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African Journal of Microbiology Research

Full Length Research Paper

Antimicrobial activity of several Brazilian medicinal plants against phytopathogenic bacteria

Cibele Maria Alves da Silva[1,2], Bruna Mirely da Silva Costa[1], Alexandre Gomes da Silva[3]*, Elineide Barbosa de Souza[4], Márcia Vanusa da Silva[3,5], Maria Tereza dos Santos Correia[1,3], da Silva Ana Paula Sant´Anna[5] and Lima Vera Lúcia de Menezes[5]

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What is currently raised as a new approach in the management of plant diseases is the development and formulation of plant based biopesticides. The objective of present study is to evaluate the antibacterial activity of aqueous extracts of twelve species belonging to seven families collected from the Northeast of Brazil against four economically important phytopathogenic bacteria. Antibacterial activities of the aqueous extracts were studied by minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Twelve aqueous extracts of twelve species were evaluated. Only three extracts were not active against *Ralstonia solanacearum* and other three extracts were not active against *Xanthomonas campestris* pv. *campestris*. *Anadenanthera colubrina* var. *cebil*, *Croton pedicellatus* and *Eugenia brejoensis* presented a broad spectrum of the inhibitory effect (MIC 3.12 to 12.5 mg/mL). According to these results, we conclude that the flora in the northeast of Brazil can be regarded as a rich source of plants with antibacterial activity. Therefore, further screening of other plant species, identifying active fractions or metabolites and *in vivo* application of active extracts are warranted.

Key words: Caatinga, Atlantic Forest, antibacterial activity, aqueous extract, anti-phytopathogenic activity.

INTRODUCTION

The plant kingdom represents an enormous reservoir of biologically active compounds with various chemical structures and disease preventing properties (Kavitha and Satish, 2013). The use of plant compounds to treat infection is an ancient practice in a large part of the world, especially in developing countries, where there is dependence on traditional medicine for a variety of diseases (Gangoue-pieboji et al., 2006). According to World Health Organization, medicinal plants would be the best source to obtain a variety of drugs. In recent years,

attention has been given to natural systems of treatment for protection and management against pathogens. Already for some decades there has been an increasing interest in plant uses and in the detection of their antibacterial activity (Harvey, 2008). Some phytopatogenic bacteria were reported to be severe phytopathogens, causing damage to carrot, potato, tomato, leafy greens, onion, green pepper, squash and other cucurbits. Furthermore, these phytopathogens cause disease in any plant tissue they invade (Ahameethunisa and Hopper, 2010).

Pesticides have been universally considered for long as the most efficient solution to control crop diseases. However, synthetic pesticides may enter the food chain and the resistance developed by plant pathogens has rendered some of them ineffective. This has highlighted the need for the use of alternatives compounds that are environmentally friendly and safe to humans.

There has been a growing interest in the research of the possible use of the plant-derived natural pesticides such as plant extracts, which can be relatively ecofriendly for disease control in agriculture (Choi et al., 2008). Besides, the plants or plant extracts have long been recognized to provide a potential source of chemical compounds or more commonly products known as phytochemicals with potent antifungal efficacy (Choi et al., 2008). Research focused on plant-derived fungicides and their possible applications in agriculture are being intensified as these are having enormous potential to inspire and influence modern agrochemical research (Duke, 1990).

Plant secondary metabolites, such as essential oils and plant extracts are known to possess insecticidal, antifungal, acaricidal, antibacterial and cytotoxic activities (Tepe et al., 2004). Therefore, they have been intensively screened and applied in pharmacology, pharmaceutical botany, medical and clinical microbiology, plant pathology and food preservation (Daferera et al., 2000). Some plant extracts (Davidson et al., 1989) and essential oils (Bakkali et al., 2008) show activity against a wide range of bacteria.

Biopesticides have been suggested as an effective substitute for chemicals (Kapoor, 2001). Reports are available on the use of several plant by-products, which possess antimicrobial properties, on several pathogenic bacteria and fungi (Bylka et al., 2004; Kilani, 2006). Here, we evaluate the potential of several plant extracts for antibacterial activity against important phytopathogenic bacteria.

Brazil is a country rich in biodiversity. To date, 44,813 species are recognized for Brazilian flora: 4,594 algae, 32,391 angiosperms, 1,529 bryophytes, 5,028 fungi, 30 gymnosperms and 1,241 ferns and lycophytes. Brazil is a country rich in biodiversity. To date, 46,096 species are recognized for Brazilian flora: 4,747 algae, 32,817 angiosperms, 1,525 bryophytes, 5,711 fungi, 30 gymnosperms and 1,266 ferns and lycophytes (Brazilian Flora, 2016). The aim of this work was to investigate the antibacterial activity of twelve species belonging to seven botanical families, collected at Parque Nacional do Catimbau (PNC) and Reserva de Floresta Urbana Mata de Camaçari (RFUMC), located in the Northeastern Brazil (State of Pernambuco, Brazil) against phytopathogenic bacteria.

MATERIALS AND METHODS

Collection of plant material

Twelve species of plant, belonging to seven families, were collected from the various phytophysiognomy at the PNC and RFUMC in Northeastern Brazil (Pernambuco, Brazil) (Table 1). The species were collected based on their popular use by the local community, giving priority to species that had reproductive organs to facilitate their identification. As a part of a wider screening program, plants were randomly collected to increase the chance of finding plants with bioactive extracts. The plants were identified at the Herbarium from Instituto Agronômico de Pernambuco (IPA) and the scientific names were checked in the International Plant Names Index (http://www.ipni.org/ipni/plantnamesearchpage.do) and Brazilian Flora Checklist (http://floradobrasil.jbrj.gov.br/jabot/listaBrasil/ConsultaPublicaUC/ConsultaPublicaUC.do). Each collected plant sample was oven dried at 45°C with forced air for 72 h. The dry materials were ground to a fine powder.

Preparation of aqueous plant extracts

Extracts were prepared from dried plants' parts according to methods described by Azmir et al. (2013). The powdered plant materials were extracted at room temperature using water by maceration successively. Aqueous extraction was achieved by adding 100 ml distilled water to 10 g of plant powder and boiled for 72 h. The extract was then lyophilized. A sample of extract at 100 mg/mL was bioassayed, as described in bioassay section.

Test microorganisms

Plant pathogenic bacteria such as *Acidovorax citrulli, Pectobacterium carotovorum* subsp. *carotovorum, Ralstonia solanacearum* and *Xanthomonas campestris* pv. *campestris* were sampled from the culture collection of Departmento de Agronomia, Universidade Federal Rural de Pernambuco, Brazil. All the tested bacterial species were maintained on nutrient agar media.

Determination of minimum inhibitory and minimum bactericidal concentrations

Micro-dilution susceptibility assay was performed using the Clinical and Laboratory Standard Institute (CLSI) method for the

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Table 1. Plants employed in this study and their ethnobotanical information.

Family/Species	Voucher	Common name	Distribution	Traditional uses of plants
Anacardiaceae				
Anacardium humile A.St.-Hil.	IPA 84049	Caju do sertão	Atlantic Forest, Caatinga and Cerrado	The fruit in nature are used as food against anemia and as tonic. The juice of pseudo fruit is indicated against anemia and diabetes (Agra et al., 2007, 2008). The external use against burnings and ulcers (Agra et al. 2007, 2008). The decoction of stem-bark is used as bath against vaginal and external ulcers. The internal use is indicated against diarrheas (Agra et al., 2007, 2008). The topical use of resin is indicated against warts coughs and wounds (Agra et al., 2007, 2008)
Apocynaceae				
Allamanda blanchetii A.DC.*	IPA 84112	Quatro-patacas-roxa, leiteiro	Caatinga	The latex is used as laxative, emetic, cathartic and vermifuge. One teaspoon of the latex in a cup of water. It is drunk after meals. It is referred to as poisonous (Agra et al., 2007, 2008)
Aspidosperma pyrifolium Mart.*	IPA 85734	Pereiro	Caatinga and Cerrado	The stem-bark is used against inflammations of urinary tract. A decoction of a teaspoon in a cup of water. It is used as tea until the symptoms disappear. The same recipe as above. It is used in baths. Entire plant is referred as poisonous (Agra et al., 2007, 2008)
Burseraceae				
Commiphora leptophloes (Mart.) J.B.Gillett	IPA 84037	Umburana	Caatinga and Cerrado	The stem-bark is used in treatment of grippes, coughs, bronchitis, treat urinary and liver diseases (Agra et al., 2007, 2008). A decoction of a handful in a liter of water and made with sugar as syrup. A spoonful is drunk 5-6 times a day. The external use against ulcers in washes or baths against vaginal ulcers (Agra et al., 2007, 2008)
Fabaceae				
Anadenanthera colubrina var. cebil (Griseb.) Altschul*	IPA 84039	Angico, angico de caroço	Caatinga and Cerrado	The stem-bark is used against coughs, whooping coughs and bronchitis. A maceration of a handful in a liter of wine or “cachaça”. It is drunk until three times a day until the symptoms disappear (Agra et al., 2007, 2008). The fruits are used as narcotic and poison. In maceration or infusion of a handful in a liter of water. It drunk before sleep (Agra et al., 2007, 2008)
Stryphnodendron pulcherrimum (Willd.) Hochr.	IPA 85968	Barbatimão	Amazonic Forest and Atlantic Forest	The decoction is used against inflammations of uterus and for wash external ulcers (Oral communication)
Crotalaria holosericea Nees & Mart.*		-	Caatinga	Used in agriculture as a green manure. In the dry period is given to cattle as food (Oral communication)
Euphrobiaceae				
Croton pedicellatus Kunth.	IPA 85734	Alecrim	Caatinga and Cerrado	A decoction of leaves is used as antiseptic against dermatitis (Oral communication)
Jatropha mutabilis (Pohl)Baill.Δ	IPA 84054	Pinhão-bravo, pinhão manso	Caatinga and Cerrado	The latex is used to treat snake bites (Agra et al., 2007, 2008)

Table 1. Contd.

Myrtaceae				
Algrizea minor Sobral et al.*	IPA 84346	Murta	Caatinga and Cerrado	Used in folk by practitioners the infusion of the fleshy leaves for the treatment of pain and fever (Oral communication)
Eugenia brejoensis Mazine *	IPA 84033	Cutia	Atlantic Forest	Used in folk by practitioners the infusion of the fleshy leaves for the treatment of pain and fever (Oral communication)
Turneraceae				
Turnera cearensis Urb.*	IPA 85143	-	Atlantic Forest and Caatinga	Against amenorrhea and dysmenorrheal. A decoction of a handful in a liter of water (Oral communication)

* = Endemic specie of Brazilian flora. Δ = Unknown endemism.

determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) (CLSI, 2009). Bacteria were cultured overnight at 30°C. The test samples of the extracts were dissolved in 10% DMSO. Dilutions were prepared in a 96-well microtiter plates to get final concentrations ranging from 0 to 50 mg/ml. Finally, 20 µl of inoculum (10^6 to 10^{-7} CFU/ml) was inoculated onto the microplates and the tests were performed in a volume of 200 µl. Plates were incubated at 30°C for 24 h. The standard reference drug, chloramphenicol, was used as a positive control for the tested plant pathogenic bacteria. The lowest concentrations of tested samples, which did not show any visual growth after macroscopic evaluation, were determined as MICs, which were expressed in mg/mL. Using the results of the MIC assay, the concentrations showing complete absence of visual growth of bacteria were identified; 50 µl of each culture broth was transferred to the agar plates and incubated for the specified time and temperature as mentioned above. The complete absence of growth on the agar surface in the lowest concentration of sample was defined as the MBC. Each assay in this experiment was replicated three times.

RESULTS AND DISCUSSION

The minimum inhibitory concentrations (MICs) of 12 aqueous extracts obtained by microdilution technique against the four phytophathogenic bacteria are shown in Table 2. The antibacterial activity of the aqueous extracts showed varying magnitudes. All four bacterial strains tested were sensitive to all aqueous extracts, with the MIC values ranging from 3.12 to 25 mg/mL. The MIC values of *Anadenanthera colubrina* var. *cebil*, *Croton pedicellatus* and *Eugenia brejoensis* ranged from 3.12 to 12.5 mg/mL (Table 2). The comparison of MICs and MBCs values allows a better evaluation of antibacterial effect of bioactive compounds. According to Biyiti et al. (2004), a substance is bactericidal when the ratio MBC/MIC ≤ 2, and bacteriostatic if the ratio MBC/MIC > 2. Based on these data, the 12 extracts exert bactericidal effects against all bacteria evaluated.

Phytopathogenic bacterial infections are of great concern in agricultural practices, given that some strains are responsible for severe sickness and losses in appropriate climatic conditions. *Acidovorax*, *Pectobacterium*, *Ralstonia* and *Xanthomonas* are among the main phytopathogenic bacterial genera. Plants and plant products have shown to be useful candidates for prevention and control of phytopathogenic bacteria. Several studies have shown that the crude extracts and purified components of plants possess inhibitory activity against plant pathogenic bacteria (Iwu et al., 1999; Mohana and Raveesha, 2006; Kumaran and Karunakaran, 2007; Parekh and Chanda, 2007).

