolate, petiolate, alternate-arranged, scabrous leaves, narrow at the base with acuminate apices and toothed edge (Fig. 1B-D). The lower and upper leaves of the collected specimens were 25 cm and 14 cm long, respectively (Fig. 1A-B). The leaves are dimorphic, i.e., in the same plant it is possible to observe trilobed leaves (Fig. 1B and D) and entire leaves, the latter in smaller proportion than the former (Fig. 1C). It has reticulated veins with a prominent central vein from which secondary veins arise in pinnate form, with dark green upper side, olive green underside, and has a bitter taste (Fig. 1B-D). The inflorescences are yellow capitula arranged in a panicle, with involucent bracts and black achenes-like fruits.

The morphological characteristics of *N. lobata* (L.) Cass. in both the dry and fresh states are consistent with previous works (Dieseldorff and Granados 2007). Different authors report the presence of trilobed and entire leaves in the same specimen, which exceed 24 cm in length and are generally found in the basal part of the plant (Fig. 1), fruits in the form of black achenes, yellow paniculate inflorescences, and a bitter taste characteristic of the species (Dieseldorff and Granados 2007; Lima-Ortiz and Morales-Coromac 2014). According to its distribution, it is the only species of the genus *Neurolaena* reported for Colombia between 0 and 1950 masl toward the Pacific coast region (Bernal *et al*. 2019).

Cultural Importance index

The cluster analysis showed the formation of three main groups according to the average taxonomic distance (Fig. 2), of which knowledge and use of *N. lobata* (L.) Cass. in snakebites were determinants. In this sense, in Figure 2 the most distant group C highlighted in purple corresponds to the interviewees who did not have any knowledge of the object of study, as opposed to the other two groups (A and B) who do know the plant. The latter were differentiated between those who have knowledge of the plant and who also recognize it as being used in *B. asper* bites (group A, highlighted in yellow) and the participants who do not register the use of the plant for snakebites (group B, highlighted in green) (Fig. 2). The co-phenetic value of the cluster analysis (r= 0.83036) indicates that the clustering is statistically significant because higher r values represent less disturbance in the original structure of the data (Sanchez *et al*. 2003; Sokal and Rohlf 1962).

The projections formed in the PCA are consistent with the cluster analysis. The variables with the greatest weight for each component made it possible to identify the set of characteristics that define each projection. Thus, group A (orange color) corresponds to 30% of the individuals who, in addition to knowing the plant, use it as an antidote against envenoming by *B. asper*, consider it highly medicinal and generally use the leaves. It is worth mentioning that this group includes three key interviewees who are healers in the study areas and are symbolized in the graph with red circles (Fig. 3).

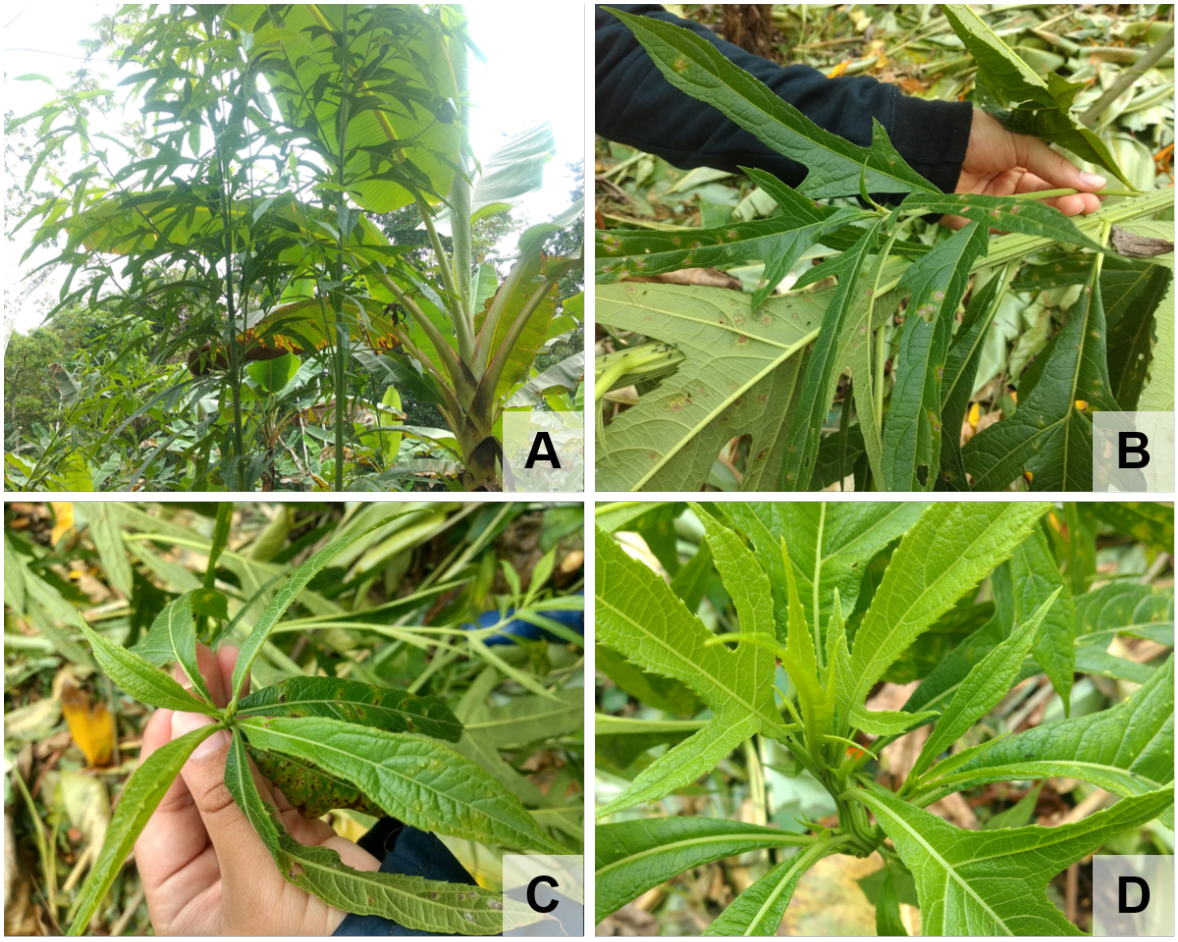

Figure 1. Macroscopic characteristics of *Neurolaena lobata* (L.) Cass. Specimen from Mar Agrícola farm, San Andrés de Tumaco. **A.** Full view of the shrub. **B.** Trilobed leaves, upper and lower side. **C.** Whole and trilobed leaves. **D.** Apex, presence of reticulate veins and toothed margins (Photographs: Rosero-Diaz, KJ and Martinez-Criollo, CA., 2021).

