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Antimicrobial, antiparasitic and antiproliferative effects of the extract of *Bacillus safensis* SG-32 isolated from a Brazilian oil reservoir

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Strains previously isolated from offshore oil reservoirs in Campos Basin (RJ, Brazil) and Potiguar Basin (RN, Brazil) were tested for production of antimicrobial compounds. Sixteen bacteria produced compounds with antimicrobial activity. The strain chosen for further study was *Bacillus safensis* SG-32 based on its ability to inhibit 76% of the strains tested. The bacterium was grown in Marine Broth (MB) and the hexane extract was investigated for minimal inhibitory concentration (MIC) (highest activity 0.4 mg/mL against *Staphylococcus aureus*), antiproliferative activity against human cancer cell lines (highest activity TGI = 69.83 µg/mL against melanoma cell lines), and antiparasitic tests (maximum activity 22.49% against *Trypanosoma cruzi*). Bioactive extract from *B. safensis* SG-32 was purified using silica column chromatography. Fractions collected were tested by bioautography in order to select the fraction with antimicrobial activity. The active molecule was identified by Nuclear Magnetic Resonance (NMR) as a diterpene of the *Cyathin* family. In addition to the antimicrobial and antitumor activity, *Cyathin* type compounds are known to stimulate the synthesis of Nerve Growth Factor (NGF), a capacity that implicates their potential as therapeutic agents to treat neurodegenerative ailments such as Alzheimer's or Parkinson's disease. In conclusion, this study showed that *B. safensis* SG-32 produces metabolites with antibacterial, antiproliferative and antiparasitic activities, and opens perspectives for further study of its potential also for nerve protection.

Key words: Antimicrobial, antiproliferative, *Bacillus safensis*, Cyathin, nerve grow factor, drug discovery.

INTRODUCTION

Cultivable microorganisms have been a source of natural compounds with significant biological activities (Bull et al., 2000). Products from secondary metabolism of microorganisms have been used in medicine, agriculture

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and industry such as antibiotics, anticarcinogenic drugs, antifungal and immunosuppressive agents, enzymes and polymers for industrial and technological applications, herbicides, insecticides, growth promoters, among others (Demain, 1999; Bull et al., 2000; Demain, 2000; Demain and Adrio, 2008; Demain and Sanchez, 2009).

However, most microorganisms have not been cultivated or even identified from many different environments, including oil fields. This suggests that the massive majority of microbial diversity remains unexplored in such hostile ecosystem, providing an invaluable source for new molecules (Kennedy et al., 2010).

There is a huge interest in new antimicrobial substances (AMS) since those in the market are becoming obsolete. The evolution of antibiotic resistance by major human pathogens has made the first antibiotics, and most of their successors, largely ineffective (Spellberg et al., 2004, 2013). For example, strains of carbapenem-resistant *Enterobacteriaceae*, penicillin-resistant *Staphylococcus aureus* and colistin-resistant *E.coli* have been detected in China and US, demonstrating the ineffectiveness of the respective compounds (Luepke et al., 2017).

Several antimicrobial substances from *Bacillus* have been described, including coagulin from *B. coagulans* (Hyronimus et al., 1998); bacthuricin F4 (Kamoun et al., 2005), thuricin 17 (Gray et al., 2006), entomocin (Cherif et al., 2003) and tochicin (Paik et al., 1997) from *B. thuringiensis;* and cerecin (Risøen et al., 2004) from *B. cereus*. Previous studies carried out by Korenblum et al. (2005a, 2005b, 2008) resulted in the isolation of three strains of *Bacillus* from Brazilian oil reservoirs, *B. subtilis* LFE-1, *B. firmus* H2O-1 and *B. licheniformis* T6-5, which were able to produce antimicrobial substances. These AMS inhibited the growth of other *Bacillus* strains, as well as the growth of sulfate-reducing bacteria consortia recovered from oil reservoirs.

Earlier research has shown the importance of microbial compounds in anticancer clinical trials (Hoskin and Ramamoorthy, 2008; Gaspar et al., 2013). Heterotrophic bacteria living in association with invertebrates have been identified, as sources of many bioactive and useful constituents (Simmons et al., 2005). In addition, there are many examples of anticancer substances isolated from bacteria, such as Curacin A (Rossi et al., 1997) and DMMC - Desmethoxymajusculamide C (Simmons et al., 2009), both isolated from cyanobacteria, Salinosporamide A (Feling et al., 2003) from *Salynospora* sp., and Thiocoraline (Romero et al., 1997) from *Micromonospora marina*. Those drugs are already in clinical trial status (Pérez and Fenical, 2017). The class of compounds of the Cyathane family have potent biological activities, with significant antitumor effects (Sennett et al., 1992; Peng et al., 2003, 2006), antimicrobial activities against Gram-negative and Gram-positive bacteria, yeasts and moulds (Allbutt et al., 1971; Shibata et al., 1998; Wachter et al., 1999), in addition to preventing neuronal death and promoting neurite outgrowth, which are essential to maintain and organize neuron functionality (Obara and Nakahata, 2002).

