Antimicrobial activity of Amazonian plant species against the causative agents of secondary infection in snakebites

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Abstract

Riverine communities in the Brazilian Amazon region use a variety of plants to treat snakebites. These plants can be effective against secondary infections, one of the main complications of snakebites. The aim of this study was to determine whether plants traditionally used to treat snakebites in the Brazilian Amazon may also have antimicrobial and antioxidant activities, and if so, which classes of chemicals may be responsible for these activities. Aqueous extracts of nine plants were tested in microdilution assays and the more active were prepared using solvents (hexane, methanol and water) and decoction, and nine assays were performed. Assays to determine the antioxidant activity of the most active species were carried out, as well as phytochemistry studies to determine the active components of this species. *Bellucia dichotoma* exhibited the greatest antimicrobial potential, particularly the hexane, methanol and decoction extracts. In comparative TLC, extracts of this species showed characteristics of terpenoids, compounds with double bonds and flavonoids. In ¹H NMR, characteristic signals of sterols such as β-sitosterol, stigmasterols or triterpenes were observed, as well as signals indicating the presence of aromatic hydrogens, characteristic of aromatic substances, and sugars. The methanol extracts and decoction were considered active in the antioxidant assay.

Keywords: *Bellucia dichotoma*. *Bothrops atrox*. Snakebites. Infections. Antimicrobial. Wound healing.

Introduction

In the Amazon region, snakebites continue to be a public health concern. The long distances that must be covered between the accident site and the nearest medical center where specialized care can be provided,
the unreliable transport (very often by river) and the frequent lack of snake antivenom reflect the distressing reality faced by patients, who may have to wait from six to more than twelve hours for medical care and may only receive a smaller dose of the antivenom than recommended or even none at all, with the consequent risk of death[1-3].

Among the snakebite envenomations reported in the region, those caused by Bothrops atrox (known locally as jararaca, jararacuçu, jararaca-do-norte, jararaca-de-rabo-branco, surucucurana and surucucu-do-barranco) are the most common[1,4]. Care is required when using popular names to report snakebites by B. atrox as the term surucucu is frequently also used for Lachesis muta, whose bite causes similar symptoms but requires different treatment[5-7].

The treatment recommended by the World Health Organization is snake antivenom, which often does not reach the patient in a satisfactory condition for several reasons, such as problems with distribution and storage in remote areas and insufficient training of healthcare staff in these areas to ensure ideal management of snakebite cases[9]. Furthermore, snake antivenom neutralizes mainly the systemic effects of the bite (such as hemorrhage, myolysis, hemolysis, paralysis, kidney injuries and abnormal coagulation) but not the local effects (such as intense pain, edema, blisters, ecchymosis and muscle necrosis), leaving the door open for complications and sequelae in the affected limb[2,8].

One of the main clinical complications caused by snakebites is secondary infection, which worsens the clinical picture, leading to abscesses, infectious cellulitis, necrotizing fasciitis, gangrene, sepsis and toxic shock syndrome, in turn very often leading to limb amputation or loss of limb function[10-12]. Secondary infection can be monomicrobial or polymicrobial, and the microorganisms that cause the infection, which are inoculated at the time of the snakebite, may come from the oral cavity of the snake or the victim’s skin. The damaged wound tissue itself may act as a source for bacterial colonization and may result in complications in the affected limb[13].

Studies have shown that the most frequently isolated bacteria that can cause secondary infections in snakebites are anaerobic bacteria from the family Enterobacteriaceae, which are found both in abscesses in snakebite victims and in the oral and cloacal cavity of snakes of the genus Bothrops (TABLE 1).