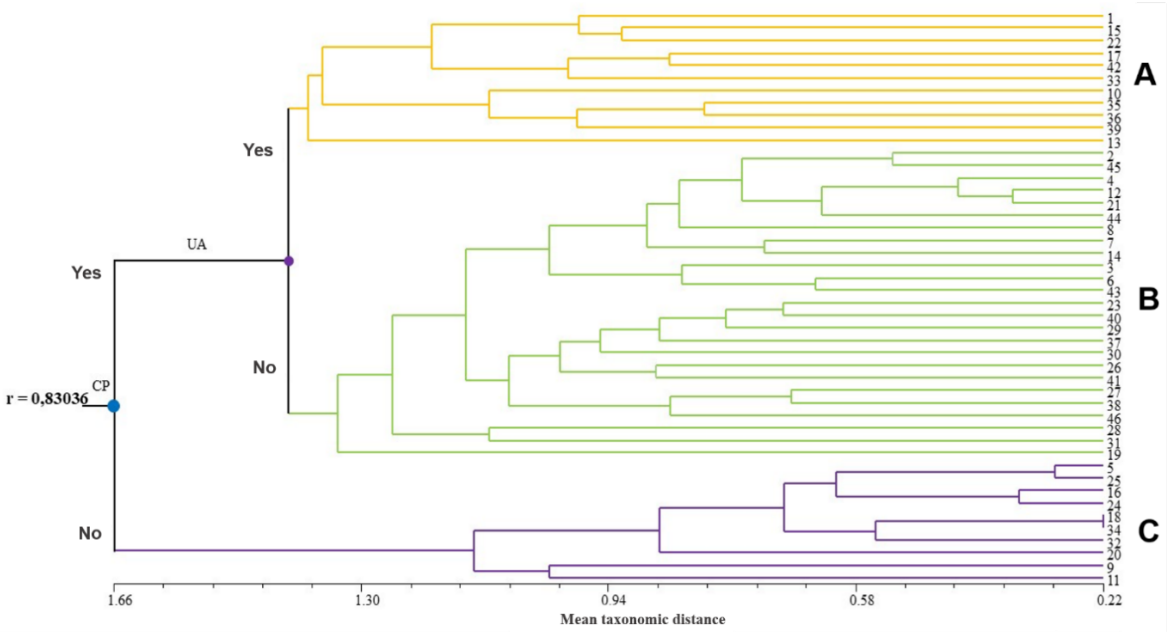

Figure 2. Similarity tree of the 46 interviewees according to the cluster analysis carried out in NTSYS version 2.1 using the UPGMA method (r= 0.83036). Group A (yellow) corresponds to individuals who know the plant, its use in snakebites and do not know other uses, group B (green) includes those who know the plant and some uses, but not in snakebites, and group C (purple) are all individuals who did not have knowledge of *N. lobata* (L.) Cass.

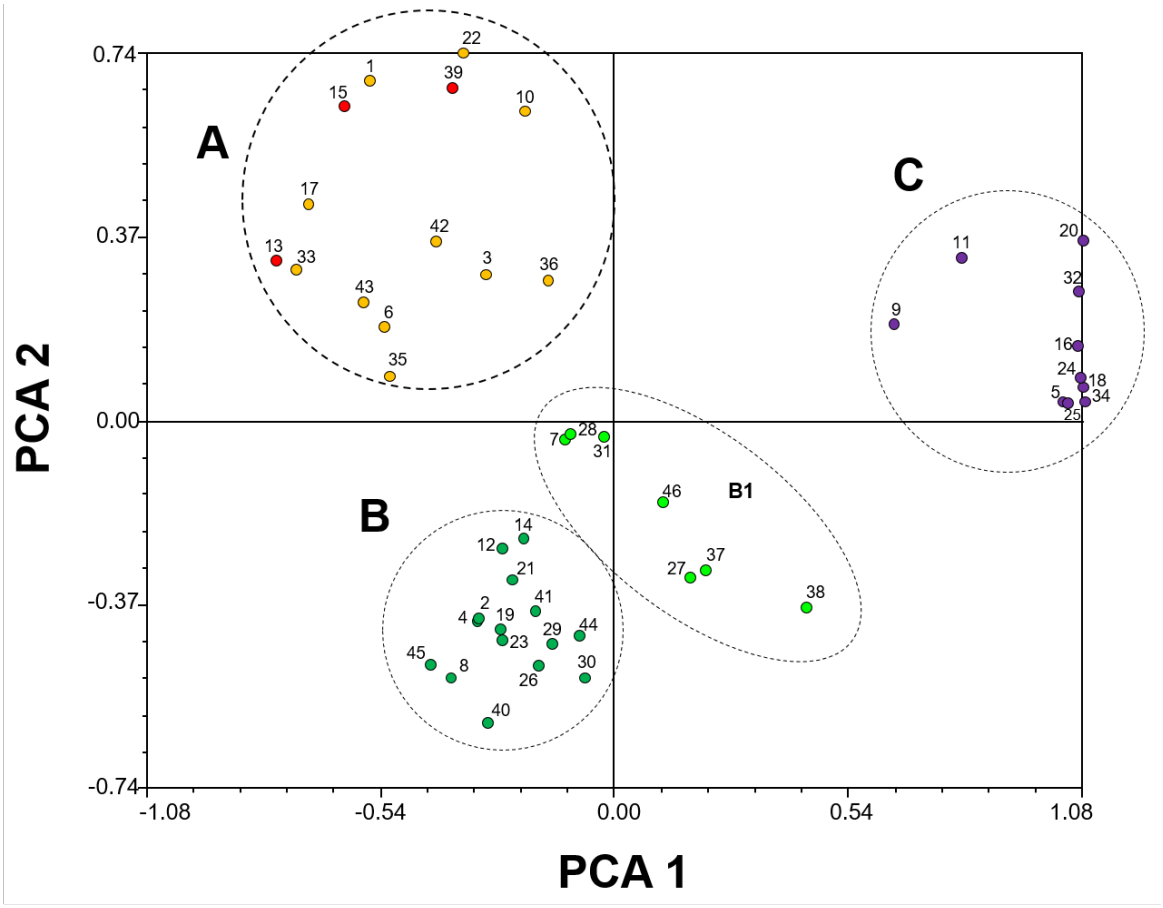

Figure 3. Principal component analysis (PCA) performed in NTSYS version 2.1. Group A, orange circles: interviewees who know the plant, its use in snakebites, use it for *B. asper* bites, use the leaves and consider it highly medicinal. Red circles: key interviewees (healers). Group B, green circles: respondents who are familiar with the plant, but do not use it in snakebites; light green circles (B1): individuals who are familiar with the plant and consider it to be highly medicinal, but without specifying its use in snakebites. Group C, purple circles: individuals who do not know the plant.

