



Effect of subinhibitory and inhibitory concentrations of *Plectranthus amboinicus* (Lour.) Spreng essential oil on *Klebsiella pneumoniae*

Thially Braga Gonçalves^{a,b,*}, Milena Aguiar Braga^a, Francisco F.M. de Oliveira^{a,b},
 Gilvandete M.P. Santiago^{c,d}, Cibele B.M. Carvalho^{b,e}, Paula Brito e Cabral^{a,b},
 Thiago de Melo Santiago^f, Jeanlex S. Sousa^f, Eduardo Bedê Barros^f,
 Ronaldo Ferreira do Nascimento^g, Aparecida T. Nagao-Dias^{a,b}

^a Departamento de Análises Clínicas e Toxicológicas, Faculdade de Farmácia, Odontologia e Enfermagem, Universidade Federal Ceará, Rua Capitão Francisco Pedro 1210, CEP 60430-370, Fortaleza, Ceará, Brazil

^b Curso de Pós-Graduação em Microbiologia Médica, Faculdade de Medicina, Universidade Federal do Ceará, Rua Monsenhor Furtado S/N, CEP 60430-270, Fortaleza, Ceará, Brazil

^c Departamento de Farmácia, Faculdade de Farmácia, Odontologia e Enfermagem, Universidade Federal do Ceará, Rua Capitão Francisco Pedro 1210, CEP 60430-370, Fortaleza, Ceará, Brazil

^d Curso de Pós-Graduação em Química, Centro de Ciências, Universidade Federal do Ceará, Cx Postal 12.200, CEP 60021-970, Fortaleza, Ceará, Brazil

^e Departamento de Patologia e Medicina Legal, Faculdade de Medicina, Universidade Federal do Ceará, Rua Monsenhor Furtado S/N, CEP 60430-270, Fortaleza, Ceará, Brazil

^f Departamento de Física, Centro de Ciências, Universidade Federal do Ceará, CEP 60451-970, Fortaleza, Ceará, Brazil

^g Departamento de Química Analítica e Físico-Química, Universidade Federal do Ceará, Campus do Pici, Bloco 940, Fortaleza, Ceará, Brazil

ARTICLE INFO

Keywords:

Antibiotic multidrug resistance
 Antimicrobial activity
Klebsiella pneumoniae
Plectranthus amboinicus

ABSTRACT

We evaluated the antimicrobial activity and some mechanisms used by subinhibitory and inhibitory concentrations of the essential oil, obtained from leaves of *Plectranthus amboinicus*, against a standard strain of *Klebsiella pneumoniae* and 5 multidrug resistant clinical isolates of the bacteria. The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC), the rate of kill and the pH sensitivity of the essential oil were determined by microdilution tests performed in 96-well plates. Subinhibitory and inhibitory concentrations of the essential oil were tested in order to check its action on *K. pneumoniae* membrane permeability, capsule expression, urease activity and cell morphology. The MIC and MBC of the essential oil were 0.09 ± 0.01%. A complete inhibition of the bacterial growth was observed after 2 h of incubation with twice the MIC of the essential oil. A better MIC was found when neutral or alkaline pH broth was used. Alteration in membrane permeability was found by the increase of crystal violet uptake when the bacteria were incubated with twice the MIC levels of the essential oil. The urease activity could be prevented when all the subinhibitory concentrations were tested in comparison to the untreated group ($p < 0.001$). Alteration of the bacterial morphology besides inhibition of the capsule expression was verified by atomic force microscopy, and Anthony's stain method, respectively. Our data allow us to conclude that the essential oil of *P. amboinicus* can be a good candidate for future research.

© 2012 Elsevier GmbH. All rights reserved.

Introduction

Since the introduction of antimicrobial agents, there has been a selective pressure of microorganisms, primarily caused by the indiscriminate use of antibiotics and chemotherapy, which has resulted in the development of resistant species (Peres-Bota et al. 2003; Pittet 2005). One of the most serious public health problems faced in the last decades has been the aggravation of antimicrobial resistance in bacterial populations, especially in nosocomial

outbreaks (Oliveira et al. 2006). An important aspect which frequently results in antibiotic resistance resides on the bacterial enzyme production, such as, beta-lactamases. The beta-lactamases of clinical interest are extended spectrum beta-lactamase (ESBL), carbapenemases (KPC), metalobetalactamase (MBL) and class C beta-lactamase (AmpC). *K. pneumoniae* ESBL is capable of inactivating third generation cephalosporins and monobactams, except carbapenems and are inhibited by beta-lactamase inhibitors such as clavulanate, sulbactam and tazobactam. The enzyme KPC was firstly described in the U.S. in 2001 and was further identified in other countries around the world. This enzyme has the ability to hydrolyze cephalosporins and aztreonam (Patterson and Bonomo 2005; Bertrand et al. 2006).

Many plants are used in Brazil in the form of crude extracts, infusions for treatment of various conditions, including bacterial

* Corresponding author at: Departamento de Análises Clínicas e Toxicológicas, Faculdade de Farmácia, Odontologia e Enfermagem, Universidade Federal Ceará, Rua Capitão Francisco Pedro 1210, CEP 60430-370, Fortaleza, Ceará, Brazil.
 Tel.: +55 85 3366 8270; fax: +55 85 3366 8292.