Moreover, AMS have been also tested as antiparasitic agents (Boulanger et al., 2002). Current drug for Chagas disease relies on antiparasitic treatment by either one of the only two registered drugs, benznidazole and nifurtimox (Bermudez et al., 2016). However, both benznidazole and nifurtimox cause severe side effects and, consequently, are not well-tolerated and are associated with poor patient compliance with treatment. Thus, as for other neglected diseases, new drugs with improved efficacy, tolerability, and safety are urgently needed (Coura and De Castro, 2002).

The search for new drugs is therefore of great importance to modern society. A promising alternative is to explore biodiversity in environments with unique characteristics, which contain microorganisms with particular physiological and metabolic properties that allow them to adapt and survive in inhospitable conditions. In this context, this work aimed to screen and characterizes AMS produced by bacteria isolated from Brazilian oil reservoirs. Numerous strains belonging to different bacterial genera were previously isolated from formation water and oil samples from production offshore reservoirs in Pampo Sul Field, Campos Basin (Da Cruz et al. 2010; Lopes-oliveira et al. 2012) and from onshore reservoirs in Potiguar Basin (Silva et al. 2013). The capabilities of novel isolates to produce AMS against other indigenous bacteria were evaluated.

MATERIALS AND METHODS

Antimicrobial activity assay

26 bacterial strains previously isolated from oil reservoirs from Potiguar Basin (Silva et al., 2013) and Campos Basin (Lopes-oliveira et al., 2012) were tested against each other as AMS producers (Table 1). The antimicrobial activity was tested using the agar diffusion method described by Anthony et al. (1972), a variation of the Burkholder test (Burkholder et al., 1966). Briefly, the pellet and supernatant of the fermented medium, obtained by centrifugation at 10,000 rpm for 10 min, were inoculated as spots (5 µL) on the surface of plates containing Luria-Bertani Agar (LB) medium. After incubation at 37°C for 48 h, bacterial cells were killed by exposure to chloroform vapor during 15 min. After evaporation of residual chloroform, the plates were covered with a layer of semi-solid medium (0.5% agar) inoculated with one of the indicator strains. Most of the indicator bacteria were *Bacillus* strains with different random amplified polymorphic DNA (RAPD) profiles (Lopes-oliveira et al. 2012). Inhibition halo formation around the spots indicated AMS production.

Cultivation of AMS-producing bacteria

B. safensis SG-32 was selected among the strains tested due to its ability to inhibit 20 strains (76%). The bacterium was grown in 8 liters of Marine Broth (MB) for 72 h at 37°C to produce biomass for

Table 1. List of bacterial strains tested against each other for antimicrobial activity.

Bacterial strain	Deposit number
Streptomyces alboniger AF43	CBMAI 1400
Marinobacter lutaoensis AF 20	CBMAI 1378
Citreicella thiooxidans AF 47	CBMAI 1377
Bacillus safensis SG 16	CBMAI 968
Micrococcus luteus P4-4	CBMAI 1366
Bacillus simplex SG 23-2	CBMAI 1323
Achromobacter xylosoxidans SG 47(1)	CBMAI 1326
Bacillus thuringiensis SG 15	CBMAI 1285
Bacillus safensis SG 61	CBMAI 986
Bacillus safensis SG 14	CBMAI 1284
Staphylococcus warneri SG 52	CBMAI 1320
Bacillus safensis SG 46	CBMAI 1295
Bacillus megaterium P2-2	CBMAI 1380
Halomonas shengliensis AF 19	CBMAI 1388
Bacillus thuringiensis SG 20	CBMAI 1287
Roseomonas cervicalis GMR9	CBMAI 1439
Georgenia muralis GMR 13	CBMAI 1441
Streptomyces albidoflavus GMR11	CBMAI 1695
Streptomyces sp. GMR12	CBMAI 1440
Pseudomonas stutzeri GMR14	CBMAI 1442
Bacillus sphaericus SG 24	CBMAI 1324
Brevundimonas diminuta GMR16	CBMAI 1444
Tessaracoccus bendigoensis GMR 15	CBMAI 1443
Bacillus safensis SG 32	CBMAI 973
Bacillus firmus P1-1	CBMAI 1316
Bacillus thurigiensis P2-1	CBMAI 1276

subsequent chemical analyses. Cells were collected by successive centrifugations at 10,000 RPM (Eppendorf 5410) and 4°C for 10 min, and stored at 5°C. The supernatant fraction (possible accumulation of antimicrobial substance) was separated for chemical analyses.