Plant extracts are rich in many phytocompounds which are the cause of their bioactivities. The mechanism of action of many antimicrobials is complex and may not be the consequence of their action on a single target. In addition, the phenomenon of membrane bleeding has been observed with several antimicrobial agents (Epand et al., 2008). For example, phenolic compounds make their actions through different mechanism, which includes membrane disruption, proteins binding, inhibition of proteins synthesis, enzyme inhibition, production of cell wall complexes, formation of disulfide bridges and intercalation with cell wall and/or DNA, among others (Bozdogan and Appelbaum, 2004). In the same manner, the antimicrobial action of alkaloids could be throughout intercalation with cell wall and/or DNA constituents; while, terpenoids display their action through membrane disruption mechanisms (Cowan, 1999).

The antimicrobial activity evaluated in this work could be attributed to the presence of different phytocompounds in variable amounts in plant extracts. The assayed antimicrobial activity from the plant species depends on the botanical species, age, part of the plant studied as well as the solvent used for the extraction procedures (Mahida and Mohan, 2006).

Table 2. Minimum inhibitory concentrations (MIC) and minimum bactericidal concentration (MBC) of aqueous extracts of medicinal plants of Caatinga against the growth of plant pathogenic bacteria.

Species	Phytopathogenic bacteria											
	Acidovorax citrulli			*Pectobacterium carotovorum* subsp. *carotovorum*			*Ralstonia solanacearum*			*Xanthomonas campestris* pv.*campestris*		
	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC
Algrizea minor	25	50	2	25	50	2	12.5	25	2	-	-	-
Allamanda blanchetii	12.5	12.5	1	12.5	25	2	-	-	-	6.25	6.25	1
Anacardium humile	12.5	12.5	1	12.5	12.5	1	-	-	-	-	-	-
Anadenanthera colubrina var. *cebil*	6.25	12.5	2	12.5	12.5	1	3.12	3.12	1	6.25	6.25	1
Aspidosperma pyrifolium	12.5	12.5		25	25	1	12.5	12.5	1	25	25	1
Commiphora leptophloes	12.5	25	2	25	25	1	12.5	12.5	1	-	-	-
Crotaria holosericea	12.5	12.5	1	12.5	25	2	6.25	12.5	2	12.5	25	2
Croton corchoropsis	12.5	12.5	1	25	25	1	3.12	6.25	2	12.5	12.5	1
Eugenia brejoensis	12.5	25	2	12.5	25	2	3.12	6.25	2	6.25	6.25	1
Jatropha mutabilis	12.5	12.5	1	12.5	25	2	-	-	-	12.5	25	2
Stryphnodendron pulcherrimum	12.5	25	2	25	25	1	6.25	6.25	1	6.25	6.25	1
Turnera cearencis	12.5	12.5	1	12.5	12.5	1	6.25	12.5	2	12.5	25	2
Chloramphenicol	0.39	0.39	1	0.39	0.39	1	0.19	0.39	2	0.39	0.39	1

Results indicate the presence of antibacterial compounds in aqueous plant extracts, which was in agreement with the results reported by authors who tested the aqueous plant extracts on different plant pathogens (Bahraminejad et al., 2011, 2012). The broad spectra of the inhibitory effect of *A. colubrina* var. *cebil* indicated that the extract of this species is potent antibacterial plants with possible potential for the control of different bacterial diseases in plants. Therefore, more research on the activity of this plant against the other plant pathogenic bacteria and fungi would be of great value.

All plants used in this study have not been tested before as inhibitor of phytopathogenic bacteria. Therefore, this is a new report. The results of the present investigation are successful in identifying the antibacterial activity of selected medicinal plants which will help in further identifying the nature of the bioactive principle and its solubility, isolation and characterization of the active principle responsible for the activity.

Field existences of antibiotic resistant phytopathogenic bacteria are increasing in recent years. The World Health Organization (WHO) banned many agriculturally important pesticides due to wide range of toxicity against non-target organisms including humans which are known to cause pollution problem (Barnard et al., 1997). Some of the developing countries are still using these pesticides despite their harmful effects. Exploitation of naturally available chemicals from plants, which retard the reproduction of undesirable microorganism, would be a more realistic and ecological method for plant protection and it will have a prominent role in the development of future commercial pesticides (Verma and Dubey, 1998; Gottlieb et al., 2002). Many reports of antibacterial activity of plants extract against human pathogens and their pharmaceutical application are available (Cowan, 1999; Gibbons, 2005), but not much has been done on the antibacterial activity of plants extract against plant pathogens (Satish et al., 1999). This is mainly due to lack of information on the screening/evaluation of diverse plants for their antibacterial potential.

Considering the rich diversity of Brazilian flora, it is expected that screening and scientific evaluation of plant extracts for their antimicrobial activity may provide new antimicrobial substances; hence in the present investigation the antibacterial investigation of all pants has been demonstrated for the first time against phytopathogenic

bacteria. Thus the present study reveals that *A. colubrina* var. *cebil*, *C. pedicellatus* and *E. brejoensis* is a potential candidate plant that could be successfully exploited for management of the diseases caused by different phytopathogens which are known to cause many diseases in wide variety of crops, causing considerable losses in yield and quality in an eco-friendly way.

These results and the encouraging percentage of plants with antibacterial activity (85% in this research) confirmed that plant extracts originated from Northeast in from Brazil can be used directly to develop new and effective classes of natural bactericide to control severe bacterial diseases. These findings persuaded us to continue screening more plant species.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Bacteriological quality and metal levels of boreholes and hand-dug well within the Golden Star Wassa mining areas in Ghana

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This study seeks to assess the bacteriological, physicochemical and some trace metals levels in water samples from seven boreholes and a hand-dug well within communities of Golden Star Wassa Mine Limited in Ghana. Five of the sampling sites were slightly acidic and were below the lower limit of the acceptable World Health Organization (WHO) and Ghana Standard Authority (GSA) permissible guidelines for pH. However, sampling from Kubekro well BH04, Akyempim BH07 and at New Akosombo BH06 reported pH mean of 6.9±0.352, 6.7±0.696 and 6.6±0.283, respectively. Well BH02 and that of BH06, respectively reported True Colour mean values of 36.5±6.097 and 17.4±1.930 true colour units (TCU) which were above the WHO/GSA permissible value of 15 TCU. Electrical conductivity, total dissolved solids (TDS), alkalinity, and total hardness were below their respective WHO/GSA permissible limits in the sampling sites with 100% compliance. Pb, Zn, As, Hg, Cu, and Fe recorded a marginal degree of non-compliance with their respective WHO/GSA guideline values of 0.01 mg/L for Pb, 3.0 mg/L for Zn, 0.01 mg/L for As, 0.006 mg/L for Hg and 0.3 mg/L for Fe in all the sampling sites except Cu. All the boreholes indicated a non-detectable microbial load (total coliforms and *Escherichia coli*) except the hand-dug well at Kubekro Well (BH02) which showed some amount of total coliform bacteria load of 6±6.594 coliforms forming unit (CFU). The mechanised borehole groundwater sources within the study area, except the well at BH02 is good for drinking, and hence have not been adversely impacted by mining operations baring the continuous impoundment of the gold processing tails or slurry.

Key words: Groundwater, physicochemical, microbial and trace metal parameters, sampling sites.

INTRODUCTION

Potable and adequate water supply services are a prerequisite for public health and water-quality index is also one of the most effective tools used in passing information on the quality of water to the concerned citizens and policy makers (Atulegwu and Njoku, 2004). Potability is therefore an important parameter for the

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assessment and management of water (Fagbote et al., 2014). Many mining companies recognize this as a critical commodity, but water use, which ultimately depends on the needs, the area, quantity and availability, has immense implication for the community. Groundwater quality evaluations in different parts of the world have been studied by various researchers in the last two decades (Gallardo and Tase, 2007; Partey et al., 2010). Shah et al. (2008) have compared groundwater quality in Gandhinagar Taluka in India with standard values given by World Health Organization (WHO, 2011) and have come up with a water-quality index for that area. Good quality of water resources depends on a large number of physico-chemical parameters and biological characteristics (Medudhula, 2012).

Many water resources in developing countries are unhealthy, because they contain harmful physical, chemical, and biological agents as a result of geological formation which may impact negatively on the water quality and thus affect human health (Aghazadeh and Mogaddam, 2010). In fact, heavy metal contamination of ground, stream and river water ecosystem is a worldwide environmental problem (Sekabira et al., 2010). Anthropogenic activities tend to impact negatively on aquatic ecosystems through alterations in hydrology, introduction of toxic chemicals and changes to other physicochemical and microbiological traits of water (Paul and Meyer, 2001; Asonye et al., 2007).

Water can be a risk issue for mine sites and its reliability in terms of scientific evaluation may be questioned (Brown, 2010).

Most mining communities in Ghana, depend mostly on well and boreholes as their main source of water. Generally, mining in Ghana is faced with a lot of environmental challenges such as water pollution arising from poor handling of ore processing tailings facilities and waste dump sites, land degradation through loss of vegetation cover and soil erosion (Smedley et al., 1996). As a result, there is growing awareness of mining activities that have been undertaken but with little concern for the environment. Groundwater quality may also be compromised as a result of anthropogenic activities close to boreholes and shallow hand dug wells. Poor sanitation, improper waste disposal, seepage of agrochemicals and mining have been observed to affect the quality of groundwater (Fianko et al., 2010; Jain et al., 2009). To date, no studies have specifically addressed these threats in relation to pit latrines spatial differences and groundwater quality. In view of this, there is still the need to qualitatively ascertain whether the ground water around the catchment communities of Golden Star Wassa Limited (GSWL) mine site is potable for drinking and useful for other domestic purposes.

The area under this study (Golden Star Wassa Limited Catchment Areas) has not been scientifically studied even though the communities around the catchment area largely depend on borehole water installed by the company for drinking and other domestic activities. The importance of the groundwater resources in the area should not be underestimated, because they are the only water resource for drinking. Despite the importance of boreholes and hand dug wells in the Golden Star Wassa Limited Mining Catchment Areas, little is known about the natural phenomena that govern the hydrochemical and bacteriological composition of the groundwater. Thus, there is the need to continuously assess the water quality of boreholes and hand dug wells in these catchment areas. The study focuses on groundwater quality provided by the GSWL to the communities in the catchment areas in order to address its suitability as drinking water with the standards provided by the WHO and that of the Ghana Standard Authority (GSA).

Table 1. Sample sites at Golden Star Wassa Limited (GSWL) used for water sampling.

Site code	Name of sampling location
BH02	Kubekro well
BH03	New Borehole at Kubekro
BH04	New Borehole at Togbekrom
BH05	Akyempim New Borehole
BH06	Borehole at New Akosombo
BH07	Akyempim Borehole near JSS school
BH08	Akyempim Borehole near station

MATERIALS AND METHODS

Golden Star Wassa Mine is located at 62 km north of Daboase, 35 km northeast of Tarkwa and 40 km east of Bogoso, in the Mpohor Wassa East District of the Western Region of Ghana. The project vicinity is predominantly rural and there are no main urban settlements within 50 km. The villages of Akyempim, Kubekro, Togbekrom, and the hamlet of Akosombo lie within the closest vicinity of the mine.

Sample collection

Sampling was conducted during the months of November and December, 2012 through to January, February, March and April 2013 from eight different sampling locations around the Golden Star Wassa (Akyempim) Limited (GSWL) listed in Table 1.

All the chemicals and reagents used were of analytical grade, BDH chemicals Limited, United Kingdom. Samples were collected in 500 ml capacity polythene bottle having doubly stopper. Prior to the collection, the well cleaned sample bottles were rinsed thoroughly with the sample water to be collected. Each sample bottle was clearly labeled and relevant details recorded. Water samples collected for metal analysis were preserved with 50% HNO_3 to attain a pH of 2 in order to keep the metal ions in the dissolved state and also to prevent microbial influence (APHA, 2005). At each sampling site, two samples were collected into 500 ml sterilized bottles. These samples were stored in an ice chest containing ice cubes and transported to the Ghana Water laboratory, Takoradi for analysis within 24 h.

Table 2. Physicochemical properties of water samples from seven boreholes and a hand-dug well in the Golden Star Wassa (Akyempim) mine catchment area.

Parameter		BH01	BH02	BH03	BH04	BH05	BH06	BH07	BH08	GSA/WHO Guidelines
pH	Mean	6.300	5.700	6.000	6.900	5.900	6.600	6.700	6.000	6.5-8.5
	SD	±0.434	±0.469	±0.322	±0.352	±0.494	±0.283	±0.696	±0.746	
EC (µS/cm)	Mean	298.300	309.700	208.300	207.000	330.600	207.000	146.100	245.000	1500
	SD	±93.698	±163.318	±52.355	±36.858	±150.421	±36.858	±38.391	±109.625	
TDS (mg/L)	Mean	346.800	105.100	172.800	194.000	168.900	454.000	73.600	135.700	1000
	SD	±99.438	±17.245	±38.627	±61.671	±58.826	±77.876	±29.366	±82.172	
TH (mg/L)	Mean	88.000	50.000	31.000	56.000	73.800	14.300	10.600	28.000	150
	SD	±13.251	±9.066	±4.136	±12.285	±11.414	±2.577	±1.524	±14.531	
Alkalinity (mg/L)	Mean	64.000	44.000	23.600	42.000	18.90	19.000	14.000	14.000	500
	SD	±12.163	±14.230	±2.925	±3.155	±3.637	±1.556	±3.042	±5.355	
True Colour (TCU)	Mean	8.400	36.500	11.800	9.700	14.300	17.400	4.500	12.000	15
	SD	±3.407	±6.097	±0.934	±0.607	±4.360	±1.930	±0.941	±6.758	

SD: Standard deviation.