Groups B and B1, represented in green, comprise people who know the plant, use the leaves, but do not use it as an alexiteric plant, and together account for 48% of the people interviewed; however, they differ from each other in terms of medicinal consideration, being very high for group B and high for group B1. Finally, to the right of the PCA1 component, 22% of the people who do not know or use the plant for the treatment of snakebites are located in group C (purple color). However, individuals 9 and 11 stand out since, although they do not have any ethnobotanical information on *N. lobata* (L.) Cass., they reported that they recognize it (Fig. 3).

The results of this work show that rural communities confer cultural importance to the species *N. lobata* (L.) Cass. as an alexiteric plant, as suggested by Suárez (2015) and Otero *et al*. (2000). This was evident not only in the calculated C.I. but also in the cluster analysis, showing the conformation of the orange group (Fig. 2) corresponding to the interviewees who distinguish *N. lobata* (L.) Cass. as an alexiteric plant.

This research also showed that some participants recognize different medicinal uses of *N. lobata* (L.) Cass., which was observed in the composition of the green group (Figs. 2 and 3), which includes individuals who mentioned uses of the plant for liver and kidney complications, diabetes, influenza, and malaria.

The Cultural Importance index calculated for the demographic characteristics of the sample revealed that *N. lobata* (L.) Cass. was most culturally important for the rural area, female gender, and age range 41-50 years, with values of 0.0544, 0.0343, and 0.0874 respectively obtained using equation described in the materials and methods section (Table 3).

The Cultural Importance index presented in Table 3 for each of the categories analyzed allows us to infer how important *N. lobata* (L.) Cass. is for the community of Tumaco according to age range, locality, and gender. Although the percentage of respondents was higher in the urban area, the high rate of cultural importance of the species in the rural area (approximately double compared to the urban area) could be explained because it is an area that stands out for the presence of healers, peasants, Afro-descendants, and indigenous people who have extensive knowledge of medicinal plants (Salcedo-Jurado 2018). In addition, the rural areas of Tumaco are the areas with the highest occurrence of snakebites, with 68% of cases, and due to the difficult access to health systems and therefore to antivenom, the population often resorts to the use of plants as medicinal alternatives to counteract the effects of snakebites envenoming (Otero *et al*. 2000; Urieles-Sierra 2020).

Table 3. Cultural Importance index (CI) of *Neurolaena lobata* (L.) Cass. according to locality, gender, and age. For the definition and calculation of CI, see materials and methods section.

Category	Subcategory	CI of *N. lobata*
Location	Rural	0.0544
	Urban	0.0207
Gender	Female	0.0343
	Male	0.0239
Age	< 30	0.0072
	31-40	0.0376
	41-50	0.0874
	51-60	0.0105
	61-70	0.0259
	> 70	0.0334

Likewise, the greater cultural importance of *N. lobata* (L.) Cass. for the age range of 41 to 50 years may be explained by the fact that it corresponds to people who are still active in farm work and, therefore, are more likely to suffer snakebites (Correa-Orobio *et al.* 2019). It is relevant to mention that the ethnobotanical knowledge of the population of Tumaco is mainly found in older people, thus revealing a lack of interest on the part of young people under 30 years of age, which endangers the flow and conservation of traditional knowledge between generations (Ramos-Hernández *et al*. 2007; Yates and Ramírez Sosa 2004). Moreover, the influence of the educational system and the use of new technologies to which young people have access lead them to think about care in health centers instead of traditional practices. In addition, young people who are in the countryside are no longer mainly dedicated to agricultural duties and are looking for other work options (e.g., motorbike taxis, mobile phone sales, and commerce in general.) (Montaño-Blandón and Moreno-Quiñones 2019).

On the other hand, the fact that *N. lobata* (L.) Cass. was culturally more important for women than for men could be explained by the social context in which many of the families in Nariño live, in which men are generally engaged in agricultural activities outside the houses, whereas women are largely responsible for transmitting traditional knowledge about the different medicinal and ornamental uses of plants. (Angulo *et al*. 2012; Hurtado-Ulloa and Moraes 2010).

Structure used and method of application

69.57% of the interviewees reported using *N. lobata* (L.) Cass. leaves in the form of poultice and infusion as a medicinal alternative to treat different conditions, including snakebite. On the other hand, 23.91% reported the joint use of leaves and stems; finally, 6.52% of the interviewees did not report any structure used.

The ethnobotanical analysis also revealed a preference for the use of leaves compared to other plant structures. This is because in traditional medicine the structure of the plant used is generally associated with the condition and the treatment; in this sense, leaves and flowers of medicinal plants are more frequently used than stems and roots (Guzmán-Maldonado *et al*. 2017). Particularly for *N. lobata* (L.) Cass., the use of leaves prepared mainly in infusion and poultice as an antidote to treat different conditions has been reported (Carbonó-Delahoz and Dib-Diazgranados 2013; Saravia-Otten *et al*. 2021).

Phytochemical characterization

The phytochemical screening for different kinds of metabolites in *N. lobata* (L.) Cass. extract was positive for tannins, flavonoids, alkaloids, saponins, and sesquiterpene lactones, as shown in Table 4 and Supplementary Figs. S3-S8.

Our results agree with previous works which described the presence of pyrrolizidine alkaloids (Passreiter *et al*., 1998) and flavonoids (Kerr *et al*. 1981), sesquiterpene lactones in this plant, which have been reported to have anti-inflammatory, antiplasmodial, antileishmanial and cytotoxic activities (McKinnon et al. 2014; Walshe-Roussel *et al*. 2013; Berger *et al*. 2001; François *et al*. 1996), and also agree with another phytochemical report of the same species (Shivananda *et al*. 2014).

