African Journal of Microbiology Research

Volume 11 Number 39 21 October, 2017

ISSN 1996-0808

Academic Journals

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academicJournals

Vol. 11(39), pp. 1459-1467, 21 October, 2017
DOI: 10.5897/AJMR2017.8470
Article Number: E45691766470
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Endophytic bacteria isolated from ipê mirim (*Tecoma stans* Bignoniaceae) and its application for plant growth promotion

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Received 3 February, 2017; Accepted 28 March, 2017

Endophytes are usually protected from soil environment competitiveness and stress. However, the presence of heavy metals can negatively affect the structure and diversity of endophyte communities. The aim of the present study was evaluate the diversity of endophytic bacteria from ipê mirim (*Tecoma stans* Bignoniaceae) grown in an area of Atlantic rainforest contaminated with metals and evaluate the ability of these bacteria to promote plant growth and seed germination. Results show that endophytic bacterial density in plants was stable among sites with different level of metals; however, bacterial richness was lower in plants from sites with low level of metals. At least 28 genera were isolated, where *Methylobacterium* (21.32%), *Bacillus* (19.12%), *Pseudomonas* (11.03%) and *Curtobacterium* (7.35%) the dominant groups. Isolates were selected from Rhizobiales order and the capability of this dominant group in plant growth promotion was evaluated. Results showed that *Methylobacterium* spp. and *Rhizobium* sp. increased germination and improved seedling growth of tomato Santa Cruz Kada Gigante. Therefore, results show that the endophyte cultivable community is not influenced by the presence of low metal concentration, and plant growth promoter bacteria that can be used on tomato seedlings production were successfully selected and on future phytoremediation studies.

Key words: *Methylobacterium* spp., *Rhizobium* sp., *Tecoma stans,* microbial diversity, metals, plant growth promoting bacteria (PGPB), tomato, growth promotion.

INTRODUCTION

The endophytic bacteria colonize the inner plant tissues without causing disease and without visible external structures (Hardoim et al., 2008, 2015). This relationship can be beneficial for both plant and microorganisms; studies

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have shown that these bacteria or fungi also can be used as a source for new drugs (Strobel et al., 2004), enzymes, antibiotics (Martinez-Klimova et al., 2016; Braga et al., 2016), and allow the development of new bioremediation and agricultural techniques (Kobayashi, 2000; Dourado et al., 2013).

The diversity of endophytic bacteria may be impacted by biotic (plant genotypes and microbial community) and abiotic (nutrients, water, temperature, pH and pollutants) factors (Hardoim et al., 2008). Although endophytes are protected of stress and competitiveness from soil environment (Gans et al., 2005), the presence of heavy metals may negatively affect the structure and diversity of microbial communities. Thus, it is important to understand the factors that affect the endophytic community and its impact on the biotechnological potential of this community.

Methylobacterium spp. has been isolated from several plant species (Omer et al., 2004), actively colonizing the leaf surface or the inner plant tissues, such as soybean, sugarcane, cotton, citrus, eucalyptus, bamboo, *Catharanthus roseus*, tobacco, strawberry, mangrove plants and different Fabaceae species (Madhaiyan and Poonguzhali, 2014; Dourado et al., 2015). Inside the host plant *Rhizobium* and *Methylobacterium* could produce phythormones (IAA and citokinine) or interact with the microbial community (endophytes and pathogens) (Madhaiyan et al., 2006; Dourado et al., 2015; Azevedo et al., 2016). In addition, *Methylobacterium* spp. can promote the seed germination and increase leaf area and plant height (Madhaiyan et al., 2006; Kumar et al., 2011; Bogas et al., 2016; Gopalakrishnan et al., 2015), the stomata number, chlorophyll and malic acid content (Cervantes-Martinez et al., 2004), and fixing N_2, inducing the formation of nodules on the host plant (Sy et al., 2001; Gopalakrishnan et al., 2015), suggesting that these bacteria can change the plant physiology during interaction.

The aim of this study was to isolate and characterize endophytic bacterial community from *T. stans,* known as Ipê-mirim, yellow trumpetbush or yellow bells, grown in an Atlantic Rain Forest with a history of heavy metal contamination; and evaluate the diversity and the application of these bacteria in promoting tomato plant (*Lycopersicon esculentum* Mill) growth.

MATERIALS AND METHODS

Plant and soil samples

Endophytic bacteria were isolated from Ipe-Mirim (*Tecoma stans*, Bignoniaceae) plants grown in an Atlantic Forest area of Nagib Najar Park (Mogi das Cruzes, São Paulo), with a history of metals contamination. The plants and soil were sampled randomly in four points in the Park: Site 1 (46°12´49.6´´W-23°31´03.6´S), site 2 (46°2´49.0´W; 23°31´08.1´´S), site 5 (46°12´44.2´´W; 23°31´09.9´´S) and site 6 (46°12´39.5´´W; 23°31´19.1´´S) in April of 2007 and May of 2008. For each sampling site, three plants were collected and immediately transported to the laboratory for isolation of the endophytic bacterial community.

Physical and chemical soil analysis

The physical and chemical soil analysis was performed at the Núcleo de Ciências Ambientais (NCA) at University of Mogi das Cruzes, under the responsibility of Prof. Dr. Andrew Fernando de Oliveira. The levels of metals were compared to soil quality reference values determined by CETESB (São Paulo State Environmental Company) (http://www.cetesb.sp.gov.br).

Isolation of endophytic bacteria from branches

Stems (5 mm of diameter) were surface disinfected (70% ethanol for 30 s; 2% sodium hypochlorite for 2 min and sterile distilled water for 30 s), macerated in PBS buffer (NaCl, 8 g l^{-1}; KCl, 0.2 g l^{-1}; Na_2HPO_4, 1.44 g l^{-1}; KH_2PO_4, 0.24 g l^{-1}) and appropriate dilutions (10^{-1}, 10^{-2} and 10^{-3}) were plated with the controls (triplicate of 200 µl water culture from the last disinfection step) on culture medium TSA (Tryptic Soy Agar - Oxoid) 5%, amended with benomyl (50 $mg.mL^{-1}$) and incubated at 28°C for 30 days. After growth, the colonies were counted and randomly picked out for further identification. Means were compared by analysis of variance (ANOVA) with Bonferroni test, comparison of independent samples and the Pearson correlation method using the Bioestat software (v.5.0, 2015).

Molecular identification and diversity analysis

The total DNA of the isolates were extracted, the gene 16S rRNA was amplified with primers 968F and 1401R (Araújo et al., 2002) and sequenced by HUG-CELL center (http://genoma.ib.usp.br/en). All 140 bacterial sequences presented in this study were submitted to GenBank (accession numbers KX914446 - KX914662).

The sequences were classified in RDPquery (http://simo.marsci.uga.edu) using only type-strain sequences from the RDP database (http://rdp.cme.msu.edu/). A phenetic tree was built using the MEGA 6.06 software (www.megasoftware.net) using the neighbor-joining method and Jukes-Cantor model, with a consistency test with 1000 bootstrap replicates. The resultant tree was edited with ITOL program (http://itol.embl.de/). The sequences were grouped into OTUs (Operations Taxonomic units), as 97% (species) and 95% (genus) similarity criteria with the Mothur program (Schloss et al., 2009). The richness (Ace and Chao1) and diversity indexes (Simpson and Shannon-H') were evaluated and the communities from each sampling sites were compared by ∫-Libshuff method.

In vitro Rhizobiales colonization assay

Since *Methylobacterium* and *Rhizobium* (Rhizobiales) were the most abundant group it was selected to proceed the analysis, testing it biotechnological potential in plant experiment.

Rhizobium sp. and *Methylobacterium* spp. tomato germination assay

The colonization of tomato Cherry and Santa Cruz Kada Gigante for 47 isolates of Rhizobiales (*Rhizobium* and *Methlylobacterium*)

Table 1. Soil content of bioavailable metal and soil pH on the sampling placing of *T. stans* plants.

Metal	Soil content of bioavailable metal (µmol/g)			
	Site 1	Site 2	Site 5	Site 6
Soil pH	7.25	7.5	5.5	7.25
Ca	6.4	9.6	6.8	5.5
Mg	2.8	6.9	7.2	4.8
Fe	0.3	0.3	1.9	0.3
Mn	0.08	0.12	0.05	0.24
Al	0.3	-	7	-
Cu	0.05	0.08	0.02	0.95
Zn	0.1	0.2	0.5	0.4
Cd	-	-	-	0.01
Characteristics	Low metal	Low metal	Low pH, high Al	High metal (Cu, Cd)

was evaluated *in* plates. For this, the bacteria were cultured in TSB medium (Oxoid) for 72 h at 28°C under agitation (150 rpm), washed and suspended in PBS. Approximately 200 seeds were inoculated with 2 ml of this bacterial suspension (OD 600 nm = 0.8) for 60 min, and further rinsed in PBS and incubated in a humid chamber at 25°C for up to 6 days. The endophytic colonization was assessed by re-isolation of the inoculated isolates from surface disinfected plant tissues on TSA 5%.

Rhizobiales tomato germination assay

Experiment was performed as reported above only with the 29 *Rhizobiales* strains able to colonize tomato plants. These 29 strains were screened for direct plant growth promotion (PGP) abilities. They were inoculated in seeds of Cherry and Santa Cruz Kada Gigante tomato and plant germination rate was measure five days after planting.

Rhizobiales- tomato plant greenhouse assay

One *Rhizobium* sp. and two *Methylobacterium* spp. strain able to colonize the inner plants tissues were evaluated for their effect on the germination rate and plant growth promotion. For this, bacteria were inoculated into tomato seeds, as described previously, and sown in trays (expanded polystyrene with 128 cells at a depth of 0.5 cm) containing PlantMax® HT substrate. Ten seeds were sown by tray cell, and five cells (repetitions) per treatment, with completely randomized blocks. The control group was sown with distillated water (without bacteria). After 20 days in the greenhouse (temperature average was 25°C and relative humidity average was 80%), the germination rate was evaluated and 5 seedlings of each replication/ treatment were transferred to plastic pots with PlantMax® HT substrate and evaluated after 10 days according to seedling height, size of root system, leaves number. For all analysis, two independent experiments were performed.

Phosphate solubilization and nitrogen fix testes in plant growth *Rhizobiales* strains

Three *Rhizobiales* bacteria (two *Methylobacterium* and one *Rhizobium*), used in the previous experiment, were grown in plates withn culture medium with inorganic phosphate (glucose 10 g l^{-1}, NH_4Cl 5 g l^{-1}, NaCl 1 g l^{-1} and $MgSO_4.7H_2O$ 1 g l^{-1}, pH 7.2) for 48 h at 28°C and after growth, the presence of clear halo around the colony indicated the phosphate solubilization (Verma et al., 2001). The same three *Rhizobiales* isolate were screened for the ability to fix N_2, inoculated into tubes containing Nfb (Döbereiner et al., 1995) and incubated at 28°C for 10 days. Isolates that grew in this medium were re-inoculated in the same condition for three consecutive times. Isolates that grew in all inoculated tubes were considered positive.

RESULTS

Physical and chemical soil analysis

The pH and bioavailability of Ca (calcium), Mg (magnesium), Fe (Iron), Mn (manganese), Al (aluminum), Cu (cuprum), Zn (zinc) and Cd (cadmium) was assessed in an Atlantic Rain Forest soil. The pH was similar for sites 1, 2 and 6, ranging from 7.25 to 7.5, but was significantly different for site 5, which present acid soil with pH 5.5 (Table 1).

The concentration of bioavailable Mg, Fe and Zn ranged from 2.8, 0.3 and 0.1 for site 1 to 7.2, 1.9 and 0.5 µmol.$g^{-1}$for site 5, respectively. The higher concentration of Mn, Cu and Cd was observed in site 6 (Table 1). The bioavailability of Ca ranged from 5.5 to 9.6 µmol.g^{-1} in site 6 and site 2, respectively.

Isolation of endophytic bacteria from branches

Isolation of cultivable endophytic bacteria from *T. stans* revealed a large discrepancy in bacteria counts. Plants grown in sites 5 and 6 showed the highest bacterial density, while plants from site 2 presented the lowest value (Table 2). However, Pearson analysis showed no correlation between the metal concentration and bacterial counts ($p = 0.47^{ns}$ for Fe; $p = 0.13^{ns}$ for Zn; $p = 0.73^{ns}$ for Cu; $p = 0.97^{ns}$ for Mn).

Table 2. Richness and diversity analyzes of endophytic bacteria of *T. stans* at similarity level of 97%.

Sampling site	Log_{10} CFU.g steam[1]	# OTUs	Richness		Diversity	
			CHAO 1	ACE	Shannon H'	Simpson
1	2.4	21	56 (30.6-147.5)	70.1 (33.7-211.2)	2.4 (2.1-2.7)	0.1209 (0.0748-0.167)
2	1.8	22	79 (39.1-212.7)	99.8 (43.2-307.7)	3.0 (2.7-3.3)	0.0154 (-0.0047-0.0355)
5	3.7	28	203.5 (83.6-581.3)	406 (96.1-2125.9)	3.3 (3.0-3.5)	0.0025 (-0.0039-0.0088)
6	3.1	26	118 (55.2-315.2)	182 (37.1-2210.4)	3.2 (2.9-3.5)	0.0053 (-0.0039-0.0145)

Molecular identification and diversity analysis

A total of 140 endophytes were isolated, being 58 from Site 1, 25 from Site 2, 30 from site 5 and 27 from site 6. The endophytic cultivable bacterial community associated of *T. stans* steam was composed by at least 28 genera: *Actinoplanes, Aquabacterium, Bacillus, Brevibacterium, Citrobacter, Curtobacterium, Enterobacter, Hymenobacter, Kineococcus, Klebsiella, Massilia, Methylobacterium, Microbacterium, Mycobacterium, Pandoraea, Pantoea, Patulibacter, Plantibacter, Pseudomonas, Rhizobium, Rhodococcus, Sphingobium, Sphingomonas, Staphylococcus, Stenotrophomonas, Terracoccus, Williamsia, Xanthomonas* (Figure 1). The genus *Methylobacterium* (21.32%), *Bacillus* (19.12%), *Pseudomonas* (11.03%) and *Curtobacterium* (7.35%) were the dominant groups, and represented 58.82% of the total culturable bacterial community inside the *T. stans* stems. However, it was observed that both *Methylobacterium* and *Bacillus* were predominantly isolated from site 1 (with low metal content) and *Pseudomonas* presented higher number in plants from site 5 (with low pH).