Water analysis

All the samples were analyzed in the laboratory employing standard methods for physicochemical parameters (pH, electrical conductivity (EC), total dissolved solids (TDS), total alkalinity, total hardness and true colour), microbial properties (Total coliforms and *Escherichia coli*) and some trace metals (Pb, Zn, Hg, Fe, Cu and As). EC, TDS, and pH were measured using potable Orion 5 star sensor multiparameter analyzer from Orion instruments (Model No. Orin 5 Star, S/N: A03158). The physical and chemical analysis of water samples were based on APHA (2005). Heavy metals were analyzed using the Perkin Elmer Optima 5300 DV for Inductively Coupled Plasma-Atomic Emission Spectrometry analysis.

Statistical analysis

Statistical analyses were carried out using AqQA (version 1.1.1) water-quality software and SAS (version 9.2), MINITAB (version 14) and Rockworks (version 15), respectively.

RESULTS AND DISCUSSION

Physicochemical analysis

The physicochemical analysis of the groundwater samples of the eight sampling sites of Golden Star Wassa mine catchment communities was carried out and their mean variation concentrations are shown in Table 2 and Figures 1 and 2. The results showed that TDS mean value ranged from 73.6±29.366 mg/L at BH07 to 454.0±77.876 mg/L at BH06. Even though a health-based value has not been proposed by the WHO, however, a TDS above 1000 mg/L may be objectionable to consumers (Amoako et al., 2011). The EC showed a maximum EC of 330.6±150.421 µS/cm at the BH05 site with a minimum of 146.1±38.391 at BHO7. The low conductivity level is an indication of low levels of dissolved ions in the ground water within the project vicinity. Mean values reported for EC and TDS, respectively are much below the WHO (2011) and GSA (2009) compliance limit of 1500 µS/cm and 1000 mg/L, respectively (Table 2), giving a compliance percentage of 100% for both parameters. The large variation in TDS values may be attributed to the variation in geological formations, hydrological processes, and the prevailing mining conditions in the region (Liu et al., 2012).

The mean total hardness ranges from 10.6±1.524 mg/L at BH07 to 88.0±13.251 mg/L at site BH01. Singh et al. (2012) have stated that hardness of water mainly depends upon the amount of calcium or magnesium salt or both. It is also an important criterion for determining the usability of water for domestic, drinking and many industrial supplies (Mitharwal et al., 2009). The relative lower values recorded in this study for the hardness of water may be due to the presence of lower concentrations of dissolved calcium and magnesium in these water sources.

Ayers and Westcot (1985) reported that the pH of the water is always an I ndicator of its quality and normally

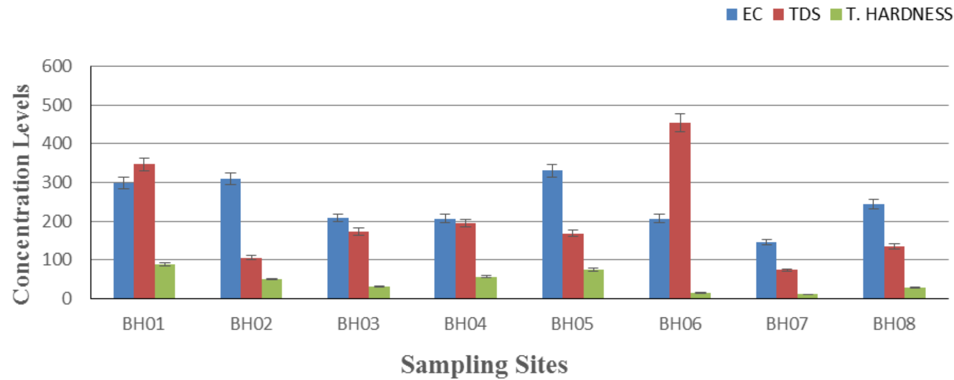

Figure 1. Variation of mean Electrical Conductivity, Total Dissolve Solids and Total Hardness concentration in boreholes and hand dug well.

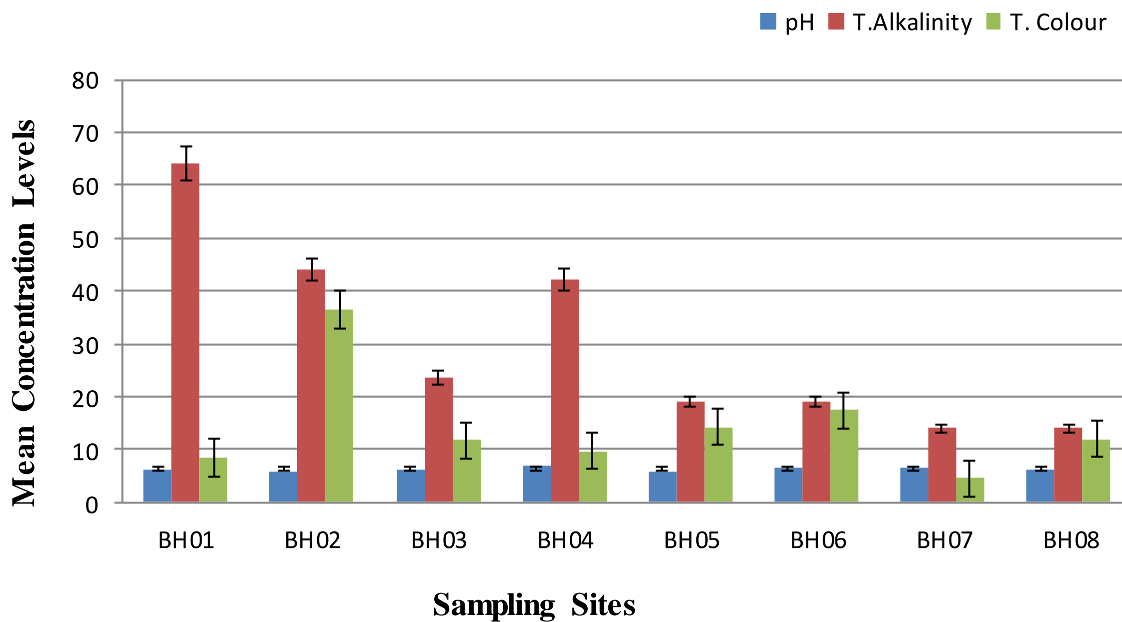

Figure 2. Variation of mean pH, True colour and Total Alkalinity in boreholes and hand dug well.

ranges from 6.5 to 8.4. The study showed that pH varied between 5.7±0.469 at BH02 and 6.9±0.283 at BH04 (Table 2 and Figure 2). Five of the eight sampling sites were slightly acidic and were below the lower limits of WHO (2011) and that of GSA (2009). This could largely be attributed to the geology of the area since the baseline assessment confirms acidic nature of the area, hence, a non-compliance regime of 62.5%. Mitharwal et al. (2009) also reported that the pH of water is a very important indicator of its quality and provides information in many types of geochemical equilibrium.

Results from the analysis of the true colour indicated that two out of the eight sampling sites showed a mean concentration values of 36.5±6.097 TCU at BH02 and 17.4±1.9930 TCU at BH06 (Table 2 and Figure 2). The maximum permissible concentration of true colour for drinking water is 15TCU, based on taste considerations (WHO, 2011). Alkalinity ($CaCO_3$) is the capacity of a solution to neutralise acids. The present study revealed that the mean values for total alkalinity ($CaCO_3$) measured ranges from 14.0±3.042 and 14.0±5.355 mg/L, respectively at BH07 and BH08 to 64.0±12.163 mg/L at BH01. Generally, all the sampling sites analyzed achieved 100% compliance with the WHO (2000) and GSA (2009). This is expected since pH is positively correlated with conductivity and total alkalinity (Figures 1 and 2). Specifically, the results from the environmental baseline assessment of the Wassa mine suggest that the

Table 3. Trace metal levels in water samples from seven boreholes and a hand-dug well in the Golden Star Wassa (Akyempim) mine catchment area.

Parameter		BH01	BH02	BH03	BH04	BH05	BH06	BH07	BH08	GSA/WHO Guidelines
Pb	Mean	0.001	0.001	0.001	0.020	0.040	0.001	0.002	0.004	0.01
	SD	±0.000	±0.001	±0.000	±0.000	±0.011	±0.000	±0.000	±0.000	
Zn	Mean	1.540	3.760	2.340	4.670	0.200	1.890	0.140	0.042	3.0
	SD	±0.363	±1.630	±0.932	±1.780	±0.115	±0.157	±0.103	±0.029	
Hg	Mean	0.010	0.001	0.010	0.020	0.001	0.001	0.001	0.001	0.006
	SD	±0.000	±0.000	±0.000	±0.000	±0.000	±0.000	±0.000	±0.000	
Fe	Mean	0.010	3.800	0.001	0.250	0.025	0.020	7.480	0.010	0.3
	SD	±0.000	±1.686	±0.000	±0.215	±0.014	±0.011	±2.977	±0.000	
Cu	Mean	1.800	1.750	1.200	0.120	0.100	0.020	0.340	0.020	2.0
	SD	±0.543	±0.868	±0.497	±0.093	±0.056	±0.000	±0.268	±0.014	
As	Mean	0.020	0.001	0.010	0.010	0.001	0.010	0.001	0.001	0.01
	SD	±0.006	±0.000	±0.000	±0.000	±0.000	±0.000	±0.000	±0.000	

SD: Standard deviation.

soils have been mostly developed on weathered products on lower Birimian rocks (GSWL EMP, 2010). Domenico (1972) reported that hydrochemical properties of groundwater also depend on lithology, regional flow pattern of water, and resident time.

Trace metal analysis

The concentration of trace metals (Pb, Zn, Hg, Fe, Cu and As) was analyzed in the groundwater samples collected from the sampling sites (Table 3). For the protection of human health, guidelines for the presence of heavy metals in drinking water have been set by different International Organizations such as USEPA, WHO, EPA, and the European Union Commission (Min et al., 1996; Anyakora and Momodu, 2010). Heavy metals have maximum acceptable concentration in drinking water as specified by these organizations. In this study, only Cu showed 100% compliance with the GSA (2009) and WHO (2011) permissible guidelines. High levels of copper in drinking water can cause vomiting, abdominal pain, nausea, diarrhea and have been reported that copper leached into drinking water from copper pipes (DTMRP, 2001). On the other hand, Pb, Zn, Hg, Fe and As indicated traces of non-compliance (Table 3).

According to Smedley et al. (2002), under natural conditions, the greatest range and highest concentrations of As are found in groundwater as a result of the favourable conditions for As mobilization and accumulation. In the present investigation, three of the sampling locations analyzed (BH01, BH03 and BH04) reported a non-compliance mean Hg concentration of 37.5% (Table 3). These sites are reportedly associated with the historical gold ore processing with mercury from anti-sanal activities in the study area. Asklund and Eldvall (2005) reported that sorption can considerably lower the metal concentration like Fe in ground water. The high concentration of Fe in the BH02 and BH07 samples is evidence of richness in naturally occurring Fe concentration in those locations (geology) which through dissolution and infiltration gets into underground water. Even though 75% of all the sampled locations reported compliance with the Maximum Compliance Limit (MCL) of WHO (2011) and GSA (2009) of 0.010 mg/L for Pb, there were marginally high levels of Pb recorded in BH04 and BH05. These results raise much concern since lead is known to be a poisonous metal that can damage nervous connections and cause blood and brain disorders (Ehi-Eromosele and Okiei, 2012). Nkansah et al. (2011) report that lead in water resources is mostly attributed to haphazard disposal of waste from lead containing substances. The mean concentration of Zn for six sampled locations showed values of 75% compliance with the WHO (2011) and GSA (2009) maximum permissible limit of 3.0 mg/L for Zn. Recorded exceedance of Zn in boreholes at BH02 and BH04 might be as a result of the mobilization of Zn in seepage from waste dump and surface water sources which are facilitated by high levels of iron presence.

Table 4. Mean concentrations of metals in water sampled from seven boreholes and the hand-dug well.

Parameter (CFU/100 ml)		BH01	BH02	BH03	BH04	BH05	BH06	BH07	BH08
Total coliforms	Mean	ND	6.000	ND	ND	ND	ND	ND	ND
	SD	-	±6.594	-	-	-	-	-	-
E. coli	Mean	ND	ND	ND	ND	ND	ND	ND	ND
	SD	-	-	-	-	-	-	-	-

ND: Not detected.