Soxhlet extraction

Cell biomass was extracted by a soxhlet extraction system. Three different solvents with different polarities were used: hexane, ethyl acetate and methanol. First, cells were extracted with 200 mL of hexane for 24 h. The crude hexane extract (EBH) was removed from the soxhlet system and saved in an Erlenmeyer flask. Cells were then submitted to extraction with 200 mL of ethyl acetate (EBA) for 24 h. This extract was kept separately. Finally, the bacterial cells were subjected to soxhlet extraction with 200 mL of methanol (EBM) for 24 h and extract was kept separately. The crude extracts were concentrated separately under vacuum in a rotary evaporator at 40°C until complete dryness. The extracts were weighed to calculate the yield of each extraction and distributed into volumetric flasks for further pharmacological tests.

Extraction of the culture medium, liquid-liquid partition

The extraction was performed on the supernatant of the microbial culture in a 2000 mL separating funnel (1000 mL of microbial broth + 500 mL of hexane). Extraction was performed three times with hexane and, subsequently with ethyl acetate. The extracts obtained from the extraction with hexane and ethyl acetate were concentrated separately under vacuum. The extracts were weighed for yield calculations, and samples of 10 mg were used for subsequent analysis.

Chromatographic purification of crude extract

Separations by column chromatography were performed using crude extracts obtained by soxhlet and liquid-liquid partition extraction. The chromatographic separations were performed in a glass column filled with silica gel 60 (Merck, 0.063 to 0.200 mm). A ratio of 1 g of sample to 30 g of silica was used for purification. The columns were eluted with 100 mL of solvent with an increasing polarity gradient, starting with 100% of hexane, followed by 90% hexane: 10% methanol and so forth until 100% of methanol was obtained. Fractions (50 mL) monitored by thin layer chromatography (TLC) were grouped accordingly. After solvent evaporation, fraction yield was determined and 10 mg samples were separated into microtubes for further analysis.

Analysis by thin layer chromatography (TLC)

TLC analyses were performed in chromatoplates of Silica gel 60 F254 (Merck 1.05554). Mixtures of dichloromethane/methanol (98:2) and hexane/ethyl acetate (50:50) were used as eluents. Irradiation with UV lamp at 254 nm and anisaldehyde reagent were used for compound detection [acetic acid solution: Sulfuric acid: anisaldehyde (48: 1: 1)], followed by further heating at 105 °C for 10 min.

Nuclear magnetic resonance (NMR)

The nuclear magnetic resonance (NMR) 1D (^{1}H, ^{13}C and Distortionless Enhancement by Polarization Transfer DEPT) and 2D (COSY, HSQC, HMQC) experiments were obtained with Inova 500 spectrometer operating at 500 MHz for ^{1}H and 125 MHz frequency for ^{13}C. The residual signal of the solvent or tetramethylsilane (TMS) was used as internal reference. Deuterated chloroform ($CDCl_3$, Isotec - INC) was used for sample dissolution to obtain spectra.

Bioautographic test

Assays for antimicrobial activity were performed using the bioautography method according to Slusarenko (Slusarenko et al., 1989), on TLC plates. After dissolution in hexane, 3 µL of the extracts at 10 mg/mL concentration were applied to plates in duplicate using a mixture of dichloromethane: methanol 98: 2 as eluent. One plate was exposed to anisaldehyde and the other submitted to microbiological tests. The standard inoculum of *B. subtilis* ATCC 6051 was grown until a final concentration of approximately 10^8 CFU/mL was obtained. The microbial suspension was inoculated in marine broth (MB) culture medium, using a dilution ratio of 1:1000 (v/v). An aliquot of 0.5 mL of triphenyl tetrazolium chloride (TTC) solution (1 mg/mL) was added to the inoculated medium, as respiratory activity indicator. Then, TLC plate containing the extract was placed on Petri dish and covered with agar medium containing the microbial indicator. The material was incubated at 37°C for 24 h. Bacterial growth was scored when cells with respiratory activity were stained red (Choma and Grzelak,

2011).