At 97% similarity level, the bacterial community contained 92 OTUs. The highest richness appeared in plants from Site 5 followed by Site 6 (Table 2). For diversity (described by *H'*), the highest value was obtained in plants from site 5 too and the lowest value appeared in plants from site 1. Although the Pearson correlation test showed no correlation, the metal accumulated in site 5 (Mg, Fe, Al and Zn) and P6 (Mn, Cu and Cd) seems to result in higher bacterial density (Table 1), richness and diversity (Table 2) in cultivable endophytic bacterial community isolates from *T. stans* steam. In addition, using ∫-Libshuff analysis with the Bonferroni correction as significance criterion, it was observed that the endophytic bacterial communities of *T. stans* present in site 1 and 2 are similar ($p = 1.00$), but are significantly different from sites 5 and 6 ($p < 0.0001$), and Site 5 is different from site 6 (Figure 2A and B); site 5 and 6 present higher metal concentrations, where site 5 present lower pH and higher Al content while Site 6 present toxic metals: Cu and Cd. Although the bacterial community isolated from plants growth in sites 1 and 2 exhibit greater similarity, these points differ significantly, each being composed of a low specific community overlapping (Figure 2A and Figure B -red bars), probably due to the low concentration of metal.

Screening of *Rhizobiales* strains for induction of tomato seed germination

From the total of 47 tested isolates, we were able to re-isolate from tomato only 29 isolates classified as *Methylobacterium* and *Rhizobium*, which were used to proceed the studies. To select *Rhizobiales* strains that directly promote plant growth, one *Rhizobium* sp. and two *Methylobacterum* spp. isolates were screened for direct plant growth promotion (PGP) abilities. These *Rhizobiales* were inoculated in seeds of *Cherry* and *Santa Cruz Kada Gigante* tomato, resulted in a germination rate that ranged from 90 to 98% and from 30 to 84%, respectively. However, as the germination rate was high for seeds of tomato *Cherry*, the effect of *Methylobacterium* spp. inoculation was not observed (Figure 3). In tomato *Santa Cruz Kada Gigante*, four isolates (A40, A59, B74 and B76) significantly reduced the germination rate, while six isolates (A21, A76, B40, B61, C2 and LGM86) increased the germination rate (Figure 3).

Rhizobiales- tomato plant greenhouse assay

Based on results of seed germination, strains A76, B61 and C2 were selected for evaluation of effect on tomato *Santa Cruz Kada Gigante* growth (germination rate, number of leaves, shoot and root length) in greenhouse experiments.

In the tomato *Santa Cruz Kada Gigante* plants inoculated with strain C2, the germination rate increased statistically ($p<0.05$) up to 13.64% compared to control. The strains A76 and B61 did not exert any significant difference in the evaluated conditions. For plant growth promotion, the highest values were obtained with the inoculation of C2 strain, which promoted a significant ($p<0.05$) increasing in shoot (17.3%) and root (17.8%) length (Figure 4).

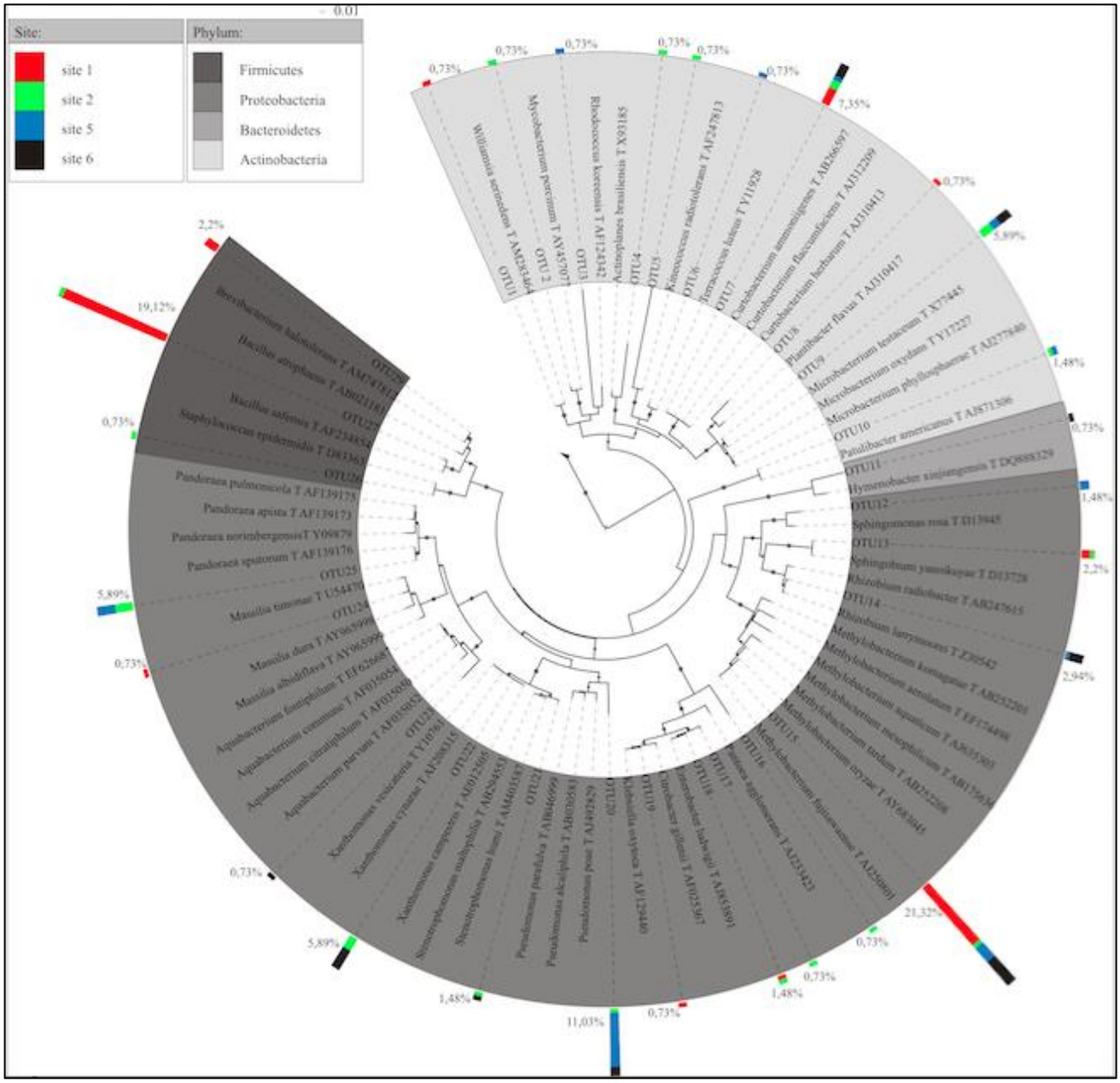

Figure 1. Phylogenetic tree of endophytic bacteria found in *T. stans.* Built by Neighbor-Joining method, Jukes-Cantor model. With Bootstrap of 1000. Colored bars indicate the isolation place and its height is proportional to the OTU frequency.

In contrast, a negative effect on root length was observed in plants inoculated with strain A76. The effect of bacteria inoculation on number of leaves was also evaluated and ranged from 8 to 12 leaves. However, no bacteria showed a statistical difference when compared with the control plants (Figure 4).

Considering that, the isolated C2 significantly increased the germination rate and shoot and root length after seed inoculation for tomato *Santa Cruz Kada Gigante*, this isolate was also inoculated in seeds of Tomato *Cherry*. Although an increase in germination rate was not observed in tomato seedling *Cherry* resulting from inoculated seeds, the shoot (13.57%) root (21.91%) length and leaves number (8%) increased statistically ($p<0.05$) compared with the control seedlings.

Phosphate solubilization and nitrogen fix assays in plant growth *Rhizobiales* strains

In order to understand the mechanisms involved in

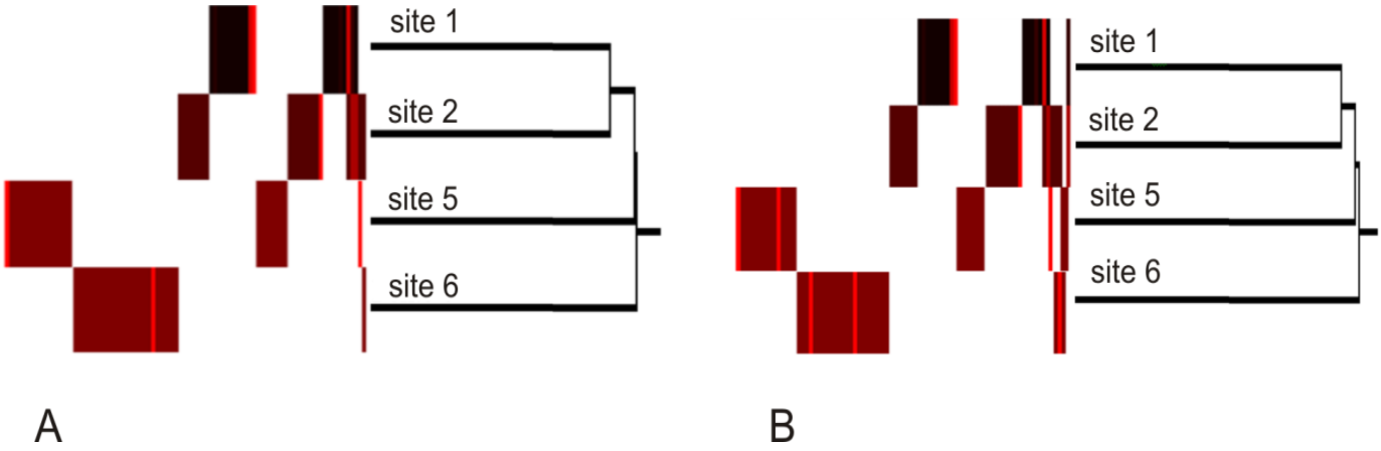

Figure 2. *Heatmap* and *Cluster* based on Jaccard index built from sequences of isolate from contaminated places. (A) Sequences at 97% similarity level (species); (B) Sequences at 95% similarity level (genus). The red color indicates the highest frequency of OTUs and black indicates the lowest.

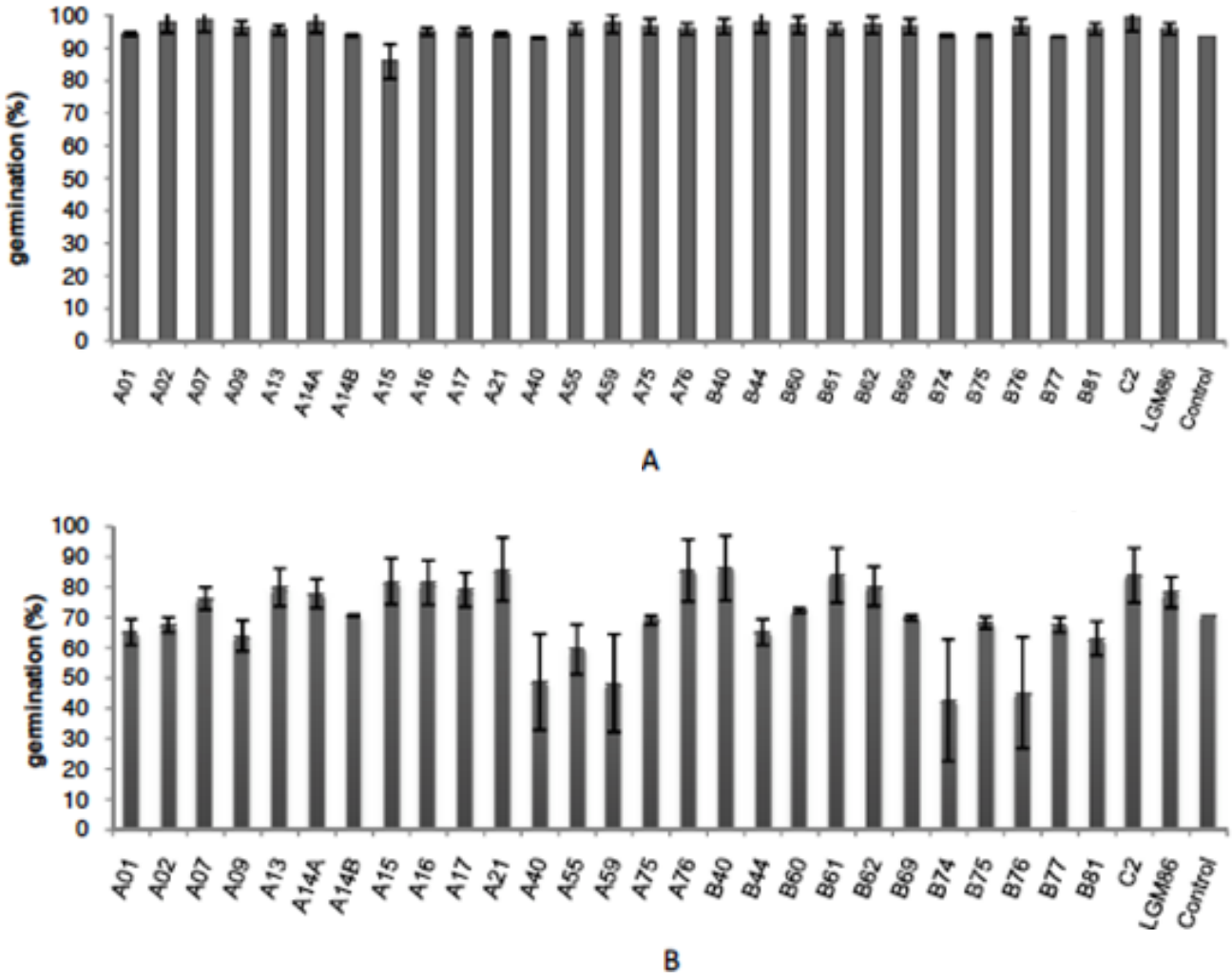

Figure 3. Influence of endophyte Rhizobiales inoculation in tomato germination. (A) Cherry; (B) Santa Cruz Kada Gigante.