Bacteriological analysis

The results of the bacteriological analysis revealed that no coliform was detected in the seven sampled boreholes (Table 4). However, the hand-dug well at BH02 recorded a mean total coliform value of 6±6.594 CFU/100 ml, indicating non-conformity with the WHO (2011) and GSA (2009) guidelines of 0 CFU/100 ml. This might be due to septic pits and latrines in the vicinity that had extended their influence on water qualities. Cairncross and Cliff (1987) have shown that soakage pits and pit latrines can extend their influence on ground-water quality up to 10 m or more as groundwater flow is either lateral or vertical. This is of concern since water from the hand-dug well may contain a microbiological agent that may pose a health problem and that some action is needed (Brian, 2012). It is however, important to note that coliform bacteria are widely found in nature and do not necessarily indicate faecal pollution (Binnie et al., 2002; Griffith et al., 2003). Water from deep boreholes is normally free of pathogenic microbial contaminants due to the relatively slow subsurface movement of water compared to water sources like deep wells which are open and heavily polluted (Zvidzai et al., 2007).

Conclusion

The results showed that only Cu showed 100% compliance with the WHO (2011) and GSA (2009) permissible guidelines while Pb, Zn, Hg, Fe and As indicated traces of non-compliance as follows, respectively; 25% for Pb, Zn and Fe, respectively, 37.5% for Hg and 12.5% for As. It was also observed that the mechanised borehole groundwater sources within the study area is good for drinking in its bacteriological, chemical and metal content and hence have not been adversely impacted on by the mining operations baring the continuous impoundment of the gold processing tails or slurry. In spite of this, the hand dug well at BH02 should be monitored regularly in terms of spatial difference between the refuse dump and the proximity of the closest household latrine near the well site to ensure compliance with the WHO/GSA guideline for quality and potable water.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Studies on leukocidins toxins and antimicrobial resistance in *Staphylococcus aureus* isolated from various clinical sources

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Staphylococcus aureus **toxins represent a public health challenge all over the world. This study aims to analyze the prevalence of genes encoding the staphylococcal leukocidins and their correlation with antimicrobial susceptibility and the source of isolation. For this purpose, the susceptibility of 75** ***S. aureus*** **isolates to 12 antimicrobial agents was investigated. The leukocidins genes (*lukD, lukE, lukF* and *lukS*) were detected by polymerase chain reaction (PCR). The ability to express these genes was assessed among 20 isolates by RT-PCR. The most prevalent *luk* genes were *lukF* gene (73.3%), followed by *lukE* (64%), *lukD* (44%) and *lukS* (34.7%). Expression of *lukD, lukE* and *lukS* genes were variable. *lukF* gene was not expressed by any of the tested isolates. A statistically significant association was found between *lukF* occurrence and burn isolates. Besides that, *lukF* gene was more prevalent among amoxicillin-clavulenic acid and amikacin resistant isolates, while *lukE* was predominant with gentamicin resistant isolates. High expression level of *lukD* was found in MRSA and MDR isolates.**

Key words: *Staphylococcus aureus*, leukocidins, antimicrobial sensitivity.

INTRODUCTION

Staphylococcus aureus is one of the important human pathogens that causes wide varieties of diseases, ranging from skin infection to bacteraemia and infective endocarditis, beside toxin-mediated diseases (Lowy, 1998). More than 30 extracellular products are produced by *S. aureus* (Rogolsky, 1979). Almost all strains secrete a group of cytotoxins and enzymes such as nucleases, haemolysins, lipases, collagenases, proteases and hyaluronidase. Some strains produce additional exoproteins, which include leukocidins, toxic shock syndrome toxin (TSST-1), the exfoliative toxins and the staphylococcal enterotoxins. Leukocidins and γ-haemolysin are members of a toxin family known as synergohymenotropic toxins, as they act by the synergy of two proteins to form a pore on cell membranes (Dinges et al., 2000).

γ-Hemolysins (*Hlg*) and leukocidins (*Luk*) consist of two classes: F class (molecular weight of about 34 kDa)

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HlgB, *LukD*, *LukF*, *LukF-R*, *LukF-PV*, *LukM* and S class (molecular weight of about 32 kDa) *HlgA*, *HlgC*, *LukE*, *LukS*, *LukS-PV* and *LukS-R*. The two classes are important for toxin biological activity (Choorit et al., 1995; Gravet et al., 1998; Qiu et al., 2010).

Most *S. aureus* strains cause necrotizing pneumonia and primary skin infections harbor the Panton-Valentine leukocidin (PVL) determinant (Lina et al., 1999; Gillet et al., 2002). PVL genes were detected of strains associated with finger-pulp infection, cutaneous abscess, cellulitis and furunculosis while they were absent in superficial folliculitis and impetigo associated strains (Lina et al., 1999).

Staphylococcal leukocidins are leukolytic toxins active against human and rabbit macrophages, monocytes and polymorphonuclear cells forming cation-selective transmembrane pores in leukocytes and macrophages. Its mechanism is as follow: three S class molecules of the toxin bind to the specific receptor in the cell membrane leading to conformational changes that allow three F class molecules to bind to the formed S subunit-receptor complex to form a hexameric pore. The formed pore leads to the influx of divalent cations into the cell which stimulates enzymes and inflammatory mediators' secretion then cell lysis (Konig et al., 1994, 1997). The spread of bacterial infection is mediated by the inhibition of phagocyte and macrophage activity due to simultaneous secretion of various leukocidins and hemolysins. Staphylococcal leukocidins has leukotoxic activity (Konig et al., 1997).

In this study, the authors aimed to (i) detect leukocidins genes in *S. aureus* by PCR and evaluate their distribution among isolates from different clinical sources; (ii) determine Leukocidins expression by RT-PCR and (iii) assess the correlation between presence of leukocidins genes and antimicrobial susceptibility. A complete survey on leukocidins and their expression has not been recorded in the Egyptian available literature.

MATERIALS AND METHODS

Study population

In this study, 75 strains of S. aureus were collected during a period of 10 months between January 2014 – October 2014 [19 isolates from Mansoura University Children Hospital (MUCH), 11 isolates from Burn and Cosmetic Center (BCC), 31 isolates from Internal Medicine Hospital (IMH) and 14 isolates from Microbiology and Immunity Unit, Faculty of Medicine, Mansoura University]. These isolates were isolated from wounds, burns and sputum.

Following isolation, identification of isolates according to Collee et al. (1996). In this respect, isolates were tested for growth on mannitol salt agar, Gram reaction, catalase production, free and bound coagulase production. The experimental protocol conducted in this study was approved by the Ethics Committee of Faculty of Pharmacy, Mansoura University with code (2015-60). Participants provided their written informed consent to participate in this study. The age of participants for the study ranged between 13-45 years. Written informed consent was obtained from the next of kin, caretakers or guardians on behalf of the minors/children enrolled in our study. Ethics Committee of Faculty of Pharmacy, Mansoura University approved the usage of minors under the age of 18 in the study and approved the consent protocol used for them.

Antibiotic susceptibility test

S. aureus isolates were screened for susceptibility to 12 antimicrobial discs namely; penicillins (oxacillin, 1 µg), cephalosporins (cefoxitin 30 µg, cefotaxime 30 µg, ceftazidime 30 µg, cefepime 30 µg, cephalothin 30 µg), carbapenems (Imipenem 10 µg), β-Lactams combinations (amoxacillin-clavulanic 30 µg, ampicillin-sulbactam 10 µg), aminoglycosides (gentamicin 10 µg, amikacin 30 µg) and quinolones (ciprofloxacin 5 µg) using the standard disc diffusion method and Muller Hinton agar plates (Hoseini Alfatemi et al., 2014). All discs and media were supplied by Oxoid products, UK. Iinterpretation of data was done according to CLSI (2014).

For methicillin resistant *S. aureus* (MRSA) detection, cefoxitin (30 µg) disk was used, where isolates with inhibition zone diameter ≥ 21 were identified as MRSA (CLSI, 2014).

Molecular techniques

Preparation of the total DNA content of S. aureus isolates

Isolates were grown overnight at 37°C, then a single colony from each isolate was mixed with 100 µl DNase/RNase-free water to obtain a turbid suspension that was held in a boiling water-bath for 10 min, chilled on ice and centrifuged. For PCR reactions, 5 µl of extracted template DNA were used (Englen and Kelley, 2000).

PCR for leukocidins and mecA genes detection

The leukocidins toxins genes (*lukF, lukS, lukD* and *lukE*) and methicillin resistance gene (*mecA*) were detected using singleplex PCR reactions (FPROGO2D, Tchne LTD, Oxford Cambridge, U.K.) and specific primers listed in Table 1. The PCR reaction was performed as described previously in Hassan et al. (2012). The program was started with initial denaturation step at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing (at the specified temperature for each primer as indicated in Table 1 for 40 s, and extension at 72°C for 1 min followed by final extension at 72°C for 5 min. Negative control was included in each reaction using, ddH$_2$O instead of DNA extract. The amplified genes were visualized using 2% agarose gel electrophoresis stained with ethidium bromide and compared with a 100 base pair plus (bp) DNA ladder (Thermo scientific). The presence of a band at the expected product size was considered a positive result.

RNA isolation

RNA was isolated from 20 isolates. Isolates that harbored 2 or more of the tested toxin genes as detected by PCR and isolates sharing the same resistance and toxin gene pattern were selected. RNA was isolated according to Abdel-Rhman (2016) using glass beads and TRI Reagent (Sigma-Aldrich).

The concentration and the purity of RNA for each strain were determined spectrophotometrically by NanoDrop (ND-1000 Spectrophotometer, NanoDrop Technologies, Wilmington, Delaware, USA) using A260 and A260/280 nm ratio respectively. RNA was considered pure if it has a A260/280 ratio of 1.8:2.1.

Expression of Luk genes

Isolated RNA was used for preparation of complementary DNA

Table 1. Oligonucleotide primers sequences used to amplify the tested genes.

Gene name	Sequence		Annealing temperature (°C)	Amplicon size (bp)	References
lukE	Fw	5'-TGCGTAAATACCAGTTCTAGGG-3'	60	199	This study
	Rv	5'-TCCAACAGGTTCAGCAAGAG-3'			
lukD	Fw	5'-ACCAGCATTTGAACTACTTTGT-3'	60	240	This study
	Rv	5'-TCTAATGGCTTATCAGGTGGAT-3'			
lukF	Fw	5'-TGTGCTTCTACTTTCCACCAT-3'	54	225	This study
	Rv	5'-TGTGACTGACTTTGCACCA-3'			
lukS	Fw	5'-GGTCCATCAACAGGAGGTAAT-3'	57	267	This study
	Rv	5'-AGGATTGAAACCACTGTGTACT-3'			
mecA	Fw	5'-TGCTATCCACCCTCAAACAGG-3'	57	286	(Kondo et al., 2007
	Rv	5'-AACGTTGTAACCACCCCAAGA-3'			
nuc	Fw	5'-GCGATTGATGGTGATACGGTI-3',	55	267	Brakstad et al., 1992
	Rv	5'-CCAAGCCTTGACGAACTAAAGC-3'			

Fw: Forward primer Rv: reverse primer.

using Quanti-Tect Reverse Transcription kit (QIAGEN, Germany). RT-PCR was performed using Rotor Gene Q thermocycler (QIAGEN, Hilden, Germany) and 5X FIREPol Eva Green, qPCR Mix, ROX Dye (Solis Bio-Dyne, Tartu, Estonia) using the same primers described previously. Program was performed as follows: 95°C for 15 min, then 35 cycles x (denaturation at 95°C for 15 s, annealing as the specified temperature for 30 s and extension at 72°C for 1 min). Target genes expression was normalized to the reference gene *nuc* (encoding nuclease enzyme) expression. The gene expression level in samples was calculated relative to the housekeeping gene using a calibrator sample by the comparative (ΔΔct) method (El-Mowafy et al., 2014). All measurements were performed in triplicate.

Statistical analysis

Correlations between data were statistically analyzed using the Graphpad Instat 3. Fisher's exact test was used to evaluate these correlations where a P value ≤0.05 was considered statistically significant.

RESULTS

Bacterial isolates

A total of 200 samples were collected from different Mansoura hospitals. Seventy five isolates were identified as *S. aureus*. The clinical origins of these isolates were wound (W, 47 isolates), burn (B, 16 isolates) and sputum (S, 12 isolates).

Antimicrobial susceptibility test

In the present study, *S. aureus* isolates showed variable resistance towards the 12 antimicrobial agents used as shown in Figure 1. The most effective antibiotic was imipenem as 83% of isolates were sensitive, while ceftazidime was the least effective one as only 8% of isolates were sensitive to it. Methicillin resistance was recorded in 55 isolates (73.3%). Thirty nine isolates were multidrug resistant (MDR) (resistant to 3 or more classes of antimicrobials). The number of antimicrobials resistance per isolate is illustrated in Table 2.

PCR detection of tested genes

The virulence genes *luk D, luk E, luk S, luk F* and resistance gene *mecA* were amplified from total DNA extracts. Results showed that both *lukF* and *mecA* genes were the predominant genes as they were found in 55 isolates (73.3%), *LukE, lukD* and *lukS* genes were harbored by 64, 44 and 34.6%, respectively.