**TABLE 1**: Bacteria isolated from abscesses caused by the bite of Bothrops sp. and from the oral and cloacal cavities of snakes of this genus.

| Bacteria                        | Family                    | Abscesses (Ref.) | Cavities (Ref.) |
|---------------------------------|---------------------------|------------------|-----------------|
| Aeromonas hydrophila           | Aeromonadaceae            | [14,15,16]       |                 |
| Aeromonas sp.                   | Aeromonadaceae            |                  |                 |
| Bacillus megaterium            | Bacillaceae               |                  |                 |
| Bacillus sp.                    | Bacillaceae               |                  |                 |
| Bacillus subtilis or Bacillus licheniformis | Bacillaceae | [15] | [17-19] |
| Bacteroides sp.                 | Bacteroidaceae            |                  |                 |
| Ochrobactrum sp. or Brucella sp.| Brucellaceae              |                  |                 |
| Clostridium biferrantis         | Clostridaceae             |                  |                 |
| Clostridium sp.                 | Clostridaceae             |                  |                 |
| Citrobacter braakii            | Enterobacteriaceae        |                  |                 |
| Citrobacter diversus           | Enterobacteriaceae        |                  |                 |
| Citrobacter freundii           | Enterobacteriaceae        |                  |                 |
| Citrobacter sp.                | Enterobacteriaceae        |                  | [18-19]         |
| Citrobacter youngae            | Enterobacteriaceae        |                  |                 |
| Enterobacter aerogenes          | Enterobacteriaceae        |                  |                 |
| Enterobacter cloacae           | Enterobacteriaceae        | [14,19]          | [15,19]         |
An analysis of epidemiologic and clinical data on snakebite envenomations in the interior of the state of Amazonas between 1989 and 1996 found that 8.3% of the patients had secondary infections\(^1\). Between 2007 and 2017, 18.1% of the snakebites reported in the state of Amazonas resulted in secondary infection\(^2\). In a study carried out between 2014 and 2016 in the state of Amazonas, 40% of snakebite victims presented with secondary infection and did not respond to preemptive treatment with amoxicillin clavulanate, the antibiotic recommended by the Infectious Diseases Society of America (IDSA) to prevent secondary infections caused by bacteria that are resistant to these antibiotics. The table below lists the bacteria isolated from the wounds and surrounding tissues of snakebite victims. These bacteria are known to cause secondary infections and are part of the normal flora of the skin and mucous membranes. The table also includes the families to which these bacteria belong, which can help determine the appropriate antibiotic therapy.

| Bacteria                          | Family               |
|----------------------------------|----------------------|
| Enterobacter sp.                 | Enterobacteriaceae   |
| Escherichia coli                 | Enterobacteriaceae   |
| Klebsiella oxytoca               | Enterobacteriaceae   |
| Klebsiella pneumoniae            | Enterobacteriaceae   |
| Kluyvera sp.                     | Enterobacteriaceae   |
| Morganella morganii              | Enterobacteriaceae   |
| Proteus mirabilis                | Enterobacteriaceae   |
| Proteus penneri                  | Enterobacteriaceae   |
| Proteus sp.                      | Enterobacteriaceae   |
| Proteus vulgaris                 | Enterobacteriaceae   |
| Providencia retgeri              | Enterobacteriaceae   |
| Providencia sp.                  | Enterobacteriaceae   |
| Salmonella enterica              | Enterobacteriaceae   |
| Salmonella sp.                   | Enterobacteriaceae   |
| Salmonella typhimurum            | Enterobacteriaceae   |
| Serratia liquefaciens            | Enterobacteriaceae   |
| Serratia marcescens              | Enterobacteriaceae   |
| Serratia sp.                     | Enterobacteriaceae   |
| Yersinia enterocolitica          | Enterobacteriaceae   |
| Yokenella regensburgei           | Enterobacteriaceae   |
| Enterococcus faecalis            | Enterococcaceae      |
| Enterococcus sp.                 | Enterococcaceae      |
| Kocuria kristinae                | Micrococcaceae       |
| Kocuria varians or Kocuria palustris | Micrococcaceae   |
| Micrococcus sp.                  | Micrococcaceae       |
| Acinetobacter anitratus          | Moraxellaceae        |
| Paeenbacillus glucanolyticus     | Paenbacillaceae      |
| Chryseomons violaceum           | Pseudomonadaceae     |
| Pseudomonas aeruginosa           | Pseudomonadaceae     |
| Pseudomonas fulva                | Pseudomonadaceae     |
| Pseudomonas paucimobilis         | Pseudomonadaceae     |
| Pseudomonas pickettii            | Pseudomonadaceae     |
| Pseudomonas putida or Stenotrophomonas maltophilia | Pseudomonadaceae |
| Pseudomonas sp.                  | Pseudomonadaceae     |
| Rhizobium radiobacter            | Rhizobiaceae         |
| Shewania putrefaciens            | Shewanelaceae        |
| Sphingomonas paucimobilis        | Sphingomonadaceae    |
| Staphylococcus arletae           | Staphylococcaceae    |
| Staphylococcus aureus            | Staphylococcaceae    |
| Staphylococcus coag.             | Staphylococcaceae    |
| Staphylococcus kloosii           | Staphylococcaceae    |
| Staphylococcus sciuri            | Staphylococcaceae    |
| Staphylococcus sp.               | Staphylococcaceae    |
| Staphylococcus warneri or Staphylococcus pasteuri | Staphylococcaceae    |
| Beta hemolytic Streptococcus     | Streptococcaceae     |
| Group A Streptococcus            | Streptococcaceae     |
| Group B Streptococcus            | Streptococcaceae     |
| Streptococcus viridans           | Streptococcaceae     |
| Stenotrophomonas maltophilia     | Xanthomonadaceae     |