Secondary metabolites present in plant extracts are characterized by inducing pharmacological effects, in addition to the physiological effects normally produced by primary metabolites. For this reason, they are employed in the production of phytomedicines (Nabavi and Silva 2018). In this sense, the ethanolic extract of *N. lobata* (L.) Cass. may contain active pharmacological principles, with the potential for inhibition of snake venom toxic effects (Alvarado-Chávez 2007).

Table 4. Qualitative test-tube tests for secondary metabolites.

METABOLITE	TEST	REAGENT	RESULT
Tannins	Control +	Tannic acid	+
	Control -	Extract only	-
	Test 1	Gelatin	+
		Gelatin-Salt	+
		$FeCl_3$	+
Flavonoids	Control +	Quercetin	+
	Control -	Extract only	-
	Test 2	H_2SO_4	+
		$FeCl_3$	
		HCl concentrated	
		NaOH	
		Shinoda (Mg + HCl)	-
		Boric acid/ acetic anhydride	-
Alkaloids	Control +	Atropine solution 1%	+
	Control -	Extract only	-
	Test 3	Mayer's reagent - extract	+
		Dragendorff - extract	
		Wagner - extract	
Saponins	Control +	Ginseng extract	+
	Control -	Distilled wáter	-
	Test 4	Extract	+
Sesquiterpene lactones	Control -	Extract only	-
	Test 6	Legal test for α, β-unsaturated lactones	-
	Control -	Extract only	-
	Test 7	Ferric hydroxamate	+
TLC for sesquiterpene lactones	Control -	Extract only	-
	Test 5	H_2SO_4	+
		Vanillin- H_2SO_4	+

In the case of tannins, it has been reported that the main biological activity corresponds to their antioxidant power, due to their ability to stabilize free radicals with oxygen-free electrons in the OH groups present in phenolic structures (Rosero-Ortiz 2021). They are also capable to neutralize proteolytic and phospholipase A_2 (PLA_2) activities (Vásquez *et al*. 2013) because they can chelate metal ions, forming complexes with zinc and calcium in the active sites of metalloproteinases and PLA_2 of *Bothrops* venom (de Moura *et al*. 2018).

Unlike what was reported by Lima-Ortiz and Morales-Coromac (2014), we detected alkaloids and saponins in the plant extract. For the former, therapeutic and bio-enhancing properties (synergistic action with other components) have been reported (Sreevidya and Mehrotra 2003), as well as expectorant activity (Espinoza 2014), and some of them such as atropine are used against toxins isolated from elapid snake venoms due to their activity as a cholinergic blocker (Lee *et al.* 1982).

Regarding saponins, previous studies have reported surfactant and hemolytic properties due to their amphipathic structural features (Mena-Valdés *et al.* 2015). On the other hand, Assafim *et al*. (2006) and Mauricio *et al*. (1997) described that saponins such as glycyrrhizin exhibit antithrombotic properties *in vivo* against snakebite envenoming.

The positive results for the presence of sesquiterpene lactones agree with those reported in previous works (François et al. 1996; Saravia-Otten *et al*. 2021); this group of compounds is widely distributed in the Asteraceae family. Walshe-Roussel *et al*. (2013) isolated five sesquiterpene lactones: germacranolide sesquiterpene lactones (neurolenin B, and D) and furanoheliangolide sesquiterpene lactones (lobatin B and 9a-hydroxy-8b-isovalerianyloxy-calyculatolide) which have anti-inflammatory activity as a result of inhibition of TNF-α production. Being compounds of terpenoid character they could contribute to covalent bond modifications that can neutralize myotoxic activity of venoms (Núñez *et al*. 2005; Vásquez *et al.* 2013). In this study, the result was negative for the legal test for alpha- and beta-unsaturated lactones, possibly due to their low concentration; the other tests for the determination of sesquiterpene lactones were positive.

Neutralization of lethal activity

The control of the ethanolic extract of *N. lobata* (L.) Cass. was lethal for 33% of mice, hence doing the analysis of inhibition of venom lethality difficult, whereas no deaths were recorded in mice injected with Tween 80-PBS control group. In contrast, Saravia-Otten (2001) did not report toxicity of *N. lobata* (L.) Cass. extract, possibly reflecting differences in the chemical composition of the extracts in samples from different geographical locations. In addition, our data show that the extract was not able to inhibit venom's lethal effect at the three extract: venom ratios tested. In contrast to our findings, previous works have demonstrated inhibition of the lethal effect of *B. asper* and *B. atrox* venoms (Saravia-Otten 2001; Vásquez-Escobar 2012; Yarlequé 2012). It is suggested that future studies should identify the toxic metabolites of this extract, in order assess the inhibitor activity of isolated plant extract fractions devoid of toxicity of *B. asper* and *B. atrox* venoms.

Neutralization of coagulant activity

The extract was able to prolong the clotting time of plasma, as compared to the clotting time of venom control, evidencing an inhibitory effect. The dose-response curve data were fitted to a lineal trend line with a highly significant coefficient of determination R^2=0.946. According to the regression analysis, the ED corresponds to 2047.3 ± 147.05 mg of extract/mg venom. No coagulation was observed in the extract control or the PBS-Tween 80 (10%) control.

Our results are congruent with those described by Saravia-Otten *et al*. (2001), who reported a partial neutralization by the extract of *N. lobata* (L.) Cass. of coagulant activity of *B. asper* venom from Guatemala. This neutralization could be due to the presence of secondary metabolites in the *N. lobata* (L.) Cass. extract capable of interacting with serine proteinases and some metalloproteinases of *B. asper* venom (Magalhães *et al*. 2011; Rengifo-Ríos 2017), which are responsible for the coagulant effect. The presence of flavonoids in *N. lobata* (L.) Cass. extract, confirmed in Table 4, may be responsible for the effect, since these metabolites can inhibit the enzymatic activity of metalloproteinases by blocking the active site of the enzyme (Cuccioloni *et al.* 2009; Jedinák *et al*. 2006; Saravia-Otten *et al*. 2017). Similarly, secondary metabolites of plant origin, such as saponins, may be also involved in this inhibitory activity; previous studies have indicated that glycyrrhizin-type saponins possess antithrombotic properties against *B. jararaca* envenoming (Assafim *et al*. 2006; Mauricio *et al*. 1997). Noteworthy, previous studies have demonstrated an anticoagulant activity of extracts from this plant (Saravia-Otten *et al*. 2021). This may have influenced our results, an issue that demands further investigation.

