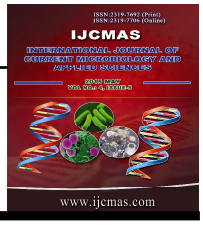
International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 4 Number 5 (2015) pp. 459-472**
http://www.ijcmas.com



Original Research Article

Screening of Antimicrobial Metabolite Yeast Isolates Derived Biome Ceará against Pathogenic Bacteria, Including MRSA: Antibacterial Activity and mode of Action Evaluated by Flow Cytometry

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Keywords

Staphylococcus aureus, MRSA, Antibacterial activity, Ceará

A B S T R A C T

Antimicrobials benefited the history of medicine by revolutionizing the treatment of infectious diseases. However, its consequent and continuous use has led to the emergence of microorganisms which once were considered safe, but have become feared as potentially lethal pathogens (Bowler et al., 2012). *Staphylococcus aureus* infections are highlighted for being a major cause of systemic infections, and for being the microorganism that has the highest morbidity and mortality rates in hospital infections (Smith et al., 2009). In addition, these strains have a remarkable ability to acquire antimicrobial resistance, with methicillin (MRSA) resistance being an increasing public health issue(Rodriguez-Noriega and Seas, 2010). In Brazil, in a study by Santos et al. (2010) in a tertiary hospital, a high incidence of MRSA colonization was reported, in which the acquisition rate was of 5.5/1.000 patient-days for adults. In another study performed in northeastern Brazil with hospitalized patients, Almeida et al., (2014) reported that among the colonized wounds by S. aureus, 32% were MRSA. Within this context, the emergence of strains with reduced sensitivity accompanied by a limited antimicrobial therapy causes the need of a search for new therapeutic options (Boucher et al., 2009). Antibacterial agents are the most important natural products, in which prokaryotes organisms and some fungi deserve special mention for playing an important role in the production of secondary metabolites with potential therapeutic applications (Singh and Macdonald, 2010). Throughout the world, approximately 99.000 fungi species were described (Kirk et al. 2008), of which approximately 3.7% of the world's diversity exist in Brazil, and the Brazilian Northeastern region has the largest number of records related to fungal diversity (Maia and Carvalho Junior 2010). In this study, isolate yeasts from the northeastern region of Brazil, more specifically from the state of Ceará, were analyzed. This area ecosystem is diverse and little investigated regarding the isolation of yeasts that produce metabolites with antimicrobial activity. Thus, this research aims to contribute to increase knowledge about the activity of extracts derived from isolated yeasts from Ceará with antimicrobial potential, as well as to contribute with a possible description of their mechanisms of action.

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In Brazil, in a study by Santos et al. (2010) in a tertiary hospital, a high incidence of MRSA colonization was reported, in which the acquisition rate was of 5.5/1.000 patient-days for adults. In another study performed in northeastern Brazil with hospitalized patients, Almeida et al. (2014) reported that among the colonized wounds by *S. aureus*, 32% were MRSA. Within this context, the emergence of strains with reduced sensitivity accompanied by a limited antimicrobial therapy causes the need of a search for new therapeutic options (Boucher et al., 2009).

Antibacterial agents are the most important natural products, in which prokaryotes organisms and some fungi deserve special mention for playing an important role in the production of secondary metabolites with potential therapeutic applications (Singh and Macdonald, 2010). Throughout the world, approximately 99.000 fungi species were described (Kirk et al. 2008), of which approximately 3.7% of the world's diversity exist in Brazil, and the Brazilian Northeastern region has the largest number of records related to fungal diversity (Maia and Carvalho Junior 2010).

In this study, isolate yeasts from the northeastern region of Brazil, more specifically from the state of Ceará, were analyzed. This area ecosystem is diverse and little investigated regarding the isolation of yeasts that produce metabolites with antimicrobial activity. Thus, this research aims to contribute to increase knowledge about the activity of extracts derived from isolated yeasts from Ceará with antimicrobial potential, as well as to contribute with a possible description of their mechanisms of action.

Material and Methods

Material collection/Plating of samples /Purification of microorganisms

Samples were collected in some regions of the state of Ceará (Figure 1). Samples were diluted in milli-Q water at a ratio of 1:5 (m/v). After this step, 1mL of the homogenate was plated on potato dextrose agar plates (BDA). After plating the samples, plates were stored in an incubator at 35 °C for 7 days. Yeast selection was performed by differences in morphology aspects of each colony, which were observed by naked eye. Strains purification was done on Sabouraud agar plates plus Chloramphenicol (5%) by streaking technique, until strains were completely pure (Wagner-Dobler et al., 2003).

Molecular identification

Genomic DNA was purified from the yeast strains using a cetyltrimethylammonium bromide (CTAB)-based protocol as previously described (Warner, 1996). The

nuclear DNA region comprising the internal transcribed spacers (ITS1 and ITS2) andthe5.8SrRNA gene was amplified by polymerase chain reaction (PCR) using the primers ITS4 (5□-TCCTCCGCTTATTGA TATGC-3□) and ITS5 (5□-GCAAGTAA AAGTCGTAACAAGA-3□), as suggested by White et al (1996).Once the specificity of the amplifications was confirmed, the PCR products were purified from there maining reactions using the GFX PCR DNA and Gel Band Purification kit (GE Healthcare Bio-Sciences, Piscataway, NJ, USA). The concentrations of the purified PCR products were determined by measuring the absorbance of a ten-fold dilution at 260 nm. DNA sequencing was performed at Macrogen Inc. (Seoul, South Korea) using Sanger□s dideoxy chain termination method. The determined sequences were compared to those previously deposited in the GenBank data base using the BLAST program (Altschul et al., 1990).