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by animal bites. It is therefore extremely important to find new substances with antibacterial activity given the high incidence of bacteria resistant to commercial antibiotics. This bacterial resistance is a consequence of the excessive, incorrect use of antibiotics by humans and in the livestock industry and the inadequate disposal of antibiotics (which very often are not fully metabolized), leading to contamination of the environment, where mutations and transmission of resistance genes can occur. In 2015, the WHO published a global action plan to contain the damage caused by bacterial resistance. Among the objectives of this plan were investment in the development of new drugs and vaccines and other interventions.

As a result of the difficulties they face, the Amazonian population, particularly the riverine population, which lives far from major centers and is most affected, ends up using alternative or complementary treatments such as those based on plants in the form of infusions, tinctures and other herbal preparations to treat snakebites. An ethnomedical study by Moura et al. of healers and inhabitants in areas with a high incidence of envenomations in the Santarém-PA region identified 24 plants used to treat snakebites. In vitro and in vivo tests of some of these plants showed that they can potentially be used to treat the effects of Bothrops atrox and Bothrops jararaca venoms. The discovery of a plant species, or even a combination of plant species, that could block the local effects of snake venom and inhibit or reduce growth of the microorganisms responsible for secondary infection would be a major step forward as such plants could be used to complement conventional treatment. In this study we evaluated the potential antimicrobial activity of aqueous extracts of nine plants used in folk medicine (TABLE 2) that have one or more anti-snakebite properties.

### TABLE 2: Plant species from the Santarém-PA region of Brazil used in folk medicine and their anti-snakebite properties.

| Scientific name | Family | Popular name | Method of preparation | Part | Biological activity |
|-----------------|--------|--------------|-----------------------|------|--------------------|
| Aniba fragrans Ducke (syn. A. parviflora Mein Mez.) | Lauraceae | Macacaporanga | Tea | Leaves | AH |
| Annona montana Macfad. | Annonaceae | Araticum | Juice | Leaves | - |
| Bellucia dichotoma Cogn. | Melastomataceae | muúba, goiaba-de-anta | Tea | Bark | AP₂, AH, AE, AC, AO |
| Crataeva berthamii (syn. Crataeva tapia L.) | Capparaceae | Catauari | (Topical) tincture | Leaves | - |
| Connarus favosus Planch. | Connaraceae | Verônica | (Oral) tincture and tea | Bark | AP₂, AC, AH, AO, AM |
| Dipterix odorata (Aubl.) Forsyth f. | Fabaceae | Cumaru | Tea | Seeds | - |
| Kalanchoe brasiliensis Cambess. | Crassulaceae | Corama | Macerated Leaves | AH |
| Philodendron megaphyllum Schott. | Araceae | cipó de tracuá | paste (oral) Lianas | AH |
| Plathymenia reticulata Benth. | Fabaceae | Vinhático | (Oral) tincture | Roots | AP₂, AC, AH, AE |