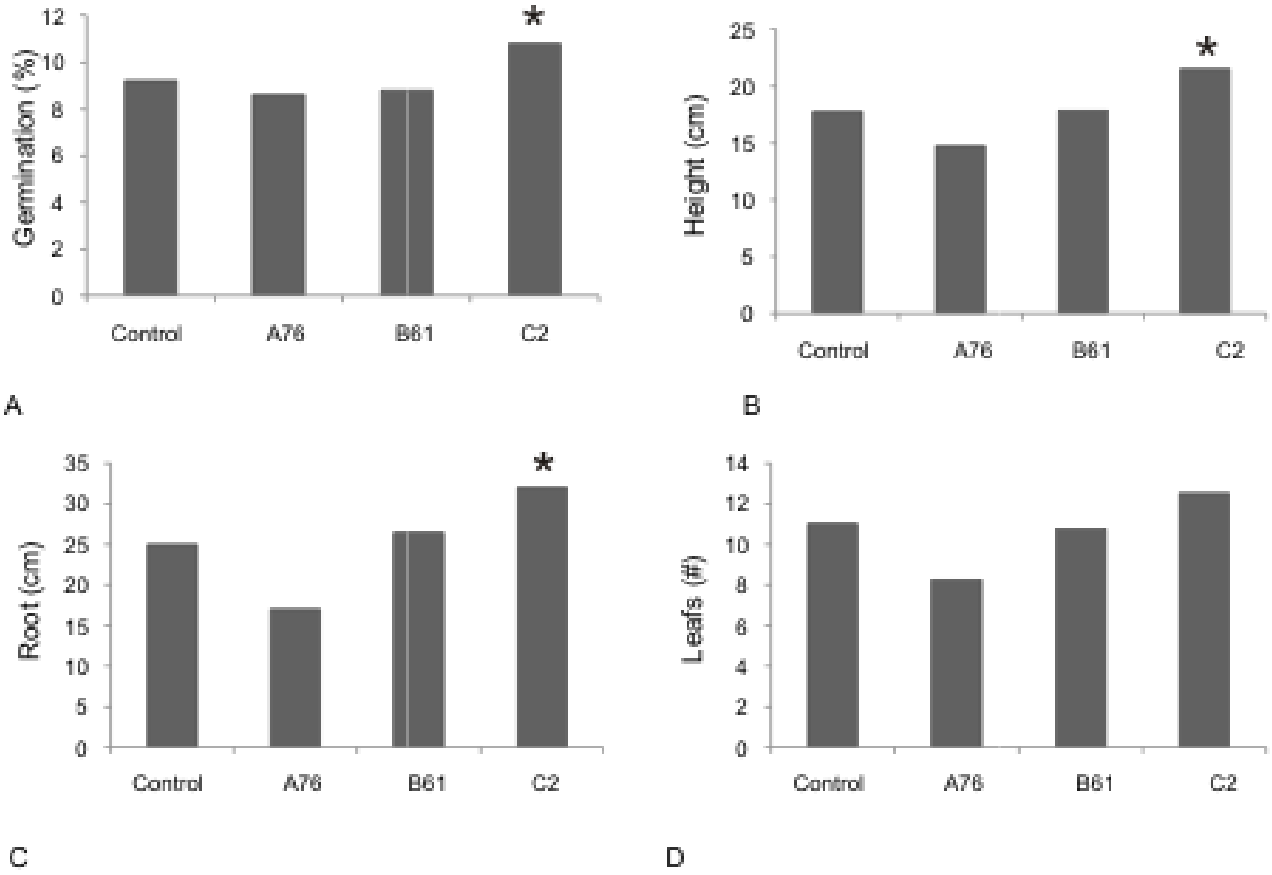

Figure 4. Response of tomato plant *Santa Cruz Kada Gigante* in greenhouse with *Methylobacterium* sp. (strains A76, B61) and *Rhizobium* sp. (C2) inoculation. (A) Germination; (B) Shoot height; (C) Root length and (D) number of leaves (*statistically different at p<0.05).

bacteria plant interaction, the *Methylobacterium* sp. strains A76, B61 and C2 were screened for direct PGP traits. The N_2-fixing ability of endophytic strains was screened by growth on N_2-free media. The strains C2 and B61 showed the capacity to grow in nitrogen-free conditions, while strains A76 and B61 formed a halo around the colonies on medium containing inorganic phosphate and were considered positive for phosphate solubilizing. In the present conditions, only the strain B61 was able to fix N_2 and solubilize inorganic phosphate.

DISCUSSION

Abiotic stress can select the adapted microbiome and consequently decrease bacteria diversity, indicating negative effects of the environment on the host plant and in endophytic bacterial community (Hardoim et al., 2015; Truyens et al., 2016). Previous reports show that the diversity and richness of endophytic community in *Arabidopsis thaliana* seeds decreased in plants exposed to cadmium (Truyens et al., 2016). In the present study, low metals levels did not affect the density of endophytic bacteria within the *T. stans* stems grown in the contaminated soil.

The analysis also did not demonstrate variation in bacterial density in endophytic *T. stans* isolates, suggesting that the plant must adjust the bacterial density inside, independent of the effects of soil conditions. Plant parts in direct contact with the ground (roots and rhizosphere) suffer more influence because of the higher concentration of metals from soil. Moreover, endophytes were reported to tolerate higher heavy metal levels than rhizosphere bacteria (Idris et al., 2004).

In addition, using ∫-Libshuff analysis with the Bonferroni correction as significance criterion, it was observed that the endophytic bacterial communities of *T. stans* present in site 1 and 2 are similar (p = 1.00), but are significantly different from sites 5 and 6 (p <0.0001). These differences in bacterial community may be associated with soil variations and non-environmental study of the area, which are located in the same park, with similar

humidity, temperature and rainfall. Sites 1 and 2 also exhibit similar levels of metals (both presented low metal concentration), although it can be suggested that there is correlation between the levels of individual metals and bacterial densities, as well as richness.

Furthermore, previous studies show that after heavy metals application, the microbial community decreases, but after a period of adaptation, it can be restored to previous levels (Rajapaksha et al., 2004) due to the selection of metal tolerant species (Baath, 1989), and also due the resilience of this microbial community under transitory impact. Endophytic bacteria present a key role on phytoremediation since it is able to enhance heavy metal pytoextraction (Rajkumar et al., 2009; Muehe et al., 2015). In this context, a higher density of endophytic bacteria of *Methylobacterium* genera can be explained by its heavy metal tolerance. Dourado et al. (2012) observed that *Methylobacterium* isolated from mangrove with oil contamination and high levels of heavy metal, showed high tolerance to cadmium, arsenic and lead. This high tolerance can reduce metal toxicity and promote tomato plant growth (Madhaiyan et al., 2007).

Methylobacterium and *Rhizobium* strains promoted tomato plants (Cherry and *Santa Cruz Kada Gigante*) growth and germination. Which was previously reported by other authors (Sy et al., 2001; Madhaiyan et al., 2006, 2007; Bogas et al., 2016). The mechanisms involved are: (1) phytohormone production: Mainly auxin and cytokinines (Dourado et al., 2015; Kwak et al. 2014); (2) Stress decrease: Heavy metal tolerance (Dourado et al., 2015) and ACC deaminase production (Bogas et al., 2016). Bacterial ACC deaminase uses the ethylene precursor: ACC (Aminocyclopropane-1-carboxylic acid) as source of nitrogen, decreasing the production of ethylene, increasing plant growth (Hardoim et al., 2008, 2015); (3) Nutrient uptake: Phosphate solubilization (Glick, 1995), nitrogen fixation and plant nodulation (Sy et al., 2001).

Rhizobium sp. strain C2 was able to fix N_2 and promote tomato seedlings Santa Cruz Kada Gigante and Cherry growth, indicating that it can be used to increase tomato production. Nitrogen fixer can help plant and mycorrhiza to improve vegetation of heavy metal-rich industrial sites (Ogar et al., 2015).

Finally, the present study shows that the presence of low metal concentration does not influence in endophyte cultivable community due to the niche of endophyte and well as its great plasticity. Furthermore, we were able to select plant growth promoter bacteria that can be used on tomato seedlings production and on future phytoremediation studies.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

This work was supported by a grant from the FAPESP - Foundation for Research Assistance of São Paulo State, Brazil (Proc. 2005/54617-2 and 2015/11563-1). The authors extend their thanks to FAPESP for the fellowship to A.J.F. and M.N.D. (Proc. 2013/17314-8) and CNPq by the Productivity Fellowship (1B) to W.L.A.

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academicJournals

Vol. 11(39), pp. 1468-1473, 21 October, 2017
DOI: 10.5897/AJMR2017.8667
Article Number: F70D0CB66475
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Bacterial and fungal etiologies of meningeal irritation syndrome in an emergency department

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Received 4 August, 2017; Accepted 26 September, 2017

Meningeal irritation syndrome denotes to a range symptoms that require emergency management. Clinically, it is an expression of an irritation of the meninges combining with headache, fever, neck stiffness, altered consciousness and vomiting. The aim of this prospective study was to contribute to the management of medical emergencies. Cerebrospinal fluid (CSF) samples recovered from patients presenting signs of meningeal irritation syndrome were submitted to bacteriological, biochemical and fungal analyses. 2656 patients were received during the study period with 47 (1.8%), which had signs of meningeal irritation syndrome. 17% of cases were bacterial meningitis while 10.6% were fungal meningitis. Microbes isolated were *Streptococcus pneumoniae, Streptococcus agalactiae, Haemophilus influenza* and *Cryptococcus neoformans*. All bacteria were found to be sensitive to amoxicillin + clavulanic acid, gentamycin, chloramphenicol and ceftriaxone, and resistant to penicillin G, ampicillin and cotrimoxazol. All cases of cryptococcal meningitis were HIV (+) subjects. Other causes of meningeal irritation syndrome were HIV-related encephalitis (19.2%) and malaria (19.2%). Bacterial and fungal meningitis accounted for about 1/3 of the cases of meningeal irritation syndrome. The bacteria isolated presented a satisfactory sensitivity profile to the usual antibiotics. Although the prevalence of meningeal irritation syndrome appears to be low, it would be interesting to pay particular attention to it given the high representation of immunocompromised patients.

Key words: Meningeal irritation syndrome, bacterial meningitis, fungal meningitis, Cerebrospinal fluid (CSF) analysis, antibiotic susceptibility.

INTRODUCTION

Meningeal irritation syndrome denotes to arange symptoms (headache, vomiting, neck stiffness, fever and altered consciousness) that require emergency management. Clinically, it is an expression of an irritation

of the meninges. One cause can be meningitis, an inflammation of the meninges usually owing to a bacterial infection, viruses, fungi, protozoans, or other causes that cannot be determined (Bruneel and Wolf, 2000; Desmettre et al., 2007).

Bacterial meningitis remains the most dangerous. Early clinical diagnosis and prompt treatment are the hallmark for survival. Empirical antimicrobial treatment is recommended to limit mortality (Loukili et al., 2015). The choice of molecule depends largely on the local epidemiology and the pharmacology of available agents. Infectious meningitis are particularly challenging in developing countries due firstly to poor-socioeconomic conditions that facilitate the spread of the pathogens and poor access to care, and secondly to the HIV epidemic which has changed the pattern of meningitis in African countries in particular (Hakim et al., 2000; Schutte et al., 2000; Traore et al., 2014). In these countries, meningitis remains one of the most serious infections occurring in individuals with HIV infection.

With the view to contribute in the management of medical emergencies, a study on the clinical and biological presentation of patients with meningeal irritation syndrome was conducted at the Yaounde Central Hospital Emergency Unit. The specific objectives were: to determine the prevalence of bacterial or fungal meningitis among patients with meningeal irritation syndrome, to identify the microbes involved and to evaluate the susceptibility to common antibiotics, of isolated bacteria. An attempt to correlate biochemical characteristics of a cerebrospinal fluid sample and the etiology of a meningeal irritation syndrome was also made.

MATERIALS AND METHODS

This study was performed from January to May 2009, in the emergency unit of the Yaounde Central Hospital. It was approved by the National Ethics Committee. Patients aged between 16 to 81 years, presenting with signs of meningeal irritation syndrome were included. Then, a clinical examination was performed by a physician.

Sociodemographic and clinical data were collected using a questionnaire. CSF samples were collected and submitted to cyto-bacteriological, biochemical and fungal analyse.