Cultivation and chemical extraction of yeast cultures

To obtain the extracts, purified yeast strains were inoculated into 250mLErlenmeyer flasks containing 50mL of medium (0.4% glucose, 1% malt extract, 0.4% yeast extract). The cultures were incubated at 35 °C for 48h (Ding et al., 2008). Isolated fungi were inoculated and grown in different nutrient media. 5 mL aliquots of the 48 hour cultures were placed on two 1000 mL Erlenmeyer flasks containing 400mL of two different culture media: (I) (1% glucose, 0.25% yeast extract, 1% peptone); (II) (1% glucose, 1% yeast extract, 0.25% peptone). Cultures were maintained at 28 °C for 14 days (Xiong et al., 2009). After growth in liquid medium, each yeast extracts were extracted with ethyl acetate (EtOAc) and concentrated on a rotary evaporator. The dry mass was resuspended in DMSO (0.1%), where they got ready for antimicrobial activity screening tests (Xiong et al., 2009).

Microbial strains

The standard control strains used in these experiments were *Escherichia coli* (ATCC® 8739™), *Pseudomonas aeruginosa* (ATCC® 9027 ™), *S. aureus* (ATCC® 6538 ™), *Bacillus subtilis* (ATCC® 6633 ™) and MRSA (ATCC® 65398 ™). In this study, 11 MRSA strains that were biochemically identified and that belonged to the Yeasts Bioprospecting and Experimentation Laboratory (LABEL) of the Federal University of Ceará were used.

Antimicrobial activity assessment (Agar Diffusion Method)

Tests were performed according to the M02-A11 (CLSI, 2012) document, with modifications. Wells with 6mm diameter were made in the agar overlay of the Petri dish, as described by Shen et al. (2014). In these wells, a volume of 20 µl (30 mg/mL) of the obtained extracts was applied. The plates were incubated for 20 hours at 35 °C. The solvents and diluents used in the extracts dissolution were used as negative control. The antibiotic disks used in antimicrobial sensitivity tests were Oxacillin 1µg (OXA), Cefepime 30µg (CPM), Amikacin 30µg (AMI), Cefoxitin 30µg (CFO) and Clindamycin 2µg (CLI) (Microbiological Specialized Diagnostics - DME, BRA) (CLSI, 2013).

Determination of Minimum Inhibitory Concentration (MIC)

MIC determination was performed according to the M07-A9 (CLSI, 2012) document. The extracts were evaluated in the concentration range of 1024-0.5 µg/mL. Vancomycin (Sigma-Aldrich Co., USA) was used as positive control in the range of 0.25-

64 μg/mL and DMSO was used as negative control in the range of 0039-10% v/v. The plates were incubated at 35 °C for 20 hours. Inhibition of microbial growth was determined visually (CLSI, 2013).

Exposure of MRSA to extract

To determine membrane integrity, DNA fragmentation and phosphatidylserine exposure, a single strain of MRSA (MRSA 1) was used. Cell suspensions were prepared from cultures in the exponential growth phase. The cells were harvested by centrifugation (1600 g for 10 min at 4°C), washed twice with 0.85% saline solution (1200 g for 5 min at 4°C) and then resuspended (~10^6 cells/mL) in HEPES buffer (pH 7.2) supplemented with 2% glucose. A bacteria suspension was incubated at 35 °C for 20 hours with the active extracts, at a concentration of 2x MIC (Silva et al., 2011; Shi et al., 2007)

Determination of membrane integrity

The analysis of membrane integrity of the bacterial strains were performed essentially as described by Silva et al. (2011) and Shi et al., (2007).A total of 10.000 events per experiment (n = 2) were assessed, with cellular debris omitted from the analysis. Cell fluorescence was then determined by flow cytometry on a Guava EasyCyte™ Mini System cytometer (Guava Technologies, Inc., Industrial Blvd. Hayward, CA, USA) and analyzed by the 4.1 CytoSoft software.

Analysis of DNA fragmentation

For analysis of DNA fragmentation, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assays were performed essentially as described by Dwyer et al. (2012). Two hundred cells were counted per sample to determine the percentage of positive cells.

Analysis of phosphatidylserine exposure

The analysis of annexin v staining was performed essentially as described by Dwyer et al. (2012). For each experiment (n = 3) 10,000 events were analyzed and cell debris were omitted from the analysis.

Leukocytes isolation and cultures

The leukocytes isolation and culture were performed essentially as described by da Silva et al. (2014). After 24 h, cells were treated with the test compounds.

Extract yeast toxicity to leukocytes

The cytotoxicity of tested compounds to leukocytes was evaluated by the MTT (3-(4,5 Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) (Sigma-Aldrich Co., USA) assay. Compounds were dissolved in 1% DMSO (Sigma Chemical) at 0.19 – 100 μg/mL and resulting solutions were added to wells. After 72 h of exposure, the supernatant was replaced by fresh medium containing MTT (0.5 mg/mL). After 3 h, the MTT formazan product was dissolved into DMSO and the absorbance was measured at 595 nm (Beckman Coulter® DTX-880 spectrometer) (da Silva et al., 2014)

Results and Discussion

Samples Isolation: Using the described selective media and culture conditions, eleven yeast strains of several samples (soil, flowers, leaves, fruits, decaying wood and water) of the Ceará biome were isolated, as seen in table 1.

Yeast growth in different nutritional conditions: Two different culture media were tested in order to assess their previous effects on the growth and production of bioactive compounds. By analyzing table 2, most of the yeast grew similarly in nutritional conditions.

Extracts antimicrobial activities: A total of 22 organic extracts were tested on their antimicrobial activity using the agar diffusion method. According to Table 3, it was found that 59% (13) of extracts appear to have *in vitro* activity to strains of Gram positive bacteria. However, seven of them seem to have a larger spectrum of action, as they were able to inhibit the growth of gram negative bacteria. These showed inhibition zones of above 30 mm compared to the negative control (0.1% DMSO). Because of this, these extracts were selected for tests against four strains of MRSA. The results are detailed in Table 3, where it was observed that all the tested extracts provided a growth inhibition of resistant strains.