The toxin gene profile is illustrated in Table 3. The 75 isolates demonstrated 13 different toxin patterns. The most common pattern was T8 which was represented by 16% of isolates followed by patterns T10 and T12 (12 and 10.6% of isolates, respectively).

RT-PCR analysis of *S. aureus* leukocidins

Relative expression of luk genes were evaluated among tested isolates. The standard curve of the housekeeping gene *nuc* and all expressed genes including *lukD, lukE, lukF* and *lukS* showed R^2 values of 0.97-0.99. They showed the same melting profile of pure amplicons which indicated the assay specificity. The relative expression levels of *luk* genes were analyzed using the comparative method ($2^{-\Delta\Delta Ct}$) method.

The relative expression of these genes was investigated in 20 isolates. All isolates showed expression of the housekeeping gene (*nuc*). *lukD* gene

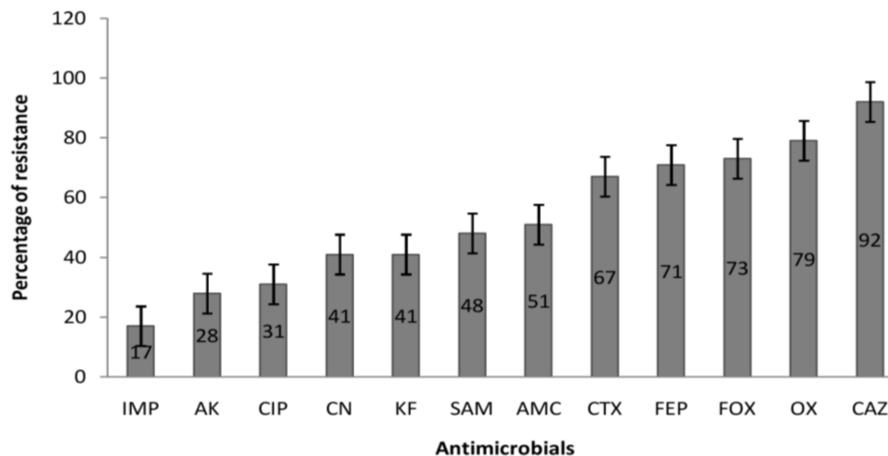

Figure 1. The percentage of resistance to different antimicrobials. AK, Amikacin; AMC, Amoxacillin-Clavulanic; CAZ, Ceftazidime; CIP, Ciprofloxacin FEP, Cefepime; CN, Gentamicin; CTX, Cefotaxime; FOX, Cefoxitin; IPM, Imipenem; KF, Cephalothin; OX, Oxacillin; SAM, Ampicillin-Sulbactam.

Table 2. Multidrug resistance profile of MSSA and MRSA to different antimicrobials.

Number of antimicrobials / isolate	No of isolates		Total	P value
	MSSA (n=20)	MRSA (n=55)		
1	0	0	0 (0%)	
2	2	0	2 (2.6%)	0.0685
3	6	2	8 (10.6%)	0.0037*
4	5	6	11 (14.6%)	0.1498
5	2	12	14 (18.6%)	0.3281
6	4	8	12 (16%)	0.7225
7	1	11	12 (16%)	0.1636
8	0	4	4 (5.3%)	0.5683
9	0	1	1 (1.3%)	1.0000
10	0	2	2 (2.6%)	1.0000
11	0	4	4 (5.3%)	0.5683
12	0	5	5 (6.6%)	0.3160

was expressed in 14/19 isolates (Figure 2). *lukE* gene was expressed in 13/16 of isolates (Figure 3). *lukS* was expressed in 10/13 of isolates (Figure 4). Although, *lukF* was detected in the 20 isolates tested by PCR, it was not expressed by any of them.

DISCUSSION

S. aureus is an important human pathogen causing nosocomial and community acquired infections (Taiwo et al., 2005). The resistance to antimicrobial agents among *S. aureus* is a growing problem worldwide. Multidrug-resistant staphylococci is a problem for human health. Infections caused by MRSA is a challenge for healthcare institutions (Kurlenda et al., 2009). Besides that, the emergence of virulent MRSA is a serious problem in the treatment and control of staphylococcal infections (Livermore, 2000; Zapun et al., 2008; Duran et al., 2012).

In this study, a total of 75 isolates were isolated from various clinical sources (wound, burn and sputum). These isolates were investigated for their antimicrobial sensitivity to 12 antimicrobials.

For β-lactam class, our results illustrated that imipenem showed the highest activity as only 17% of isolates were resistant in contrast to 64% resistant isolates reported by Kholeif and Mohamed (2009). Cephalosporins showed different activity on *S. auerus* according to its generation. The first generation cephalosporins (cephalothin) showed higher activity toward the isolates (41% of isolates were

Table 3. The toxin gene profile among *S. aureus* isolates.

Toxin pattern	Toxin profile	MDR	NMDR	Total
T1	No toxin	2	4	6
T2	*lukD*	2	1	3
T3	*lukE*	1	3	4
T4	*lukF*	4	3	7
T5	*lukD, lukE*	2	4	6
T6	*lukD, lukF*	1	1	2
T7	*lukE, lukS*	1	1	2
T8	*lukE, lukF*	9	3	12
T9	*lukF, lukS*	2	3	5
T10	*lukD, lukE, lukF*	7	2	9
T11	*lukD, lukF, lukS*	2	2	4
T12	*lukE, lukF, lukS*	4	4	8
T13	*lukD, lukE, lukF, lukS*	2	5	7

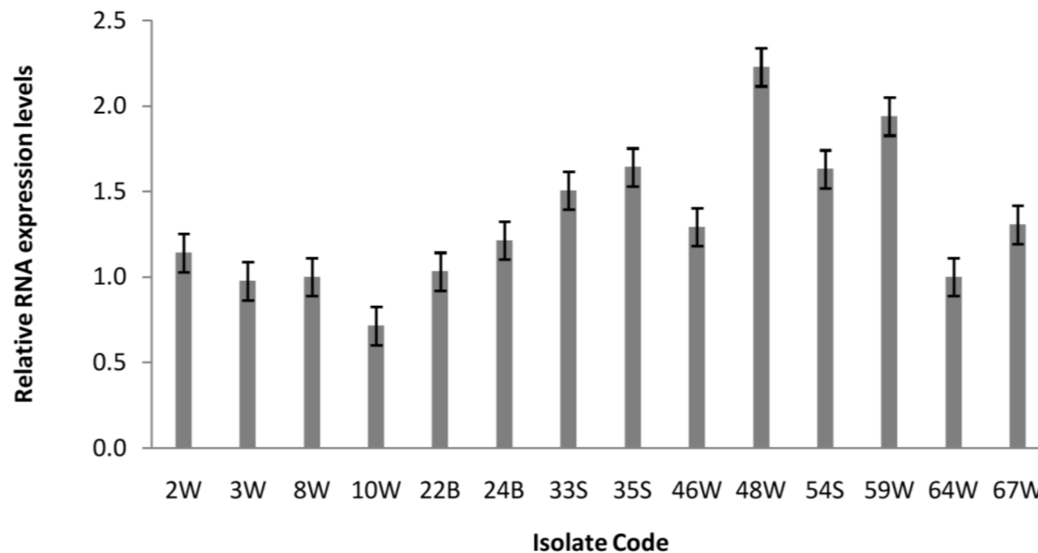

Figure 2. Relative *lukD* gene expression levels calculated by the comparative (ΔΔct) method using *nuc* gene as endogenous reference gene.

resistant) and the resistance increases with higher generations of cephalosporin as ceftazidime and cefotaxime (2nd generation) showed high resistance (92 and 67%, respectively). For the 4th generation (cefepime), 71% of isolates were resistant. A previous study was consistent with our results as it showed that 68.4% of isolates were resistant to cefotaxime (Onelum et al., 2015). The study conducted by Kholeif and Mohamed (2009) reported similar results concerning cefepime and cefotaxime while for cephalothin, it showed higher resistance (67%) than ours. On the other hand, another study reported a lower resistance level to cefepime (8%) (Siddiqui et al., 2013).

The resistance of isolates towards β-lactam combinations (ampicillin-sulbactam and amoxicillin – clavulenic acid) was intermediate (48 and 51% respectively). However, Duran et al. (2012) reported low percentage of resistance (23%) to amoxicillin–clavulenic acid. For ampicillin-sulbactam, all tested isolates were sensitive in the study carried out by Ali et al. (2013).

In this study, amikacin showed higher activity toward isolates than gentamicin (28:41%). Thirty one percentage isolates were resistant to ciprofloxacin. This was in accordance with Duran et al. (2012) who reported similar results concerning gentamicin and ciprofloxacin. In contrast, Kitara et al. (2011) showed that all *S. aureus* isolates were sensitive to gentamicin and only 1.6% were resistant to ciprofloxacin.

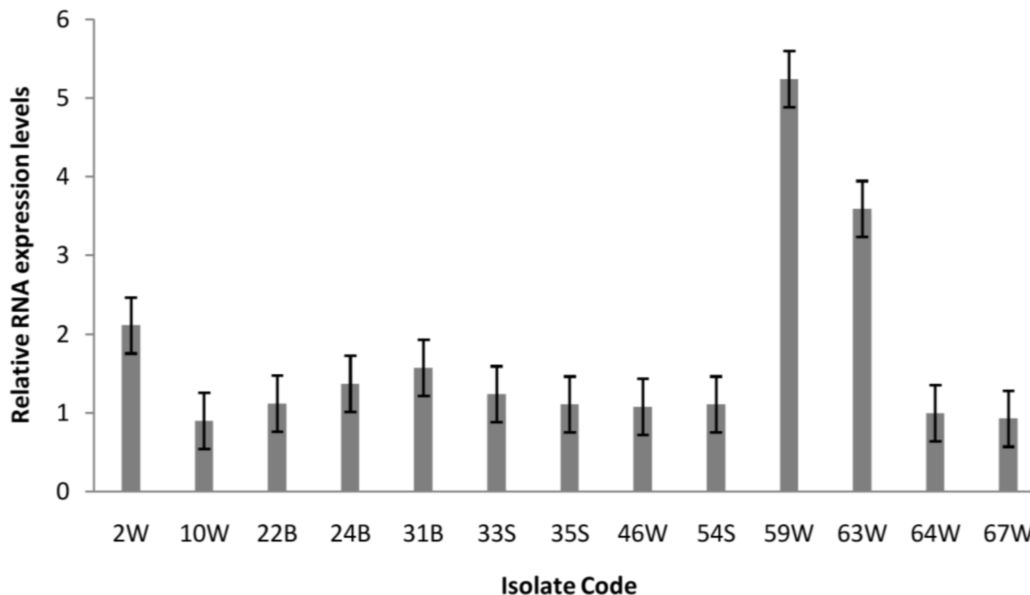

Figure 3. Relative *lukE* gene expression levels calculated by the comparative (ΔΔct) method using *nuc* gene as endogenous reference gene.

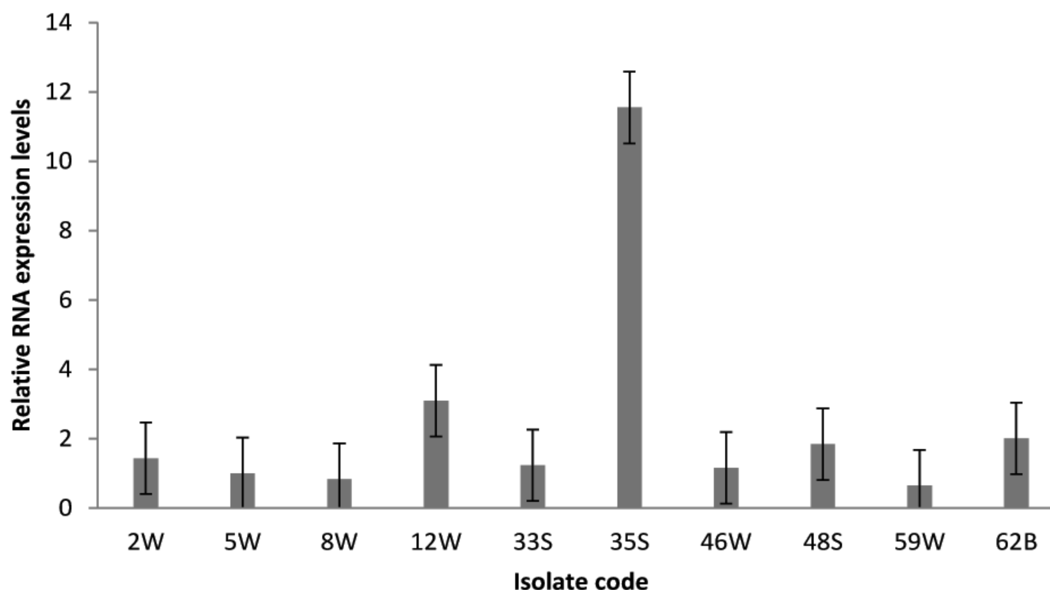

Figure 4. Relative *lukS* gene expression levels calculated by the comparative (ΔΔct) method using *nuc* gene as endogenous reference gene.