Pharmacological assays for determining in vitro antiproliferative activity

The following human tumor cell lines were used for *in vitro* tests: melanoma (UACC-62), ovarian cancer (OVCAR-03), resistant ovarian cancer (NCI-ADR / RES), breast cancer (MCF-7), lung cancer (NCI-460), kidney cancer (786-O), prostate cancer (PC-03), colon cancer (HT-29), glioblastoma (U251) and leukemia (K-562), provided by the NCI (National Cancer Institute), and a normal cell line as control (epithelial cell from green monkey kidney, VERO).

Stock cultures were grown in medium containing 5 mL RPMI 1640 medium (GIBCO BRL) supplemented with 5% fetal bovine serum. Cells in 96-well plates (100 µL cells/well) were exposed to different samples of bacterial extract concentrations in dimethyl sulfoxide (DMSO)/RPMI (0.25, 2.5, 25, and 250 g/mL) and incubated at 37°C with 5% of CO_2 for 48 h. Final DMSO concentration did not affect cell viability. Cells were fixed with 50% trichloroacetic acid and cell proliferation was determined by spectrophotometric quantification (540 nm) of cellular protein content using sulforhodamine B assay. Doxorubicin chloridrate (0.1 mg/mg; Europharma) was used as a positive control.

Three measurements were obtained: at time zero (T0, at the beginning of incubation) and 48 h post-incubation for compound free (C) and tested (T) cells. Cell proliferation was determined according to the equation 100 × [(T−T0)/C−T0], for T0<T ≤C, and 100 × [(T−T0)/T0], for T≤ T0. A concentration response curve for each cell line was plotted using software Origin 8.0 (OriginLab Corporation). From the concentration-response curve for each cell line, total growth inhibition (TGI or cytostatic effect) value was determined by non-linear regression analysis using the software Origin 8.0® (OriginLab Corporation) (Shoemaker 2006).

Antimicrobial activity by disc diffusion test and minimum inhibitory concentration (MIC)

The hexane extract of *B. safensis* SG-32 was solubilized in 5% DMSO solution. Petri dishes containing Muller Hington medium were seeded with 100 µL of the indicator strain at a concentration 10^8, by the spread-plate technique. Then 3 µL (1 mg) of extract sample was spot-shaped on the disc, followed by incubation at 35°C. The absence of growth of the indicator strain showed the antimicrobial activity.

MIC tests were carried out according to CLSI guidelines. The following strains were used: *Escherichia coli* ATCC 11775; *Micrococcus luteus* ATCC 4698; *S. aureus* ATCC 6538; *B. subtilis* ATCC 6051 and *C. albicans* ATCC 10231 The stock solution of the extract was diluted and transferred into the first well, and serial dilutions were performed so that concentrations in the range of 1.6 to 0.012 mg/mL were obtained. The inoculum was added to all wells and cell concentration was adjusted to approximately $5x10^5$ cells per mL, followed by incubation at 37ºC during 24 h (bacteria) or at 30ºC for 48 h (yeast). Antimicrobial activity was detected by adding 20 µL of 0.5% TTC aqueous solution. MIC was defined as the lowest concentration that inhibited visible growth, as indicated by the TTC staining (Eloff, 1998).

Anti-parasitic assay

Anti-parasitic assay was performed as described by Moraes et al. (2014), using *Trypanosoma cruzi* strain Y. For the single-concentration primary screening, samples were tested at 10 µg/mL; 100 µg/mL was the highest concentration in the confirmatory dose-response assay, with ten 2-fold-dilution points; 104 µg/mL was the highest concentration for the reference compound benznidazole. The Operetta high-content automated imaging system (Perkin Elmer) was used to acquire and analyze images. The analysis output was based on several parameters: host cell number, ratio of infected cells and number of parasites per infected cell. The ratio of infected cells to total number of cells was then calculated, and defined as the Infection Ratio (IR). The raw data for IR values were normalized to negative – DMSO (mock)-treated infected cells – and positive (non-infected cells) controls to determine the normalized antiparasitic activity, expressed as a percentage of activity in comparison to wells. Sample cytotoxicity effect was determined by the cell ratio (number of cells in the test well divided by the average number of cells in negative control wells).