E-mail address: thially@gmail.com (T.B. Gonçalves).

and fungal infections (Gurgel et al. 2009). Essential oils have long been used due to their many therapeutic properties, including antiviral, antispasmodic, analgesic, antimicrobial, wound healing, expectorant, soothing, antiseptic airway, larvicide, antihelmintic and antiinflammatory activities (Costa et al. 2010; Oyedeji and Afolayan 2006).

The genus *Plectranthus* involves about 300 species occurring naturally in Africa, Asia and Australia (Lukhoba et al. 2006). Species of this genus exhibit biosynthetic sesquiterpenes, diterpenes and phenolic compounds, some with proven relevant biological properties (Lukhoba et al. 2006).

Plectranthus amboinicus (Lour.) Spreng (Labiatae), also known as *Coleus amboinicus* Lour., *Coleus aromaticus*, *Plectranthus aromaticus* (Benth) (Murthy et al. 2009), and popularly known as “malvarisco”, is an aromatic perennial herb cultivated all over the North and Northeast regions of Brazil (Matos 2000). It is widely used in Brazilian folk medicine for therapeutic purposes against colds, asthma, constipation, headache, cough, fever and digestive diseases (Morais et al. 2005).

This present work proposed to investigate the antimicrobial activity of the essential oil against *K. pneumoniae* standard strains and multiresistant clinical isolates, and also to evaluate the ability of subinhibitory and inhibitory concentrations of *P. amboinicus* essential oil in altering the membrane permeability, the capsule expression and the urease activity of *K. pneumoniae*, its rate of kill and its capability on growing in different pH conditions.

Materials and methods

Plant material

Leaves of *Plectranthus amboinicus* (Lour.) Spreng were collected from the Horto de Plantas Mediciniais Professor Francisco José de Abreu Matos (Fortaleza, Ceará, Brazil). A voucher specimen (EAC40080) has been deposited at the Herbário Prisco Bezerra (EAC), Universidade Federal do Ceará, Brazil.

Extraction of the essential oil

The fresh leaves of *P. amboinicus* were subjected to hydrodistillation in a Clevenger-type apparatus for 2 h to afford pale yellow oil. The isolated oil, after drying over anhydrous sodium sulfate and filtration, was stored in sealed glass vials and maintained under refrigeration until further analysis. The yield (w/w) was calculated based on the fresh weight of the leaves.

The essential oil was obtained in three different periods and analyzed by gas chromatography (GC) and GC/MS in order to compare the chemical constituents from each extraction. The essential oils were named S1, S2 and S3.

Chemical analysis of the essential oil

Gas chromatography (GC) analysis was performed on a Shimadzu GC-17A gas chromatograph equipped with a flame

ionization detector using a non-polar DB-5 fused silica capillary column (30 m × 0.25 mm × 0.25 μm film thickness). Hydrogen was used as carrier gas at a flow rate of 1 ml/min and 30 psi inlet pressure; split ratio 1:30. The column temperature was programmed from 35 °C to 180 °C at a rate of 4 °C/min, then heated at a rate of 17 °C/min to 280 °C and held isothermal for 10 min; both injector and detector temperatures were 250 °C.

The GC/MS analysis was carried out on a Hewlett-Packard Model 5971 GC/MS instrument, using a non-polar DB-5 fused silica capillary column (30 m × 0.25 mm × 0.1 μm film thickness); carrier gas helium, flow rate 1 ml/min and with split mode. The injector and detector temperatures were 250 °C and 200 °C, respectively. The column temperature was programmed from 35 °C to 180 °C at 4 °C/min and then 180 to 250 °C at 10 °C/min. Mass spectra were recorded from 30 to 450 m/z.

Individual components were identified by matching their 70 eV mass spectra with those of the spectrometer database using the Wiley L-built library and two other computer libraries. Mass spectrometry (MS) searches using retention indices as a pre-selection routine, as well as by visual comparison of the fragmentation patterns with those reported in the literature (Alencar et al. 1990; Adams 2007).

Microbial strains

The *Klebsiella pneumoniae* (ATCC 700603) was obtained from the Instituto Nacional de Controle de Qualidade em Saúde (INCQS), Instituto Oswaldo Cruz, Rio de Janeiro, Brazil. Five clinical isolates were obtained from the Hospital Universitário Walter Cantídio (Fortaleza, Ceará, Brazil). The clinical isolates were analyzed by standard methods for identification of *Enterobacteriaceae* (Koneman 2008). Susceptibility profile was tested by disc-diffusion agar (CLSI 2009) with the following drugs: amikacin, ampicillin, ampicillin + sulbactam, cephalothin, cefoxitin, cefpime, caeftazidime, ceftriaxone, gentamicin, imipenem, meropenem, piperacillin, quinolones, and sulfazotrim ticarcillin + clavulanic acid (Table 1).