MIC determination of the most active extracts against MRSA strains: The extracts that showed inhibitory activity against MRSA strains had their MIC determined. Table 4 shows a potential antimicrobial activity of seven extracts against MRSA clinical isolates, with MICs ranging between 34-512 µg/mL. The results have shown to be promising. Therefore, it was decided to investigate the possible mechanism of action of these extracts on MRSA strains using flow cytometric techniques.

Damage to the MRSA plasma membrane after exposure to the extracts: Figure 2 shows that in the presence of the propidium iodide (PI) marker, part of the cells became PI-positive when treated with extracts after 20 h of exposure when compared to the control group ($p < 0.05$), demonstrating that the extracts cause damage to the cell membrane of MRSA strains. Treatments with HVNJ-27 (I) andHVNJ-27 (II) extracts resulted in higher percentages of cells stained with PI.

DNA fragmentation induced by the extracts in MRSA strains detected by TUNEL: Figure 3 shows that after exposure of extracts, a significant increase ($p < 0.05$) was observed in the percentage of TUNEL positive cells compared to the control group. In this experiment, 62.0 ± 2.4% and 62.0 ± 4.3% of MRSA cells treated with HVNJ-130 (I) and HVNJ-27 (I) extracts were TUNEL-positive, and these extracts were the ones that caused more damage to DNA.

Phosphatidylserine externalization in MRSA: It can be seen in figure 4 that only 11.5 ± 3.6% of cells that have not received treatment with the extracts (control group) showed positive staining for annexin V. However, when the cells were treated with the extracts, a statistically significant increase ($p < 0.05$) happened in the number of cells with positive staining for annexin V, comparing with the control group. HVNJ-130 (I) and HVNJ-27 (II) extracts were those with a higher percentage of annexin-positive cells, with values of 57 ± 11.4% and 56 ± 6.2%, respectively.

Cytotoxic activities: The cytotoxic activity of the most active extracts was assessed against human leukocytes (Table 5). It is Interesting to highlight that approximately 67% (5) of the obtained extracts showed low cytotoxicity, with CI 95% values ranging between 84.9 and 95.3 µg/mL, respectively, while two extracts were considered inactive.

Considering the results in Table 1, most of the yeasts described in this study are cosmopolitan, where a higher number of yeasts, about 54.5%, were isolated through local vegetation. According to Mautone

(2008), the external surface of the leaf (phylloplane) is an important substrate for yeast development.

Table.1 Yeast strains isolated from environmental samples in the state of Ceará

Isolate (accession #)	Source	Latitude	Longitude	Strains	GenBank (NumberAcession)
HVNJ-27	Leaves	-3,82329	-38,6465	*Candida tropicalis*	KJ740185
HVNJ-70	Trunk	-4,29845	-38,9087	*Candida tropicalis*	KJ740182
HVNJ-72	Leaves	-4,28922	-38,9283	*Candida tropicalis*	KJ740181
HVNJ-82	Trunk	-4,2413	-38,9328	*Candida tropicalis*	KJ740180
HVNJ-95 B	Leaves	-4,20817	-38,9672	*Exophialadermatitidis*	KJ740171
HVNJ-115 A	Leaves	-4,20848	-38,9673	*Candidaparapsilosis*	KJ740190
HVNJ-115 B	Trunk	-4,22508	-38,9263	*Candidaparapsilosis*	KJ740189
HVNJ-120	Stone	-4,21568	-38,9616	*Candida albicans*	KJ740178
HVNJ-130	Leaves	-4,24134	-38,9328	*Trichosporonasahii*	KJ740172
HVNJ-138	Soil	-4,21238	-38,9454	*Exophialadermatitidis*	KJ740170
HVNJ-189	Leaves	-4,24134	-38,9328	*Trichosporonasahii*	KJ740173

Table.2 Yeast grown in culture medium with different components for 14 days. G, glucose; Y, yeast extract; P, peptone. The growth was estimated visually (+ + + best growth; + + average growth; + weak growth). This code is also used in Tables 3–6 and Figures 1–4

Code	Carbounsource (10g/L)	Nitrogen Source (g/L)	Growth	Color of broth
HVNJ-27 (I) [a]	Glucose	10Y+0.25P	++	Blue
HVNJ-27 (II) [b]	Glucose	0.25Y+10P	++	Yellow
HVNJ-70 (I)	Glucose	10Y+0.25P	++	Yellow
HVNJ-70 (II)	Glucose	0.25Y+10P	++	Yellow
HVNJ-72 (I)	Glucose	10Y+0.25P	++	Yellow
HVNJ-72 (II)	Glucose	0.25Y+10P	++	Yellow
HVNJ-82 (I)	Glucose	10Y+0.25P	++	Yellow
HVNJ-82 (II)	Glucose	0.25Y+10P	++	Yellow
HVNJ-95B (I)	Glucose	10Y+0.25P	++	Yellow
HVNJ-95B (II)	Glucose	0.25Y+10P	++	Yellow
HVNJ-115A (I)	Glucose	10Y+0.25P	+++	Yellow
HVNJ-115A (II)	Glucose	0.25Y+10P	++	Yellow
HVNJ-115B (I)	Glucose	10Y+0.25P	+++	Yellow
HVNJ-115B (II)	Glucose	0.25Y+10P	++	Yellow
HVNJ-120 (I)	Glucose	10Y+0.25P	++	Yellow
HVNJ-120 (II)	Glucose	0.25Y+10P	++	Yellow
HVNJ-130 (I)	Glucose	10Y+0.25P	++	Yellow
HVNJ-130 (II)	Glucose	0.25Y+10P	++	Yellow
HVNJ-138 (I)	Glucose	10Y+0.25P	++	Yellow
HVNJ-138 (II)	Glucose	0.25Y+10P	+	Yellow
HVNJ-189 (I)	Glucose	10Y+0.25P	++	Yellow

