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Original Article

In vitro antibacterial effects of *Zanthoxylum tingoassuiba* root bark extracts and two of its alkaloids against multiresistant *Staphylococcus aureus*

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ABSTRACT

The emergence of multiresistant strains of bacteria reinforces the need to search for new compounds able to combat resistant organisms. Medicinal plants are a great resource of bioactive substances, providing the possibility of obtaining molecules with potential antimicrobial activity. The aim of the present study is the evaluation of the antibacterial activity of extracts and alkaloids isolated from the root bark of *Zanthoxylum tingoassuiba* A. St.-Hil., Rutaceae, against four resistant clinical isolates and *Staphylococcus aureus* ATCC 25923. The dichloromethane and methanol extracts were fractionated by chromatography on silica gel, leading to the isolation of dihydrocheleryhtrine and *N*-methylcanadine, identified by Nuclear Magnetic Resonance spectroscopy. The antibacterial activity of the extracts and isolated compounds was evaluated by the disc diffusion method and the minimum inhibitory concentration was determined. The dichloromethane extract was the most active against all the tested strains and the two pure alkaloids were more active than the extracts. The anti-MRSA activity of the two benzophenanthridine alkaloids is demonstrated for the first time in this study. These compounds appear as potential leads for the development of new anti-MRSA compounds and could be responsible for the antibacterial activity, justifying the ethnobotanical use of *Z. tingoassuiba* and other species for the treatment of various infectious diseases.

Introduction

The evolutionary adaptation of microorganisms has caused an increase of bacteria resistant to the known antibiotics. The emergence of those drug resistant strains has turned the management of infectious diseases more precarious, and there is an urgent need for new active compounds (Demain and Sanchez, 2009).

Staphylococcus aureus commonly causes lower respiratory tract and surgical site infections, being the second cause of nosocomial infections, bacteremia, cardiovascular infections and pneumonia, usually in people admitted to intensive care units. Due to the widespread use of methicillin in the 1960s, several isolates of *S. aureus* have become resistant to a wide range of β-lactam antibiotics (Podoll et al., 2013; Ghidey et al., 2014). Infections caused by methicillin resistant *S. aureus* (MRSA) can be fatal, and it has been classified by the Centers for Disease Control and Prevention (CDC) as one of the eighteen multidrug-resistant (or "superbug") microorganisms. Today some of these strains are not limited to hospitals and have become widespread in community (Butler et al., 2013; Kali, 2015).

Medicinal plants are a great resource of bioactive substances, and in the last decade a great number of works have been dedicated all over the world to the study of the antimicrobial properties of plants, providing the possibility of obtaining molecules that could be employed as new alternative treatments of microbial infections caused by multiresistant bacteria (Meléndez and Capriles, 2006; Sasikumar et al., 2007; Meléndez et al., 2008; Bussmann et al., 2010; Mirzaei et al., 2013; Reddy et al., 2014).

The genus *Zanthoxylum*, Rutaceae, with more than 550 species worldwide is mostly found in tropical and subtropical areas, varying in size from shrub to trees (20 m high) (Patiño et al., 2012). The chemical composition of a large number of these species has been studied in the search for new bioactive compounds as well as for the identification of chemosystematic markers such as benzylisoquinoline alkaloids, characteristic compounds of the proto-rutaceae group (Negi et al., 2011).

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More than 25 species are endemic to Brazil, among which *Z. tingoassuiba* A. St.-Hil., also known as *tinguaciba*, is relevant in folk medicine, being used as antiparasitic and anti-inflammatory agent. The plant is described in the first edition of the Brazilian Pharmacopeia (bark extract) for the treatment of inflammation and of abdominal pain and has been commercialized since 1923 as an active component of a phytotherapeutic formulation prescribed for muscle cramps and spasms (Oliveira et al., 2002; Matu and van Staden, 2003; Tatsadjieu et al., 2003; Mbaze et al., 2007; Goud et al., 2008; Silva et al., 2008; Hohlemwerger et al., 2012; Patiño et al., 2012). Previous studies have shown that *Z. tingoassuiba* essential oil obtained from the leaves displays antibacterial activity against *S. aureus* and MRSA (Detoni et al., 2009).

Considering the importance of the genus *Zanthoxylum* for the discovery and identification of bioactive natural substances capable of inhibiting mechanisms of bacterial resistance, the present work reports the antibacterial evaluation of *Z. tingoassuiba* root bark extracts and two of its alkaloids against multiresistant clinical isolates of *S. aureus.*

Material and methods

Plant material

Fresh roots from *Zanthoxylum tingoassuiba* A. St.-Hil., Rutaceae, were collected in April 2004 in Jaiba, Feira de Santana, Bahia, Brazil (12° 12′ 52.560″ S; 38° 52′ 46.205″ W). The voucher specimens were identified and deposited at the ALCB – Herbário Alexandre Leal Costa, Instituto de Biologia-UFBA (voucher n° 678894).