RESULTS

Antimicrobial activity assay

Bacterial strains previously isolated from formation water and crude oil samples from reservoirs located in Potiguar (RN, Brazil) and Campos basins (Macaé, RJ, Brazil) (Lopes-Oliveira et al., 2012; Silva et al., 2013), were tested one against another as antimicrobial producers. Among the 26 bacteria strains recovered from oil samples, 16 inhibited growth of at least one of the strains tested. The antimicrobial activity and inhibition halo size of isolates are provided in Table 2. Seven strains of *Bacillus* species, including *B. firmus* P1-1, *B. thurigiensis* SG-20 and SG-15, and *B. safensis* SG-16, SG-14, SG-32 and SG-61, were the main antibiotic producers, inhibiting at least 11(42%) of the other bacteria. Moreover, these strains showed a broad antibacterial spectrum against Gram-negative and Gram-positive target bacteria.

B. safensis SG-32 inhibited 76% of the tested strains (Table 3). *B. safensis* SG-32 extracts were therefore tested against several microorganisms by both the disc-diffusion and microdilution assay. The results presented in Table 3 shows that the soxhlet hexane extract retained antibiotic activity against *M. luteus* and *E. coli,* whereas the other extracts did not present inhibitory activity.

The antimicrobial potency of SG-32 hexane crude extract was semi-quantitatively assessed by the presence or absence of inhibition zones (Figure 1) and quantitatively assessed by MIC values (Table 4). Maximal inhibition zones for the microorganisms sensitive to the extract of *B. safensis* SG-32 were in the range of 7 to 25 mm.

MIC values were in the range of 0.4 to 1.6 mg/mL. The highest antibacterial activity was against *S. aureus* ATCC 6538 with a MIC of 0.4 mg/mL, followed by the activity against *B. subtilis* ATCC 6051 with MIC of 0.8 mg/mL and of 1.6 mg/mL against *E. coli* ATCC 11775 and *M. luteus* ATCC 4698. No activity was observed against *C. albicans*.

In vitro antiproliferative tests

Tumor growth inhibition (TGI) values in different fractions

Table 2. Screening for antimicrobial activity of strains isolated from oil wells against each other.

	Indicator strains																			
Producer strain	***B. firmus* P1-1**	***B. thuringiensis* P2-1**	***B. megaterium* P2-2**	***H. shingelensis* AF 19**	***S. alboniger* AF 43**	***C. thiooxidans* AF 47**	***B. thuringiensis* SG 20**	***M. lutaoensis* AF 20**	***B. safensis* SG 16**	***B. safensis* SG 14**	***B. safensis* SG 46**	***S. warneri* SG 52**	***B. safensis* SG 32**	***B. thuringiensis* SG 15**	***B. safensis* SG 61**	***A. xylosoxidans* SG47(1)**	***M. luteus* P4-4**	***S. albidoflavus* GMR 11**	***R. cervicalis* GMR 9**	***B. simplex* SG 23-2**	***B. diminuta* GMR 16**
B. firmus P1-1	-	+++	-	-	-	-	+++	+++	+++	+++	+++	-	+++	-	+++	+++	+++	-	-	-	-
B. thuringiensis P2-1	-	-	-	-	-	-	-	-	-	-	-	-	++	-	-	-	-	-	-	-	-
B. thuringiensis SG20	-	-	-	+	+	+	-	+	+	+	-	-	++	+	+	+	+	-	-	-	-
M. lutaoensis AF20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
B. safensis SG16	-	+	-	+	++	-	-	+	-	+	++	+	++	+	+	-	+	+	-	-	-
B. safensis SG14	-	+	-	+	+	+	+	+	-	-	+	+	++	+	+	-	+	+	-	-	-
B. safensis SG46	-	+	-	+	-	-	+	+	-	+	-	-	-	-	-	-	+	+	-	-	-
S. warneri SG52	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-		-	-	-
B. safensis SG32	+++	++	+++	++	+	+	++	+	+	+	+	+++	-	+	+	+	++	+	+	+	++
B. thuringiensis SG15	+	+	-	-	+	+	-	+	-	+	+	+	+	+	-	+	+	+	-	-	-
B. safensis SG61	-	-	-	+	+	+	-	+	+	-	+	+	+	+	-	-	-	-	-	+	+
M. luteus P4-4	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
S. albidoflavus GMR11	+	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-
R. cervicalis GMR9	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-
B. simplex SG23-2	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
B. sphaericus SG24	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. stutzeri GMR14	-	-	-	-	-	-	-	-	-	++	-	-	-	-	+	-	-	-	-	-	-
B. diminuta GMR16	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-

*No zone of inhibition is represented by - ; + means zone of inhibition just above the spot; ++ means a clear zone of inhibition <3 mm; +++ means clear zones of inhibition >3 mm.

with anticancer activity were also assessed (Table 4). Values below 250 indicate anticancer activity and the closer to zero the higher the inhibitory capacity of the compound. Values above 250 indicated no activity and are represented only as > 250.