### Materials and Methods

**Plant material**

The plant species studied were collected in the Santarém region (PA) close to the Vila de São Pedro (02°32'08.9″S, 54°54'23.9″W), Cucurunã (02°27'21.0″S, 54°47'45.7″W) and Alter do Chão (02°30'53.3″S, 54°37'00.1″W) communities and on the Curauá Experimental Farm (02°33'99.3″S, 54°36'61.2″W). The
species are used in folk remedies by the local communities and have been studied for their anti-snakebite properties. The following voucher specimens are stored at the EMBRAPA herbarium in Belém-PA: *Aniba fragrans* (synonym: *A. parviflora*) (herbarium registration no. 184897), *Annona montana* (185214), *Bellucia dichotoma* (1852213), *Crataeva benthamii* (184823), *Connarus favosus* (185216), *Dipterix odorata* (186494), *Kalanchoe brasiliensis* (184695), *Philodendron megalophyllum* (184899) and *Plathymenia reticulata* (185215). The plant material collected was dried, pulverized in a cutting mill, used in a decoction and lyophilized.[9,36]

**Antimicrobial assay**

Minimum inhibition concentration of the extracts was determined by the broth microdilution method (Mueller-Hinton Broth) and spectrophotometry at 625 nm. Each assay was performed in triplicate and expressed as mean ± standard deviation of the mean. The means were compared by two-way ANOVA followed by the post hoc Tukey test using p<0.05. The following microorganism strains were used for the assays: AB (*Acinetobacter baumanii* ATCC 19606), AH (*Aeromonas hydrophila* IOC/FDA 110-36), CA (*Candida albicans* ATCC 10231, CBS 6431), CP (*Candida parapsilosis* ATCC 22019, CBS 604), CF (*Citrobacter freundii* ATCC 8090), ET (*Edwardsiella tarda* ATCC 15947), EnCl (*Enterobacter cloacae* ATCC 13047), EnF (*Enterococcus faecalis* ATCC 29212), EC (*Escherichia coli* ATCC 11775), KP (*Klebsiella pneumoniae* ATCC 13883), MM (*Morganella morganii* ATCC 00082), PA (*Pseudomonas aeruginosa* ATCC 10145), PF (*Pseudomonas fluorescens* ATCC 13525, NCTC 10038), SE (*Salmonella enterica* ATCC 13076), SM (*Serratia marcescens* ATCC 13880), StAu (*Staphylococcus aureus* ATCC 12600), PR (*Propionibacterium acnes* ATCC 6919) and YE (*Yersinia enterocolitica* ATCC 9610). The positive control was oxytetracycline 125 g/mL and the negative control was Mueller-Hinton broth medium.

**Phytochemical study of Bellucia dichotoma**

The extracts were prepared from 675 g of bark powder. Of the 675 g, 275 g were used for decoction with a bark mass-to-water volume ratio of 1:10 following the protocol described by Mourão et al.[35] and 400 g were extracted in an ultrasound bath for 20 minutes with 12 L of each solvent (1 L at a time for every 200 g repeated 6 times) in the following order: hexane, methanol and water. After extraction with hexane, the plant material was left to dry at room temperature. The following day the material was used for extraction with methanol and dried again, and the next day the residue was used for extraction with distilled water. The hexane and methanol extracts were concentrated in a rotary evaporator, and the aqueous extracts were dried in a freeze dryer.

The crude extracts were analyzed by comparative thin layer chromatography (CTLC) to determine the chemical classes present. Using glass capillary tubes, aliquots of crude extracts were run on silica gel chromatography plates with UV 254 fluoroscent indicator. To separate the different chemical compounds effectively, these were eluted with different combinations and proportions of organic solvents. The chromatoplates were viewed under 254 and 365 nm UV light (the physical developer) and with chemical developers (iodine, ceric sulfate, NP, PEG and anisaldehyde). The crude extracts were also analyzed by 1H nuclear magnetic resonance (NMR) in a 300 MHz spectrometer (Bruker Fourier 300).
Antioxidant activity

The antioxidant activity of the *Bellucia dichotoma* extracts was determined by two methods: (i) measurement of the extract's ability to sequester Fe$^{3+}$ free radicals and (ii) the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. In both cases the results were expressed as ascorbic acid equivalents [39].