Neutralization of hemorrhagic activity

This activity was reduced by 32% at the 50 mg EX/mg VE ratio. However, none of the remaining extract/venom ratios tested (25 and 100 mg EX/mg V) reduced the area and intensity of hemorrhagic lesions compared to the venom control. The extract control did not induce hemorrhagic activity. Therefore, at the experimental conditions tested, the extract did not reach 50% inhibition of the effect.

Although the results of the phytochemical screening revealed the presence of flavonoids (Table 4), considered as antihemorrhagic metabolites due to their metal ion chelating capacity (Castro *et al*. 1999), the low abundance of these compounds in the ethanolic extract of *N. lobata* (L.) Cass. from the geographical area of study could explain its limited antihemorrhagic capacity (Mors *et al*. 2000). Previous works have reported that the extract of this plant can only partially neutralize the hemorrhagic effect of *B. asper* venom (Castro *et al*. 1999; Saravia-Otten *et al*. 2001).

Neutralization of myotoxic activity

The control of extract induced a significant increase in the plasma CK activity, as compared to the value of plasma of mice injected with the Tween 80-PBS control (Fig. 4). This indicates that the extract exerts direct myotoxicity. In agreement with this finding, the CK activity of mice receiving mixtures of extract and venom showed plasma CK activities higher than in samples from mice injected with venom alone (Fig. 4). These findings do not necessarily imply that the extract does not have metabolites able to inhibit myotoxic activity of the venom, but instead that the presence of myotoxic metabolites in the extract does not allow the detection of this potential anti-myotoxic activity. Previous works have demonstrated that this and other plant extracts inhibit the phospholipase A_2 activity of *B. asper* and other *Bothrops* sp venoms (Lobo *et al*. 2010; Fernandes *et al*. 2011; Saravia-Otten *et al*. 2021).

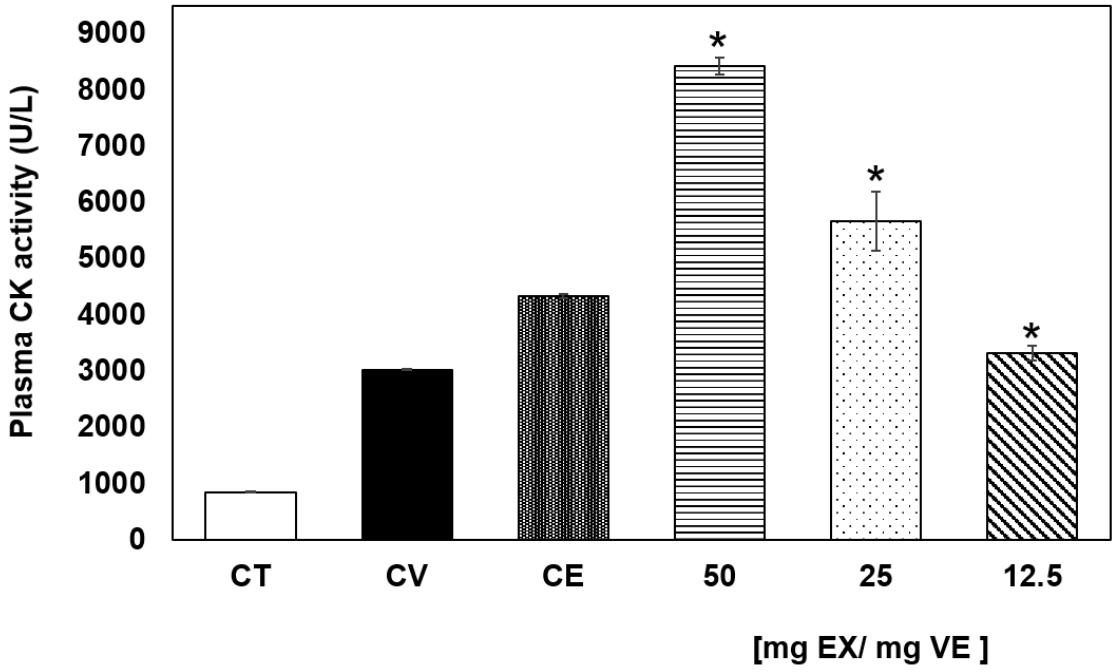

Figure 4. Neutralization of myotoxic activity of *B. asper* venom by ethanolic extract of *N. lobata* (L.) Cass. in mice. Animals were injected, by the intramuscular route, with mixtures of venom and plant extract. Controls included mice injected with either venom, plant extract, or Tween80-PBS. Mice were bled 3 hr after injection and the CK activity of plasma was determined and expressed as U/L (see materials and methods for details). **CT**: Tween 80-PBS control, **CE**: extract control, **CV**: venom control. Results are presented as mean ±standard deviation (n = 5). Significant differences ($p<0.05$) of the venom + extract groups compared to the venom control are represented by asterisks (*).

Conclusions

This study combined ethnobotanical, phytochemical and pharmacological analyses of *N. lobata* (L.) Cass., a plant traditionally used in snakebite envenoming in Latin America. Our observations revealed hitherto unknown ethnobotanical aspects in the municipality of Tumaco-Nariño, and highlighted differences in the Cultural Important index among different groups in this community regarding this plant. A limited interest on medicinal plants in the younger population studied is likely to reflect a decline in the interest on traditional medicine; this stresses the need to document ancestral knowledge to preserve this cultural heritage. The interviews highlighted that *N. lobata* (L.) Cass. is traditionally used not only for snakebite envenoming, but also for other diseases, an observation that deserves further studies.

In our experiments, the ethanolic extract of *N. lobata* (L.) Cass. tested was able to inhibit the coagulant activity of *B. asper* venom and partially reduced its hemorrhagic activity. However, it failed to neutralize lethality and myotoxicity induced by this venom, and in addition it showed toxicity in these experimental models. Overall, our findings do not support the view that *N. lobata* (L.) Cass. crude extracts are effective in the neutralization of this venom. However, further studies are required to assess whether other protocols for administering the extract are effective, as well as to test alternative extraction protocols with different solvents. Likewise, since the secondary metabolites of the plant may vary geographically, it is necessary to test

extracts from specimens collected in other regions and at different times of the year. Additional efforts should involve the isolation of active compounds from this plant responsible for the inhibition of coagulant and hemorrhagic activities.