CSF sample collection and analysis

Two to five milliliters of CSF were collected in a sterile container, according to good clinical practices and were immediately processed. Each CSF sample was subjected to macroscopic examination. It was then centrifuged at 2000 rpm for 15 min. The supernatant was used for biochemical, cytological and immunological tests whereas the pellet was used for microbiological analysis (Joffin and Leyral, 2005).

Biochemical analysis

Concentrations of proteins, glucose and chlorides in CSF samples were determined using respectively, the Biuret (Falkner and Metter, 1982), Trinder (Trinder, 1969) and Tiezt methods (Tietz et al., 1999).

Glucorachy was considered normal when it was greater than or equal to 0.5 g/l (50% of the patient's blood sugar) and abnormal in the opposite situation. Chlororachy was lower, normal and greater for values respectively, less than 110 mmol/l, between 110 and 130 mmol/l and greater than 130 mmol/l. Proteinorachy was lower, normal and higher for values respectively, of less than 0.25 g/l, between 0.25 and 0.45 g/l and greater than 0.45 g/l.

Immunological analysis

Soluble bacterial antigens of *Streptococcus agalactiae*, *Haemophilus influenza* b, *Streptococcus pneumoniae*, *Neisseria meningitidis* and *Escherichia coli* were determined, using latex agglutination tests (LAT) (Reagent Remel Wellcogen Bacterial Antigen Kit). Cerebrospinal fluid (CSF) sample was pre-heated at 100°C in a water bath for 5 min, cooled to room temperature and centrifuged at 2000 rpm for 15 min. The supernatant was then used for LAT.

Disposable reaction cards containing six separate circles with the color codes of different test latex reagents were provided with the kit. A single drop of respective samples was placed on the separate circles of the reaction card and a drop of each of five different test latex reagents was added to the separate circles. These were mixed thoroughly and manually rotated for 3 min. They were then observed for agglutination. Positive and negative controls and control latex tests were put up (Kaldor and Asznowick, 1977).

Cytological exam

Leukocytes were counted using a Nageotte counting chamber after May-Grünwald-Giemsa staining (Piaton et al., 2016).

Bacteriological and mycological analyses

After a microscopic examination of the smears prepared from the sediment, the isolation was carried out onto sheep blood agar, chocolate agar with X and V factors, and Sabouraud agar without Actidione. Bacterial growth was observed after incubation of the agar plates at 37°C during 24 to 48 h. The identification was made on the basis of biochemical and immunological characteristics (presence of catalase and of oxidase, needs in factors V and X for *H. influenzae*, sensibility to optochine for *S. pneumoniae*, oxidase test, serogrouping test for *S. agalactiae*) (Denis and Mounier, 1991; Joffin and Leyral, 2005).

Antimicrobial susceptibility testing with the following antibiotics: penicillin, ampicillin, ceftriaxone, gentamycin, chloramphenicol and cotrimoxazol were performed according to standards for

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Table 1. CSF cell count and biochemical parameters of patients.

Parameter	Patients = 47 (%)
Leukocyte count (cells/mm^3)	
≤ 4	29 (61.7)
5 - 10	6 (12.8)
11 - 99	5 (10.6)
≥ 100	7 (14.9)
Mononuclear cells (%)	
≤ 50	7 (14.9)
> 50	40 (85.1)
Polymorphs cells (%)	
≤ 50	38
> 50	9 (19.2)
Glucose (g/l)	
< 0.5	23 (48.9)
[0.5 - 0.6	24 (51.1)
Chloride (mmol/l)	
< 110	8 (17)
110 - 130	36 (76.6)
> 130	3 (6.4)
Protein (g/l)	
< 0.25	6 (12.8)
0.25 - 0.45	32 (68.1)
> 0.45	9 (19.2)
Normal CSF [(1)]	20/47 (42.6)

[(1)] Normal Cerebrospinal Fluid [leucocytes ≤ 4 cells / mm^3; glucose (0.5 - 0.6 g/l), chloride (110 - 130 mmol/l) and protein (0.25 - 0.45 g/l)] antimicrobial disk susceptibility tests described by Ferraro et al. (2003).

Statistical analysis

The data obtained were subjected to ×2 tests, which was applied for group comparison. A p value <0.05 was considered statistically significant.

RESULTS

Among the 2656 patients that were received at the emergencies, 47 (1.8%) were eligible and the informed consent to participate to the study was obtained for all. 25 (53.2%) subjects were male. Patients aged ≥18 years were found for 45 cases (95.7%).

Clinical features of study population

The delay between the onset of symptoms and consultation ranged from 1 to 30 days. The median was 4.5 days. Fever was the most predominant symptom with 85.1% cases, followed by headache (61.7%) and altered consciousness (61.7%). The percentage of neck stiffness (42.6%) and vomiting (27.7%) were relatively low, compared to other symptoms. 15 (31.9%) patients died before the end of the study.

31 patients (65.9%) were HIV positive, 4 (8.5%) were HIV negative, and 12 (25.5%) had an unknown status. Out of the HIV positive patients, 12 (38.7%) were under antiretroviral treatment.

Cytology and biochemistry of CSF

The number of leukocytes from CSF was found to be normal (≤ 4 cells /mm^3) in 29 samples (61.7%), moderate (between 5 and 100 cells/mm3) for 11 samples and high (>100) in 7 samples. Regarding the different kind of leukocytes, mononuclear and Polymorphs cells counts were respectively higher in 40 (85, 1%) and 9 (19, 2%) samples.

The level of glucose, chlorides and proteins was lower in 23 (48.9%), 8 (17%) and 6 (12.8%) samples, respectively. Levels of chlorides and proteins were found to be higher in 3 (6.4%) and 9 (19.2%) samples, respectively. With regard to the criteria that a normal Stem cell factor (SCF) should have, 20 samples (42.6%) out of the 47 analysed were normal, while 27 samples (57.4%) were abnormal (Table 1).

Prevalence of bacterial or fungal meningitis among patients with meningeal irritation syndrome

Bacterial and *Cryptococcus* meningitis accounted respectively for 17 and 10.6% of cases of meningeal irritation syndrome, presented at the Emergency Centre of the Yaounde Central Hospital.

Three other etiologies of meningeal irritation syndrome were recorded: HIV encephalitis (19.2%), malaria (19.2%), and septicemia (2.1%).

Bacteria and fungi involved in bacterial or fungal meningitis among patients with meningeal irritation syndrome

Three bacterial species were isolated and identified in six samples; namely *S. agalactiae* (2 cases), *S. pneumoniae* (2 cases) and *H. influenzae* (2 cases). Bacterial soluble antigens of *S. agalactiae* (3 cases), *S. pneumonia* (3 cases) and *H. influenza* (2 cases) were found from the different samples. *Cryptococcus neoformans* was identified in five samples.

Antibiotic susceptibility of isolated bacteria

All the isolated bacteria were found to be susceptible to

Table 2. Antibiotics sensitivity pattern of bacterial species isolated.

Antibiotics		*S. pneumoniae* (n = 2)		*S. agalactiae* (n = 2)		*H. influenzae* (n = 2) (n = 2)		
		S	R	S	R	S	I	R
Class of β-lactamines	Penicillin G	1	1	1	1	1	0	1
	Ampicillin	1	1	1	1	1	0	1
	Amoxicillin+Clavulanic acid	2	0	2	0	2	0	0
	Ceftriaxone	2	0	2	0	2	0	0
Class of aminosides	Gentamycin	2	0	2	0	1	1	0
Class of phenicoles	Chloramphenicol	2	0	2	0	2	0	0
Class of sulfamides	Cotrimoxazol	1	1	2	0	2	0	0

S, Sensitive; I, intermediary; R, Resistant.

Table 3. Laboratory diagnosed meningitis in patients in relation to their HIV serostatus.

Aetiology of meningitis	HIV positive	HIV negative	Unknown status
Cryptococcal meningitis	5 (100%)	00	00
Bacterial meningitis			
Streptococcus pneumoniae	2 (25%)	1 (12.5%)	00
Streptococcus agalactiae	2 (25%)	00	1 (12.25%)
Haemophilus influenzae	1 (12.5%)	00	1 (12.5%)

amoxicillin + clavulanic acid, gentamycin, chloramphenicol and ceftriaxone; and resistant to penicillin G, Ampicillin and Cotrimoxazol (Table 2).

Meningeal irritation syndrome and HIV

40% of patients with meningeal irritation syndrome were HIV positive. Thus, all patients with encephalitis, fungal meningitis and most of those with bacterial meningitis were HIV infected (Table 3).

Cytology and biochemistry of CSF versus etiology of a meningeal irritation syndrome

In the subgroup with normal leukocytes (29 subjects), proteinorachy and chlorurorachy were also normal in all samples. Glucorachy was normal in 24 samples and low in five. The etiologies of the meningeal irritation syndrome identified were one fungal meningitis, unclarified diagnoses, nine malaria (four with hypoglucorachy) and four HIV encephalitis.

In the subgroup with a moderate level of leucocytes (11 samples), hypoglucorachy was observed in all the samples, proteinorachy was normal in three, low in six and high in two samples. Chlorurorachy was normal in one, low in seven and high in three samples. Etiologies identified were five encephalitis (with hypoproteinorachy), four fungal meningitis, one bacterial meningitis and one malaria (with hyperproteinorachy).

In the last subgroup with high levels of leukocytes (seven samples), glucorachy was low in all samples while proteinorachy was high. Chlorurorachy was normal in six and low in one. The meningeal irritation syndrome was caused by bacteria (Table 4).

DISCUSSION

The emergency unit of the YCH receives more than 50% of emergency's cases in Yaounde. With regard to the total number of patients (2656) that were received during the study, 1.8% (47) presented meningeal irritation syndrome. Hence, the situation is not so dramatic, but should not be neglected.

Demographic data analysis of patients revealed that they were mainly males (53.2 %). This finding agrees with those of Fouad et al. (2014); Khater and Elabd (2016) who confirmed that males were more significantly affected with bacterial meningitis than females. Concerning the clinical features of enrolled patients, fever was the most predominant sign (85.1%), followed by headache (61.7%), altered consciousness (61.7%), neck stiffness (42.6%) and vomiting (27.7%). The two first signs are in accordance to those of Newman (2004) for patients with meningitis. However, we did not observed the same proportions regarding the altered consciousness

Table 4. Correlation between the etiology of meningeal irritation syndrome and the biochemical characteristics of CSF.

Leucocytes count diagnoses (cells/mm^3)		Biochemistry								
		Glucorachy (g / l)			Proteinorachy (g / l)			Chlororurachy (mmol / l)		
		Low (< 0.5)	Normal (≥ 0.5)	High (>0.5)	Low (< 0.25)	Normal (0.25 < n < 0.45)	High (> 0.45)	Low (< 110)	Normal (110 < n < 130)	High (> 130)
Normal (≤ 4) 61.7%	Malaria 31.04%	±	±			+			+	
	Fungal meningitis 3.45%	+				+			+	
	Encephalitis 13.79%		+			+			+	
	Unclarified diagnoses 51.72%		+			+			+	
Moderated (5 < n < 100) 23.4%	Malaria 9.10%	+					+	+		
	Fungal meningitis 36.35%	+			±	±		+		
	Encephalitis 45.45%	+			+			±	±	±
	Bacterial meningitis 9.10%	+					+	+		
High (> 100) 14.9%	Bacterial meningitis 100%	+					+	±	±	

and neck stiffness for which he got 46 and 70%, respectively.

Higher frequency of patients with altered consciousness was observed in our study which could be explained by the long delay between the onset of the symptoms and admission to the hospital as described by Loukili et al. (2015); indeed, the poor socio-economic conditions of the populations resulted to low health care access. The rate of mortality was high (31.9%) because of the very late admission to the emergencies; most of the patients (33.3%) were self-medicated and decided to go to the hospital because of the persistence and worsening of the clinical signs implication; when the causal agent had caused serious damages to their organism.

Analysis of the CSF allowed to distinguish 3 subgroups in which leucocytes counts were normal, moderate or high. The number of leucocytes was normal in patients suffering from malaria. It was normal or moderate for those having HIV encephalitis and fungal meningitis. This was high from those having bacterial meningitis; the last observation was in accordance with those of Khater and Elabd (2016). Patients with hypochlorurachy presented vomiting. Vomiting is a perturbation of cerebrospinal fluid (Pradat and Jan, 2007) and is inconstant at the onset of infection (Desmettre et al., 2007).

High proteinorachy and low glucorachy were observed in patients with meningitis. High proteinorachy can be explained by the increase of the hematomeningeal barrier permeability which results in the passage of many proteins and others plasma molecules *(*Maugein, 2006). Low glucorachy results from the use of glucose by infectious germs (Pradat and Jan, 2007).

The fact that detection of antigens was more efficient in diagnosing meningitis even from samples of patients under self antibiotherapy, was not a surprise. As far as immunological and bacteriological tests are concern, immunological test appeared to be more accurate to diagnose active or latent infection since it targets the consequence of the presence of the infectious agent and not the infectious agent itself. *Streptococcus pneumonia* and *S. agalactiae* were the most responsible of bacterial meningitis (75%); our results are in accordance with those of previous studies (Koulla-Shiro et al., 1997; Bruneel and Wolf, 2000; Fonkoua et al., 2001; Popovic et al., 2000; Pradat and Delattre, 2002). Traoré et al. (2014) described *S. pneumonia* as being responsible for meningitis in young adults and the elderly in Cameroon, while *S. agalactiae* was responsible for neonatal meningitis and severe opportunistic infections in immune-compromised patients, mainly those suffering from acquired immune deficiency syndrome (AIDS).