Concerning cefoxitin disc, 73.3% (55) of the isolates were found to be MRSA. For oxacillin, there were 59 isolates found to be MRSA. These results emphasize that cefoxitin is superior to oxacillin as an indicator of MRSA. Various studies illustrated that the cefoxitin is more sensitive than oxacillin for MRSA detection by disc diffusion method (Skov et al., 2003; Boutiba-Ben Boubaker et al., 2004; Velasco et al., 2005), this may be explained by cefoxitin is an inducer of the *mecA* gene resulting in increased expression of the *mecA*-encoded protein PBP2a (Velasco et al., 2005). The relation between methicillin resistance and clinical sources of *S. aureus* isolates was investigated where only isolates from wounds were significantly associated with MRSA (P value= 0.0136).

Comparing antimicrobial resistance profile of MRSA and MSSA isolates, it was found that resistance to amoxicillin-clavulenic acid, amikacin, imipenem and

cephalothin was significantly correlated to MRSA isolates with P value = 0.001, 0.0018, 0.0154 and 0.0073, respectively. Only isolates resistant to 3 antimicrobials was associated significantly to MSSA (p value= 0.0037).

S. aureus possess a remarkable ability to acquire resistance to multiple antibiotics (Jung et al., 2015). Thirty nine *S. aureus* isolates were MDR, 87% of them were MRSA. High level of antibiotic abuse leads to MDR. Many reasons lead to the high level of antibiotic abuse as the self-medication is associated with inappropriate dosage and failure to comply to treatment (Melles et al., 2006). In addition, the use of the over the counter medication with or without prescription increases the antibiotic abuse (Miller et al., 2005; Kholeif and Mohamed, 2009).

In the present study*, S. aureus* isolates were investigated for their leukocidins genes (*lukD, lukE, lukF* and *lukS*) and resistance gene (*mecA*) by PCR. The results show that 55 isolates harbored *mecA* gene. This result was consistent with cefoxitin disc results. The current gold standard for MRSA detection is identification of the *mecA* gene that cannot accurately be measured by disc diffusion or microdilution of oxacillin (Velasco et al., 2005).

In the present work, *lukD* and *luk E,* genes were amplified in 33 (44%) and 48 (64%) of isolates, respectively. The prevelance of *lukD-lukE* was variable in literatures where in a study performed in USA, it was 36.5% (Abdalrahman and Fakhr, 2015). In another study, 82.8%, of isolates were *lukD-lukE* positive (de Almeida et al., 2013).

Concerning *lukF* and *lukS* genes, they were detected in 55 (73.3%) and 26 (34.7%) isolates, respectively. This was consistent with a study performed in northeast Thailand hospitals, as nearly 50% of *S. aureus* isolates were PVL gene positive (Nickerson et al., 2009). In contrast, PVL positive isolates in Malaysia and Bangladesh were 5 and 4.3%, respectively (Neela et al., 2009). A study conducted in Egypt reported that detection of PVL gene was positive in only 23 isolates (39.7%) (Kholeif and Mohamed, 2009).

It was stated that *lukD-lukE* were present at higher percentage than PVL toxins (56:19%) (Baba-Moussa et al., 2010), while their incidence in the present study among isolates was nearly the same (31.4: 34.3%). No correlation was found between the *luk* genes and the isolation source except for *lukF* that was significantly associated with isolates of burn source (P value= 0.046). The *luk* genes were predominant in MRSA with no significant association except for *lukF* that was significantly associated with MRSA (P value= 0.0089). This was consistent with Larsen et al. (2009) who found that 69.4% of CAMRSA were PVL positive.

The correlation between the presence of *luk* genes and the resistance to antimicrobials was studied. *lukF* gene was prevalent among isolates resistant to amoxicillin-calvulenic acid and amikacin (P value= 0.0354 and 0.002, respectively). While for *lukE* gene, it was prevalent among isolates resistant to gentamicin (P value=0.029). In this study, the 75 *S. aureus* isolates demonstrated 13 different toxin patterns. The toxin patten (T1) represents isolates with no leukocidins toxins (6 isolate), while toxin pattern (T13) represent 7 isolates harboring the four toxin genes. T8 was the most common toxin pattern as represent (16%) followed the patterns T10, T12 (12 and 10.6%, respectively). The toxin patterns T4 and T13 have the same distribution between isolates (9.3%). Analysis of toxin pattern distribution among multidrug-resistant and non-multidrug-resistant isolates did not allow the determination of a clear correlation between them.

Detection of toxin genes, does not necessarily mean the ability of toxin expression, this may be attributed to mutation in the gene regulatory region (Indrawattana et al., 2013), so it was important for us to demonstrate the ability to express the studied leukocidins genes among the selected 20 isolates by real-time PCR technique. *lukD* gene was expressed in 73.6% of isolates by variable degrees (Figure 2). In addition, *lukE* gene was expressed in 81.25% isolates that harbored this gene. However, only two of these thirteen isolates showed a relatively very high expression level (Figure 3). For *lukS* gene, it was expressed in 76.9% of the isolates with only one isolate showing very high relative expression level (Figure 4). Regarding *lukF* gene, it was not expressed by any of the tested isolates. This was in accordance with a study by Yu et al. (2013) reporting that the PVL detected by qRT-PCR was expressed in all isolates harboring PVL genes by variable levels suggesting that PVL genes transcription is associated with clinical isolates. Another study showed that PVL genes expression levels are strain dependent, with more than 10-fold variance (Said-Salim et al., 2005). In contrast, a study performed by Kholeif and Mohamed (2009) showed that PVL was positive in 39.7% by real time PCR.

The relation between *luk* genes expression, MRSA and MDR was evaluated. The level of *lukD* expression in MRSA and MDR isolates was higher than the expression levels recorded for MSSA and NMDR isolates. While for *lukE* and *lukS*, thier level of expression were not correlated with MRSA and MDR.

Conclusion

The present study highlights the prevalence of MRSA among *S. aureus* clinical isolates. High incidence of MRSA isolates were found among isolates of wound infections. A significant association was found between MRSA and MDR isolates. MRSA isolates were significantly resistant to amoxicillin-clavulenic, amikacin, imipenem and cephalothin. These results also demonstrate high prevalence of *luk* genes including PVL genes among *S. aureus* isolates. Leukocidins genes were found to be strain dependent except for *lukF* that was significantly associated with burn isolates. *lukF* was

found to be predominant in MRSA isolates and in isolates resistant to amoxicillin-clavulenic acid and amikacin. *lukE* was prevalent in isolates resistant to gentamicin. High expression levels of *luk* genes were recorded in MRSA and MDR isolates. These results demonstrated the spread of highly resistant *S. aureus* isolates which possess leukocidins toxin in our hospitals and the utmost need for strict guidelines for controlling their spread.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Endophytic microorganisms from *Bauhinia monandra* leaves: Isolation, antimicrobial activities and interaction with galactose-specific lectin BmoLL

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***Bauhinia monandra* leaves are well known hypoglycemic agent in Brazilian popular medicine from which a galactose specific lectin (BmoLL) has been purified in milligram quantities. In this work, the antimicrobial activity of endophytes isolated from *B. monandra* leaves and the ability of BmoLL to agglutinate the microorganisms were evaluated. After disinfection, leaves of *B. monandra* were fragmented and distributed in Petri plates leading to isolation of fungi (37 strains) and both Gram-positive (26 strains) and negative bacteria (6 strains). A preliminary antimicrobial assay revealed that 62% of bacterial strains were active, while no antagonist action was detected with fungi. However, only 2 strains were able to excrete the antimicrobial compounds: *Pseudomonas aeruginosa* UFPEDA598 inhibited *Aspergillus niger*, *Fusarium moniliform*, *Fusarium oxysporum*, *Micrococcus luteus, Staphylococcus aureus*, *Bacillus subtilis* and PE(24)C1 inhibited *Candida* sp. strains, *M. luteus* and *S. aureus*. On the other hand, BmoLL did not show any antimicrobial action, but it was able to agglutinate the active strain *P. aeruginosa* UFPEDA598. The BmoLL-*Pseudomonas* interaction could promote a defense against the attack of phytopathogenic microorganisms in plants through a *P. aeruginosa* metabolic production mechanism. The lectins/endophytes interaction could be a new line to unravel defense mechanisms against phytopathogenic organisms.**

Key words: Endophytes, *Bauhinia monandra* leaf lectin, antimicrobial activities, *Pseudomonas aeruginosa*, agglutination.

INTRODUCTION

Endophytic bacteria and fungi are organisms that could live in association with plants for most, if not all, their life cycles. They live within the intercellular spaces of plants, in tissues such as roots, stems and leaves (Hormazabal and Piontelli, 2009). They invade the vegetal in different stages of development, but do not cause symptoms of

diseases. Some fungi and bacteria can be isolated from a great variety of host plant families while growing under different ecological and geographical conditions. Other endophytes are apparently restricted to host species that occur within a particular plant family (Azevedo et al., 2000; Yuan et al., 2010). Endophytic microorganisms may confer advantage to the plant, and the benefits may be reciprocal, resulting in an enhanced symbiotic system for specific plant characteristics. Therefore, the use of endophytic bacteria and fungi opens up new areas of biotechnological explorations, which leads to the necessity to isolate and cultivate these organisms. Endophytes are used for biological control of various plant diseases, to enhance agronomic plant characteristics such as increased drought tolerances and nitrogen efficiency, as bioherbicides, and pharmaceutical agents (Bacilio-Jiménez et al., 2001; Audenaert et al., 2002; Vendan et al., 2010). Several endophytes can be found in a unique species and act in plant defense against pathogenic microorganisms (Omacine et al., 2001).

Lectins are carbohydrate binding proteins or glycol-proteins that participate in various metabolic processes in cells (Coriolano et al., 2014; da Silva and Correia, 2014). There is evidence that lectins act in the recognition between cells or cells and various carbohydrate-containing molecules; they may be involved in regulating physiological functions. Lectins seem to play an important role in defense mechanisms of plants against the attack of microorganisms, pests and insects. Fungal infection or wounding of the plant seems to increase lectin concentrations (Guan et al., 2008; Charungchitrak et al., 2011). In legumes, the role of lectins in the recognition of nitrogen-fixing bacteria *Rhizobium* genus, which have sugar-containing substances, has received a special attention (Antonyuk and Evseeva, 2006). Plant lectin functions have been speculated, among them; these proteins participate as a binding factor in the interaction between plants and microorganisms (Carlini and Grossi-de-Sá, 2002; Souza et al., 2011). Seeds, roots, flowers as well as leaves may contain considerable concentrations of lectins (Coelho and Silva, 2000; Silva et al., 2014); it is believed that in these organs, lectins could favor the symbioses with endophytic organisms (Antonyuk and Evseeva, 2006; Vershinina et al., 2012).

The genus, *Bauhinia* (Fabaceae) contains a number of ornamental species which are well distributed in the tropics. Some species are important for animal nutrition because of their high protein content; they have been used as forage, as human food, in folk medicine for the treatment of diabetes and as diuretic (Macedo et al., 2007; Souza et al., 2011). Leaves of *Bauhinia monandra* (pata-de-vaca, pulse) contain a relatively high concentration of a galactose-specific lectin (BmoLL); more than 2 mg of highly purified lectin has been obtained from 5 g of leaf powder (Coelho and Silva, 2000). BmoLL already showed insecticidal action against Pyralidae and Bruchidae larvae (Macedo et al., 2007). Previous studies have also reported the isolation and characterization of lectins from *B. purpurea* and *B. monandra*, seeds and roots (Souza et al., 2011). Although the interaction between lectins and phytopathogen microorganisms have been studied deeply (Gaidamashvili and Van Staden, 2002; Charungchitrak et al., 2011), there is still scarce literature concerning their interaction with plant endophytics. The present study reports the isolation and evaluation of antimicrobial activity of endophytic microorganisms obtained from *B. monandra* leaves, and the ability of BmoLL to agglutinate them.

MATERIALS AND METHODS

Isolation of endophytes

A total of 69 endophytes (bacteria and fungi) were isolated from *B. monandra* leaves. Sample collections were performed at the campus of the Universidade Federal de Pernambuco (Recife, State of Pernambuco, Northeast of Brazil). Leaves were washed (10 min) and disinfected (70%, v/v ethanol, 1 min; 5%, v/v, sodium hypochloride, 5 min; 70%, v/v ethanol, 30 s; washed twice in sterile distilled water, 1 min). A control consisted of the last wash. Fragments of tissue (5 mm) were distributed in Petri plates with distinct culture medium. Bacterial isolation was performed using different five media supplemented with cyclohexamide: Nutrient Agar (NA; meat extract 1 g, leaven extract 2 g, peptone 5 g, sodium chloride 5 g, agar 15 g and pH 7.2 - 7.4; distilled water 1000 mL); NA 50%; Czapek-agar medium (CZP; $NaNO_3$ 3 g; K_2HPO_4 1 g, $MgSO_4.7H_2O$ 0.5 g, KCl 0.5 g, $FeSO_4$ 0.01 g, sacarose 30 g, agar 15 g; distilled water 1000 mL), Triptic Soy Agar (TSA; MERCK, 4 g in 100 mL of distilled water) and Casein Starch Agar (CSA; agar 15 g, soluble starch 10 g, $K_2SO_4(2H_2O)$ 2 g, KNO_3 2 g, NaCl 2 g, casein 0.3 g, $MgSO_4(7H_2O)$ 0.05 g, $CaCO_3$ 0.02 g, $FeSO_3(7H_2O)$ 0.01 g, distilled water 1000 mL). For fungi isolation, Sabouraud agar (SAB; DIFCO, 6.5 g in 100 mL of distilled water) and potato dextrose agar medium (PDA; potato 200 g, dextrose 15 g, agar 17 g, distilled water 1000 mL) were both supplemented with tetracycline. Samples were incubated at 28°C for 5 to 20 days. The strains were stored at 4ºC, for short-term on mineral oil in freeze (-20°C).