Declarations

List of abbreviations: Ap - Acquisition of the plant; BDM - Basic data matrix; CE - Extract control; CFW - Swiss Webster strain; CIBUC - Centro de Investigaciones Biomédicas de la Universidad del Cauca; CI - Cultural Importance index; CK - Creatine kinase; Cp - Know the plant; CT - Tween 80; CV - Venom control; Eba - Used against envenoming by *Bothrops asper*; ED_{50} - Median Effective Dose; ED - Effective Dose; EDTA - Ethylenediaminetetraacetic acid; Equ - Part of the plant used; Fap - Form of application; Faq - Method of acquisition; MCD - Minimum Coagulant Dose; MHD - Minimum Hemorrhagic Dose; Nc - Common name; LD_{50} - Lethal Dose $_{50}$; Out - Other uses; PBS - Phosphate buffer saline solution; PCA - Principal component analysis; Pum - Perception of medicinal use; PLA2 - Phospholipase A2; Qp - Preparation process; TLC - Thin-layer chromatography; Ua - Use in snakebites; Up - Use the Plant.

Ethics approval and consent to participate: The development of the study followed the ethical and legal guidelines for the development of research on traditional knowledge. The project was approved by the ethics committee of the University of Nariño (approval record number 038 of October 2020). The participation of healers was subject to the acceptance of the Free and Informed Consent Form.

Consent for publication: Not applicable

Availability of data and materials: datasets have not been deposited in public repositories. Data will be provided by the authors upon reasonable request.

Competing interests: the authors declare that there are no conflicts of interest.

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Author contributions: KRD, CMC, MSGI, JCVG, KLAC, JAGV, and DMO were responsible for the conceptualization of the work, formal analysis, writing-original draft preparation, writing-reviewing and editing. MJSS, and JMG participated in writing - reviewing and editing. All authors have read and agreed to the published version of the manuscript.

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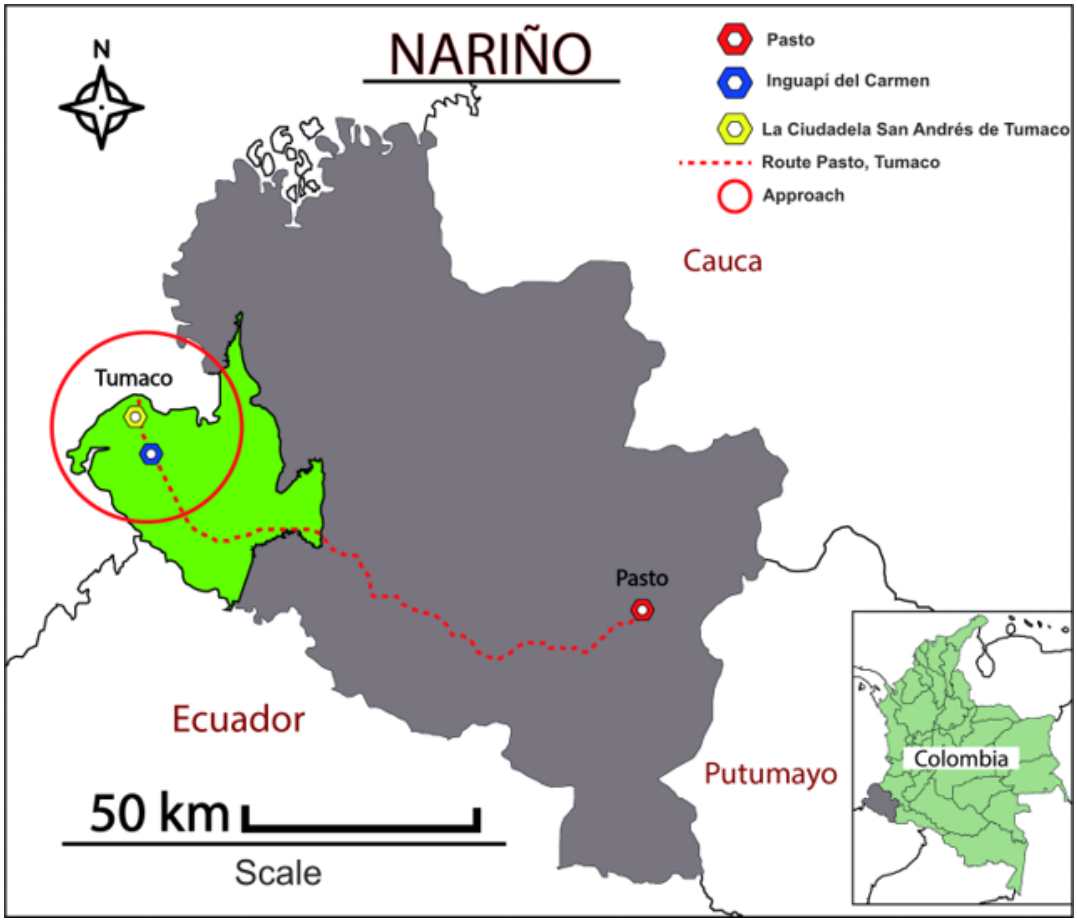

Figure S1. Geographical location of the study area. Green region: municipality of Tumaco. Blue hexagon: Inguapí del Carmen-San Andrés de Tumaco (01°40'24'' N-78° 45'12.5'' W). Yellow hexagon: La Ciudadela, Cabecera municipal- San Andrés de Tumaco (01°47'26.2 "N-78°47'02.6 "W). Red hexagon: San Juan de Pasto.