HVNJ-189 (II)	Glucose	0.25Y+10P	+	Yellow

Table 3. Evaluation of antimicrobial activity of extracts derived from yeasts isolated in Ceará,using the agar diffusion method

Code	*B.subitilis* ATCC® 6633 ™ (Halo-mm)	*S.aureus* ATCC® 6538 ™ (Halo-mm)	*E.coli* ATCC® 8739™ (Halo-mm)	*P.aeruginosa* ATCC® 9027 ™ (Halo-mm)	MRSA ATCC® 65398 ™ (Halo-mm)	MRSA 1[a] (Halo-mm)	MRSA 2 (Halo-mm)	MRSA 3 (Halo-mm)	MRSA 4 (Halo-mm)
NC [b]	NE [c]	NE	NE	NE	NE	NE	NE	NE	NE
HVNJ-27 (I)	37	39	39	37	33	37	12	13	36
HVNJ-27 (II)	37	39	37	36	35	34.5	22.5	13.5	34
HVNJ-38 (I)	17	8.3	NE	NE	NE	NE	NE	NE	NE
HVNJ-38 (II)	13	7.3	NE	NE	NE	NE	NE	NE	NE
HVNJ-70 (I)	16.7	18	NE	NE	11	NE	19	NE	18
HVNJ-70 (II)	16	12	NE	NE	9	NE	9.0	NE	10
HVNJ-72 (I)	15.7	9.0	NE	NE	6	NE	NE	NE	NE
HVNJ-72 (II)	9.7	6.7	NE	NE	9	NE	NE	NE	NE
HVNJ-82 (I)	NE	NE	NE	NE	NE	NE	NE	NE	NE
HVNJ-82 (II)	NE	NE	NE	NE	NE	NE	NE	NE	NE
HVNJ-115 A (I)	NE	NE	NE	NE	NE	NE	NE	NE	NE
HVNJ-115 A (II)	NE	NE	NE	NE	NE	NE	NE	NE	NE
HVNJ-115 B (I)	NE	NE	NE	NE	NE	NE	NE	NE	NE
HVNJ-115 B (II)	NE	NE	NE	NE	NE	NE	NE	NE	NE
HVNJ-130 (I)	36.0	37.3	34.7	33.3	18	35	11	14.5	35
HVNJ-130 (II)	35.0	35.3	38.0	37.0	18	36	11.5	15	34.5
HVNJ-138 (I)	37.3	34.7	35.3	37.7	33	36	12.5	13	32.5
HVNJ-138 (II)	NE	NE	NE	NE	NE	NE	NE	NE	NE
HVNJ-189 (I)	36.7	38.3	37.7	35.7	33	37.5	12.5	14	35.5
HVNJ-189 (II)	35.7	34.7	40.7	35.3	34	37	12.5	15	35
OXA	21	26.5	NE	NE	NE	NE	NE	NE	NE
CPM	33	26	30	30	14	26	NE	NE	14
AMI	32	23	21	24	NE	29	24	24	22
CFO	25	34	27	27	NE	14	12	NE	14
CLI	30	24	NE	NE	8.0	NE	NE	NE	NE

[a]MRSA strains isolated from biological samples.[b] **NC –** Negative control was treated with the vehicle (DMSO, 0.1%) used for diluting the test substances; **OXA–** Oxacillin, **CPM–**Cefepime, **AMI–** Amikacin, **CFO–** Cefoxitin, **CLI–** Clindamycin.[c]**NE**: No Effect. The agar diffusion method was performed according to CLSI protocol M02-A11 and break points were based on CLSI protocol M100-S23. All extracts **HVNJ** had fixed concentrations of 30 mg/ml. The halo (mm) represent the geometric means of at least three halo determined on different days.

Table 4.Minimal inhibitory concentration of extracts derived from yeasts isolated in Ceará against strains of MRSA