Results and Discussion

Of the nine plant species investigated (TABLE 2), the species that exhibited the greatest potential to inhibit microbial growth at a concentration of 1 mg/mL for gram-positive and gram-negative bacteria as well as yeasts were *Bellucia dichotoma*, *Connarus favosus* and *Philodendron megalophyllum* (TABLE 3), each of which inhibited growth by more than 60%. *Acinetobacter baumanii*, *Citrobacter freundii*, *Edwardsiella tarda*, *Enterobacter cloacae*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Morganella morganii*, *Pseudomonas fluorescens*, *Salmonella enterica*, *Serratia marcescens* and *Staphylococcus aureus* were inhibited by *C. favosus*; *A. baumanii*, *Candida albicans*, *Candida parapsilosis*, *C. freundii*, *E. tarda*, *E. cloacae*, *E. faecalis*, *E. coli*, *M. morganii*, *P. fluorescens*, *S. enterica*, *S. marcescens* and *S. aureus* were inhibited by *B. dichotoma*; and *A. baumanii*, *Aeromonas hidrophyla*, *C. albicans*, *C. parapsilosis*, *C. freundii*, *E. tarda*, *E. cloacae*, *E. coli*, *K. pneumoniae*, *M. morganii*, *P. aeruginosa*, *P. fluorescens*, *S. enterica*, *S. marcescens* and *S. aureus* were inhibited by *P. megalophyllum*.

Successive dilutions of these three most active species were made to yield concentrations of 1000, 500, 250, 125, 62.5 and 31.25 µg/mL, and a new microdilution assay was carried out to determine the inhibitory potential of these species at these concentrations. Best results were obtained with aqueous extracts of *Bellucia dichotoma* and *Connarus favosus* (GRAPHS 1, 2, 3). The means were compared by two-way ANOVA followed by Dunnett's *post hoc* test using $p<0.05$. The results in Graphs 1 to 4 are expressed as mean absorbance at 586 nm: the greater the value of absorbance, the greater the turbidity due to microbial growth, and the smaller the value of absorbance, the greater the growth inhibition caused by the extract.

New extracts of *Bellucia dichotoma* and *Connarus favosus* were prepared, this time with solvents of increasing polarity (hexane, methanol and water) and tea (decoction), and then used in new antimicrobial activity assays (GRAPH 4). Shows that the best results for antimicrobial inhibition were achieved with the hexane and methanol extracts and decoction (tea) of *B. dichotoma*.

In CTLC, the hexane extract exhibited purple spots when developed with ceric sulfate and lilac stains when developed with anisaldehyde, both indicative of terpenoids. When developed with iodine, the extract showed characteristics of double bonds, and when developed with NP-PEG at 365 nm there was an increase in the fluorescence intensity of spots characteristic of flavonoids.
TABLE 3: Results of the microbial growth inhibition assay for 1 mg/mL aqueous plant extracts expressed as percentage inhibition. Extracts: Aniba fragrans (AF), Annona montana (AM), Crataeva benthamii (CB), Connarus favosus (CF), Dipterix odorata (DO), Kalanchoe brasiliensis (KB), Philodendron megalophyllum (PM) and Plathymenia reticulata (PR). Microorganisms: Acinetobacter baumannii (AB), Aeromonas hydrophila (AH), Candida albicans (CA), Candida parapsilosis (CP), Citrobacter freundii (CF), Edwardsiella tarda (ET), Enterobacter cloacae (EC), Enterococcus faecalis (EF), Escherichia coli (EC), Klebsiella pneumoniae (KP), Morganella morganii (MM), Pseudomonas aeruginosa (PA), Pseudomonas fluorescens (PF), Salmonella enterica (SE) and Serratia marcescens (SM). Positive control (Ctrl+) (oxytetracycline 125 µg/mL); negative control (Ctrl-) (Mueller-Hinton broth).