H. influenzae was also isolated; this bacterium generally infects subjects aged from 1 month to 15 years. We did not isolate *Neisseria meningitidis* in this study; that could be explained by the fact that, bacteria are mainly involved in the cases of epidemics of meningitis in sahelian region of Cameroon. *C. neoformans* was the only microbe responsible of fungal meningitis, and in 40% of the cases of meningeal irritation syndrome

from patients with AIDS; these results are in accordance with those of previous studies (Gordon et al., 2000; Roos, 2003; Millogo et al., 2004; Békondi et al., 2006), since *Cryptococcal meningitis* has been described as the most common life-threatening opportunistic fungal infection in patients with immune-compromised status.

Conclusion

Bacterial and fungal meningitis accounted for 27 (6%) of cases of meningeal irritation syndrome presented at the Emergency Centre of the Yaounde Central Hospital. *S. pneumonia* and *S. agalactiae* were the main causative agents of bacterial infection, while *C. neoformans,* was the one responsible for fungal infection.

The bacteria isolated presented a satisfactory susceptibility profile to the usual antibiotics although, the prevalence of meningeal irritation syndrome appears to be low in the medical emergencies at Yaoundé Central Hospital. Special consideration should be given to the high representation of immunocompromised patients.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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academicJournals

Vol. 11(39), pp. 1474-1483, 21 October, 2017
DOI: 10.5897/AJMR2017.8622
Article Number: 47DD68866479
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Study of kefir biofilm associated with hydroethanolic extract of *Euterpe oleracea* Mart. (açai)

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Received 19 June, 2017; Accepted 20 July, 2017

Kefir is a microbial complex capable of producing biofilms. This study aims to obtain biofilms associated with hydroethanolic extract of Euterpe oleracea Mart. (BEHEEo) as fixing method of recovery anthocyanins in kefir biofilm. Thus, BEHEEo formation kinetics was made at different concentrations of aqueous extract of E. oleracea, of kefir grains (KG) and of brown sugar (BS). Thereafter, macro and microelements of BEHEEo were determined; the rheological and structural characteristics were analyzed using atomic force microscopy (AFM), and anthocyanins dosage in BEHEEo was obtained. The best concentration of BS to form BEHEEo was 40 g/l with 100 ml of EHEEo (12.62±0.16 g), as well as a release of the BEHEEo anthocyanins occurring after 5 min (4.3±0.6 mg/100 g) with maximum peak at 60 min (20.8±0.3). The concentration of calcium iron and magnesium was 0.089, 0.1740 and 0.1808 mg/kg, respectively, indicating the concentration of Zn2+ which was 0.533 mg/kg. The AFM analysis revealed differences in the peaks of roughness depending on the concentration of kefir grains, with presence of Lactobacillus and yeast. The concentration of anthocyanins in BEHEEo two years after the incorporation was 26 mg/100 g. Therefore, it is suggested that the BEHEEo incorporating EHEEo anthocyanins has potential for therapeutic applications in several pathologies necessary for antioxidative processes.

Key words: Biofilms, kefir, açaí, *Euterpe oleracea* Mart., anthocyanins, atomic force microscopy (AFM).

INTRODUCTION

Kefir is a beverage produced by fermenting substrates such as milk or sugar water by kefir grains (Rodrigues et al., 2005; Laureys and De Vuyst, 2014). The growth and the function of bacteria inside a population are crucial for their survival (Davey and O'toole, 2000). In kefir, there is a predominance of *Lactobacillus* which has high catabolic

capacity and is complemented by yeast with high biosynthetic capacity.

When growing kefir in water for more than twenty days, there is the formation of biofilms with therapeutic properties against pathogenic organisms. These biofilms are surfaces with a high rate of structural organization associated with bacterial colonies held together by a polymer secreted by their cells, which is called extracellular polymeric substance (EPS) (Watnick and Kolter, 2000, Donlan, 2002; Zhang et al., 2015). Among many strategies used for the study of biofilms, the one that is more highlighted is the use of the Atomic Force Microscopy (AFM), which allows measurement of the interaction forces between cells or cell-cell interactions (Dufrêne, 2015).

One of the substances that have been intensified in recent years are the anthocyanins, due to their protective and healing properties. Anthocyanins have an important role in physiological functions related to human health (Lee et al., 2013). The antioxidant effect of anthocyanins have been known and proven fact. The action mechanism of the anthocyanin is inhibiting the free radicals that promote oxidation. The antioxidant agents capable of binding to the free radicals are found in the medium.

The açaí (*Euterpe oleracea* Mart.) plant species is found in wetlands along rivers and streams, in the lowlands and wetlands, along the full extent of the Amazon (Malcher, 2011). The high anthocyanin content with its consequent antioxidant power has awakened growing interest in the study of the fruits of this plant. This fact is related to the antimutagenic effect (Galotta and Boaventura, 2005). The fruits of palm tree have high value as antioxidants, and are therefore considered a great source of antioxidants (Santos et al., 2008). This study aims to obtain kefir biofilms associated with aqueous extract of *E. oleracea* (EHEEo) as means of fixation and future use of anthocyanins.

MATERIALS AND METHODS

Study area

Kefir grains (2 kg) were obtained in the Laboratory of Drugs Research at the Federal University of Amapá, Amapá, Brazil. Five kilogram of the ripe fruit of açaí (*E. oleracea*) was collected in August, 2015 in a fragment of Amazon forest (16° 30′ to 16° 44′ S and 56° 20′ to 56°30′ W) in Mazagão city, State of Amapá, Brazil.

Sample collection and preparation

A total of 500g of the ripe fruit of açaí (*E. oleracea*) was weighed and placed in maceration at ambient temperature (23°C) in ethanol 98% acidified with 2% of formic acid for 3 days. After this procedure, the extraction solution was filtered and the mass discarded. The EHEEo was concentrated on rotaevaporator (Hikal, India), and the determination of anthocyanins was performed based on the AOAC method (2005-02).

To obtain the biofilm associated with hydroethanolic extract of *E. oleracea,* bottles with 4 L capacity were used and then placed in a laminar flow cabinet under ultraviolet UV radiation for two hours. A brown sugar (BS) solution (40 g/l) with KG (0.25, 0.5, 1, 2, 5, 10, 20, 40, 60 and 80 g/l) were added to 100 ml of EHEEo and conditioned at 25°C for 20 days. After this time, it was removed and weighed. The volume and pH of the remaining solution were measured and or the best concentration of KG was considered for the formation BEHEEo in terms of biomass consistency, in other words, the one that could be separated without loss of integrity.

KG (40 g/l) were inoculated at different concentrations of BS solution (0.25, 0.5, 1, 2, 5, 10, 20, 40, 60 and 80 g/l) in which was poured EHEEo (100 ml) and the final volume was 500 ml. The experiment was performed in triplicate. After 20 days pH, volume and weight of BEHEEo were measured; treatment being considered the best is the one that presented the highest BEHEEo biomass consistently.

The amounts of 10, 25, 50 and 100 g of EHEEo were inoculated completing the volume of 500 ml with BS solution (40 g/l) with kefir grains 20 g/l. After 20 days, the grains and BEHEEo formed were weighed separately. The pH, temperature, volume were measured and the dosage of anthocyanins in the BEHEEo and in the culture medium was performed based on the AOAC method (2005-02). The best concentration was the one that presented the highest concentration of anthocyanins.

The quantification of anthocyanins was carried out using the AOAC method (2005-02). KG was inoculated (20 g/l) in 400 ml of BS solution (40 g/l) in which was poured in 100 ml of EHEEo. After 20 days, the BEHEEo were removed and weighed on an analytical balance and further dehydrated in oven at 110°C. After that, 1 g BEHEEo was placed in 100 ml of distilled water and left to stand for 24 h protected from light at room temperature (25°C). 10 ml of this dilution was then removed to assay anthocyanin. 10 ml of the culture medium for dosage and 1 g of the EHEEo was also removed. Both samples were filtered through paper Whatmam No. 3.

The samples of EHEEo (1 g) of the culture medium with kefir and of the EHEEo (10 ml) were diluted in buffer (KCl 0.03 M, pH 1), and in sodium acetate buffer (0.4 M, pH 4.5), respectively and the volume was completed to 50 mL. Then, the readings were performed on Shimadzu UVMini 1240 (Shimadzu Corporation, Kyoto, Japan) at a wavelength of 520 and 700 nm for anthocyanin, respectively. For calculating the anthocyanin concentration, the following formula was employed:

$$TAC = A/e \times l \times MW \times DF \times M/W \times 100\%$$

Where, TAC = total anthocyanin content in %; $A = (A_{520\ nm} - A_{700\ nm})_{pH1.0} - (A_{520\ nm} - A_{700\ nm})_{pH4.5}$; MW (molecular weight) = 449.2 mol^{-1}; DF = dilution factor; W = sample weight in mg; l = optical path in cm; ε = 26.900 M extinction coefficient in L $mol^{-1}cm^{-1}$ to cyd-3-glu
The anthocyanin of the açaí fruits was expressed as cyanidin-3-glucose (Inácio et al., 2013) and this method is based on the fact that the monomeric anthocyanins undergo reversible structural change in function of the pH.

In this assay the equipment Dissolution Model 299-6 was used (New Ethics, Ltda. Sao Paulo, Brazil) with rotation speed of 75 rpm. In each vessel of the equipment was placed 1 g of BEHEEo and

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100 mL of distilled water pH 7.0, which was used as a dissolutor means. The temperature was maintained at 37±1°C. The samples were collected at 5, 10, 30 and 60 min respectively. After the end of the process, the samples were filtered through Whatman paper n°1, and then performed the determination of anthocyanins using the AOAC method (2005-02).

A total of 100 g of the BEHEEo samples were used for the determination of macro and microelements using the calcination method described by Salazar et al. (2011). The analysis was made in Laboratório of Absorção Atômica e Bioprospecção – UNIFAP by atomic absorption spectrophotometry model 6300 Shimadzu AAS.

BEHEEo structural analysis by atomic force microscopy (AFM)

The surface morphology of BEHEE was analyzed by atomic force microscopy (AFM Easyscan2/STM, Nanosurf; USA). The relative humidity during the measurements was maintained at 51%. The scan was of 30 mm × 30 mm, and the images were obtained in contact mode. The cantilever has rectangular shape with spring constant of 0.77 nN/m and constant strength of 0.2 N/m, resonance frequency of 13 KHz. For the analysis of BEHEEo, they were dehydrated and electrodeposited on slides with gold films after 03/10 scanning cycles and analyzed by cyclic voltammetry on its device.

Twenty images of different regions were examined and analyzed, and the roughness values (RMS) were calculated. The parameter analyzed was the average roughness (Rm) and its effective value (RMS), defined by the equations described by Khulbe et al. (2008);

$$R_m = \int Z(x,y)dxdy$$

Where R_m is the average roughness and Z (x, y) is the vertical profile of the area

$$RMS = \sqrt{\iint |Z(x,y)|^2 dx\, dy}$$

RMS expresses the average of the roughness square root.

Analysis of kefir biofilms through scanning electron microscopy (SEM)

The analysis of the microstructure and surface morphology of the biofilms were investigated by scanning electron microscopy (TM3030Plus, Hitachi, Japan). The accelerating voltage of 15 kV was used. The magnification used was 2.5 K.

Stability of the anthocyanin in the biofilms

It was sought to verify if the anthocyanins present in the biofilms remained stable over time. Therefore the anthocyanins incorporated in biofilms were quantified. These biofilms were produced 12 and 24 months ago using the same analytical methodology previously described. So, to evaluate if the anthocyanins present in biofilm were stable throughout the time, the anthocyanin content at 12 and 24 months after the biofilms' preparation was evaluated. The same analytical methodology was used as mentioned before. Three different biofilms (BHE1, BHE2 and BHE3) made in the same conditions as described before, were used. The biofilms were held at 25ºC, in amber flask with liner of PVC and tight closed for 24 months. The analysis was made in triplicate.

Statistical analysis

For statistical analysis of the results the Statgraphics Centurion XVI software (STATPOINT Technologies, 2012) was used. ANOVA was used followed by Tukey's test, and the average weights of BEHEEo were analyzed using the Student t test, and results with $p < 0.05$ were considered significant.

RESULTS

Obtainment of BEHEEo

It was observed the formation of BEHEEo biomass was dependent on the increase of the concentration of the KG (Figure 1). It was observed that the change in pH occurred, which was 4.42 at the beginning, and then declined in accordance with the concentration of KG (Figure 1). ANOVA showed the difference among the treatments, and according Tukey test, above 10 g/l of the KG the biomas of biofilm (13.10±0.30 g) represented no significant increases. At 40 g/l of the KG (14.52±0.45 g) BEHEEo was obtained biofilm with good integrity, however at lower concentrations it was broken up easily.

An important parameter for obtaining BEHEEo is the consistency. The consistency here is simply defined as the formation of cohesive BEHEEo in their structures. BEHEEo without consistency break up easily and disperse in the medium. The consistency was obtained only when using concentrations equal to or greater than 40 g/l of the KG (14.52 ± 0.45 g of the biomas).

Determination of the concentration of substrate to form biofilms with EHEEo

The variation of the BS concentration in EHEEo resulted in different BEHEEo cohesiveness. Without BS and only EHEEo, the BEHEEo formed showed no cohesiveness. With the BS and the BEHEEo from 20 g/l of the BS (12.44 ± 0.332 g the biomas) BEHEEo was presented with cohesiveness and the anthocyanins were incorporated to them (Figure 1). ANOVA showed a significant difference between treatments with different concentrations of BS up to 10 g/L. Through the Tukey test it was shown that only above 10 g/L (12.68 ± 0.3272 g) there were no significant differences in the treatments. However, to obtain BEHEEo cohesiveness is necessary concentration of 40 g/l (12.62 ± 0.1626 g) (Figure 1). The formation of BEHEEo was stabilized around 10 g/l of BS and the pH fell from 4.61 to 2.72 over 24 days (Figure 1).