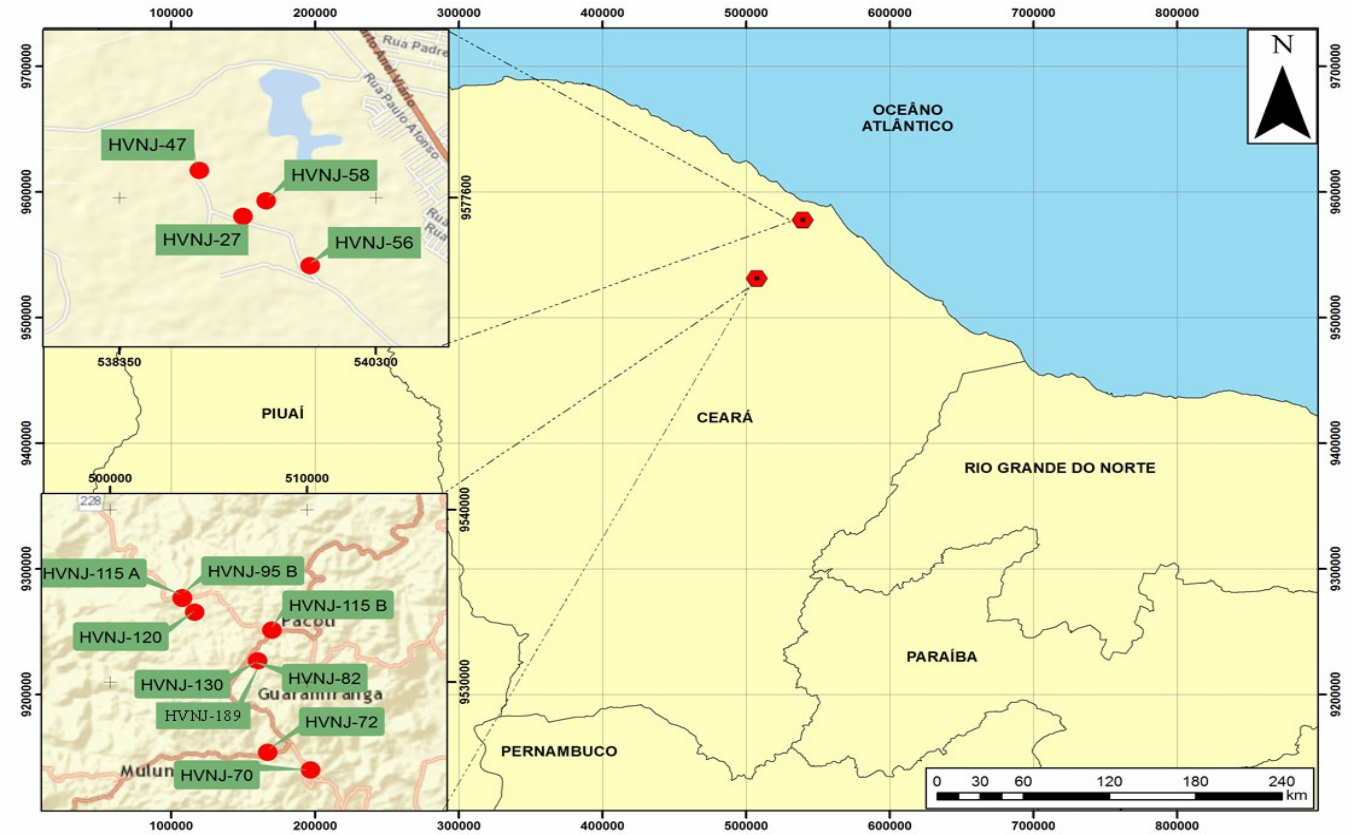
Strains[a]				**MIC**				
				Standard MIC				
	VAN [b] **(µg/mL)**	**HVNJ-27(I)**[b] **(µg/mL)**	**HVNJ-27 (II)** [b]**(µg/mL)**	**HVNJ-130 (I)** [b]**(µg/mL)**	**HVNJ-130 (II)** [b]**(µg/mL)**	**HVNJ-138 (I)** [b]**(µg/mL)**	**HVNJ-189 (I)**[b] **(µg/mL)**	**HVNJ-189 (II)** [b]**(µg/mL)**
MRSA ATCC® 65398 ™	2	128	512	512	256	256	128	512
MRSA 1*	4	136	136	132	132	72	68	34
MRSA 2	4	256	128	96	96	192	192	64
MRSA 3	4	128	128	64	96	192	96	48
MRSA 4	4	72	72	40	32	72	40	36
MRSA 5	2	128	512	512	256	256	128	512
MRSA 6	2	16	512	512	32	128	16	512
MRSA 7	2	64	256	512	32	64	16	512
MRSA 8	2	32	512	512	32	64	64	512
MRSA 9	2	64	256	512	32	128	16	512
MRSA 10	2	64	256	512	32	16	16	512
MRSA11	2	64	256	512	128	128	16	512

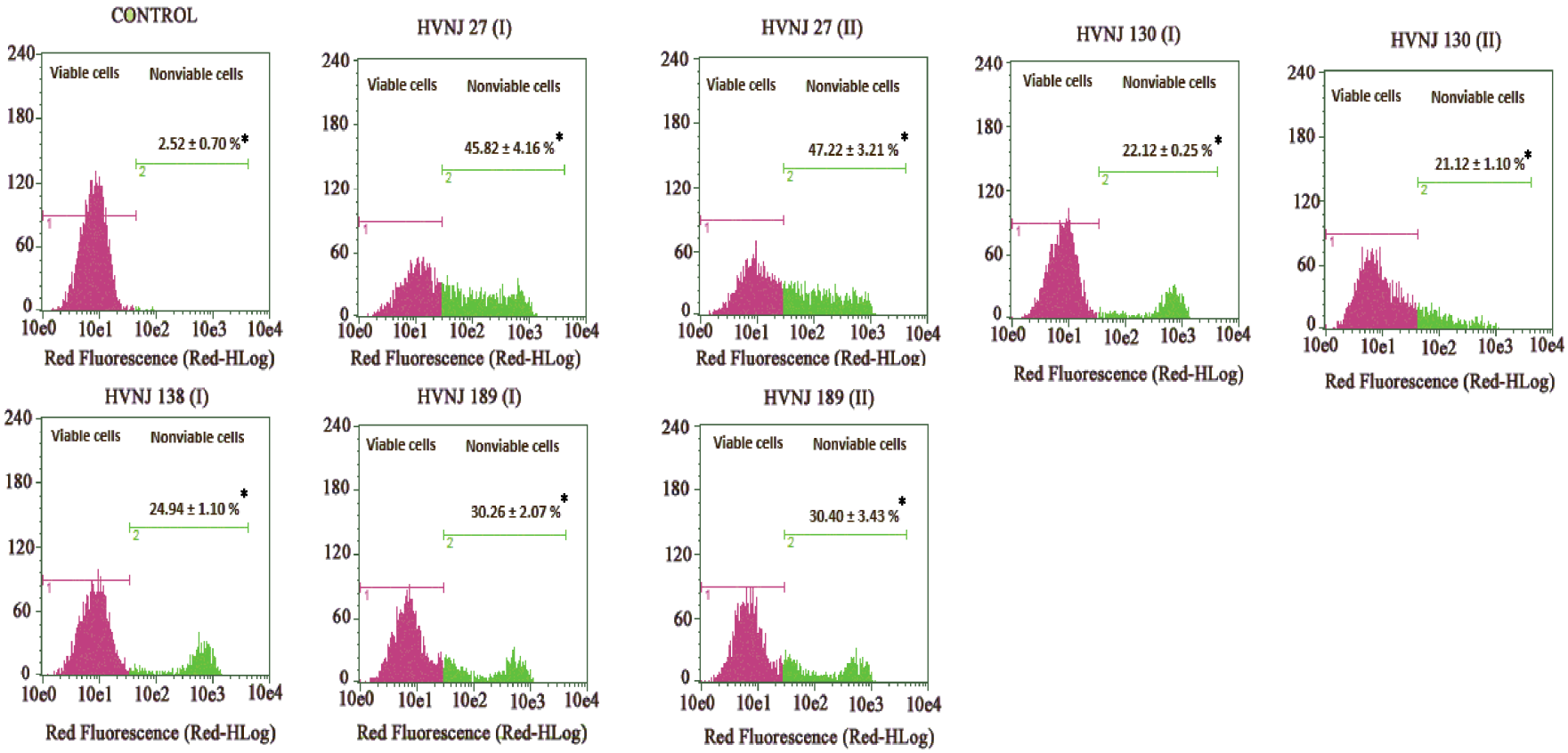
[a]MRSA strains.[b]**VAN –** Vancomycin; Extracts **HVNJ**. The MIC was defined as the lowest concentration that produced 80% reduction in the growth of bacterial cells after 20h of incubation. The microdilution in broth was performed according to CLSI protocol M07-A9. The**VAN**concentrations ranged from 0.125 to 64 µg/mL and the extracts concentrations varied from 0.5-512 µg/mL. The MICs represent the geometric means of at least three MICs determined on different days[c].*The strains were used for determination of action mechanism.