Determination of the minimal inhibitory concentration and minimal bactericidal concentration

To determine the minimal inhibitory concentration we performed microdilution tests (CLSI 2009) in 96-well microplates (Costar, USA). Each well contained 100 μl Mueller-Hinton Infusion (MHI) broth, 10 μl of inoculum (1.5×10^8 CFU/ml) and 100 μl of the essential oil, which ranged from 0.009% to 1.25% (v/v) in DMSO 3%. The inoculum bacterial concentration was determined by adjusting its turbidity (at 625 nm) to 0.5 McFarland scale. The plates were covered and incubated for 24 h at 37 °C. The lowest concentration of the test sample in which no growth could be visualized was defined as the MIC. We used ciprofloxacin (Sigma Chemical Co., St. Louis, MO, USA), ranging from 0.125 to 32 μg/ml, as the positive control and dimethyl sulfoxide (Merck, Darmstadt, Germany), ranging from 0.039 to 10% (v/v), as the negative control. After the incubation

Table 1

Table of pattern of drug resistance in *K. pneumoniae* clinical isolates: CE, clinical specimen; URO, uroculture; ATR, tracheal aspirate; BLO, blood; NT, not tested; S, sensitive; R, resistant; I, intermediate; AMI, amikacin; AMP, ampicillin; ASB, ampicillin + sulbactam; ATM, aztreonam; CEP, cephalothin; FM, cefepime; CTX, cefotaxime; CFO, cefoxitin; CAZ, ceftazidime; CEN, ceftriaxone; CIP, ciprofloxacin; COL, colistin; ERT, ertapenem; GEN, gentamicin; IPM, imipenem; MER, meropenem; PPT, piperacillin + tazobactam; TIG, tigecycline.

Clinical isolates	AMI	AMP/ASB/CEF	ATM/ERT/IPM/MER	COM/CFO/CAZ/PPT	CTX	CEN	CIPRO	COL	GEN	TIG
K2 (uro)	nt	R	R	R	R	nt	S	S	S	S
K5 (blood)	S	R	R	R	R	nt	R	S	S	R
K8 (uro)	nt	R	S	R	nt	R	S	nt	R	R
K12 (uro)	I	R	R	R	R	nt	R	S	S	S
K16 (atr)	R	R	R	R	R	nt	R	S	R	S

period, 10 μ l of sterile 0.05% resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide) (Sigma, USA) in aqueous solution were added to the plates. After 2 h of incubation, the readings were done. A color change caused by the reduction of resazurin to resorufin indicated presence (pink color) or absence (blue color) of cell viability (Palomino et al. 2002). The minimal bactericidal concentration was determined for the essential oil by subculturing 5 μ l from each tube (MIC, 2 and 4 \times MIC) onto BHI agar. The MBC was defined as the lowest concentration of the essential oil that could inhibit 99% of the bacterial growth. The tests were run in triplicate with the three samples (S1–S3) of the essential oil.

pH sensitivity assay

The effect of pH on antibacterial activity of *P. amboinicus* essential oil (S1) and ciprofloxacin against *K. pneumoniae* was done according to the procedure described by Devi et al. (2010). Cultures of the standard strain were grown on Muller-Hinton broth previously prepared and calibrated to different pH ranges (from 5.5 to 9.0). The MIC was determined.

Determination of rate of kill

One milliliter of the standard strain was adjusted to the concentration of 1.5×10^8 CFU/ml and incubated at 35 °C for 2 h, before its addition to tubes containing 0.16% (which represented two times the MIC). Control tubes were also prepared without the essential oil (S1). The tubes Test and Control were incubated at 37 °C and aliquots of 10 μ l were collected at intervals of 0, 2, 4, 6, 8 and 24 h after the addition of the plant samples. Serial dilutions were plated onto MacConkey agar to determine the number of viable cells which was expressed as log of colony forming units per ml (log CFU/ml). The bactericidal (≥ 3 -log-unit reduction in log₁₀ CFU/ml) and bacteriostatic activities (<3-log-unit reduction in log₁₀ CFU/ml) were determined (Devi et al. 2010).

Crystal violet assay

The alteration in membrane permeability was detected by crystal violet assay according to the procedure described in literature (Devi et al. 2010). Briefly, suspensions of the bacteria were prepared in Brain Heart Infusion (BHI) broth. Cells were harvested at 4500 \times g for 5 min at 4 °C. The cells were washed twice and resuspended in PBS at pH 7.4. The essential oil (S1) of *P. amboinicus* (tested at MIC and twice the MIC), the antibiotics (tested at MIC), besides ethylenediamine tetraacetic acid or EDTA (positive control) and phosphate buffered saline or PBS (negative control) were added to the cell suspensions and incubated for 30 min at 37 °C (all tests were done in quintuplicate). The cells were harvested at 9300 \times g for 5 min. After this procedure, the cells were resuspended in PBS containing 0.010 mg of crystal violet. The cell suspension was then incubated for 10 min at 37 °C and centrifuged at 13400 \times g for 2 h and the OD₅₉₀ of the supernatant was measured.