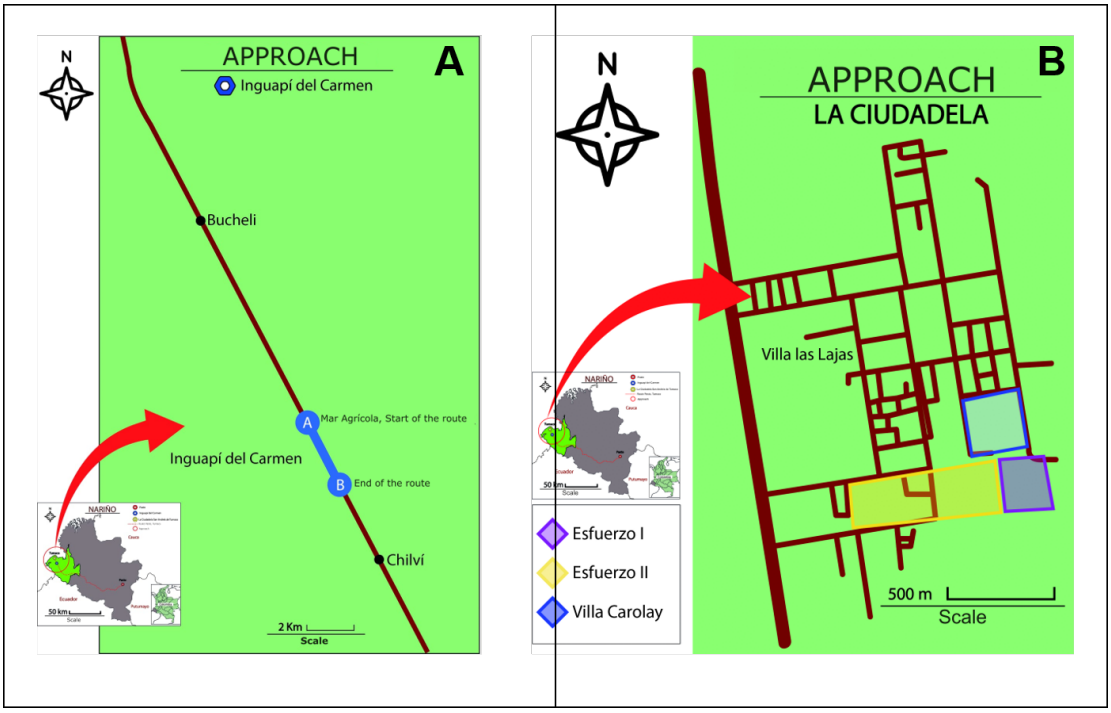

Figure S2. Geographical location of the interview sampling zones. A. Rural area, Inguapí del Carmen-San Andrés de Tumaco route. B. Urban zone, route through the neighborhoods of Esfuerzo I, Esfuerzo II, and Villa Carolay, near La Ciudadela, San Andrés de Tumaco.

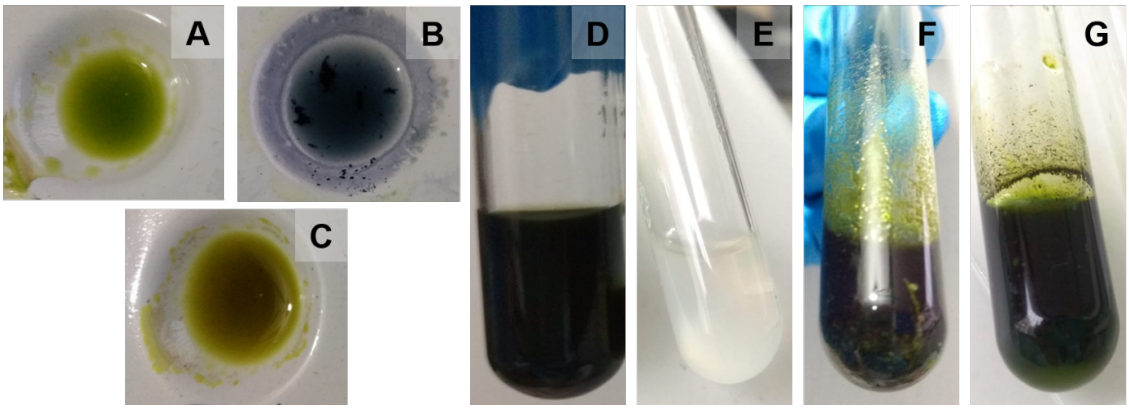

Figure S3. Tannin determination. A, D. *N. lobata* extract (control -). B, E. Tannic acid (control +). C. Assay of *N. lobata* extract and ferric chloride. F. Assay of *N. lobata* extract and gelatin. G. *N. lobata* extract and gelatin-salt assay.

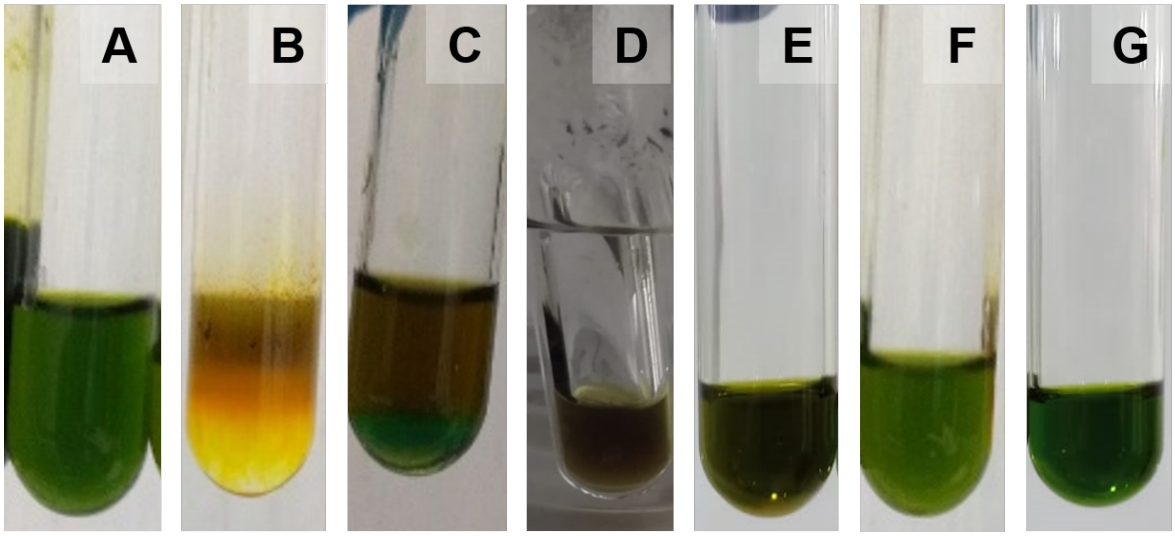

Figure S4. Determination of flavonoids. A. *N. lobata* extract (control -). B. Quercetin (control +). C. Test with extract and concentrated H_2SO_4. D. Test with extract, HCl and water bath. E. Test with Magnesium metal extract and HCl (Shinoda Test). F. Test with extract and NaOH. G. Test with extract and anhydrous boric acid. The result for ferric chloride was the same as in Figure S3.