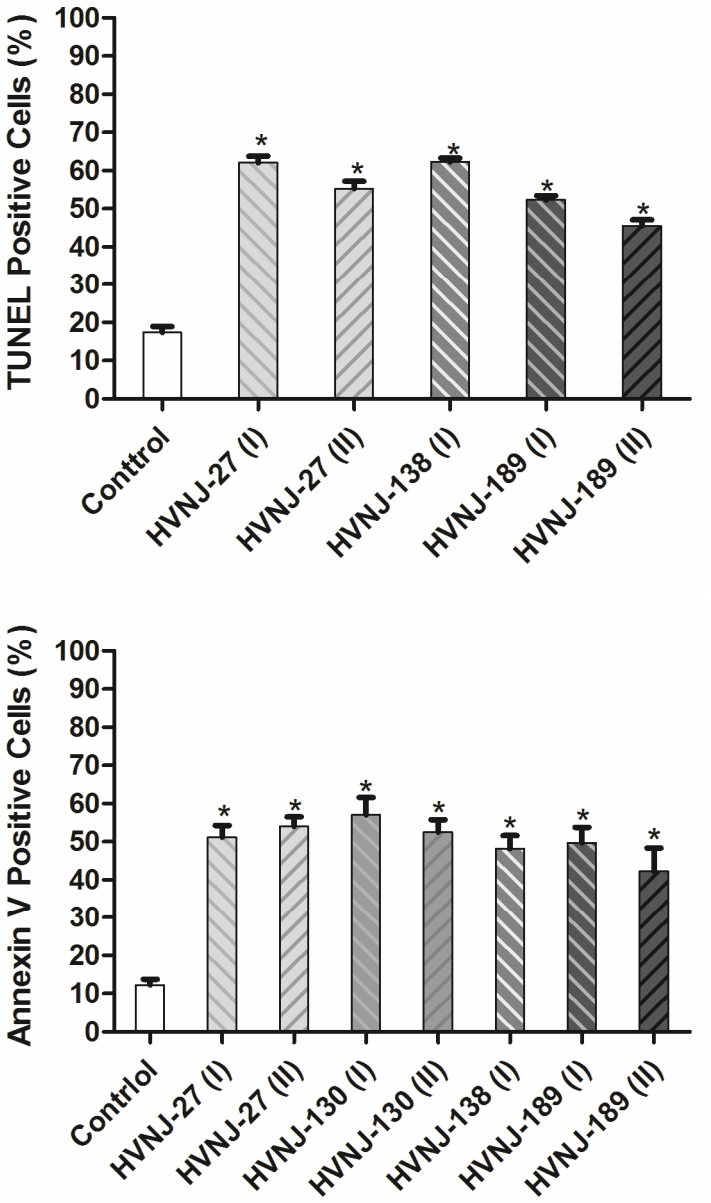
Table.5 Cytotoxic activity of secondary metabolites derived from yeasts on Leukocytes. Data are presented as IC50 values and 95% confidence interval (CI 95%) fromthree independent experiments, performed in triplicate

CI 95% (µg/mL)	
Compounds	**Leukocytes IC_{50}**
NC [a]	NE [b]
HVNJ-27 (I)	95.73 ± 0.10
HVNJ-27 (II)	86.49 ± 4.15
HVNJ-130 (I)	84.90 ± 4.07
HVNJ-130 (II)	92.59 ± 1.10
HVNJ-138 (I)	>100
HVNJ-189 (I)	91.36 ± 5.10
HVNJ-189 (II)	>100

[a]Negative control was treated with the vehicle (DMSO, 0.1%) used for diluting the test substances.
[b] NE: No Effect.