Effect of sub-MIC and MIC levels of *P. amboinicus* essential oil on urease activity

Bacterial urease activity was determined by the procedure described by Derakhshan et al. (2008). Five clinical isolates of *K. pneumoniae* and the ATCC standard were grown overnight in urea broth containing sub-MIC and MIC levels of the essential oil (S1) (1/8 to 1/1 \times MIC). Untreated cells were used as negative control. All tubes were incubated for 24 h at 37 °C. Next, the suspensions were centrifuged at 5000 \times g for 3 min and the supernatants were recovered. Color intensity was measured at 560 nm using a universal

microplate reader (Biotek, China). The samples were run in triplicate.

Effect of sub-MIC and MIC levels of *P. amboinicus* essential oil on bacterial capsule expression

The *K. pneumoniae* capsule was stained by the Anthony's capsule stain, which procedure was described in literature (Derakhshan et al. 2008). Briefly, five clinical isolates of the bacteria and the ATCC standard strain were grown in milk broth (9.5 g powder milk/l) for 24 h in the presence of different concentrations of the essential oil (S1) (1/8 to 1/1 \times MIC). Smears were prepared on glass slides and allowed to air dry. Thereafter, the slides were covered with 1% crystal violet for 2 min and rinsed gently with a 20% copper sulfate solution. The bacterial cells (not treated control) would appear purplish while the capsules would appear transparent under an oil immersion lens at optical microscopy.

Atomic force microscopy

Changes in the morphology of *K. pneumoniae* caused by the essential oil of *P. amboinicus* were analyzed by atomic force microscopy (AFM) (Braga and Ricci 1998). Suspensions of cultures were incubated in the presence of different concentrations of the essential oil (S1) (1/8 to 1/1 \times MIC) for 18 h at 37 °C. The cells were washed and centrifuged at 4500 \times g for 5 min at 4 °C, washed twice and resuspended in PBS. A volume of 10 μ l of the suspension was placed on a glass slide and dried in air for 15 min. Then the sample was examined by a Multimode Atomic Force Microscope Nanoscope III-a (Digital Instruments, Santa Barbara, CA). Scans were performed in air and amplitude images were acquired by intermittent contact mode using crystalline silicon cantilevers (Veeco-probes) with a spring constant of approximately 40 N/m, resonance frequency of 242.38 kHz and tip radius of 15 nm. The amplitude images can be used to better evidence cell borders and their shape. Cultures of not treated *K. pneumoniae* were taken as controls.

Statistical analysis

The effect of various concentrations of *P. amboinicus* essential oil on the *K. pneumoniae* urease activity and on the uptake of crystal violet by the bacteria were analyzed by one-way analysis of variance (ANOVA) and Tukey–Kramer multiple comparisons tests. The statistical significance was considered at *p*-value of 0.05.

Results

Chemical analysis of the essential oil and determination of the minimal inhibitory concentration and minimal bactericidal concentration

Three different samples of essential oil from the leaves of *P. amboinicus* were analyzed by GC and CG/MS. Table 2 shows the chemical composition of the three samples of the essential oil. Carvacrol (90–98%) is the major constituent identified in all samples. The samples of essential oil obtained in different periods have few differences in their chemical constitutions.

All tested strains (clinical and the standard) were susceptible to the essential oil (MIC mean \pm standard error, that is, $0.09 \pm 0.01\%$, *n* = 6). The MIC and MBC were the same. The tests were repeated with the other essential oil samples and the results were similar. For this reason, the following experiments were done with just one of the samples, that is, S1.

Table 2
Chemical composition of essential oil from leaves of *P. amboinicus*.

Compound	RI	RT	S1	S2	S3
4-Terpineol	1177	16.80	1.34	–	–
Carvacrol	1298	21.39	90.55	95.39	98.03
β-Caryophyllene	1417	25.46	3.09	–	0.01
α-Bergamotene	1433	25.97	1.78	–	–
Caryophyllene oxide	1583	30.97	1.36	–	0.20
p-Cimene	1024	8.28	–	–	1.65
γ-Terpinene	1059	9.55	–	–	0.01
α-trans-Bergamotene	1434	25.79	–	–	0.02
α-Caryophyllene	1454	25.49	–	–	0.02
Total identified	–	–	98.12	95.39	99.94

RI: relative retention index; RT: retention time; S: sample of essential oil of *P. amboinicus*.

Table 3
Effect of pH on antibacterial activity of *P. amboinicus* essential oil against *Klebsiella pneumoniae*.

pH	Essential oil		Cipro (μg/ml)
	%	μg/ml	
5.5	0.08	700.00	4.0
6.0	0.08	700.00	4.0
6.5	0.08	700.00	4.0
7.0	0.08	700.00	2.0
7.5	0.04	350.00	2.0
8.0	0.04	350.00	1.0
8.5	0.04	350.00	1.0
9.0	0.04	350.00	1.0

Determination of rate kill

Treatment of *K. pneumoniae* with 0.16% of essential oil, which represented two times the MIC, of essential oil was enough to kill all the bacteria after 2 h of incubation compared to control and to ciprofloxacin.

pH sensitivity assay

With respect to the pH sensitivity assay, a better MIC was found when neutral or alkaline pH broth was used (Table 3). Similar results were found when ciprofloxacin was tested.