Determination of the concentration of EHEEo to the biofilm formation

When only the EHEEo used without BS, fragmented biofilms with no cohesiveness and with the medium pH around 6.5 was obtained. With the addition of the BS to

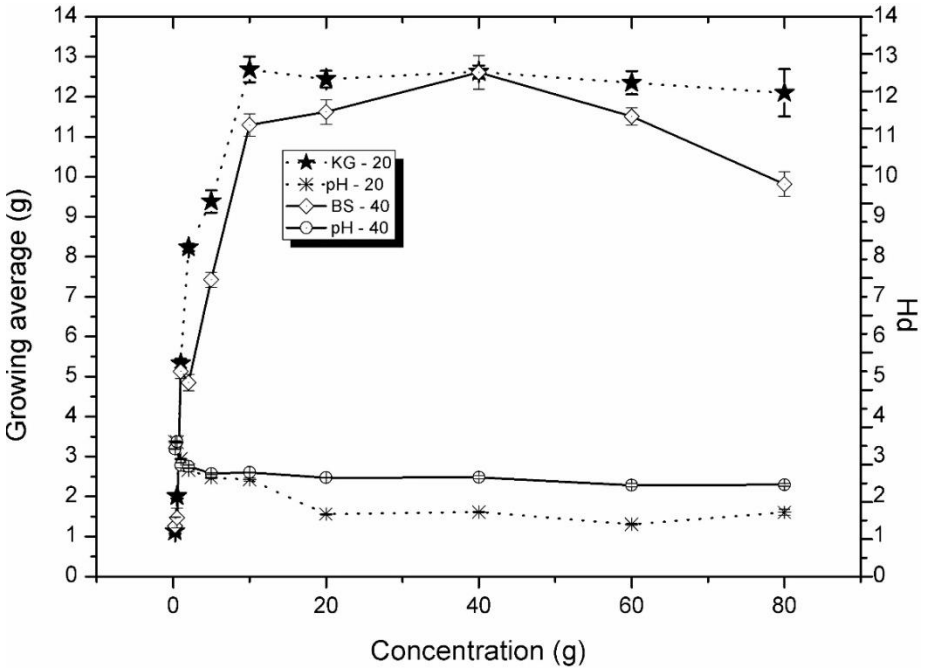

Figure 1. Growing average of the biomas of biofilms: ★ Formation of the biofilms associated with hydroethanolic extract of *E. oleracea* Mart. (BEHEEo) in different concentrations of kefir grains (KG) in brown sugar solution (BS) (40 g/l). ◇ Formation of the biofilms associated with hydroethanolic extract of *E. oleracea* Mart (BEHEEo) at different concentrations of brown sugar (BS) with kefir grains (KG) (20 g/l). * Altering of pH at variation of concentrations of kefir grains (KG) in brown sugar solution (BS) (40 g/l). ○ Altering of pH at variation of at different concentrations of brown sugar (BS) with kefir grains (KG) (20 g/l).

the culture medium in EHEEo, biofilms showed cohesiveness.

Before starting the experiment, the pH of the EHEEo was 4.6 and with the EHEEo concentration increasing, the biofilms showed higher concentration of anthocyanins.

Biomass which presented undifferentiated growth and ANOVA showed no differences between treatments ($p>0.05$). There were, however, clear increase in incorporation of anthocyanin in BEHEEo, with an increasing of EHEEo concentration in the medium. This increase was detected in both BEHEEo as in the medium.

The analysis of BEHEEo through AFM confirmed the predominance of *Lactobacillus* in lower concentrations of EHEEo (Figure 2a and c) and in higher concentrations yeast were predominant (Figure 2e). These results are in agreement with Stadie et al. (2013) which showed Lactobacillus preparing the condition for yeasts.

Profile of liberation and dissolution of anthocyanins from BEHEEo

The dissolution profile in aqueous medium showed that the release of anthocyanins from BEHEEo occurred after 5 min reaching the release peak at 60 min (Table 1).

Extraction and dosage of anthocyanin of the BEHEEo and medium of culture

Table 2 shows anthocyanin concentration in biofilm associated with hydroethanolic extract of *E. oleracea* (BEHEEo) formed with kefir grains (20 g/l) in brown sugar solution (BS) (40 g/l) with different concentrations of aqueous extract of *E. oleracea*. (EHEEo). The pH climbed from 2.23±0.02 to 3.89±002 (Table 2) in proportion to the increasing of the concentration of EHEEo. The culture medium of two months still showed anthocyanins (19±0.5 mg/100 g).

Content of macro and microelements of BEHEEo

The presence of microelements in the biofilm was evaluated. This is important because, first the calcium concentration help to maintain the consistence of the biofilm network, and secondly because these elements could contribute to the antioxidant activity of the anthocyanin (Grumbein et al., 2014). The identified elements were: Zinc 0.53 mg/kg, iron 0.089 mg/kg, magnesium 0.183 mg/kg and calcium 0.140 mg/kg. The results correlated with the results of previous researches. According to one of the studies, it has been reported that

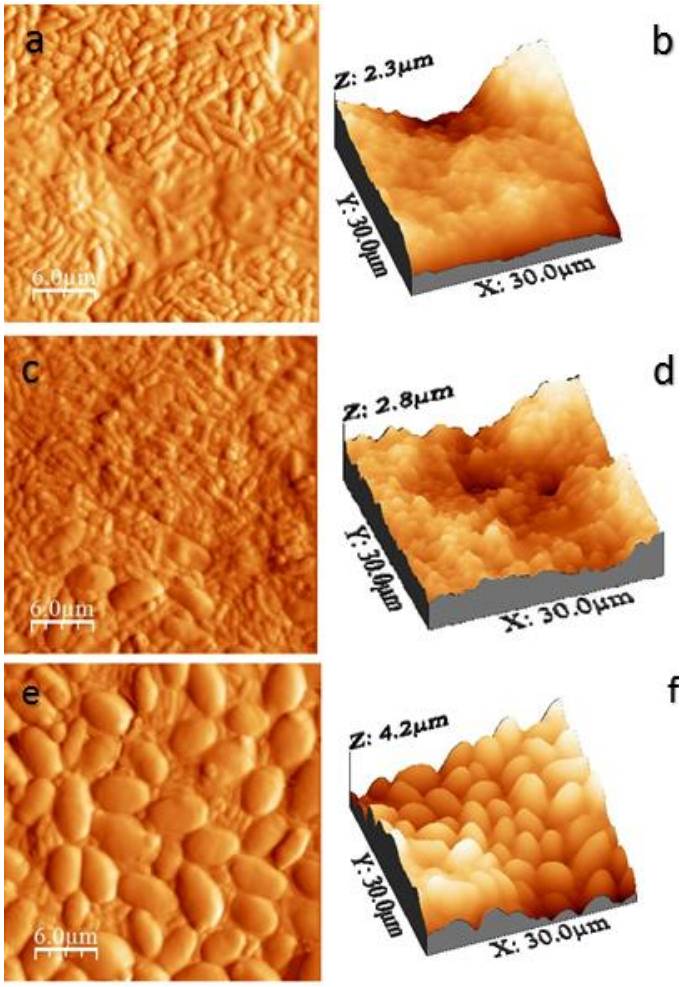

Figure 2. Graphic image by atomic force microscopy (AFM) with BEHEEo (a) (10 g/l), (c) BEHEEo (20 g/l) noteworthy is the predominant presence of *Lactobacillus* and (e) BEHEEo (60 g/l) where occur the predominant presence of yeast. Topographic image by Atomic Force Microscopy (AFM) with (b) BEHEEo (10 g/l), (d) BEHEEo (20 g/l) and (f) BEHEEo (60 g/l).

the occurrence of Zn, Fe and Ca have been observed in the biofilm matrix formed by *Lactobacillus* strains (Grumbein et al., 2014). The presence of these polyvalent elements can contribute to the rigidness of the biofilms once dried (Table 3).

BEHEEo structural analysis by atomic force microscopy

In the analysis of BEHEEo by AFM, 3D images with different concentrations of EHEEo was obtained (Figure 2b, d, e and f), which revealed different configurations of roughness (RMS), dependent of the concentration (Figures 2b, d, e and f). There is an increased in roughness in proportion to the increasing concentration of EHEEo in the culture medium. With lower concentrations (10 g/L), the predominant presence of bacillus was revealed (Figures 2a and c). They are presumed to be Lactobacillus because they constitute up to 70% of the flora present in kefir (Stadie et al., 2013).

Analysis of kefir biofilms through scanning electron microscopy (SEM)

Figure 3 show Scanning electron microscopy (SEM) microphotograph of the biofilm surface obtained when 20 g/L of the kefir grain and 40 g/L of brown sugar, loaded with anthocyanin was used.

Determination of temperature on the formation of BEHEEo

In relation to the BEHEEo formation, temperature is one of the most important parameters. With the culture medium cultivated at room temperature (25 ± 2°C), BEHEEo with a significant biomass (11.46±0.50 g) with relative cohesiveness was obtained whereas at a temperature of 15°C the formation of structures without consistency, breaking in the middle was observed. The optimum temperature was 30°C and there was no formation to 38°C.

Stability of the anthocyanin in the biofilms

In this work the content of anthocyanins of a vegetal extract was recovered and fixed in a complex matrix of a biofilm, obtained by using kefir grains (20 g/L) and brown sugar (40 g/L). In these conditions, the content of anthocyanins remained stable for two years (Table 4).

DISCUSSION

The initial adhesion of the cells is crucial in the biofilm formation period, and the characteristic of the contact surface has great influence at this stage (Pereira et al., 2015). Therefore, the biofilm formation depends on the variation of the substances concentration and on the flow conditions at the surface and on the culture medium turbulence (Perni et al., 2006).

The three most important factors to form the BEHEEo were: Time, temperature and substrate concentration (Figure 1). Biofilms may be formed in just 24 h (Alimova et al., 2006; Heydorn et al., 2000; Stoodley et al., 2002). The increase of the cells in the biofilm is part of its own maturation process, occurring up to 10 days after initial adhesion. This increase is as a result of cell division and co-adherence of other cells present in the system in their planktonic state. The BEHEEo formation occurred only after 20 days, although the formation of biofilm structures

Table 1. Profile of liberation and dissolution of anthocyanins from biofilms associated with hydroethanolic extract of *Euterpe oleracea* Mart. (BEHEEo).

Time (minutes)	Anthocyanins (mg/100 g)	Percentage
5	4.3±0.6	0.0040
15	3.8±0.8	0.0038
30	14.5±0.5	0.0145
60	20.8±0.3	0.0208

The numbers represent the average ± standard deviation of n = 3.

Table 2. Anthocyanin concentration in biofilm associated with hydroethanolic extract of *E. oleracea* Mart. (BEHEEo) formed with kefir grains (20 g/l) in brown sugar solution (BS) (40 g/L) with different concentrations of aqueous extract of *E. oleracea* Mart. (EHEEo).

Conc. of EHEEo (g/L)	Antocyanins (mg/100 g)	pH
10	0.00±0.00	2.23±0.02
25	4.93±0.40	2.32±0.01
50	13.10±0.26	3.13±0.01
100	18.06±0.21	3.89±0.02

The numbers represent the average ± SD of n = 3.

Table 3. Concentration of macro and microelements of aqueous extract of *E. oleracea* Mart. (EHEEo) and biofilm associated with hydroethanolic extract of *E. oleracea* Mart (BEHEEo) in brown sugar solution (BS).

Macro and microelements	BS (mg/kg)	EHEEo (mg/kg)	BEHEEo (mg/kg)
Mg^{2+}	0.1856	0.1823	0.1808
Zn^{2+}	0.5740	0.2438	0.5330
Fe^{2+}	0.2496	0.2446	0.089
Cu^{2+}	0.0269	0.0034	0
Ca^{2+}	0.1836	0.1572	0.1384

can already be observed from the fifth day. These structures are aggregated by a binding substance secreted in the medium that form the biofilm.

The BEHEEo were formed at ambient temperature (around 25°C). At 15°C, the biofilms were not formed, only fragments which were dispersed in the medium were observed. Asadishad et al. (2014) reported that low temperatures (below 10°C) paralyze the production of biofilms by *Bacillus subtilis*. Biofilms formed by *Listeria* sp. are more resistant to temperature of 15°C than at 37°C (Shimamura et al., 2015). BEHEEo are not formed at 37°C. The optimum temperature for the formation was 30°C (27.53±0.92 g) while at room temperature the biomass formed was 11.46±0.50 g.

When using low substrate concentrations of BS only biofilms structures were formed. These structures did not merge, remaining dispersed in the culture medium. Biofilms were formed only when concentrations above 20 g/l were used, but were not yet cohesive and fragmented with the mildest agitation of the medium. At concentrations above 40 g/l, the biofilms produced showed cohesiveness. Interestingly at this concentration, an increase in biofilm roughness was identified by atomic force microscopy (AFM).

When free nutrient concentration is increases proportionally, there is an increase on the number of microorganisms of the biofilm. This is a very important factor for the adsorption of molecules present in the biofilm, which precede the bacteria adsorption (Rubio et al., 2006). When the substrate was insufficient or when it sought to use only the EHEEo as substrate and no BS, the biofilms that were formed showed lack of cohesiveness and so only biofilms fragments were formed, which were dispersed in the medium.