| Aqueous Extracts (1 mg/mL) | AB | AH | CA | CP | CF | ET | EnCl | EnF | EC | KP | MM | PA | PF | SE | SM | StAu |
|---------------------------|----|----|----|----|----|----|------|-----|----|----|----|----|----|----|----|-----|
| AF | 36 | 15 | 37 | 83 | 61 | 66 | 40 | 49 | 50 | 57 | 48 | 65 | 57 | 60 | 72 |
| AM | 0  | 0  | 26 | 51 | 48 | 65 | 38 | 34 | 32 | 35 | 22 | 13 | 33 | 43 | 16 | 40 |
| CB | 18 | 5  | 37 | 54 | 27 | 76 | 42 | 39 | 36 | 53 | 34 | 25 | 63 | 64 | 66 | 36 |
| CF | 100| 40 | 42 | 55 | 61 | 66 | 67 | 65 | 67 | 93 | 86 | 56 | 75 | 90 | 93 | 74 |
| DO | 20 | 0  | 11 | 36 | 40 | 46 | 19 | 7  | 39 | 36 | 34 | 49 | 50 | 45 | 57 |
| BD | 77 | 51 | 78 | 84 | 65 | 74 | 72 | 100| 65 | 54 | 61 | 55 | 74 | 82 | 79 | 84 |
| KB | 0  | 8  | 7  | 37 | 46 | 30 | 2  | 16 | 15 | 0  | 2  | 32 | 36 | 20 | 39 |
| PM | 81 | 84 | 73 | 85 | 83 | 83 | 93 | 56 | 87 | 90 | 86 | 80 | 79 | 92 | 82 | 69 |
| PR | 0  | 0  | 6  | 40 | 18 | 39 | 12 | 0  | 34 | 38 | 44 | 25 | 31 | 39 | 18 | 36 |
| Ctrl+| 97 | 99 | 88 | 90 | 100| 99 | 99 | 90 | 99 | 99 | 99 | 98 | 95 | 98 | 99 | 99 |
| Ctrl-| 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  |
GRAPH 1: Growth inhibition of *Acinetobacter baumanii* (AB), *Aeromonas hydrophila* (AH), *Candida albicans* (CA), *Citrobacter freundii* (CF), *Edwardsiella tarda* (ET), *Enterobacter cloacae* (EnCl), *Enterococcus faecalis* (EnF), *Escherichia coli* (EC), *Klebsiella pneumoniae* (KP), *Morganella morganii* (MM), *Pseudomonas aeruginosa* (PA), *Pseudomonas fluorescens* (PF), *Salmonella enterica* (SE), *Serratia marcescens* (SM), *Yersinia enterocolitica* (YE) and *Propionibacterium acnes* (PrAc) by aqueous extracts (decoction) of *Bellucia dichotoma* at concentrations of 1 to 0.3 mg/mL. Values are expressed as mean absorbance at 586 nm and standard deviation. Ctrl+: oxytetracycline 125 µg/mL; Ctrl–: Mueller-Hilton broth.
GRAPH 2: Growth inhibition of *Acinetobacter baumanii* (AB), *Aeromonas hidrophylia* (AH), *Citrobacter freundii* (CF), *Edwardsiella tarda* (ET), *Enterobacter cloacae* (EnCl), *Enterococcus faecalis* (EnF), *Escherichia coli* (EC), *Klebsiella pneumoniae* (KP), *Morganella morganii* (MM), *Pseudomonas aeruginosa* (PA), *Pseudomonas fluorescens* (PF), *Salmonella enterica* (SE), *Serratia marcescens* (SM), *Staphylococcus aureus* (StAu), *Yersinia enterocolitica* (YE) and *Propionibacterium acnes* (PrAc) by aqueous extracts (decoction) of *Connarus favosus* at concentrations of 1 to 0.3 mg/mL. Values are expressed as mean absorbance at 586 nm and standard deviation. Ctrl+: oxytetracycline 125 µg/mL; Ctrl–: Mueller-Hilton broth.
GRAPH 3: Growth inhibition of Acinetobacter baumanii (AB), Aeromonas hidrophylla (AH), Citrobacter freundii (CF), Edwardsiella tarda (ET), Enterobacter cloacae (EnCl), Enterococcus faecalis (EnF), Escherichia coli (EC), Morganella morganii (MM), Pseudomonas aeruginosa (PA), Pseudomonas fluorescens (PF), Salmonella enterica (SE), Serratia marcescens (SM), Staphylococcus aureus (StAu) and Propionibacterium acnes (PrAc) by aqueous extracts (decoction) of Philodendron megalophyllum at concentrations of 1 to 0.3 mg/mL. Values are expressed as mean absorbance at 586 nm and standard deviation. Ctrl+: oxytetracycline 125 µg/mL; Ctrl–: Mueller-Hilton broth.
GRAPH 4: Growth inhibition of Acinetobacter baumanii (AB), Aeromonas hidrophyla (AH), Candida albicans (CA), Citrobacter freundii (CF), Edwardsiella tarda (ET), Enterobacter cloacae (EnCl), Enterococcus faecalis (EnF), Escherichia coli (EC), Klebsiella pneumoniae (KP), Morganella morganii (MM), Pseudomonas aeruginosa (PA), Salmonella enterica (SE), Serratia marcescens (SM) and Staphylococcus aureus (StAu) by hexane, methanol and aqueous extracts and decoction of Bellucia dichotoma and Connarus favosus at a concentration of 1 mg/mL. Values are expressed as mean absorbance at 586 nm and standard deviation. Ctrl+: oxytetracycline 125µg/mL; Ctrl–: Mueller-Hilton broth.
In the $^1$H NMR spectrum of the hexane extract, signals characteristic of sterols such as β-sitosterol (0.67) and stigmasterols or triterpenes (0.69) were observed. The spectra of the methanol and aqueous extracts and decoction were very similar and showed shifts in the 6.5 to 8 ppm region characteristic of aromatic hydrogens, suggesting the presence of aromatic substances; shifts characteristic of sugars were also observed in the 3 to 4 ppm region.

