Biomedicine & Pharmacotherapy 83 (2016) 323–329

Available online at
ScienceDirect
www.sciencedirect.com

Elsevier Masson France
EM|consulte
www.em-consulte.com/en

Activity of pyrrolizidine alkaloids against biofilm formation and *Trichomonas vaginalis*

Themístocles da Silva Negreiros Neto[a], Dale Gardner[b], Fernando Hallwass[c], Ana Jéssica Matias Leite[a], Camila Guimarães de Almeida[d], Laura Nunes Silva[e], Alan de Araújo Roque[f], Fernanda Gobbi de Bitencourt[g], Euzébio Guimarães Barbosa[a], Tiana Tasca[g], Alexandre José Macedo[e], Mauro Vieira de Almeida[d], Raquel Brandt Giordani[a,*]

[a] *Departamento de Farmácia, Universidade Federal do Rio Grande do Norte, Gustavo Cordeiro de Faria, SN, CEP 59010-180, Natal, RN, Brazil*
[b] *USDA, ARS, Poisonous Plant Research Laboratory, 1150 East 1400 North, Logan, UT 84341, USA*
[c] *Departamento de Química Fundamental, Universidade Federal de Pernambuco, Avenida Professor Moraes Rego, 1235, CEP 50670-901, Recife, PE, Brazil*
[d] *Departamento de Química, Instituto de Ciências Exatas, Universidade Federal de Juiz de Fora, Campus Martelos, CEP 36036-330, Juiz de Fora, MG, Brazil*
[e] *Centro de Biotecnologia and Faculdade de Farmácia, UFRGS, Av. Ipiranga, 2752, 90610-000, Porto Alegre, RS, Brazil*
[f] *Herbário da UFRN, Universidade Federal do Rio Grande do Norte, Campus Universitário Lagoa Nova, CEP 59078-970, Natal, RN, Brazil*
[g] *Laboratório de Pesquisa em Parasitologia, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Av. Ipiranga, 2752, 90610-000, Porto Alegre, RS, Brazil*

ARTICLE INFO

Article history:
Received 23 April 2016
Received in revised form 10 June 2016
Accepted 20 June 2016

Keywords:
Usaramine
Monocrotaline
Pyrrolizidine alkaloids
Biological activity

ABSTRACT

Crotalaria genus belongs to the subfamily Papilionoideae comprising about 600 species spread throughout tropical, neotropical and subtropical regions. In this study, seeds of *Crolatalaria pallida* were used to the isolation of usaramine, a pyrrolizidine alkaloid. Thus, *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* were utilized as strains to test some activities of this alkaloid, such as antibiofilm and antibacterial. Meanwhile, monocrotaline obtained from *Crotalaria retusa* seeds, was used as the starting material for synthesis of necine base derivatives with anti-*Trichomonas vaginalis* potential. Alkaloids were characterized by 1D and 2D NMR techniques and GC–MS analysis. Usaramine demonstrated a highlighted antibiofilm activity against *S. epidermidis* by reducing more than 50% of biofilm formation without killing the bacteria, thus it could be assumed as a prototype for the development of new antibiofilm molecules for pharmaceutical and industrial purposes. Monocrotaline activity against *T. vaginalis* was evaluated and results indicated inhibition of 80% on parasite growth at 1 mg/mL, in addition, neither cytotoxicity against vaginal epithelial cells nor hemolytic activity were observed. On the other hand, retronecine showed no anti-*T. vaginalis* activity while azido-retronecine was more active than monocrotaline killing 85% of the parasites at 1 mg/mL. In conclusion, pyrrolizidine alkaloids are suggested as promising prototypes for new drugs especially for topical use.

1. Introduction

Crotalaria genus belongs to Fabaceae, subfamily Papilionoideae comprising about 600 species spread throughout tropical, neotropical and subtropical regions. In Brazil, 49 species have been found so far. *Crotalaria* spp. are an important source of pyrrolizidine alkaloids (PAs). The most common pyrrolizidine alkaloid chemical skeletons are formed by a necine base nucleus esterified with necic acids. It is known that PAs, with unsaturation at C1-C2 (dehydro-pyrrolizidines), are converted through hepatic metabolism to toxic pyrrole derivatives. Thus, other biological properties have been poorly studied because of the poisonous