Crystal violet assay

The uptake of crystal violet was considered to be significant when twice the MIC of essential oil was used (Table 4), compared to the not treated group ($p < 0.01$) or to ciprofloxacin ($p < 0.01$) and to EDTA ($p < 0.05$). EDTA is a chelating agent which increases the membrane permeability of the bacterial cells.

Effect of sub-MIC and MIC levels of *P. amboinicus* essential oil on urease activity

All the concentrations of the essential oil tested could significantly reduce the urease activity, compared to the not treated group ($p < 0.001$), as shown in Fig. 1. The dashed line in Fig. 1 is the best exponential fit of the data.

Effect of sub-MIC levels of *P. amboinicus* essential oil on bacterial capsule

As shown in Fig. 2, smaller colonies with reduced expression of capsules could be found when the bacteria were treated with $1/8 \times$ MIC of the essential oil compared to the not treated controls (Fig. 2a and b). However, when the bacteria were treated with $1/4$

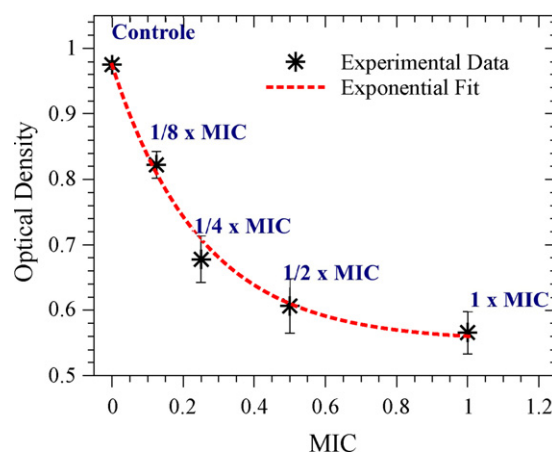


Fig. 1. Effects of sub-inhibitory ($1/8$ to $1/2$) and inhibitory concentrations of *P. amboinicus* essential oil on *Klebsiella pneumoniae* urease activity. It shows the absorbance averaged over five samples vs the MIC. The dashed line is the best exponential decay fitting of the experimental data {optical density = $0.552 + 0.423 \exp(-\text{MIC}/0.251)$ }. This expression indicates that for sufficient high values of MIC the absorbance presents a saturation value, near 0.552.

or higher subinhibitory concentrations of the essential oil almost no bacteria could be found, only cellular debris (Fig. 2c–e).

Atomic force microscopy

According to the atomic force microscopy (Fig. 3A and B), the morphology of untreated *K. pneumoniae* was smooth and regular with an intact cell membrane and individual bacteria were readily distinguishable. Fig. 3A shows after 24 h of treatment with $1/8$ and $1/4 \times$ MIC essential oil (Fig. 3Ab and Ac), it was observed an increasing loss of the membrane integrity with a consequent altered morphology of the bacteria. Finally, a severe cell damage was verified when $1/2 \times$ MIC and the inhibitory concentrations ($1 \times$ MIC and $2 \times$ MIC) were used (Fig. 3Ad, Ae and Af). Fig. 3B shows the three dimensional aspect of the not treated bacteria compared to those treated with twice the MIC of the *P. amboinicus* essential oil.

Discussion

It is known that the composition of the essential oils varies according to the geographical region, age of the plant, methods of extraction (Murthy et al. 2009). For instance, comparing the literature data, carvacrol comprised 70% (Castillo and González 1999), 58–65% (Dorman and Deans 2000), 50% (Pino et al. 1990), and, 28.65% (Senthilkumar and Vankatesalu, 2010), of the *P. amboinicus* essential oil, meanwhile our extraction contained 90–98% of carvacrol. Others found 94.3% of thymol as the major constituent of the essential oil (Singh et al. 2002).

Table 4
Crystal violet uptake by *Klebsiella pneumoniae* previously incubated with *Plectranthus amboinicus* essential oil. The results were expressed by mean of OD₅₉₀ values ± standard error. The samples were tested in quintuplicate.

Groups	PBS	EDTA	CIPRO	Essential oil	
				MIC	2 × MIC
X _{Abs} ± EM	0.412–0.013	0.383–0.019	0.412–0.037	0.315–0.029	0.254–0.016
CI ₉₅	0.371–0.454	0.329–0.437	0.308–0.517	0.234–0.396	0.209–0.299
p	N.S.	N.S.	N.S.	N.S.	<0.01 (vs PBS and CIPRO) <0.05 (vs EDTA)