B. safensis extract presented antiproliferative activity against UACC62 (TGI = 69.83 µg/mL), NCI/ADR (TGI = 179.13 µg/mL), OVCAR (TGI = 249.86 µg/mL), HT29 (TGI = 168.10 µg/mL), 786-0 (179.93 µg/mL) and a cytotoxic activity against non-tumor cell VERO (TGI = 80.78 µg/mL). Other

Table 3. Antimicrobial activity of *B. safensis* extracts.

Sample	Initial volume/mass	Extract yield	Solvent	Extraction method	Antibiogram *Micrococcus/E. coli*	
B. safensis SG-32	8 L	1.087 g	Ethyl acetate	Partition liquid/liquid	-	-
	30 g of cell	128.5 mg	Hexane	Soxhlet	+	+
	30 g of cell	519 mg	Methanol	Soxhlet	-	-

Table 4. TGI values of the crude extracts tested against tumor cell lines. The fraction highlighted in yellow was the one that showed antimicrobial activity.

Extracts/cancer cell lines	TGI (µg/mL)[a]									
	UACC62	MCF7	NCI/ADR	NCI460	PCO_3	OVCAR	HT29	K562	VERO	786-0
[c]Ext. part. Hexane *B. safensis*	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250
Ext. part. Ethyl Acetate *B. safensis*	229.15	>250	212.83	>250	>250	>250	>250	>250	>250	>250
dExt. Sox. Hexane *B. safensis*	69.83	>250	179.13	>250	>250	249.86	168.10	>250	80.78	179.93
Ext. Sox. Ethyl Acetate *B. safensis*	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250
Ext. Sox. Methanol *B. safensis*	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250
Doxorubicin[b]	1.38	>250	20.12	0.43	2.44	24.72	23.98	>250	38.41	1.41

Cancer cell lines, UACC-62 (melanoma); MCF-7 (breast); NCI-DR/RES (ovarian-resistant); NCI, H460 (lung); PC-03 (prostate); OVCAR-3 (ovarian); HT-29 (colon); K562 (leukemia); VERO (normal epithelial renal cell line, green monkey); 786-0 (kidney). [a] TGI, Total growth inhibition – concentration that inhibited cell growth by 100%. The coefficients of variation obtained in these analyses were below 5%. [b] Positive control. [c] Ext. part., Extraction with partition liquid/liquid. [d] Ext. Sox, Extraction of the cells pellet on soxhlet.

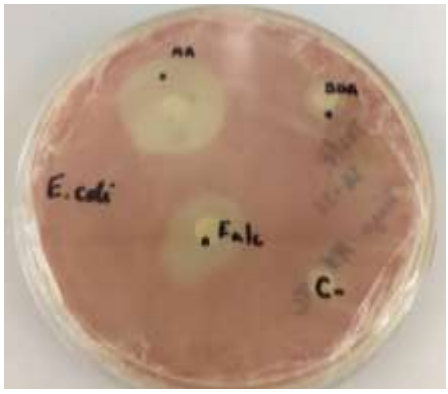

Figure 1. Inhibitory halo produced by 1 mg/mL extract of *Bacillus safensis* SG-32 (producer) against *E. coli* (red stained overlayer). MA, disc containing the *B. safensis* hexane-extract; BDA, disc containing *B. safensis* hexane extract produced in Potato Dextrose Agar (PDA); Falc, disc containing the *B. safensis* extract produced in small aliquot in falcon tube; C, negative control disc containing only the solvent.

extracts presented no activity or activity at a TGI higher than 210 µg/mL.

Antiparasitic assay

Antiparasitic assay was performed against *T. cruzi* Y strain. The extract samples were tested at the single concentration of 10 µg/mL, while the reference drug benznidazole was tested at 104 µg/mL (400 µM) as a positive control. Sample SG-32-HEX was considered sufficiently active for further testing in dose-response as it presented normalized antiparasitic activity superior to 50% and cell ratio greater than 0.5 (Figure 2).

Preliminary characterization of the antimicrobial compound

B. safensis SG-32 extract was fractionated and purified by silica column chromatography. The solvent which best separated the active compounds was hexane. Fractions with antimicrobial activity were identified by bioautography assay, resulting in one fraction with antimicrobial activity denominated fraction 3 (Figure 3). The fraction was then analyzed by nuclear magnetic resonance spectroscopy. More details about molecular characterization are in (Appendix A: Attachment 1- 8 supplementary materials).