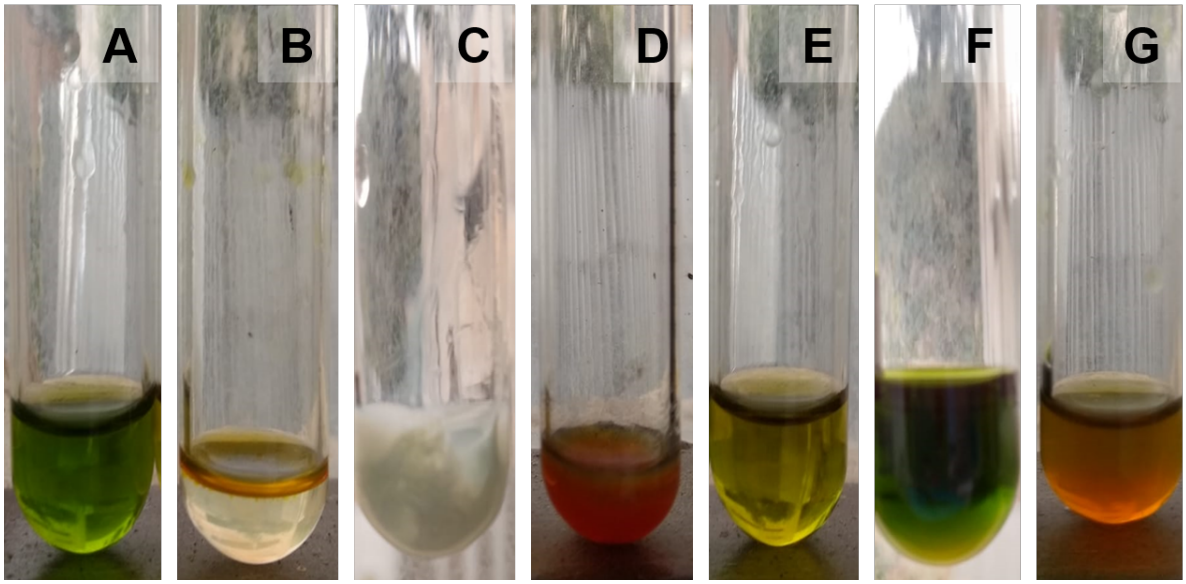

Figure S5. Determination of alkaloids. A. *N. lobata* extract (control -). B. Dragendorff's reagent (control +). C. Mayer's reagent (control +). D. Wagner's reagent (control +). E. Dragendorff - extract. F. Mayer - extract. G. Wagner - extract.

Figure S6. Determination of saponins. A. Ginseng extract (control +). B. Distilled wáter (control -). C. *N. lobata* Extract.

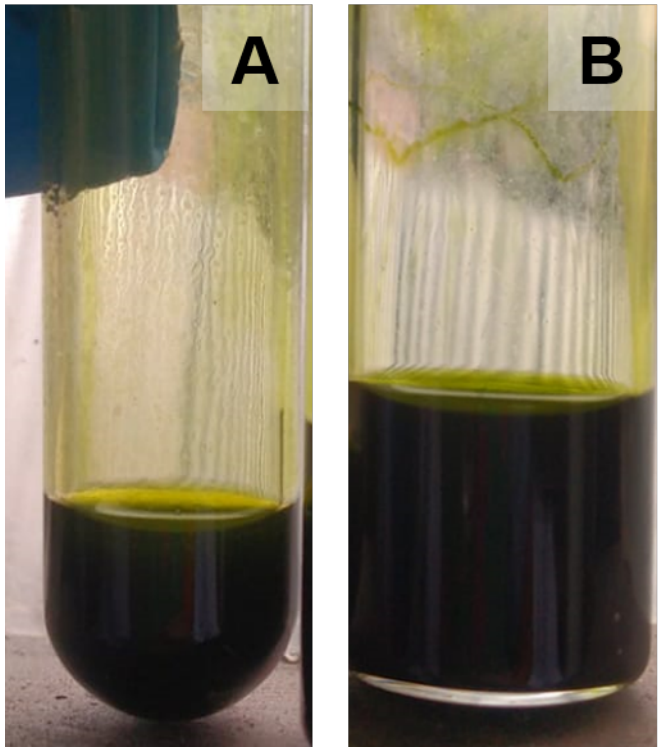

Figure S7. Determination of α, β-unsaturated lactones by legal test. A. *N. lobata* extract alone (control -) and B. Assay with *N. lobata* extract, pyridine, sodium nitroprusside and potassium hydroxide.

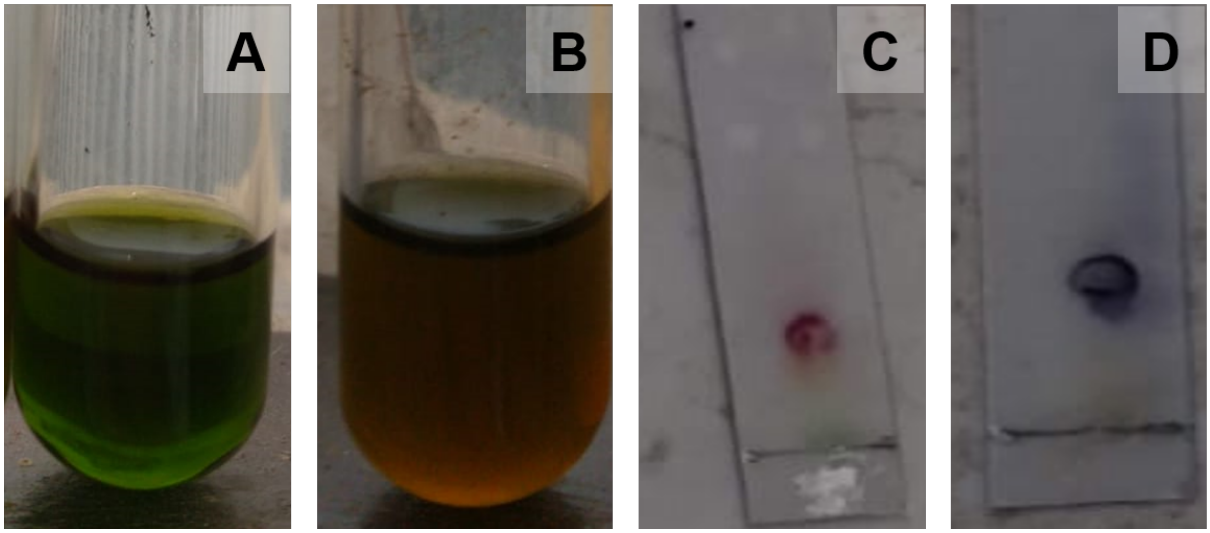

Figure S8. Determination of sesquiterpene lactones by ferric hydroxymate test. *N. lobata* extract alone (control -) (A) and assay with extract and ferric hydroxymate (B). Determination of sesquiterpene Lactones for TLC. Revelation with sulfuric acid (C) and disclosure with vanillin-sulfuric acid (B).