The results of the antioxidant assays are expressed in equivalent values in relation to the standard (ascorbic acid) using the scale proposed by Martins et al.[39], which compares the activity of 1 mg of extract with the activity of 1 mg of ascorbic acid. On the scale, <1.0 corresponds to very active; >1.1 and <2.0 corresponds to active; >2.1 and <3.0 to moderately active; and >3.1 to inactive. In the antioxidant activity assay (TABLE 4), the methanol extracts and tea (decoction) had values of between 1 and 2 and were both considered active.

### TABLE 4: Results of the antioxidant assays with *Bellucia dichotoma* extracts.

| Extract   | Method using DPPH | Method using Fe$^{3+}$ |
|-----------|-------------------|------------------------|
|           | Mean values       | Mean values            |
|           | [DABS$^{a,b}$]    | [AA]$_{eq}$            |
|           | Equiv.            | [Fe$^{3+}$]            |
|           |                   | [AA]$_{eq}$            |
|           |                   | Equiv.                 |
| Hexane    | 0.013             | 0.102                  | 75.065 |
|           |                   |                        | -0.025 | 0.114 | 49.841 |
| Methanol  | 0.271             | 2.928                  | 2.180  |
|           |                   |                        | 1.551  | 2.709 | 1.846  |
| Water     | 0.064             | 0.540                  | 23.504 |
|           |                   |                        | 0.865  | 1.580 | 3.169  |
| Tea       | 0.299             | 2.540                  | 1.972  |
|           |                   |                        | 1.802  | 3.123 | 1.602  |

DABS: Absorbance of extract obtained by DPPH method; AA: Ascorbic acid equivalent.

The antimicrobial activity of nine plant species used in folk medicine to treat snakebites was tested against gram-positive and gram-negative strains of bacteria and two yeasts that can be found in the oral cavity of snakes and in abscesses caused by snakebites.

Although snake venom itself has antimicrobial properties, the bacterial microbiota in the oral cavity of snakes varies. Some studies have shown that the composition of this microbiota reflects the fecal flora of the snake’s prey, generally rodents, amphibians, reptiles and small birds, as these normally defecate when they are being ingested[40,41,11,42,43,12,44]. According to other studies, the composition of the oral microbiota in snakes is related to the species of snake rather than its diet. In addition to suggesting that bacteria can play an important role in the production of enzymes for snakes to digest their prey, these studies showed that adult individuals had the same microbiota composition as neonates before their first meal and that, in captivity, different species of snake fed the same food in the same environment had different oral microbiota[45]. Interestingly, snakes of the species *Bothrops insularis*, which are endemic to the isolated Queimada Grande Island in Brazil and have distinct eating habits, have very similar microbiota to that found in *Bothrops* sp. species on the mainland[23].

Many cases of secondary infection can be attributed to the oral microbiota of snakes. A particular cause for concern is the recent publication of studies describing bacterial strains isolated from snakes or snakebites that are resistant to the antibiotics normally prescribed in such cases[17,49].