The substrate concentration increasing (BS) was accompanied by pH decay much more significant than the change in concentration of KG grains, which suggests the occurrence of the increase on the microorganism activity over the substrate, resulting in final products that make the medium acidified by the presence of lactic and

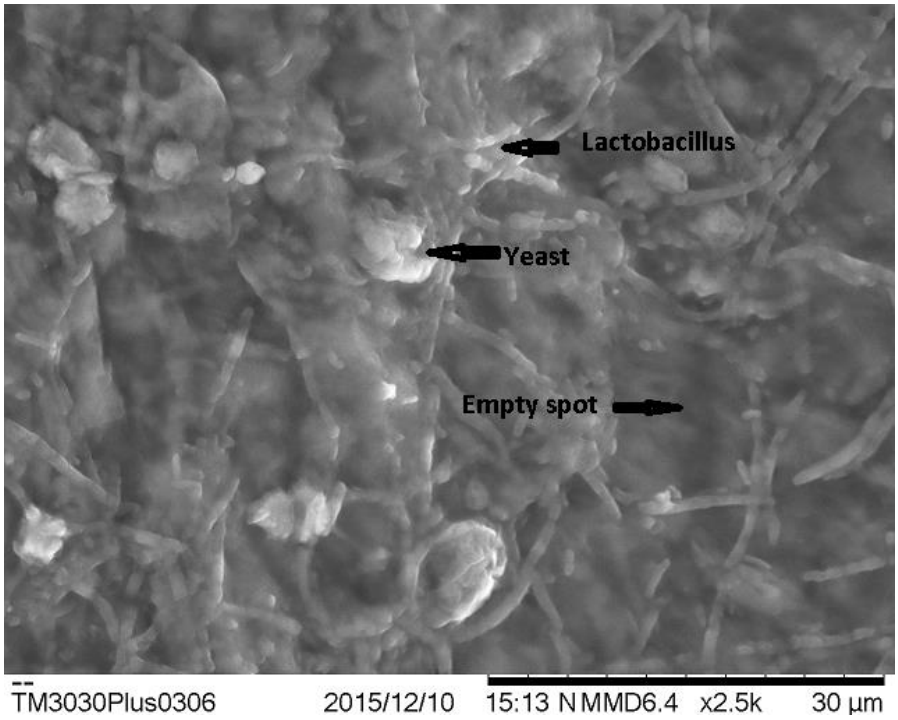

Figure 3. SEM microphotograph of the biofilm surface obtained when 20 g/L of the kefir grain and 40 g/L of brown sugar loaded with anthocyanin was used.

Table 4. Anthocyanin content evaluated along the time of 24 month.

Time (month)	BHE1	BHE2	BHE3
0	70.30 ± 2.52	71.36 ± 2.25	64.30 ± 1.25
12	71.50 ± 1.70	69.26 ± 1.70	62.50 ± 0.70
24	70.33 ± 1.57	71.33 ± 2.57	65.33 ± 0.57

ANOVA test F = 233.25; p = 0.0000 BHE, biofilm loaded with anthocyanin, n = 3. BHE1 batch one, batch 2 and so on.

acetic acid. The culture medium with kefir after 18 months remained with no unchanged pH (2.81) and free from contaminants. According to Abe et al. (2013) low pH constitutes a protection against contamination. Although pH below or above neutral directly affects the biofilm development by interfering with the motor proton force, which is the force used by the bacteria to generate its electrochemical gradient (Pereira, 2001) low pH did not interfere in the formation of the BEHEEo.

The microorganisms present in kefir are incorporated in the biofilm (Figures 2a, c and e). Thus, they ensure a contaminant-free environment. The pH drops until a protection level was reached against contaminants. Probiotics such as kefir are organisms that live in consortium repelling pathogenic organisms (Santos et al., 2003).

The anthocyanins present in the EHEEo were incorporated into BEHEEo which remained stable 24 months after formation. These anthocyanins suffered BEHEEo release in aqueous medium at pH 7.0 without losing its characteristics. This fact is important because the acai (*Euterpe oleracea* Mart.) anthocyanins inevitably suffer degradation from the harvest until reach the consumer. Rogez et al. (2012) concluded that the degradation kinetics of the anthocyanin results in a half-life of 50 h, occurring, concomitantly, with the infection by microorganisms such as yeast, mesophilic bacterias and fecal coliforms. The anthocyanins degradation can occur during the extraction process or during the storage of food and drugs (Rogez et al., 2012). A major challenge has been to find a matrix where anthocyanins can remain stabilized. These anthocyanins may then be used for incorporation into foods and pharmaceuticals. In this work the content of anthocyanins of a vegetal extract was recovered and fixed in a complex matrix of a biofilm, obtained by using kefir grains (20 g/L) and brown sugar (40 g/L). In these conditions, the content of anthocyanins remained stable for two years (Table 4). The ANOVA test

showed statistical significant differences among the mean of the biofilm mass, for the three batches used. The Tukey HSD test showed that was no statically significant differences between the time 0 and 24 month, for the three batch. In this work a simple technique is used to extract and stabilize the anthocyanins. In this sense this result suggest that this biofilm can be used for recovery and stabilizing anthocyanin for further utilization both in food, as a possible probiotic by the content of yeast and lactobacteria, and for pharmaceuticals as a proved antioxidant.

The release of the anthocyanin from the BEHEEo occurred from 5 min reaching a peak after 60 min. This time-dependent kinetics of release is an important factor for application of BEHEEo, since the gradual increase in anthocyanin release suggests that there may also be an efficiency increase over time.

In the culture medium of kefir, the anthocyanins were stable for at least two months. After this period, they started to be degraded along with the culture medium. Bobbio et al. (2000) described two types of EHEEo in anthocyanins, cyanidin-3-arabinoside and cyanidin-3-arabinoside-arabinosyl, and considered them as the two major anthocyanins of the *E. oleracea* fruits peel, with tenor of 50.00 ± 5 nmg/100 g in the peel of the fruit. They concluded that the fruit as a whole must have 263 mg/100 g of peel, which is only 19% of the fruit and the *E. oleracea* anthocyanins are concentrated only in the peel.

In this study, 110 mg/100 g of anthocyanin was found in the EHEEo. In the BEHEEo, 36 mg/100 g was found. It is noteworthy that the dosage was performed in BEHEEo with six months of acquisition. Açaí is a source of carbohydrates, therefore, the possibility that the formation of biofilms could occurred with the use exclusive of açai as the source of carbon was sopposed. In fact the formation of a biofilm occurred, but also an unpleasant smell of these biofilms fragmented easily. The culture medium without BS containing just the EHEEo as substrate presented biofilms with low cohesiveness, fragmented and with pH of 6.7, suggesting actions of different kinds of microorganisms

The release and dissolution profiles of BEHEEo synthesized after 12 months released 34.5±0.70 mg/100 g of cyanidin-3-glucose, while BEHEEo synthesized after 24 months released 33.87±0.57 mg/100 g of cyanidin-3-glucose. In relation to the presence of macro and microelements in EHEEo and BEHEEo, 0.553 mg/kg of zinc was found, and Rogez (2000) found 7 mg/100 g in extract and Menezes et al. (2008) found 2.82 mg/100 g in lyophilized açai extract.

The biofilms may use the absorption capacity of some metals in its ionic form, such as zinc, copper, iron and aluminum contained in its matrix to avoid erosion forces (Grumbein et al., 2014). These ionic metals are toxic for planktonic forms of the bacteria, components of the biofilm, but its toxicity is suppressed in the biofilm matrix (Grumbein et al., 2014).

The analysis by AFM can be used to evaluate the morphological progression of biofilms (Chatterjee et al., 2014; Pereira et al., 2015) as well as providing an understanding of the biophysical mechanisms of the impact of the organizational structure of the poly-saccharide capsule in biofilm formation (Wang et al., 2015).

The AFM makes mechanical nano measures providing data that show how the capsular organization influences in the adhesion of cells and consequently in the formation of biofilms. In this study, through AFM, the presence of *Lactobacillus* at high quantity in the biofilms was recorded (Figures 2a and c) especially at lower concentrations of EHEEo (10 g/L). At higher concentrations (60 g/l) the predominant presence of yeast was noted (Figure 2e). The *Lactobacillus* acidify the medium and thus provide optimum conditions for the metabolism of yeast (Stadie et al., 2013) that appeared in BEHEEo with increasing concentration of EHEEo to 60 g/L. In kefir, the yeasts provide the essential amino acids and vitamins, as folic acid to the lactobacilos and the *Lactobacillus* reduce the pH creating ideal conditions for the yeast, in a mutualistic relationship enlightened by Stadie et al. (2013).

It is known that the substrate influences biofilm formation, and also the extent of microbial colonization increases with increasing surface roughness (Characklis, 1984). An increase in the concentration of the substrate causes a biofilm growth with the consequent increase in roughness (Viana, 2009) and with the analysis by AFM; it is possible to detect these roughness changes on the surface of the biofilm (López-Jiménez et al., 2015). The variation of the concentration of EHEEo in the culture medium was accompanied by changes in the roughness of the BEHEEo (Figures 2b, d and f). The increase in the roughness is a very important factor for indicating increased retention capacity of the cells and incorporation of substances like anthocyanins in this study. The AFM analysis confirmed the increased roughness of the biofilm depend on the concentration of the amount of kefir grains. The presence of *Lactobacillus* and yeast in the biofilms loaded with anthocyanins was observed.

Conclusion

The BEHEEo formed showed capacity to retain and preserve the anthocyanins. The analysis by AFM confirmed the increased roughness of BEHEEo dependent on the concentration of EHEEo, and showed *Lactobacillus* and yeast in large quantities in these biofilms. Also, the BEHEEo retained anthocyanins of EHEEo for relatively long periods, and, the anthocyanins of BEHEEo were released without losing its characteristics *in vitro* conditions (pH 7.0). In the present study, it was established that biofilms incorporated anthocyanins at a satisfactory level and that fermentation with kefir grains did not reduce the amounts of

anthocyanins. In biofilms incorporation of the anthocyanins took 20 days of cultivation. It is suggested that the biofilms with incorporated anthocyanins have potential for therapeutic applications in several pathologies wherein the antioxidative processes is necessary. This work suggests a solution to the problem of anthocyanins instability. They remain stable in biofilms for at least 24 months. Biofilms, therefore, constitute vehicles for the transposition of bioactive compounds, notably anthocyanins.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGMENTS

Authors would like to thank CAPES (nº 3292/2013 AUXPE), and CNPq Proc. 402332/2013-0 (Biotec) for the financial support. The author, Antonio Ferreira de Oliveira1 would also like to thank too the CNPq doctoral scholarship (Process: 140282/2014-9). The authors extend their thanks the referees for their comments, which helped to improve the earlier version of the manuscript.

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Vol. 11(39), pp. 1484-1494, 21 October, 2017
DOI: 10.5897/AJMR2016.7986
Article Number: E77E78266484
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Comparative evaluation of the antimicrobial profile of *Moringa* leaf and seed oil extracts against resistant strains of wound pathogens in orthopedic hospitals

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Received 27 February, 2016; Accepted 9 May, 2016

The antimicrobial profile of oil extracts from *Moringa oleifera* leaves and seeds on orthopaedic wound pathogens was tested and compared with the antimicrobial activity of some antibiotics. The pathogens were characterized using biochemical and morphological tests. Antimicrobial susceptibility test was done on the pathogens using paper discs diffusion method. Plasmid curing was done on the isolates that showed resistance to antibiotics and the *Moringa* extracts. Time of kill assay was done with modified plating technique. *Staphylococcus aureus* accounted for 43% of the pathogens followed by *Proteus* spp. (16%), *Klebsiella* spp. (15%), *Citrobacter* spp. (11%), *Escherichia coli* (8%) and *Pseudomonas aeruginosa* (6%). Over 80% of the organisms were resistant to the tested antibiotics and their resistance were of plasmid origin. The methanolic leaf oil extract showed highest antimicrobial activity. The activity of the aqueous seed oil extract was significantly higher than the methanolic and ethanolic seed oil extracts ($P > 0.05$). Viable cell counts of *S. aureus* and *Klebsiella* spp. were reduced by the methanolic leaf and aqueous seed oil extracts. The antimicrobial activity of the methanolic leaf and aqueous seed oil extracts compared favourably with the reference antibiotics and can be used as alternatives for treatment of orthopaedic wound infections.

Key words: Orthopeadic, antibiotics, plant extracts, plasmid, wound, infection, antimicrobial, *Moringa oleifera.*

INTRODUCTION

Antibiotic resistance, especially among wound containing bacteria is an important issue of discussion in the

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treatment of chronic wound infections. Most wounds have been reported to be contaminated by pathogens and body commensals ranging from bacteria, fungi to other parasites (Sani et al., 2012). The common gram positive organisms found in most wounds include β- haemolytic *Streptococcus- Streptococcus pyogenes* and *Staphylococcus aureus*. Others such as the gram negative aerobic rods, *Pseudomonas aeruginosa* have also been implicated in wound infections (Enweani, 1991). The facultative anaerobes like the Enterobacteriaceae species, such as *Escherichia coli*, *Klebsiella* and *Proteus* spp. have also been reported to constitute a serious problem in several wound infections (Mulugeta and Bayeh, 2011). The widespread use of antibiotics, together with the length of time over which they have been available, have led to major problems of resistant organisms, contributing to high levels of morbidity and mortality in patients (Akinjogula et al., 2010). The control of wound infections is becoming more challenging due to widespread bacterial resistance to antibiotics and to a greater incidence of infections caused by methicillin resistant *S. aureus* (MRSA) and polymicrobic flora (Cheesbrough, 2006). Increased antimicrobial resistance among pathogens of wound infections can result to complications and increase in the cost associated with procedures and treatments. Due to this rising resistance among wound pathogens, there is a need to search for alternative sources of treatment of wound infections in natural compounds from medicinal plants such as *Moringa oleifera*.