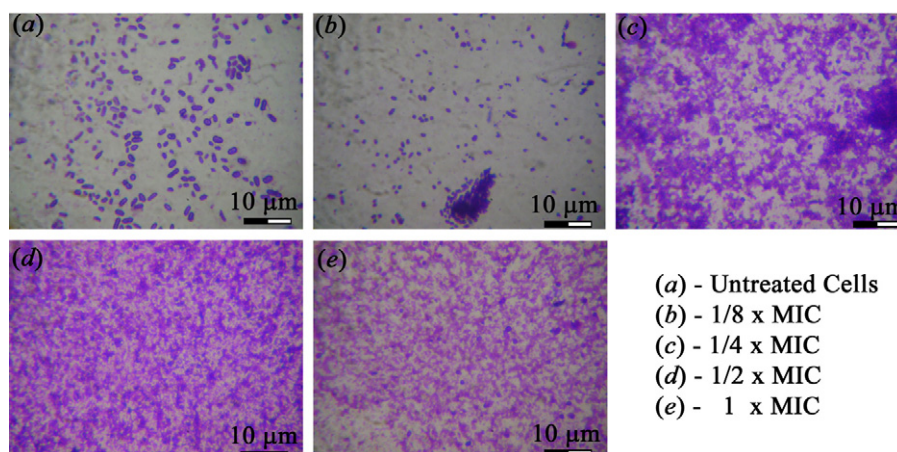


Fig. 2. Effects of sub-inhibitory (1/8 to 1/2), inhibitory and twice the inhibitory concentrations of *P. amboinicus* essential oil on *Klebsiella pneumoniae* capsule expression. The bacteria were stained with the Anthony capsular staining method. The colony morphology and capsule visualization was done under light microscopy (magnification 1000× with 3× optical zoom).

Carvacrol is a monoterpene phenol which is known to be either bactericidal or bacteriostatic agent, depending on the concentration used (Nostro et al. 2004). Previous studies reported that carvacrol had the property of destroying the cellular membrane of *Escherichia coli*, *Salmonella typhimurium*, *Shigella flexneri* (Dorman and Deans 2000).

P. amboinicus essential oil presents bactericidal effect when a concentration of twice the MIC is added to *K. pneumoniae* suspension. After 2 h of incubation, there is a complete loss of bacterial viability. The data shown is relevant by the fact *K. pneumoniae* is one of the main pathogens associated with nosocomial infections; also, as the pH sensitivity test showed, the essential oil was

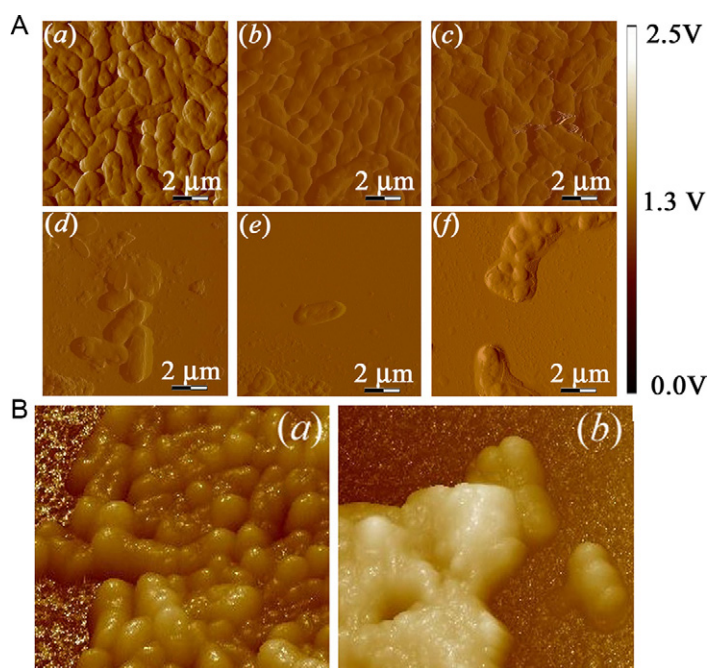


Fig. 3. (A) AFM images of *Klebsiella pneumoniae* exposed to 1/8 × MIC to 2 × MIC of *P. amboinicus* essential oil. Amplitude images show the morphology of cells. Control (a), treated cells with 1/8 × MIC (b), 1/4 × MIC (c), 1/2 × MIC (d), 1 × MIC (e) and 2 × MIC (f). The scale bar in volts corresponds to changes in the oscillation's amplitude of the free cantilever. (B) AFM images of *Klebsiella pneumoniae*. Three-dimensional aspect of the untreated bacteria (a) compared to those treated with twice the MIC of the *P. amboinicus* essential oil (b).

more active in higher pH ranges. These data are valuable, once *K. pneumoniae* can grow in the small intestine, where pancreatic juice and bile make the environment pH highly alkaline (Koneman 2008).

Klebsiella pneumoniae is a nonmotile enteric organism that ferments lactose, decarboxylates lysine, hydrolyzes urea, and forms large mucoid colonies, due to the presence of capsule (Koneman 2008). The capsule of *K. pneumoniae* consists of thick and dense bundles of fine fibers, which contributes to its high virulence capacity.