* Corresponding author.
E-mail addresses: theminegreiros@yahoo.com.br (T. da Silva Negreiros Neto), dale.gardner@ars.usda.gov (D. Gardner), hallwass@ufpe.br (F. Hallwass), ana@analeite.org (A.J.M. Leite), camilagdealmeida@gmail.com (C.G. de Almeida), laransilva@gmail.com (L.N. Silva), alan.ufrn@gmail.com (A. de Araújo Roque), fe_gobbi@hotmail.com (F.G. de Bitencourt), euzebiobg@gmail.com (E.G. Barbosa), tiana.tasca@ufrgs.br (T. Tasca), alexandre.macedo@ufrgs.br (A.J. Macedo), mauro.almeida@ufjf.edu.br (M.V. de Almeida), raquebg@hotmail.com (R.B. Giordani).

http://dx.doi.org/10.1016/j.biopha.2016.06.033
0753-3322/

effects in animals and humans after ingestion of plants containing these compounds [1–3]. However, clinical studies with topical preparations of Comfrey (*Symphytum* sp.), a plant containing PAs, showed remarkable activity in treatment of ankle distortions [4], contusions and distortions of the knee joint [5], acute supraspinatus tendon syndrome [6], osteoarthritis of the knee [7], wound healing [8], myalgia [9], muscular symptom and functional locomotors disturbances [10]. No toxic effects were reported in these clinical trials. Therefore, PAs could be useful as therapeutic alternatives in different applications like topical use or surface coating of biomaterials with medical interest. Taking into account that the search for new compounds to impair infectious diseases are of current importance we selected two target assays in order to exploit the potential of PAs as leads for topical drugs: the bacterial biofilm and the parasite *Trichomonas vaginalis*.

Bacterial adhesion plays a pivotal role in the surface colonization by microorganisms, constituting the first step in the development of structured surface-associated communities of bacteria, called biofilms [11]. Biofilm growth and its persistence within wounds have been suggested as being contributing factors towards the delay or even prevention of the healing process [12]. As well, the biofilm formed under medical devices represents a health concern where natural products could contribute as biofunctionalized agents. As problems of antibiotic resistance increase, a continuing search for effective bioactive wound dressings that target bacterial virulence (such as the biofilm formation), rather than cell growth, represents an alternative approach to control biofilm-forming pathogens.

Trichomonas vaginalis is an unicellular eukaryotic protist that parasitizes the human genitourinary tract and causes trichomoniasis which is recognized as the most prevalent non-viral sexually transmitted disease (STD) [13]. The clinical presentation is mainly problematic in women, where it may be asymptomatic or cause severe vaginitis and cervicitis [14]. The complexity of trichomoniasis pathogenesis is illustrated by the interaction of the parasite with human cells, tissues, and the immune system [15]. Systemic metronidazole and other 5-nitroimidazoles have been the standard therapies for the treatment of trichomoniasis for more than 30 years [16]. Taking into account the increased prevalence of metronidazole-resistant infections, alternative drugs are necessary for the treatment of trichomoniasis.

This study addresses two important issues: (i) the potential activity of the pyrrolizidine alkaloid usaramine, isolated from *Crotalaria pallida,* against two important pathogens, *Pseudomonas aeruginosa* and *Staphylococcus epidermidis*; and (ii) the anti-*Trichomonas vaginalis* potential of monocrotaline and two semi-synthetic derivatives. Overall, our results point out that PAs are potential leads for developing drugs for topical use.

2. Methods and materials

2.1. General

The GC–MS data were acquired on a Shimadzu GC-2010 gas chromatograph coupled with a mass spectrometer with electron impact ionization source (Shimadzu, Kyoto, Japan). The ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectra were recorded on a Bruker Advance DRX spectrometer (Bruker, Billerica, MA, USA) or on a Agilent spectrometer operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C. All spectra were measured at 25 °C and samples were dissolved in $CDCl_3$ or CD_3OD. The chemical shifts are given on the δ (ppm) scale and were referenced to residual $CHCl_3$ (δ_H 7.24 and δ_C 77.23 ppm) and CHD_2OD (δ_H 3.30 and δ_C 49.00 ppm). All reagents used were analytical grade.

2.2. Plant material

Seeds of *Crotalaria pallida* and *Crotalaria retusa* were collected in Natal, Rio Grande do Norte, Brazil, in March, 2013. They were identified by Msc. Alan de Araújo Roque, Federal University of Rio Grande do Norte, and a voucher specimen was deposited at the Herbarium of the Federal University of Rio Grande do Norte, Brazil, under the reference number 16084 and 16083, respectively. The authorization for harvesting the plant material was conceded by SISBIO (number 32749-2) and the permission to access the Brazilian genetic patrimony was allowed by CNPq (number 010142/2012-6).