Chemicals

All solvents (analytical grade) were purchased from Sigma–Aldrich® and used without further purification. Silica gel 60 UV 254 (Macherey-Nagel), Silica gel 60 (70–230 mesh ASTM, Merck), and silica octadecyl-functionalized (C_{18}) (Aldrich) were used for the chromatographic separations. Deuterated solvents used for NMR analysis, $CDCl_3$ and CD_3OD, were obtained from TEDIA. Chloramphenicol ≥98% was purchased from Sigma Aldrich®.

Preparation of extracts and fractions

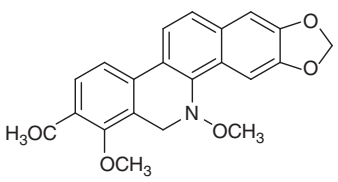
The dried powdered bark from the roots of *Z. tingoassuiba* (217.7 g) was extracted by maceration in dichloromethane (DCM) (CH_2Cl_2, 1 l) for three weeks and then in methanol (MeOH, 1 l) for the same period. The extracts were concentrated under vacuum, not exceeding the temperature of 50 °C, and kept in a desiccator until constant weight was recorded. The dried extracts were stored in a freezer at −20 °C.

The CH_2Cl_2 extract (DCM) (18.11 g) was fractioned by vacuum column chromatography on silica gel using chloroform ($CHCl_3$, 500 ml), ethyl acetate (EtOAc, 500 ml), diethyl ether (Et_2O, 500 ml) and methanol (MeOH, 500 ml) as successive eluents.

Purification and identification of the alkaloids

Compound **1** crystallized spontaneously from the $CHCl_3$ fraction and was recrystallized from MeOH, affording 676 mg of yellow crystals. The pure substance was analyzed by ^1H and ^{13}C NMR spectroscopy (Gemini 500 Hz, $CDCl_3$) and identified as dihydrochelerythrin based on comparison with literature data (Krane et al., 1984; Ming Ng et al., 1987).

An aqueous solution of acetic acid (3%, v/v, 500 ml) was added to the methanol extract (73.9 g) and the resulting mixture was extracted with $CHCl_3$ (3× 50 ml). The organic layer was concentrated under vacuum and fractioned on a C_{18} column chromatography eluted with an isocratic system of acetonitrile and phosphate buffer pH = 4.0 (1:1, v/v). Nineteen fractions were collected. Fractions 7, 8 and 9 were combined after TLC analysis, allowing the isolation of compound **2** (19.0 mg) which was identified as *N*-methylcanadine by comparison of its ^1H and ^{13}C NMR spectra with literature data (Binutu and Cordell, 2000).



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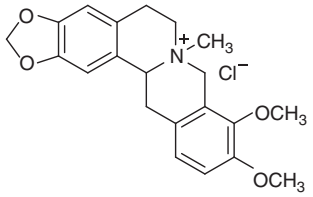

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Antibacterial assay

Microorganisms

Staphylococcus aureus standard strain ATCC 25923 (American Type Culture Collection) was used, as well as *S. aureus* multiresistant strains isolated from clinical samples. The susceptibility profile was determined by the disc diffusion method. Strains 2 and 3 were resistant to the eight tested antibiotics (amoxicillin, ampicillin, oxacillin, clindamycin, erythromycin, ciprofloxacin, levofloxacin, and ofloxacin). Strain 1 was susceptible to levofloxacin and strain 4 to ampicillin, oxacillin, levofloxacin, and ofloxacin. All microbial isolates were stored in the culture collection of the Laboratório de Pesquisa em Microbiologia Clínica (LPMC, UFBA).

Qualitative screening

Qualitative test was performed according to protocol M02-A8 adapted for natural products (CLSI, 2015). Filter paper discs (6 mm diameter) were impregnated with 10 μl of 300 μg/μl solution in dimethylsulfoxide (DMSO) of the DCM and methanol extracts or of the pure compounds **1** and **2**. The discs were placed in Petri dishes containing Muller Hinton agar (MHA) seeded with bacteria suspension of 1.5×10^8 CFU (0.5 McFarland density). After 24 h of incubation at 35 °C, the diameter of the inhibition zone was measured. All experiments were performed in triplicate. Chloramphenicol (30 μg – CECON) was used as positive control against *S. aureus* ATCC 25923 and a disc impregnated with 10 μl of DMSO was used as negative control.