Comparison of the experimental results based on NMR and literature data (Allbutt et al., 1971; Ayer et al., 1984;

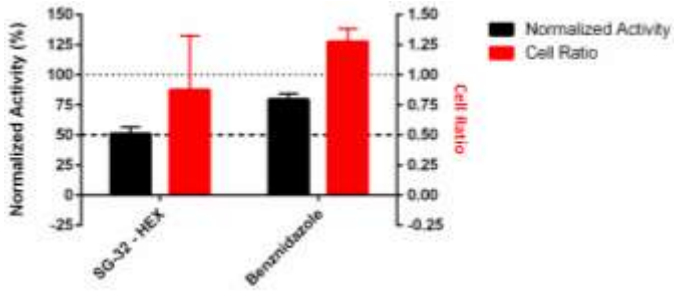

Figure 2. Samples activity profile against osteosarcoma-derived human cell line U2OS cells infected with *T. cruzi* amastigotes: normalized activity (in % - black columns -) and cell ratio (red columns). The samples tested concentration was 10 µg/mL. Error bar indicates standard deviation from 2 independent experiments.

Figure 3. Bioautography test against *B. subtilis* indicator strain (overlayer stained in red). Blue circle shows the inhibition spot of sample 3.

Nicoletti et al., 1996; Kenmoku et al., 2002; Kamo et al., 2004; Marcotullio et al., 2007; Urzúa et al., 2008; Dong et al., 2009; Enquist and Stoltz 2009; Kobayakawa and Nakada, 2014) suggested that the compound obtained in fraction 3 of the cultivation extract of *B. safensis* SG-32 is a diterpene with a "Cyathin"-like basic skeleton (Appendix A: Attachment 1- 8 supplementary materials).

DISCUSSION

In this study, we have focused on extremophiles isolated from oil environments as a potential source for novel antimicrobial compounds. The use of classical antimicrobials for application in pharmacological tests has been well documented, but the need for novel antimicrobial compounds appears particularly urgent as many pathogenic bacteria become resistant to the conventional antibiotics (Martinez et al., 2009; Davies and Davies, 2010). Antibiosis screening has long been used for screening potential drugs. It is an old fashion way to prospect new compounds but, as said by Lewis (2012), “we should reactivate the platform used by Waksman (the discoverer of streptomycin) for the detection of new antibiotics”. The strategy involves reworking with antibiosis, employed in the 1950s, for antibiotic screening.

The capacity of *Bacillus* species to produce substances with antimicrobial activity against a wide variety of microorganisms is well documented (Walker and Abraham, 1970; Nakano and Zuber, 1990; Leifert et al., 1995; Korenblum et al., 2005a). Of the strains tested, *B. safensis* SG-32 was selected for further characterization due to its ability to inhibit more than 70% of the indicator strains tested. Strain SG-32 was preliminarily identified as *B. pumilus* by 16S rRNA gene sequencing (Da Cruz et al., 2010), but recently, sequencing of DNA gyrase subunit B gene allowed the correct identification of isolate SG-32 as belonging to the *B. safensis* group (Lopes-oliveira et al., 2012).

The hexane extract using the soxhlet extraction method presented a broad range of activities against indicator bacterial strains. However, MIC assay did not present a powerful activity even though there was activity detected in the disc diffusion tests. The highest activity was against *S. aureus*, but not at a concentration considered practical for antibiotic use (Ling et al., 2015). One explanation could be the use of hexane (non-polar solvent) as extraction solvent, making it difficult to dissolve the active compound in aqueous culture medium for the MIC experiment.

According to Demain (1999), most of the important antitumor compounds used for chemotherapy are microbially-produced antibiotics (Oki, 1977; Tomasz, 1995). These include actinomycin D, mitomycin, bleomycins, and the anthracyclines; daunorubicin and doxorubicin (Zhang and Demain, 2005). A recent active molecule, taxol (= paclitaxel), was originally discovered in plants (Wall and Wani, 1995), but has also been found to be a microbial metabolite (Stierle et al., 1993).