Antimicrobial activity

To evaluate antimicrobial activity, two assays were accomplished with each isolated endophyte: one in solid (agar plug assay) and

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another in liquid culture medium (fermentation broth assay). In all assays, 11 microorganisms were used: *Staphylococcus aureus* (UFPEDA 01), *Micrococcus luteus* (UFPEDA 06), *Bacillus subtilis* (UFPEDA 16), *Candida albicans* (UFPEDA 1007 and UFPEDA 2224), *Aspergillus niger* (UFPEDA 2003), *Colletotrichum gramminicola* (UFPEDA 2403), *Fusarium moniliforme* (UFPEDA 2409), *Fusarium oxysporum* (UFPEDA 3505), *Candida* sp. (UFPEDA 1315 and UFPEDA 1316) (Table 1).

Agar plug diffusion assay (primary screening)

For the preparation of the agar plug, bacterial strains were cultivated in NA and TSA plates, while SAB and PDA plates were used to inoculate fungi. After incubation, agar plugs (5 mm) were placed on the agar medium seeded with test microorganisms. After incubation during 16-18 (bacteria), 24-48 (yeast) or 72-96 h (fungi), the inhibition diameter zones (IDZ) formed around the fungal agar plugs were measured. Isolates exhibiting significant activities were then subjected to secondary screening.

Assay with fermentation broth

The endophytic bacteria with highest spectrum activity in agar plug assay were cultured overnight (28°C, shaked at 180 rpm) in 50 mL of tryptic soy broth (TSB, DIFCO, 3 g in 100 mL of distilled water). Here, 2.5 mL of each pre-inoculum was cultured in a 250 mL Erlenmeyer flask containing 100 mL of M1 (soybean meal 1 g, glucose 1 g, $CaCO_3$ 0.1 g, NaCl 5 g and 100 mL of distilled water, pH 7.0) and TSB. The endophytic fungi were grown (48 h) in 50 mL of SAB as pre-inoculum and 100 mL of SAB and M1 as fermentation media, shaked at 180 rpm, 30°C. Aliquots of fermentation broth (30 µL) were added to Petri plates seeded with each pathogenic strain to be assayed in the respective culture media. All experiments were made in triplicate.

Assays with BmoLL

Purification of BmoLL

Fresh leaves of *B. monandra* were harvested from the Germplasm Bank of Medicinal Plants at the Experimental Station of Itapirema (Goiana, State of Pernambuco Brazil) or from ornamental trees in the cities of Olinda and Recife (State of Pernambuco, Northeast of Brazil). A sample of the collected material is archived as voucher specimen number 57462, IPA, at the herbarium "Dárdano de Andrade Lima" (Empresa Pernambucana de Pesquisa Agropecuária, Recife, Brazil). Petioles were removed from the fresh leaf material; the blades were well washed in tap water followed by distilled water, and allowed to dry at room temperature. Dried blades were powdered in a multiprocessor and extract (10% w/v) was obtained by overnight gentle shaking at 4°C in a 0.01 mol L^{-1} citrate phosphate buffer (pH 6.5) containing 0.15 mol L^{-1} sodium chloride (the selected buffer). This extract was passed through gauze and centrifuged at 11,270 g for 15 min to give preparation P1, which was submitted to 60% (w/v) ammonium sulphate fractionation (F 0-60%) by addition of solid salt. After 4 h at room temperature, the resuspended precipitate was dialyzed against distilled water, followed by the selected buffer (preparation P2). An affinity matrix was made by cross-linking refined gum guar (guaran; Sigma Chemical Co., St. Louis, MO, USA) with epichlorohydrin in a mixture of water: 2-propanol (Gupta et al., 1979). A sample (140 mg) of P2 containing BmoLL was applied to a 10 mL guar gel column. The affinity column was washed with buffer until the absorbance at 280 nm was zero, and then a galactose solution (0.05 mol L^{-1}) prepared in the buffer was used to irrigate the gel. The fractions with high activity were bulked and dialyzed with three changes of 0.01 mol L^{-1} citrate phosphate buffer (pH 6.5) containing 0.15 mol L^{-1} sodium chloride (1 mL/100 mL/h). Hemagglutinating activity (HA) was determined (Correia and Coelho, 1995); protein concentration was measured according to Lowry et al. (1951) and by absorbance at 280 nm. The material was stored at 20°C until used.

Assay of antimicrobial activity to lectin

A previous assay of antimicrobial activity to lectin (Ye and Ng, 2001) was carried out in Petri plates (100 x 15 mm) containing 10 mL of NA medium or 10 mL of SAB. Around a plug of bacterial or fungi (0.5 cm in diameter) grown previously in specific culture medium, at a distance of 1 cm away from it, were placed sterile blank paper disks of the same size. Aliquots (10 µL) containing 30 to 300 µg of BmoLL (in selected buffer) was added to a disk. The plates were incubated at 28°C for 24 h for bacteria, to analyze the development of microorganisms on plates. The pathogenic microorganisms were used in this assay, as well the endophytics bacteria strains PE(23)C1, PE(24)C1, UFPEDA598, PE(63)C1, PE(64)C1 and PE(67)C1 (chosen since they showed a good performance in primary antimicrobial screening).

Agglutination assay

To evaluate the agglutination activity of BmoLL, two Gram-positive (PE(17)C1 and PE(24)C1) and two Gram-negative (UFPE-DA598 and PE(65)C1) endophytics isolated from *B. monandra* leaves were used as test organisms. Additionally, *S. lutea* (Waksman), *P. aeruginosa* (IT 2633 and ATCC 27853), *E. coli* (ATCC 25922) and *S. aureus* (Waksman) were used as controls. The bacterial strains were cultured in TSB broth (50 mL) and incubated overnight under shaking (180 rpm) at 28°C. Aliquots (5 mL) were transferred to Erlenmeyers containing 100 ml^{-1} of medium and were incubated at 28°C (180 rpm). After 48 h, bacterial cells were centrifuged at 750 g, 7 min at 4°C, washed three times in NaCl 0.15 mol l^{-1}, two times in citrate-phosphate buffer 5% (v/v) pH 6.5 for 7 min at room temperature and resuspended in selected buffer. The turbid suspensions were adjusted to approximately 10^8 cells per mL^{-1}.

The agglutination assay was performed in microtitre plates (96 wells). In this assay, 50 µL of 0.15 mol L^{-1} NaCl, 50 µL of a bacterial suspension (10^8 cell mL^{-1}) and a serial dilution of 50 µL highly purified BmoLL or Con A (a comparison lectin) preparation (0.96 mg mL^{-1}) were mixed in the plate. The control did not contain lectin. Agglutination activity was observed and photographed after 24 h, using an OLYMPUS BH-2 microscope.

Inhibition assays were performed with a solution containing 100 µL of 100 mmol L^{-1} galactose in 0.15 mol L^{-1} sodium chloride mixed with 100 μL^{-1} of lectin preparation (0.96 mg mL^{-1}), and 50 µL of this mixture was distributed in the wells. After 15 min at room temperature, 50 µL of bacterial solution was added in a final volume of 100 µL. The result was recorded visually after 45 min at room temperature.

RESULTS

Isolation and antimicrobial screening of *B. monandra* endophytic microorganisms

A total of 69 endophytic strains were isolated from

Table 1. Antimicrobial activity of *Bauhinia monandra* leaves endophytes in NA medium.

Microorganism assayed	Endophytic in NA medium					
	PE23C1	PE24C1	UFPE-DA598*	PE63C1	PE64C17	PE67C1
Staphylococcus aureus (UFPEDA 01)[a]	--	+++	+++++	--	+	+++
Micrococcus luteus (UFPEDA 06)[a]	+++	++++	++++	--	+	--
Bacillus subtilis (UFPEDA 16)[a]	--	--	++++	--	--	--
Candida albicans (UFPEDA 1007)[a]	--	--	++	--	--	--
Aspergillus niger (UFPEDA 2003)[a]	--	--	+++	--	--	--
Candida albicans (UFPEDA 2224)[m]	++	+	--	--	+	--
Colletotrichum gramminicola (UFPEDA 2403)[a]	--	+++	+++++	+++	--	--
Fusarium moniliforme (UFPEDA 2409)[a]	--	--	+	++	--	+
Fusarium oxysporum (UFPEDA 3505)[m]	--	--	++	+	+	+++
Candida sp. (UFPEDA 1315)[a]	+	+	++++	--	+	--
Candida sp. (UFPEDA 1316)[a]	--	+++	+++	--	--	--

Inhibition zone: --, 0-5 mm; +, 5-10 mm; ++, 10-15 mm; +++, 15-20 mm; ++++, 20-25 mm; +++++, above 25 mm. [a] Collection from the Department of Antibiotics, [m] collection from the Department of Mycology, * *Pseudomonas aeruginosa*. NA: Nutrient Agar.

Table 2. Antimicrobial activity of *Bauhinia monandra* leaves endophytes in TSA medium.

Microorganisms	Endophytics in TSA medium					
	PE23C1	**PE24C1**	**UFPE-DA598***	**PE63C1**	**PE64C1**	**PE67C1**
Staphylococcus aureus (01)[a]	--	++++	++++	--	+	++
Micrococcus luteus (06)[a]	++++	++++	++++	--	+	++
Bacillus subtilis (16)[a]	--	--	+++++	--	+	+
Candida albicans (1007)[a]	--	--	++	--	--	--
Aspergillus niger (2003)[a]	--	--	+++	--	--	--
Colletotrichum gramminicola (2403)[a]	--	++++	+++++	+++	--	--
Fusarium moniliforme (2409)[a]	--	--	++	++	--	++
Fusarium oxysporum (3505)[m]	--	--	+++	+	+	+
Candida sp. (1315)[a]	--	+	+++	--	++	--
Candida sp. (1316)[a]	++	+	++++	--	--	--

Inhibition zone: --, 0-5 mm; +, 5-10 mm; ++, 10-15 mm; +++, 15-20 mm; ++++, 20-25 Inhibition zone: --, 0-5 mm; +, 5-10 mm; ++, 10-15 mm; +++, 15-20 mm; ++++, 20-25 mm; +++++, above 25 mm. [a] Collection from the Department of Antibiotics, [m] collection from the Department of Mycology, * *Pseudomonas aeruginosa*. TSA, Triptic Soy Agar (MERCK, 4 g in 100 mL of distilled water).

leaves of *B. monandra*, of which 32 were bacteria (Gram-positive: 26 strains; Gram-negative: 6 strains) and 37 fungi. The best medium was PDA for isolation of fungi, and NA for bacteria isolation (data not shown). These organisms were subsequently submitted to a preliminary antimicrobial screening on solid medium against 11 pathogenic microorganisms, where 62% of bacterial strains were active and displayed inhibition zone ranged from 5 to 25 mm. The best antimicrobial activity was found using NA plates (IDZ ranged from 20 to 30 mm). On the other hand, the endophytic fungi did not inhibit any pathogenic microorganism. Six strains showed the best activities as they inhibit more than two pathogenic microorganisms or showed one IDZ value higher than 15 mm (Tables 1 and 2). From those, two strains were selected for the fermentation assay (PE24C1 and UFPEDA 598), as they showed the best antimicrobial potential. The strain PE24C1 inhibited the growth of *S. aureus, M. luteus, C. albicans*, two strains of *Candida* sp. (isolated from clinical material) and *C. gramminicola.* The strain UFPEDA598 inhibited all tested microorganisms and it was identified as *P. aeruginosa* UFPEDA598 (King et al., 1954).