Determination of the minimal inhibitory concentration (MIC)

The MIC was determined using the broth microdilution method in 96-well microplates according to protocol M07-A10 (CLSI, 2015) for DCM and methanol (MeOH) extracts and for compound **2** (*N*-methylcanadine). Due to its poor solubility, the MIC for compound **1** (dihydrochelerythrin) was determined by the agar macro dilution method. Initial bacterial suspensions were prepared in sterile saline solution (0.85% NaCl), adjusted to the turbidity 0.5 McFarland (1.5×10^8 CFU/ml) and diluted to final density of 5×10^4 CFU/ml.

DCM, MeOH extracts and compound **2** were dissolved in DMSO and twofold serial dilutions were made with broth MHA to obtain a concentration range from 15 to 480 μg/ml and 38.4 to 1231.1 μM, respectively. DMSO final concentration was less than 0.5% (v/v). In the same way, twofold serial dilutions of compound **1** were performed to obtain concentrations varying from 42.9 to 973 μM in MHA.

The plates were incubated at 35 °C for 18 h and 15 μl of an aqueous solution (0.5%, v/v) of 2,3,5-triphenyltetrazolium chloride (TTC-NUCLEAR) were added in each well to visualize bacterial growth as a red color. The MIC was defined as the lowest concentration able to inhibit the growth of bacteria. For both techniques,

Table 1
Antibacterial activity (diameter of the inhibition zone (mm) and SD) of methanol (MEOH) and dichloromethane (DCM) extracts from *Zanthoxylum tingoassuiba.*

Staphylococcus aureus	Extract		Compounds		Control
	MeOH	DCM	**1**	**2**	Chloramphenicol
ATCC 25923	21.0 ± 0	20.3 ± 0.58	16.0 ± 0	16.0 ± 0	23.0
Strain 1	23.3 ± 0.58	19.7 ± 0.58	14.0 ± 0.0	18.7 ± 0.58	11.0
Strain 2	19.3 ± 0.58	14.0 ± 0	11.0 ± 0.0	15.7.0 ± 0.58	25.0
Strain 3	22.0 ± 0	13.3 ± 0.58	11.7 ± 0.58	17.7 ± 0.58	20.0
Strain 4	18.3 ± 0.58	15.3 ± 0.58	14.7 ± 0.58	14.7 ± 0.58	11.0

chloramphenicol was used as positive control. DMSO was used as negative control. Culture medium alone was used to ensure the sterility of the medium. Bacterial suspension in culture medium was used as control for the growth of the microorganism.

The experiments were realized in triplicate. Data are expressed as means ± SD (standard deviation).

Results

Yields of extraction and purification

The extraction yield (%, w/w) was calculated based on the initial amount of dry material. The yield obtained for the MeOH extract was higher than the yield of DCM extract (34% and 8.31%, respectively). Dihydrochelerythrine **1** was obtained in 3.73% yield from the DCM extract, after purification by recrystallization. *N*-Methylcanadine **2** was recovered in 0.03% from the methanol extract.

Antibacterial activity

As shown in Table 1, both methanol and DCM extracts were effective against all strains tested, with inhibition zones varying from 18.3 to 21 mm and 13.3 to 20.3 mm, respectively. All the bacteria were also sensitive to dihydrochelerythrine **1** and *N*-methylcanadine **2**, with inhibition halos ranging from 11 to 16 mm and 14.7 to 18.7 mm, respectively.

Minimum inhibitory concentration (MIC)

The results detailed in Table 2 show that the DCM extract displayed a higher inhibitory activity than the MeOH extract against four of the tested strains (ATCC 25923, strain 1, 2 and 4), with MIC values varying from 60 to 240 μg/ml and 120 to 480 μg/ml, respectively.

Compounds **1** and **2** were also capable of inhibiting the growth of all the tested bacteria (MIC values ranging from 85.8 to 171.7 μM and 76.9 to 307.8 μM, respectively). Compound **1** displayed better activity than chloramphenicol against *S. aureus* ATCC 25923 and strain 4. Compound **2** was less effective than the control against all strains with the exception of strain 1.