Doxorubicin used in this study as a positive control showed a strong positive effect as antiproliferative drug against most of the cell lines tested. Nevertheless, doxorubicin is a pure substance and is therefore expected to present better activity in comparison with complex mixtures of substances, such as crude extracts. According to Fouche et al. (2008), extracts of natural products with anti-proliferative activity can be classified as inactive (TGI> 50 µg/mL), low activity (15 µg/mL <TGI< 50 µg/mL), moderate activity (6.25 µg/mL <TGI <15 µg/mL), and potent activity (TGI <6.25 µg/mL). The anti-proliferative activities of *B.safensis* SG-32 showed results above 50 µg/mL. The low activity of the crude extract might be explained by the fact that the substance responsible for the activity is present at a low

concentration in the crude extract. Comparison with pure compounds such as doxorubicin chemotherapy should be performed, when the entity in the crude extract has been isolated.

The antiparasitic test was performed against *T. cruzi*, the causative agent of Chagas disease (American trypanosomiasis), which is a chronic tropical infectious disease endemic in Latin America. The protozoan parasite *T. cruzi* occurs throughout the American continent and is transmitted by the triatomine bug insect vector, infecting a variety of mammals, including humans. Antiparasitic activity of SG-32 HEX was sufficiently promising in primary screening at single concentration; however more studies are necessary for further confirmation.

B. safensis possesses properties of biotechnological interest, for example, plant growth-promoting traits (Kothari et al., 2013), production of various industrially applicable enzymes (Tomova et al., 2013; Kumar et al., 2014) and secondary metabolites (Khaneja et al., 2010; Domingos et al., 2015). In addition, as its pathogenicity has never been evidenced, it may be considered a potential target for further biotechnological exploitation (Lateef et al., 2015).

The structural characteristics of the antimicrobial compound produced by *B. safensis* SG-32 was determined as a diterpene of the Cyathin-type. According to Enquist and Stoltz (2009), this class of diterpenes has been isolated from various organisms, such as fungi and sponges, but there are no reports so far of this class of compound being produced by bacteria. Diterpenoids, composed by four isoprene units, include the cyathane family of natural products (Dixon et al., 2011). Members of the cyathane family are related to each other by a characteristic 5-6-7 tricarbocyclic fused-core structure, derived from the cyclization of geranylgeranyl diphosphate (Enquist and Stoltz, 2009). Besides the antimicrobial and antitumor activities, this class of compounds has shown ability to stimulate the synthesis of Nerve Growth Factor (NGF), a capacity that implicates their potential as therapeutic agents to treat neurodegenerative ailments such as Alzheimer's or Parkinson's disease (Kawagishi et al., 1994; Kawagishi et al., 1996; Connor and Dragunow, 1998; Kita et al., 1998; Ma and Liu, 2005; Ma et al., 2010). These factors are important for the development, growth, and maintenance of the central and peripheral nervous systems. Studies have indicated that supplemental NGF consumption can offer cytoprotection and stimulate the outgrowth of neuritic projections (Wright et al., 1999). It is assumed that functional deficiency of NGF is related to Alzheimer's disease and plays a role in the etiology of the disease process (Allen and Dawbarn, 2006). NGF is expected to be used in the future for the treatment of Alzheimer's disease (Takei et al., 1997). Further tests must be carried out to check which kind of Cyathin is the main one for *B. safensis*.

More biotechnological studies in untapped environments such as oil reservoirs are desirable. *B. safensis* is a strain with the ability to produce metabolite(s) with biological potential. The production of Cyathin-like compound using bacteria, based on simpler handling and lower cost methods should be exploited. Further improvements to obtain yield increase of compound(s) need more research

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Appendix A

Supplementary materials

Attachment 1: Spectrum observed by ^{13}C NMR (125,6MHz) of the major component of fraction 3
Attachment 2: Fraction 3 of *B. safensis* SG-32, ^{1}H NMR spectrum (499.88 MHz) in $CDCl_3$ for major compound
Attachment 3: Fraction 3 of *B. safensis* SG-32, RMN^{13}C spectrum (125,69 MHz) obtained from DEPT pulse.
Attachment 4: Spectrum ^{1}H NMR (499.88 MHz) x ^{13}C (125.69 MHz) in $CDCl_3$ obtained in the HMBC pulse sequence for the major fraction of compound 3 of *B. safensis* extract.
Attachment 5: Spectrum ^{1}H NMR (499.88 MHz) x ^{13}C (125.69 MHz) in $CDCl_3$ obtained in the HSQC pulse sequence for the major fraction of compound 3 of *B. safensis* extract
Attachment 6: NMR Spectrum ^{1}H x ^{1}H (499.88 MHz) in CDCl3 obtained in the pulse sequence for the major fraction of compound 3.
Attachment 7: NMR^{1}H spectral data of the isolated compound and ^{13}C (11 Tesla, $CDCl_3$).
Attachment 8: The hypothetical skeleton of Cyathin compound.