2.3. Extraction and isolation

The seeds of *Crotalaria pallida* (733 g) were dried in an air circulating oven at 45 °C, crushed and subjected to exhaustive maceration with 96% ethanol at room temperature. The crude extract was filtered, dried under reduced pressure and solubilized in 10% HCl. The acidic solution (pH 1) was extracted with $CHCl_3$ (300 mL) to provide extract A. Basifying the aqueous solution to pH 9 with concentrated NH_4OH and extracting with chloroform (300 mL), drying the chloroform extract over anhydrous sodium sulfate, filtering, and evaporation of the solvent under reduced pressure, gave extract B (0.538 g). Preparative thin layer chromatography (Si-gel; $CHCl_3/CH_3OH$ – 90:10, v/v) was used to isolate usaramine (33 mg = 0.0045%) from extract B. Usaramine was isolated as a brown amorphous solid; NMR data are presented in Table 1. EIMS *m/z* 351 $[M]^+$ (3), 320 (4), 248 (5), 220 (8), 178 (4), 138 (37), 136 (96), 121 (65), 120 (100), 119 (88), 109 (20), 108 (15), 106 (18), 95 (53), 94 (70), 93 (80), 80 (25), 53 (13).

The seeds of *Crotalaria retusa* (315 g) were extracted using identical procedure described to *Crotalaria pallida*. Extract B (crude alkaloid fraction) was submitted to recrystallization with methanol to give monocrotaline (3 g). Its structure was confirmed in comparison with literature data [17,18]. ^{1}H NMR ($CDCl_3$, 500 MHz): δ (ppm) 1.23 (3 H, d, J = 12 Hz), 1.36 (3H, s), 1.45 (3H, s), 2.10 (2H, m), 2.61 (1H, q, J = 14 Hz), 2.81 (1H, q, J = 12 Hz), 3.24 (1H, m), 3.49 (1H, dd, J = 17.5, 9.5 Hz), 3.92 (1H, dd, J = 24,3 Hz), 4.41 (1H, m),4.70 (1H, d, J = 20 Hz), 4.92 (1H, d, J = 20 Hz), 5.06 (1H, m), 6.05 (1H, s); ^{13}C NMR ($CDCl_3$, 125 MHz) δ (ppm) 13.6, 17.7, 21.9, 33.5, 44.4, 53.6, 60.6, 61.3, 75.1, 76.8, 76.9, 78.7, 132.7, 134.2, 173.5, 174.0.

2.4. Synthesis of monocrotaline derivatives

Retronecine **2**. Monocrotaline **1** (3 g, 9.2 mmol) and barium hydroxide octahydrate (10 mmol) were refluxed in 15 mL of water for 2 h. After cooling, carbon dioxide was added to saturate the solution and the barium carbonate precipitate was filtered. The filtrate was dried under reduced pressure and submitted to classic column chromatography (Si-gel) using dichloromethane:methanol (4:1, v/v) as mobile phase. Thus, 90% yield of brown oil was obtained. The ^{1}H and ^{13}C NMR analyses confirmed its structure. ^{1}H NMR (CD_3OD, 500 MHz): δ (ppm) 1.98 (2 H, m), 2.80 (1H, m), 3.26 (1H, m), 3.30 (1H, m), 3.45 (1H, dd, J = 2, 13 Hz), 3.88 (1H, d, J = 15 Hz), 4.21 (3H, m), 4.33 (1H, m), 5.70 (1H, s); ^{13}C NMR (CD_3OD, 125 MHz): δ (ppm) 36.8, 54.9, 59.8, 63.1, 71.9, 79.5, 125.5, 140.0.

Azido-retonecine **4**. Retronecine (0.2 g, 1.2 mmol) was solubilized in 5 mL of pyridine and then slowly added to tosyl chloride (0.3 g, 1.5 mmol) under cooling (ice bath) and kept stirred at laboratory temperature for 3 h. The solvent was removed under reduced pressure. The dry powder was submitted to classic column chromatography (Si-gel) using diclorometha ne:methanol (9:1, v/v) as mobile phase to yield tosyl-retronecine **3**. A mixture of tosyl-retronecine (0.3 g, 0.1 mmol) and sodium azide (0.50 g, 7.8 mmol) in 15 mL of water:ethanol (1:5, v/v) was refluxed for 3 h. The