Generally, subinhibitory concentrations of an essential oil, although not able to kill bacteria, can modify the architecture of their outermost surface and may interfere with some bacterial functions (Derakhshan et al. 2008). In our study, when *K. pneumoniae* was tested in the presence of 1/8 MIC of *P. amboinicus* essential oil, an alteration of its morphology besides reduction of capsule expression was found. When higher subinhibitory concentrations (1/4 and 1/2) of the essential oil were tested, almost no bacteria could be visualized, but only cellular debris. This could mean that subinhibitory concentration of 1/8 × MIC could reduce the bacterial capacity of capsule expression. Besides this observation, alteration in membrane permeability was detected by the increase of crystal violet uptake when the bacteria were incubated with twice the MIC levels of *P. amboinicus* essential oil. Loss of membrane integrity and leakage of intracellular components could be demonstrated by the atomic force microscopy, when 1/2 × MIC levels of the essential oil was used, and a complete destruction of the cells at the inhibitory concentrations (MIC and 2 × MIC) of the plant sample.

Many microorganisms need to use urease in order to obtain nitrogen for their growth (Maroncle et al. 2006). Urease catalyzes the hydrolysis of urea to ammonia and carbamate and has been found to be implicated in kidney and bladder stone formation and to the pathogenesis of several diseases. Urease inhibitors are potential alternatives for the prevention of *K. pneumoniae* from colonizing the gastrointestinal tract, where is plenty of urea (Koneman 2008; Hostacká 2000; Maroncle et al. 2006). Imipenem and ofloxacin, do not affect the enzyme activity (Hostacká 2000). Our data showed a strong inhibition of urease activity by the *P. amboinicus* essential oil in all concentrations tested compared to the not treated bacteria ($p < 0.001$). Although 1/8 MIC was only able to reduce 15.7% of the urease activity, 1/4 × MIC to 1 MIC reduce from 30.5% to 42%, respectively. These percentages of the enzyme inhibition were superior to β-lactam and aminoglycoside actions (20% of inhibition), and equivalent or superior to acetohydroxamic acid (30% of inhibition), which is frequently used to inhibit urease activity (Maroncle et al. 2006; Rauf et al. 2011). On the other hand, it has been recently shown novel barbituric and thiobarbituric acid-derived sulfonamides with ability to inhibit more than 88% of the urease activity when the drugs were tested at 500 μM concentrations (Rauf et al. 2011).

Conclusion

The results of our data clearly demonstrated that essential oil of *Plectranthus amboinicus* possesses a potential antibacterial activity against *Klebsiella pneumoniae*. Subinhibitory concentrations of the essential oil could affect the capsule expression, alterations in membrane integrity, inhibition of the urease activity. At bactericidal concentrations, a complete destruction of the bacteria was found. Our data allow us to conclude that the essential oil of *P. amboinicus* could be a good candidate for future research.

Conflict of interest

No conflict to disclose.

Acknowledgements

This research was financially supported by the CNPq (Processes 579437/2008-6 and 554970/2010-4) and FUNCAP/PPSUS (Process 09100057-2).