Discussion

After fractionation of the plant extracts by chromatography on silica gel, two alkaloids, dihydrochelerythrine **1** and *N*-methylcanadine **2** were isolated in low yield (3.73 and 0.03%, respectively). The structures of the isolated compounds were determined by comparison of the NMR spectra with data from the literature. These isoquinoline alkaloids have been previously reported, in several *Zanthoxylum* species, among other compounds (chelerythrin, norchelerythrine, arnortianamide, and methyl predicentin) and appear as promising antibacterial compounds (Stermitz et al., 1980; Kato et al., 1996; Facundo et al., 1999; Oliveira et al., 2002; Silva et al., 2008; Hohlemwerger et al., 2012; Luo et al., 2012; Hoa et al., 2015).

According to criteria described in the literature (Aligiannis et al., 2001; Holetz et al., 2002; Morales et al., 2008) the antibacterial activity of extracts is considered good when they display MIC less than 100 μg/ml, moderate from 100 to 500 μg/ml, and weak between 500 to 1000 μg/ml. Extracts with MIC values above 1000 μg/ml do not show antimicrobial effect (Aligiannis et al., 2001; Holetz et al., 2002; Morales et al., 2008). The MeOH extract from *Z. tingoassuiba* showed moderate activity against all the strains (MIC value between 120 and 480 μg/ml), while the DCM extract displayed a good activity against *S. aureus* ATCC 25923 and strain 1 (both MIC 60 μg/ml), being moderately effective against strains 2–4 (MIC ranging from 120 to 240 μg/ml). Although the antimicrobial activity of *Z. tingoassuiba* has been reported against standard strains of *S. aureus*, its promising activity against multiresistant strains is described for the first time in this study.

The antibacterial activity of dihydrochelerythrine **1** against *S. aureus* ATTC strain determined in the present work is in agreement with the literature data. Luo et al. (2012) found that benzophenanthridine alkaloids isolated from the methanol root extract of *Z. capense* were active against *S. aureus* ATCC 6538, with a MIC value < 143.11 μM for dihydrochelerythrine. However, they did not find activity against *S. aureus* ATCC 25923 below this concentration, while in the present study dihydrochelerythrine inhibited the growth of bacteria at 85.8 μM.

The anti-MRSA activity of compound **1** is related here for the first time. Dihydrocheleryhtrine displayed activity against the four tested clinical isolates (MIC ranging from 85.8 to 171.7 μM) and was more active than chloramphenicol against strain 4 and ATCC 25923.

Table 2
Minimum inhibitory concentration (MIC) of methanol (MEOH) and dichloromethane (DCM) extracts from *Zanthoxylum tingoassuiba.*

Staphylococcus aureus	Extract (μg/ml)		Compounds (μM)		Control (μM)
	MeOH	DCM	**1**	**2**	Chloramphenicol
ATCC 25923	120.0[a]	60.0[a]	85.8[b]	307.8[a]	>185.6b/>49.51[a]
Strain 1	240.0[a]	60.0[a]	171.7[b]	76.9[a]	>92.8b/>198.1[a]
Strain 2	480.0[a]	120.0[a]	171.7[b]	153.9[a]	>92.8b/>24.76[a]
Strain 3	240.0[a]	240.0[a]	171.7[b]	153.9[a]	>92.8b/>24.76[a]
Strain 4	480.0[a]	120.0[a]	85.8[b]	307.8[a]	>185.6b/>198.1[a]

[a] Value for microdilution method.
[b] Value for macrodilution method.

Even though the presence of *N*-methylcanadine **2** has been detected in *Zanthoxylum* species and other plants such as *Hypecoum erectum* (Su et al., 2011) or *Macleaya microcarpa* (Qing et al., 2015), a survey of the literature shows only two works related to its antibacterial activity (Su et al., 2011; Cheng et al., 2014). The MIC value obtained for *N*-methylcanadine in the present work against *S. aureus* ATCC strain (307.8 μM) is higher than the one reported by Cheng et al. (MIC 80.3 μM), but much lower than the one found by Su et al. (1.28 mM).

A wide number of works report the anti-MRSA activity of extracts and fractions obtained from plants, but in many cases, the individual substances did not have activity against the same strain. This work demonstrates that benzophenanthridine alkaloids dihydrochelerythrine and *N*-methylcanadine, along with other phytochemicals, are responsible for the antibacterial activity of some plants and that their presence might justify the ethnobotanical use of several species of *Zanthoxylum* genus for the treatment of various infectious diseases.

These two compounds are described for the first time as promising anti-MRSA and they appear as a potential lead for the design of new bioactive compounds for the treatment of MRSA infections responsible for serious public health issues.

Authors' contributions

RSC (MSc student) contributed in collecting plant sample, running the laboratory work, data analysis and drafted the paper. MOL (MSc student) and TFB contributed to biological studies. MLH contributed to data analysis and drafted the paper. ESV designed the study, supervised the laboratory work and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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