Endophytic efficiency in fermentation broth

Both strains which showed highest spectrum activity in plug agar (PE(24)C1 and *P. aeruginosa* UFPEDA598), were cultured in fermentation broth M1 and TSB and the

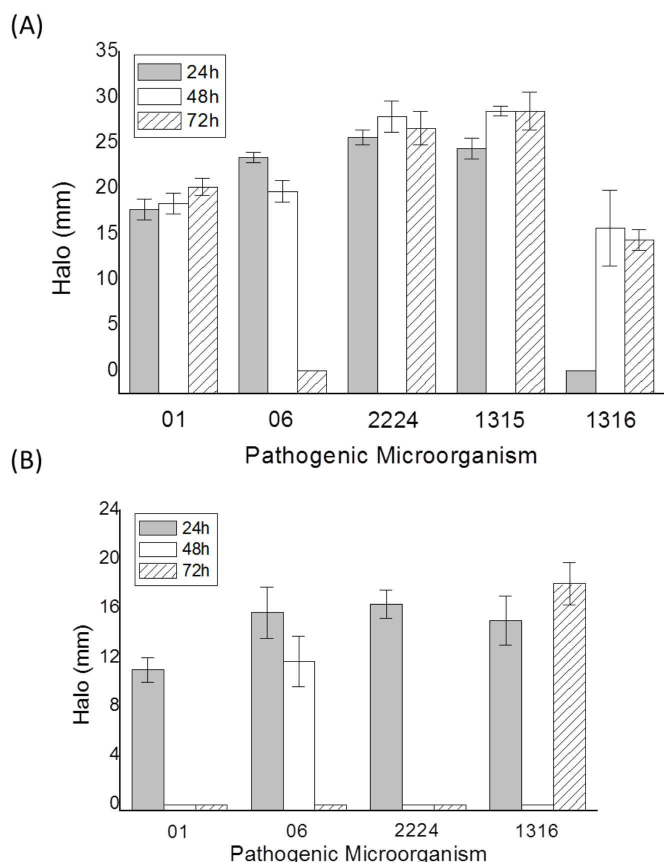

Figure 1. Antimicrobial activity of endophytic strain PE(24)C1 growth in medium M1 (A) and TSB (B). Microorganisms: *S. aureus* (UFPEDA 01), *M. luteus* (UFPEDA 06), *C. albicans* (UFPEDA 2224), *Candida* sp. (UFPEDA 1315 and UFPEDA 1316).

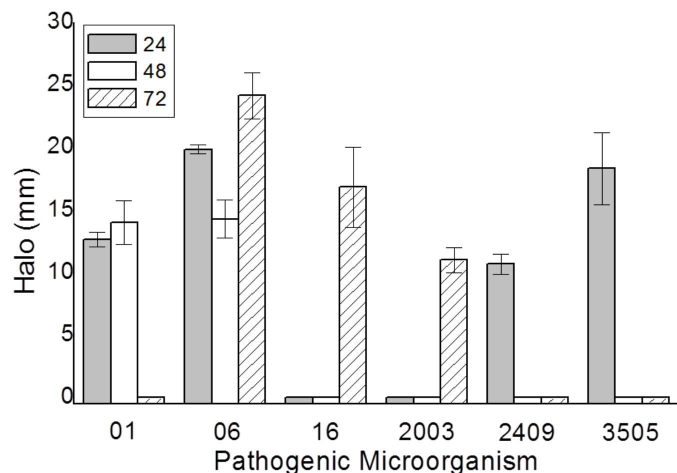

Figure 2. Antimicrobial activity of endophytic strain UFPEDA598 growth in medium M1. Microorganisms: *S. aureus* (UFPEDA 01), *M. luteus* (UFPEDA 06), *B. subtilis* (UFPEDA 16), *A. niger* (UFPEDA 2003), *F. moniliforme* (UFPEDA 2409) *F. oxysporum* (UFPEDA 3505).

antimicrobial activity was evaluated after 24, 48 and 72 h. PE(24)C1 displayed high activity in M1 (Figure 1A) and TS broth (Figure 1B). In M1 broth, the activity was found against the same microorganisms than in agar assay in all times, except *B. subtilis* and *Candida* sp. 1316, for them, no inhibition was observed at 72 or 24 h, respectively. When growth in TSB, PE(24)C1 only inhibited *S. aureus* and *C. gramminicola* (at 24 h), *B. subtilis* (24 and 48 h) and *Candida* sp. 1316 (24 and 72 h). On the other hand, *P. aeruginosa* UFPE-DA598 was only active in TSB, inhibiting fungi (*A. niger*, *F. moniliform* and *F. oxysporum*) and bacteria strains (*M. luteus, S. aureus* and *B. subtilis*) (Figure 2).

Assays with BmoLL

Agglutination assays were performed to investigate the possible interaction of BmoLL, a highly purified galactose specific lectin prepared in milligram quantities (Coelho and Silva, 2000), with endophytes. Bacterial agglutination was expressed as the degree of agglutinin-bacterial solution to the bottom of microtitration plate different from that of a bacterial control. Aggregation was observed visually after overnight incubation of plates clearly indicating the minimal concentration of agglutinins eliciting the bacterial aggregation (Figure 3A and B). Observation of plates revealed agglutination between BmoLL and *P. aeruginosa* UFPEDA598, with a titer of 16^{-1} (Figure 3A). No agglutination was detected in the inhibition assay containing BmoLL, 100 mM of galactose and endophytic. BmoLL did not show an inhibitory effect against six tested bacterial endophytes and negative results were also obtained with all tested microorganisms (data not shown).

DISCUSSION

Infusions of leaves from *B. monandra* are broadly used in popular medicine to treat diabetes and antioxidant activity was revealed in leaf preparations (Argolo et al., 2004). A galactose specific lectin was obtained in milligram quantities from leaves of *B. monandra* and termed *B. monandra* leaf lectin (BmoLL) (Coelho and Silva, 2000). *B. monandra* is an ornamental plant whose leaves are always very healthy. In fact, Macedo et al. (2007) detected an insecticidal activity of BmoLL against *Anagasta kuehniella* (Lepidoptera: Pyralidae), *Zabrotes subfasciatus* and *Callosobruchus maculatus* (Coleoptera: Bruchidae). Another galactose-specific lectin from *B. monandra* secondary roots (BmoRoL) also purified in milligram quantities, showed significant antifungal and

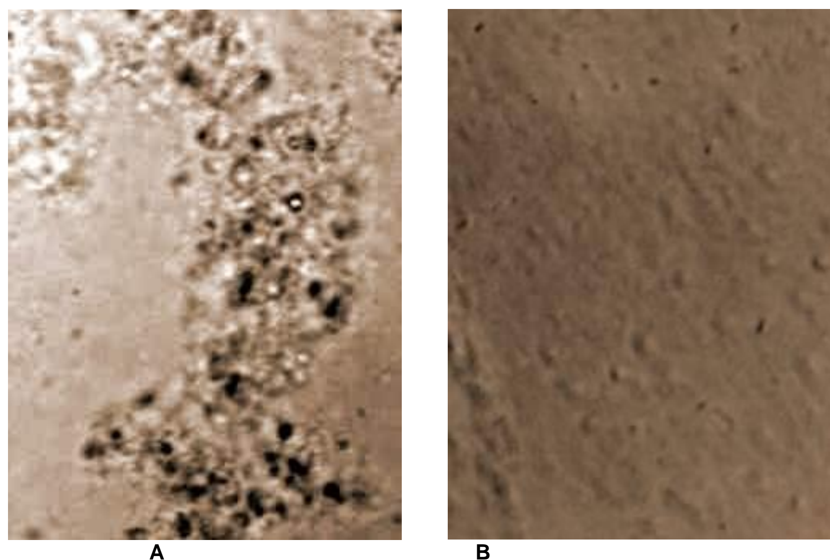

Figure 3. Agglutination of *P. aeruginosa* (UFPEDA598) by BmoLL. A. Suspension of bacteria with 50 μL of BmoLL; B. Suspension of bacteria without BmoLL.

termiticidal activities (Souza et al., 2011).

Endophytic microorganisms have been isolated from different plant species and they are reported as agents in biological control of plant diseases and plagues (Hormazabal and Piontelli, 2009; Vendan et al., 2010; Jin et al., 2014). Endophytes have potential applications in agriculture, industry and medicine (Brader et al., 2014). Bacterial (32) and fungi (37) strains of endophytics were isolated from *B. monandra* leaves, which were assayed for antimicrobial activity against 14 pathogenic micro-organisms. The endophytic strains assayed, with antagonism against phytopathogens, could validate the applicability of endophytic microorganisms as sources of new antibiotic production, or agents for biological control (Yuan et al., 2010). The performance of PE(24)C1 and *P. aeruginosa* (UFPE-DA598) strains against pathogenic microorganisms may allow the speculation of plant defense action by endophytic bacteria, and confirm the production of compounds with antibiotic function by endophyte microorganisms.

P. aeruginosa (UFPE-DA598) displayed antimicrobial activity against fungi *A. niger*, *F. moniliform* and *F. oxysporum*, as well as bacteria *M. luteus, S. aureus* and *B. subtilis*. Most strains of *P. aeruginosa* endophyte shows *in vitro* antagonism to fungi and bacteria. *Pseudomonas* strains have been extensively used in biological control, antibiotic production, rhizobacteria-mediated induced systemic resistance (ISR) or as a considerable tool to plant-defense mechanism evaluation (Nomura et al., 2005). Audenaert et al. (2002) mentioned that tomato with a patented strain, *P. aeruginosa* 7NSK2, induced systemic resistance by secondary metabolites production against *Botrytis cinerea*; this fungus attacks several species of cultivated plants of economic importance. Production of metabolite with antimicrobial activity, or competition for nutrients and exclusion from the ecological niche of colonizing microorganisms, has been suggested as a possible mechanism (Bacilio-Jiménez et al., 2001). It has been proposed that endophytically resident bacteria may be strategically available at the right place and at the right time for suppression of vascular wilt diseases and probably have the unique ability to survive inside plants with little or no microbial competition; the endophytic bacterium and its host plant can coevolve in a communicative and co-operative manner that leads to inhibiting and suppressing vascular (wilt) diseases (Antonyuk and Evseeva, 2006; Bright and Bulgheresi, 2010; Vershinina et al., 2012).

BmoLL did not show any inhibitory effect against six tested bacterial endophytes and negative results were also obtained with all tested microorganisms. Positive results have already been mentioned for other lectins, which agglutinated pathogen microorganisms (Charungchitrak et al., 2011; Souza et al., 2011) or symbiotic root endophytes (Antonyuk and Evseeva, 2006). Gaidamashvili and Van Staden (2002) worked with lectin-like proteins from medicinal plants of South African; they observed antibacterial effects against two Gram-positive bacteria pathogens, *S. aureus* and *Bacillus subtilis*. In roots of transgenic rice (*Oriza sativa* L. cv. Murasaki), the expression of two legume symbiotic lectin genes, psl and gs52, promoted rhizobial colonization (Sreevidya et al., 2005). N-Glycolylneuraminic acid-specific lectins from leaves of mulberry (*Morus alba*) showed anti-bacterial activity against *P. syringae pv mori* a phytopathogenic bacteria of mulberry leaves (Ratanapo

et al., 2001). The high concentration of BmoLL in *B. monandra* leaves allowed obtaining more than 2 mg of pure lectin from 5 g of leaf powder (Coelho and Silva, 2000). BmoLL (96 µg ml^{-1}) agglutinated only one endophytic bacterium: the strain UFPE-DA598, identified as *P. aeruginosa*. *Pseudomonas* strains have been reported to bind lectins (Boteva et al., 2005; Nomura et al., 2005); the agglutination activity allows speculating a possible relationship between BmoLL and endophytics from *B. monandra* leaves. Many plant lectins have interacted with several pathogens (Macedo et al., 2007; Souza et al., 2011); also, the symbiotic relationships between plants and root endophytic bacteria have been explored (Antonyuk and Evseeva, 2006). There is no plausible mechanism suggested for interaction of lectins with leaf endophytics. Plant–associated *Pseudomonas* are known to use one or more systems to regulate the production of antibiotics and secondary metabolites, exoprotease activity, and cell-surface features to contribute to their persistence on plants and effectiveness as biological control agents or potential pathogen (Nomura et al., 2005; Antonyuk and Evseeva, 2006).

The function of milligram quantities of BmoLL in plant leaves is unclear; this lectin could interact with endophytic microorganisms as an inhibitor, symbiotic or stimulating factor. Lectin functions in plants as carbohydrate binding proteins and regulatory molecules (Santos et al., 2014). Gao et al. (2003) showed that higher plants produce compounds that specifically stimulate or inhibit response in bacteria. The interaction between plants and microorganisms has been extensively explored, and a symbiotic mechanism in roots, through plant lectins, has been deeply studied (Antonyuk and Evseeva, 2006). Plant lectins have been involved in recognition of bacteria in roots (Sreevidya et al., 2005) or agglutination, *in vitro*, to phytopathogens (Macedo et al., 2007; Charungchitrak et al., 2011; Souza et al., 2011). The interaction between lectins and *Pseudomonas* strains has been extensively studied (Boteva et al., 2005). The potential interaction, BmoLL-*Pseudomonas* in leaves, could promote a defense against the attack of phytopathogenic microorganisms in plants through a *P. aeruginosa* metabolic production mechanism (since this strains was active against phytopathogenic strains). The interaction of plant lectins and endophytes could be a new line to unravel agricultural defense mechanisms against phytopathogenic organisms in biological control. In this way, metabolites of *B. monandra* endophytics are currently under investigation in our laboratory to search for antibacterial products.

Conflict of Interests

The authors have not declared any conflict of interests.

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