CONTROL
Viable cells
Nonviable cells
2.52 ± 0.70 %*
HVNJ 27 (I)
45.82 ± 4.16 %*
HVNJ 27 (II)
47.22 ± 3.21 %*
HVNJ 130 (I)
22.12 ± 0.25 %*
HVNJ 130 (II)
21.12 ± 1.10 %*
HVNJ 138 (I)
24.94 ± 1.10 %*
HVNJ 189 (I)
30.26 ± 2.07 %*
HVNJ 189 (II)
30.40 ± 3.43 %*
240
180
120
60
0
10e0 10e1 10e2 10e3 10e4
Red Fluorescence (Red-HLog)



The yeast ability of colonizing different plants contributes to the diversity of species (Mautone, 2008; Ding et al., 2008). Within this context (Table 1), it was possible to evaluate diversity among yeast species. However, a prevalence of *Candida* genus was observed. Several publications, including state, regional and general lists, point to the convergence of *Candida* genus species with Ceará native plants of (Embrapa Genetic Resources and Biotechnology. 2010; Ceará, 2013).

During the screening of isolated yeasts, as to their antimicrobial activity, extracts in general have shown higher activity against Gram-positive bacteria. According to Motamedi and Taghi Ronagh (2010) and Darabpour et al. (2012), this fact can be explained by some reasons, such as: low permeability provided by the presence of

multiple membranes in Gram-negative bacteria and by the fact that they have enzymes in the periplasmic space, which are able to break foreign molecules to the microorganism.

In the current study, the activity of the most active extracts was assessed against bacterial strains with multi-drug resistance (MDR); which were MRSA clinical isolates in this case. The results of this study show that tested extracts showed effects against MRSA strains, with prominence to HVNJ-189 (I) and HVNJ 189 (II) extracts. Data of this study are in accordance with those found by Darabpour et al. (2012).

In this study it was observed that MRSA cells that were exposed to bioactive extracts showed an increased PI uptake, and this finding may indicate that these extracts promote a significant damage ($p < 0.05$) to bacterial cells membrane. The plasma membrane is a latent target for antibiotic action, as molecules that act at this site have a greater biological potential, due to demonstrating a rapid and extensive antimicrobial effect, besides being more difficult to develop resistance (Mehravar and Sardari, 2011).

Programmed cell death (PCD) plays a critical role in the life cycle of several bacterial species, and this has been proposed as a potential target for new antibacterial strategies (Tanouchi et al., 2013). Taking the current findings together, it is demonstrated that these extracts promote cell death by apoptosis in MRSA cells. Data of this study match with data from Dwyer et al. (2012), who demonstrated cell death similar characteristics in *E. coli* strains after treatment with antibacterial antibiotics.

Drugs derived from natural products regularly have a limited solubility in aqueous solvents and/or exhibit a considerable cytotoxicity, resulting in narrow therapeutic indexes (Cragg et al., 2014). In cytotoxic tests using the MTT test on human leukocyte cells, the secondary metabolites showed low cytotoxicity.

These data support the results of this study, because according to Bertrand et al., (2014), natural products extracts are highly complex mixtures, what further complicates the assessment of bioactivity, as there can be toxic substances in excess, a fact that was not observed on the findings of this paper, which can generate an advantage to subsequent pharmacological tests.

Within this context, the antimicrobial activity and the low cytotoxic potential demonstrated by these extracts reveal a class of promising chemical compounds to the development of new antibiotics. However, more pharmacological studies will be done to confirm this hypothesis.

Acknowledgments

This research was supported by grants and fellowships from CNPq, CAPES/Brazil, and FUNCAP/Ceará. We declare no conflicts of interest concerning this article.

References

Almeida, G.C., dos Santos, M.M., Lima, N.G., Cidral, T.A., Melo, M.C., Lima, K.C. 2014. Prevalence and factors associated with wound colonization by *Staphylococcus* spp. and *Staphylococcus aureus* in hospitalized patients in inland northeastern Brazil: a cross-sectional study. *BMC Infect. Dis.*, 14: 328. doi: 10.1186/1471-2334-14-328.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J. 1990. Basic local alignment search tool. *J. Mol. Bio.*, 215(3): 403–410,

Bertrand, S., Bohni, N., Schnee, S., Schumpp, O., Gindro, K., Wolfender, J.L. 2014. Metabolite induction viamicroorganism co-culture: A potentialway to enhance chemical diversity for drug discovery. *Biotechnol. Adv.,* 32(6): 1180–204.

Boucher, H.W., Talbot, G.H., Bradley, J.S., Edwards, J.E., Gilbert, D., Rice, L.B., Scheld, M., Spellberg, B., Bartlett, J., Bugs, B. 2009. No drugs: no ESKAPE! an update from the Infectious Diseases Society of America. *Clin. Infect. Dis.,* 48(1): 1–12.

Bowler, P.G., Welsby, S., Towers, V., Booth, R., Hogarth, A., Rowlands, V., Joseph, A., Jones, S.A. 2012. Multidrug-resistant organisms, wounds and topical antimicrobial protection. *Int. Wound J.,* 9(4): 387–96.

Ceará. Statistical Yearbook. 2011. http://www2.ipece.ce.gov.br/publicacoes/anuario/anuario2011/index.htm. Acessado em 11/03/2013.

Clinical and Laboratory Sandards Institute (CLSI), 2012. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M07-A9, CLSI, Wayne, Pa, USA, 9th edn.

Clinical and Laboratory Sandards Institute (CLSI), 2013. Performance standards for antimicrobial susceptibility testing. Approved standard M100-S23, CLSI, Wayne, Pa, USA, 23th edn.

Clinical and Laboratory Sandards Institute (CLSI). 2012. Performance standards for antimicrobial disk susceptibility tests. Approved standard M02-A11, CLSI, Wayne, Pa, USA, 11th edn.

Cragg, G.M., Grothaus, P.G., Newman, D.J. 2014. New horizons for old drugs and drug leads. J. Nat. Prod., 77(3): 703–23.

da Silva,. R., de Andrade Neto, J.B., de Sousa Campos, R., Figueiredo, N.S., Sampaio, L.S., Magalhães, H.I., Cavalcanti, B.C., Gaspar, D.M., de Andrade, G.M., Lima, I.S., de Barros Viana, G.S., de Moraes, M.O., Lobo, M.D., Grangeiro, T.B., NobreJúnior, H.V. 2014. Synergistic effect of the flavonoid catechin, quercetin, or epigallocatechingallate with fluconazole induces apoptosis in *Candida tropicalis* resistant to fluconazole. *Antimicrob. Agents Chemother.,* 58(3): 1468–78.