References

- Adams, R.P., 2007. Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry. Allured Publishing Corporation, Carol Stream, IL.
- Alencar, J.W., Craveiro, A.A., Matos, F.J.A., Machado, M.L.L., 1990. Kovats indices simulation in essential oils analysis. *Quimica Nova* 13, 282–284.
- Bertrand, S., Weill, F.-X., Cloeckert, A., Vrints, M., Mairiaux, E., Praud, K., Dierick, K., Wildemaue, C., Godard, C., Butaye, P., Imberechts, H., Grimont, P.A.D., Collard, J.-M., 2006. Clonal emergence of extended-spectrum beta-lactamase (CTX-M-2)-producing *Salmonella enterica* serovar Virchow isolates with reduced susceptibilities to ciprofloxacin among poultry and humans in Belgium and France (2000 to 2003). *Journal of Clinical Microbiology* 44, 2897–2903.
- Braga, P.C., Ricci, D., 1998. Atomic force microscopy: application to investigation of *Escherichia coli* morphology before and after exposure to cefodizime. *Antimicrobial Agents Chemotherapy* 42, 18–22.
- Castillo, R.A.M., González, V.P., 1999. *Plectranthus amboinicus* (Lour.) Spreng. *Revista Cubana de Plantas Medicinales* 4, 110–115.
- CLSI, 2009. Performance Standards for Antimicrobial Susceptibility Testing; 18th Informational Supplement. CLSI M07-A8. Clinical and Laboratory Standards Institute, Wayne, PA, pp. 15–18.
- Costa, J.G., Campos, A.R., Brito, S.A., Pereira, C.K.B., Souza, E.O., Rodrigues, F.F.G., 2010. Biological screening of araripe basin medicinal plants using *Artemia salina* Leach and pathogenic bacteria. *Pharmacognosy Magazine* 6 (24), 331–334.
- Derakhshan, S., Sattari, M., Bigdeli, M., 2008. Effect of subinhibitory concentrations of cumin (*Cuminum cyminum* L.) seed essential oil and alcoholic extract on the morphology, capsule expression and urease activity of *Klebsiella pneumoniae*. *International Journal of Antimicrobial Agents* 32, 432–436.
- Devi, K.P., Nisha, S.A., Sakthivel, R., Pandain, S.K., 2010. Eugenol (an essential oil of clove) acts as antibacterial agent against *Salmonella typhi* by disrupting the cellular membrane. *Journal of Ethnopharmacology* 130, 107–115.
- Dorman, H.J.D., Deans, S.G., 2000. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *Journal of Applied Microbiology* 88, 308–316.
- Gurgel, A., Silva, J.G., Grangeiro, A.R.S., Xavier, H.S., Oliveira, R.A.G., Pereira, M.S.V., Souza, I.A., 2009. Antibacterial effects of *Plectranthus amboinicus* (Lour.) Spreng (Lamiaceae) in methicillin resistant *Staphylococcus aureus* (MRSA). *Latin American Journal of Pharmacy* 28 (3), 460–464.
- Hostacká, A., 2000. The *in vitro* effect of imipenem and ofloxacin on enzymic activity of *Klebsiella* strains. *Folia Microbiologica* 45, 387–390.
- Koneman, E.W., 2008. Diagnóstico Microbiológico – Texto e Atlas Colorido. Guanabara Koogan, Rio de Janeiro, pp. 209–290.
- Lukhoba, C.W., Simmonds, M.S.J., Paton, A.J., 2006. *Plectranthus*: a review of ethnobotanical uses. *Journal of Ethnopharmacology* 103, 1–24.
- Maroncle, N., Rich, C., Forestier, C., 2006. The role of *Klebsiella pneumoniae* urease in intestinal colonization and resistance to gastrointestinal stress. *Revista de Microbiologia* 157, 184–193.
- Matos, F.J.A., 2000. Plantas medicinais: guia de seleção e emprego de plantas usadas em fitoterapia no Nordeste do Brasil, vol. 2, pp. 36–42.
- Morais, S.M., Dantas, J.D.P., Silva, A.R.A., Magalhães, E.F., 2005. Plantas medicinais usadas pelos índios Tapebas do Ceará. *Revista Brasileira de Farmacognosia* 15, 169–177.
- Murthy, P.S., Ramalakshmi, K., Srinivas, P., 2009. Fungitoxic activity of Indian borage (*Plectranthus amboinicus*) volatiles. *Food Chemistry* 114, 1014–1018.
- Nostro, A., Blanco, A.R., Cannatelli, M.A., Enea, V., Flamini, G., Morelli, I., Roccaro, S., Alonzo, V., 2004. Susceptibility of methicillin-resistant staphylococci to oregano essential oil, carvacrol and thymol. *FEMS Microbiology Letters* 230, 191–195.
- Oliveira, J.F.P., Cipullo, J.P., Burdmann, E.A., 2006. Nefrototoxicidade dos aminoglicosídeos. *Revista Brasileira de Cirurgia Cardiovascular* 21 (4), 444–452.
- Oyediji, A.O., Afolayan, A.J., 2006. Chemical composition and antibacterial activity of the essential oil isolated from South African *Mentha longifolia* (L.) L. subsp. *capensis* (Thunb.) Briq. *The Journal of Essential Oil Research* 18, 57–59.
- Palomino, J.C., Martin, A., Camacho, M., Guerra, H., Swing, J., Portaels, F., 2002. Resazurin microtiter assay plate: simple and inexpensive method for detection of drug resistance in *Mycobacterium tuberculosis*. *Antimicrobial Agents and Chemotherapy* 46 (8), 2720–2722.
- Patterson, D.L., Bonomo, R.A., 2005. Extended-spectrum beta-lactamases: a clinical update. *Clinical Microbiology* 18, 657–686.
- Peres-Bota, D., Rodriguez, H., Dimopoulos, G., 2003. Are infections due to resistant pathogens associated with a worse outcome in critically ill patients? *Journal of Infection* 47, 307–316.
- Pino, J., Rosado, A., Borges, P., 1990. Volatile components in the essential oil of wild oregano (*Coleus amboinicus* Lour.). *Food Nahrung* 34, 819–823.

- Pittet, D., 2005. Infection control and quality health care in the new millennium. *American Journal of Infection Control* 33, 258–267.
- Rauf, A., Ahmed, F., Qureshi, A.M., Aziz-ur-Rehman, Khan, A., Qadir, M.I., Choudhary, M.I., Chohan, Z.H., Youssoufid, Z.M.H., Haddad, T.B., 2011. Synthesis and urease inhibition studies of barbituric and thiobarbituric acid derived sulphonamides. *Journal of the Chinese Chemical Society* 58, 528–533.
- Singh, G., Singh, O.P., Prasad, Y.R., Lamposona, M.P., Catalan, C., 2002. Studies on essential oils. Part 33. Chemical and insecticidal investigations on leaf oil of *Coleus amboinicus* Lour. *Flavour and Fragrance Journal* 17, 440–442.
- Senthilkumar, A., Vankatesalu, V., 2010. Chemical composition and larvicidal activity of the essential oil of *Plectranthus amboinicus* (Lour.) Spreng against *Anopheles stephensi*: a malarial vector mosquito. *Parasitology Research* 107, 1275–1278.