Brazilian researchers have suggested various measures to address the problem of envenomation by animals (such as snakes and scorpions) in the Amazon region, including support for studies on the use of phytomedicines as alternative or complementary therapies[8]. Various plant species used by the local
population have been studied and have shown good results against the local and systemic effects of the venom of snakes of the genus *Bothrops* sp., which are common in the region [32-35,9,36-38]. In the present study we sought to evaluate plant species that not only show activity against the *Bothrops* venom, but also can act on bacterial strains that cause secondary infection in snakebite envenomations.

After antimicrobial screening assays with nine species whose anti-snakebite potential has been tested previously in other studies (*Aniba fragrans* (synonym: *A. parviflora*), *Annona montana*, *Bellucia dichotoma*, *Crataeva benthamii*, *Connarus favosus*, *Dipterix odorata*, *Kalanchoe brasiliensis*, *Philodendron megalophyllum* and *Plathymenia reticulata*), *B. dichotoma* showed the greatest potential to inhibit microbial growth. Aqueous, hexane and methanol extracts of these plants inhibited growth of gram-negative microorganisms: *Acinetobacter baumanii*, *Aeromonas hidrophyla*, *Citrobacter freundii*, *Edwardsiella tarda*, *Enterobacter cloacae*, *Escherichia coli*, *Klebsiella pneumoniae*, *Morganella morganii*, *Pseudomonas aeruginosa*, *Salmonella enterica* and *Serratia marcescens*; two gram-positive microorganisms: *Enterococcus faecalis* and *Staphylococcus aureus*; and yeast: *Candida albicans*.

*Bellucia dichotoma* is a tree from the family Melastomataceae, which is native to, but not endemic in, Brazil and is found in the states of Amazonas, Amapá, Pará and Rondônia in areas with a human presence and *terra firme* forests [47-49]. It is known popularly in the region as *muúba*, *araçá-de-anta* or *goiaba-de-anta*. According to Moura et al. [9], it is used in the form of tea (decoction) by riverine communities to treat snakebites. The tea is prepared in a ratio of 100 g of plant extract to 1L of water, of which 150 mL is drunk immediately after the victim is bitten and the remainder is drunk three times a day thereafter. The anti-snakebite properties of the aqueous extract have been demonstrated in studies by Moura et al., who found that it can inhibit local effects, such as edema and the hemorrhagic and clotting activities induced by *Bothrops atrox* venom, can inactivate phospholipase A2 in the venom and is an antioxidant [35,9,37].

The CTLC and NMR assays yielded results suggestive of the presence of terpenoids such as sterols (possibly β-sitosterol), triterpenes and flavonoids in *Bellucia dichotoma* extracts prepared with hexane. Using colorimetric assays of the aqueous extract in an earlier study, Moura et al. [34], also identified terpenoids and flavonoids in *B. dichotoma*. These classes of substances were observed in various studies of species of the family Melastomataceae [48-50] and may be associated with the antimicrobial and antioxidant properties of extracts from these plants.

According to some studies, the ability of triterpenes and flavonoids to inhibit microorganisms such as *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumoniae* can be explained by the fact that these classes of metabolites can damage and disrupt the bacterial cell membrane and interfere with cell metabolism, cellular biosynthesis of essential components and the cell cycle [51,52].

In the assessment of antioxidant activity, the methanol extract and decoction (the most polar of the four preparations used here) had the greatest activity. Antioxidant activity plays an important role in healing as although generation of ROS (reactive oxygen species) is essential in the initial stages of wound healing (e.g., abscesses caused by snakebites) and the removal of microorganisms, excessive production of these oxidants can delay wound healing and cause greater tissue damage [53]. The antioxidant activity of the extracts studied here may thus help to restore tissue integrity.
Conclusion

Plant extracts used in folk medicine by inhabitants of the Brazilian Amazon region to treat snakebites, particularly those from *Bellucia dichotoma*, were able to inhibit the growth of bacteria that can cause secondary infection following snakebites. Such bacteria are typically found in abscesses caused by snakebites and in the oral cavity of snakes of the genus *Bothrops* sp. The antibacterial and antioxidant potential of these extracts may be related to the presence of terpenoids such as sterols (e.g., β-sitosterol), triterpenes and flavonoids.

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