Darabpour, E., Roayaei Ardakani M., Motamedi H., Taghi Ronagh M. 2012. Isolation of a potent antibiotic producer bacterium, especially against MRSA, from northern region of the Persian Gulf. *Bosn J. Basic Med. Sci.,* 12(2): 108–21.

Ding, G., Liu, Shuchun; Guo, L., Zhou, Y., Che, Y. 2008. Antifungal Metabolites from the Plant Endophytic Fungus *Pestalotiopsisfoedan. J. Nat. Prod.,* 71(4): 615–8.

Dwyer, D.J., Camacho, D.M., Kohanski, M.A., Callura, J.M., Collins, J.J. 2012. Antibiotic-induced bacterial cell death exhibits physiological and biochemical hallmarks of apoptosis. *Mol. Cell,* 46(5): 561–72.

Embrapa Genetic Resources and Biotechnology, 2010. Disponível em: http://pragawall.cenargen.embrapa.br/aiqweb/michtmL/fgbanco01.asp. Acesso em: 06/06/2014.

Kirk, P.M., Cannon, P.F., David, J.C., Stalpers, J.A. (Eds.). 2008. Dictionary of the fungi. CABI Publishing, Wallingford. 11th edn.

Maia, L.C., Carvalho Junior, A.A. 2010. Introduction: fungi of Brazil. Research Institute Botanical Garden of Rio de Janeiro. 1: 43–48. ISBN 978-85-8874-242-0.

Mautone, J.N. 2008. Diversity and biotechnological potential of yeasts and fungi like yeast isolated from leaves of fig trees in the Itapuã park, RS, Brazil. 62f. Dissertation (Master) - Institute of Basic Health Sciences Federal University of Rio Grande do Sul, Porto Alegre.

Mehravar, M., Sardari, S. 2011. Screening of antimicrobial membrane-active metabolites of soil microfungi by using chromatic phospholipid/polydiacetylene vesicles. *J. Mycol. Med.,* 21(3): 188–97.

Moraes Filho, M.O. 2010. The Brazilian biodiversity as a source of innovative medicines. *Strategic Partnerships. Special Edition.* 31(15): 171–192.

Motamedi, H., Darabpour, E., Gholipour, M., Seyyednejad, S.M. 2010. Antibacterial effect of ethanolic and meyhanolic extract of Plantagoovata and Oliveriadecumbens endemic in Iran against some pathogenic bacteria. *Int. J. Pharmacol.,* 2(6): 117–122.

Rodriguez-Noriega, E., Seas, C. 2010. The Changing Pattern Of Methicillin-Resistant *Staphylococcus aureus* clones. In: Latin America: implications for clinical practice in the region. *Braz. J. Infect. Dis.,* 14(2): 87–96.

Santos, H.B., Machado, D.P., Camey, S.A., Kuchenbecker, R.S., Barth, A.L., Wagner, M.B. 2010. Prevalence and acquisition of MRSA amongst patients admitted to a tertiary-care hospital in brazil. *BMC Infect. Dis.,* doi: 10.1186/1471-2334-10-328.

Shen, X.Y., Cheng, Y.L., Cai, C.J., Fan, L., Gao, J., Hou, C.L. 2014. Diversity and antimicrobial activity of culturable endophytic fungi isolated from moso bamboo seeds. *PLoS ONE.,* 9(4): e95838.

Shi, L., Günther, S., Hübschmann, T., Wick, L.Y., Harms, H., Müller, S. 2007. Limits of propidium iodide as a cell viability indicator for environmental bacteria. *Cytometry Part A.,* 8(71): 592–598.

Silva, F., Ferreira, S., Queiroz, J.A., Domingues, F.C. 2011. Coriander (*Coriandrumsativum L.*) essential oil: its antibacterial activity and mode of action evaluated by flow cytometry. *J. Med. Microbiol.,* 60(10): 1479–86.

Singh, B. K., Macdonald, C. A. 2010. Drug discovery from uncultivable microorganisms. *Drug Discov. Today.,* 15(17–18): 792–9.

Smith, T.C., Male, M.J., Harper, A.L., Kroeger, J.S., Tinkler, G.P., Moritz, E.D., Capuano, A.W., Herwaldt, L.A., Diekema, D.J. 2009. Methicillin-resistant *Staphylococcus aureus* (MRSA) strain ST398 is present in midwestern U.S. swine and swine workers. *PLoS One,* 4(1): e4258.

Tanouchi, Yu., Lee, A.J., Meredith, Hannah, You, L. 2013. Programmed cell death in bacteria and implications for antibiotic therapy. *Trends Microbiol.,* 21(6): 265–70.

Wagner- Döbler, I., Beil, W., Lang, S., Meiners, M., Laatsch, H. 2002. Integrated approach to explore the potential of marine microorganisms for the production of bioactive metabolites. Integrated approach to explore the potential of marine microorganisms for the production of bioactive metabolites. *Adv. Biochem. Eng. Biotechnol. N,* 74: 207–238.

Warner, S.A.J. 1996. Genomic DNA isolation and lambda library construction. In: Foster, G.D., Twell, D. (Eds). Plant gene isolation: Principles and practice. John Wiley & Sons. West Sussex. Vol. 1, Pp. 51–73.

White, T., Bruns, T., Lee, S., Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M.A., Gefland, D.H., Sninsky, J.J., White, T.J. (Ed.), PCR protocols: a guide to methods and applications. Academic Press, San Diego, CA. Pp. 315–322.