M. oleifera is an ancient tree that is historically known to possess numerous medicinal qualities (Posmontier, 2011). Previous studies have reported the antimicrobial properties of the various parts of *Moringa* roots, flowers, barks and stem including the seeds (Lockett et al., 2000; Walter et al., 2011). The seed kernel of *M. oleifera* has been employed in the treatment of bronchial asthma and found to show an appreciable decrease in severity of symptoms of asthma and also simultaneous improvement in respiratory functions (Fahey et al., 2001).The family Moringaceae has been found to be rich in compounds containing rhamnose, and it is rich in a fairly unique group of compounds called glucosinolates and isothiocyanates (Bennett et al., 2003). *Moringa* is also rich in a number of vitamins and minerals as well as other more commonly recognized phytochemicals such as carotenoids (Siddhuraju and Becker, 2003). *M. oleifera* has been reported to have natural antioxidant properties and thus enhance the shelf-life of fat-containing foods (Gilani et al., 1994). The roots of *Moringa* plants have been reported to possess antispasmodic activity through calcium channel blockade which forms basis for its traditional use in diarrhoea (Ghebremichael et al., 2005). The flowers show effective hepatoprotective effect due to the presence of quercetin (Costa-Lotufo et al., 2005). The seed of *Moringa* is also effective against skin papillomas in mice and the seed ointment had similar effect to neomycin against *S. aureus* pyodermia in mice (Adegoke et al., 2010). Finding an alternative source of treatment for wound infections using *M. oleifera* plant extracts could provide the needed replacement for conventional antibiotics since most wound pathogens have appeared to have developed resistance to most of the available antibiotics. The aim of the present study was to investigate the antimicrobial activity of *M. oleifera* seed and leaf oil extracts against broad spectrum antibiotic resistant microorganisms isolated from wound infections. The study also investigate if the resistance to the conventional antibiotics by some wound pathogens is plasmid associated.

MATERIALS AND METHODS

Sample collection *(M. oleifera),* identification and preparation of the plant extracts

The fresh leaves and seeds of *M. oleifera* plant (Supplementary Figure 1a and b) were collected from Dekina Local Government Area, Kogi State Nigeria. The plant samples were identified at the Department of Botany, University of Nigeria, Nsukka, Nigeria. For the methanol and ethanol extractions, 50 g each of *M. oleifera* leaves and seeds were crushed and fed to a lab-scale Soxhlet extractor fitted with a 1 L round-bottom flask and a condenser. About 250 mL of both methanol and ethanol were added to the set up and the extraction was done for 6 h. The solvents were then evaporated under reduced pressure using a rotary evaporator at 45°C.

Aqueous extracts of the fresh leaves and seeds for antimicrobial examination

One hundred grams (100 g) of fresh leaves and seeds of *M. oleifera* were weighed and crushed directly using electric blender and missed in 400 mL distilled water into a conical flask stoppered with rubber corks and shook for 30 min, after which they were left to stand for 72 h and were shaken intermittently. The mixture was filtered off using sterile filter paper into a clean conical flask. The solvents were allowed to evaporate leaving the concentrated filtrates, which were then stored at 4°C for antimicrobial activity test.

Isolation, characterization and identification of orthopaedic wound samples

Wound samples were collected from one hundred (100) patients at National Orthopaedic Hospital Enugu, using a sterile swab stick and sterile distilled water. Out of the 100 samples, 45 were from post-operative wounds while 55 were from other wound sources. The samples were transferred to the Microbiology Laboratory of the University of Nigeria within 24 h for further analysis. The collected samples were streaked on freshly prepared nutrient agar and MacConkey agar and incubated aerobically at 37°C for 24 h. Discrete colonies differing in size, shape and colour were selected from the different plates and were sub cultured into fresh nutrient agar plates. The colonies were characterized using conventional biochemical tests (indole, catalase, urease, coagulase, sulphide indole motility, sugar fermentation and oxidase tests) following established protocols (Doughari et al., 2007). Further characterization was done using colonial and cell morphology.

Antibiotic susceptibility test

Antimicrobial susceptibility of the bacterial isolates was determined using the discs diffusion method, as described by Doughari et al. (2007). Briefly, the isolates were picked using sterile inoculating loop and inoculated into a sterile test tube with 5 mL of phosphate-buffered saline. The optical density of the organisms at 600 nm wasadjusted to 0.5 McFarland (0.08 to 0.1). Sterile cotton swabs were used to spread the growth evenly on the surface of Mueller-Hilton (MH) agar. The antibiotic discs which contained the following antibiotics, streptomycin, erythromycin, cloxacillin, chloramphenicol, amoxycillin, nitrofurantoin, nalidixic acid, ofloxacid, tetracycline, augmentin, gentamycin and cotrimoxazole were placed on the surface of MH agar plates. The MH agar plates were incubated for 18 to 24 h at 37°C. All the antibiotics were tested on both the gram positive and gram negative organisms. The antibiogram was read and recorded as the diameter of zone of the inhibition (ZI). The resistant isolates were separated from the susceptible ones and further analysis was carried out on them.

Antibacterial activity of the leaf and seed oil extracts

Antimicrobial activity of the aqueous, ethanolic and methanolic oil extracts of the leaves and seeds were assayed using the paper discs diffusion method (Oluma et al., 2000). The concentrated leaf extracts were dissolved in 5% dimethyl sulfoxide (DMSO) and sterile discs, 6 mm (Hi-media, India) in size were each impregnated with 30 µL of 50 µg/mL of each extract. The discs were carefully and firmly placed on the Muller Hinton Agar (MHA) plates earlier seeded with standardized bacterial suspensions (approximately 1.5×10^6 CFU/mL). Filter paper discs dipped into sterile distilled water and allowed to dry were used as control. The plates were then incubated at 37°C for 24 h. Antibacterial activity was determined by measurement of ZI around each paper disc.

Determination of minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

Equal volumes of each oil extract (with different concentrations) and nutrient broths were mixed in 8 test tubes and 0.1 mL of the standardized inoculum was added to each of the tubes. The tubes were incubated aerobically at 37°C for 18 to 24 h. Two control tubes were maintained for each test batch. The MIC was taken as the lowest concentration that prevented bacterial growth. The MBC was determined by sampling all the macroscopically clear tubes and the first turbid tube in the series. Before being sampled, the tubes were gently mixed by flushing them with a sterile pipette, and a 100 mL aliquot was removed from each test tube. Each aliquot was placed on a single antibiotic-free nutrient agar plate in a single streak down the centre of the plate in accordance with the method established by Irkin and Korukluoglu (2007). The samples were allowed to stand for 30 min so that they can be absorbed into the agar until the plate surface appeared dry. The aliquots were then spread over the plate by lawn technique. The growth and sterility controls were sampled in the same manner. The MBC lawn plates were incubated for 24 h at 35°C. After the incubation periods, the lowest concentrations of the extract that did not produce any bacterial growth on the solid medium were regarded as MBC values for the extract (Cruishank et al., 1975).

Time of kill assay

An assay on the rate of killing of *S. aureus* and *Klebsiella* spp. by the methanol and aqueous seed oil extracts was carried out using a modified plating technique (Zhou et al., 1990). Briefly, the extracts were incorporated into 10 mL Mueller Hinton broth in McCartney bottles at MIC. Two controls, one Mueller Hinton broth without extract inoculated with test organisms and Mueller Hinton broth incorporated with the extract at the test concentrations without the test organisms, were included. Inoculum density, approximately 10^5 CFU/mL further verified by total viable count was used to inoculate 10 mL volumes of both test and control bottles. The bottles were incubated at 37°C on an orbital shaker at 120 rpm. A 100 µL aliquot was removed from the culture medium at 0, 1, 2, 3 and 4 h for the determination of CFU/mL by the plate count technique (Zhou et al., 1990). About 25 µL of each of the dilutions was plated out and incubated at 37°C for 24 h for emergence of bacterial colonies. The cell counts from the experimental cultures were compared with the controls.

Plasmid isolation and profiling

The bacterial isolates that were resistant to the antibiotic susceptibility test were selected for plasmid isolation and curing. Preparations of plasmid DNA of the resistant isolates was done by alkaline lysis method as described by Taylor and Brose (1988). A single bacterial colony was picked from the clones and grown in nutrient broth at 37°C for 24 h. The cell suspension was transferred to Eppendorf tube using a micropipette and centrifuged at 6000 rpm for 5 min at 4°C. Pelleted cells were re-suspended in 300 µL of the lysis solution (25 mM Tris, 10 mM EDTA, 0.1 N NaOH and 0.5% SDS) and incubated at room temperature for 5 min. Sodium acetate solution was added to the mixture and the solution was mixed properly by whirling before incubation at room temperature for 5 to 10 min. Cold absolute ethanol was used to precipitate the $_P$DNA and the mixture was spun for 10 min at 12000 rpm. The pelleted DNA was washed with 1 mL 70% cold ethanol, air dried for at least 10 min and re-suspended in 20 to 40 µL of distilled water for further analysis. Electrophoresis was done with 2 µL of the extracted plasmid in 0.8% agarose gel for 1 h at 90 V. The gel was stained with ethidium bromide (0.5 µg/mL) and visualized with UV light (302 nm UV transilluminator).

Plasmid DNA curing

Plasmid curing was done with acridine orange (AO) and sodium dodecyl sulphate (SDS) as described in previous studies (Bhalakia, 2005; Isibor et al., 2008). Nutrient broth was prepared and supplemented with 1g of SDS in one batch of 99 ml and 10 g of SDS in a second batch of 90 mL to achieve final concentrations of 1 and 10% (w/v) SDS, respectively. Each of the solutions was adjusted to pH 7.6 with 1 M NAOH and autoclaved at 121°C for 15 min. Selected overnight (O/N) cultures of the isolates were standardized to 0.5 McFarland turbidity standard using sterile saline. In each 5 mL SDS supplemented nutrient broth, 0.1 mL of each O/N culture was inoculated and incubated at 37°C for 24 h. Cultures were later subjected to the same antibiotics to which they were resistant to previously. The curing process was repeated with acridine orange following the standard protocols.

RESULTS

Characterization and antibiotic susceptibility test of the bacterial isolates

A total of 100 wound samples were collected from the National Orthopaedic Hospital Enugu and were identified. From the biochemical test, 43% of the bacterial isolates were *S. aureus,* 16% were *Proteus* spp., 15% were

Table 1. Biochemical characterization and the identification of bacterial pathogens from orthopaedic wound infections.

Organism	Gram reaction	Oxidase	Catalase	Urease	H_2S	Motility	Coagulase	Indole	Citrate
S. aureus	+	-	+	+	-	NT	+	-	+
Citrobacter spp.	-	NT†	+	NT	NT	+	NT	NT	+
Proteus spp.	-	NT	NT	-	+	+	NT	-	-
Klebsiella spp.	-	NT	+	+	-	-	NT	NT	+
E. coli	-	NT	+	-	-	+	NT	+	-
P. aeruginosa	-	+	NT	+	-	+	NT	+	+

Different biochemical tests were carried out to characterize and identify each orthopaedic wound samples. The biochemical analysis were able to group the othorpedic wound samples into gram positive (*S. aureus*) and gram negative organisms (*Citrobacter spp*, *Proteus spp, Klebsiella spp, E. coli* and *P. aeruginosa*). NT = Not tested; - = Negative; + = Positive.

Table 2. Descriptive statistics of the antibiotic resistance profile of the different bacterial groups identified in this study.

Antibiotic†	Concentration (µg/mL) ‡	No of resistant isolates††	Gram (-)	Gram (+)	Percentage
Cotrimoxazole	25	31	ND	31	75.6
Gentamicin	10	69	35	35	70.4
Augmentin	30	94	56	38	95.9
Tetracycline	25	91	53	38	92.8
Ofloxacin	5	46	46	ND	80.7
Nalidixic acid	30	47	47	ND	82.4
Nitrofurantoin	200	44	44	ND	77.1
Amoxycilin	25	56	56	ND	98.2
Chloramphenicol	10	32	ND	32	78.0
Cloxacillin	5	41	ND	41	100
Erythromycin	5	41	ND	41	100
Streptomycin	10	38	ND	38	92.6

† = Antibiotics used for the susceptibility study; ‡ = Concentrations of the antibiotics; †† = Number of isolates that were resistant to the antibiotics. ND = Not determined.

Klebsiella spp., 11% were *Citrobacter* spp. while *Escherichia coli* and *P. aeruginosa* were 8 and 6% respectively (Table 1). The result from the biochemical test was further confirmed with the analysis of the colonial and cell morphology of the organisms. Out of the 100 wound isolates used in this study, 98 of them were used for antibiotic susceptibility test, 88.7% of them were resistant to at least 4 or more antibiotics (Table 2). All the organisms were resistant to amoxycillin and cloxacillin.

Bacterial resistance and plasmid association

All the gram negative bacterial wound pathogens (*Proteus* spp., *P. aeruginosa*, *Klebsiella* spp. and *E. coli*) had 3 plasmid bands each. The first set of bands lie in the region of 23 kb while the two smaller sized plasmids lie within the regions of 2 kb and 500 bp (Figure 1a). For the gram positive wound pathogens which were mainly *S. aureus*, no bands were observed in two strains (lanes 13 and 72), while the three remaining strains (lanes 16, lane 22 and 45) had 3 plasmid bands each. The first set of bands was 23 kb in size while the other bands lie between the regions of 500 bp and 2 kb (Figure 1b). To analyse if the resistance observed in some multidrug resistant isolates was plasmid associated, a subset of the isolates that showed resistance to the tested antibiotics were subjected to plasmid curing. All the gram negative organisms became susceptible to most of the antibiotics they were previously resistant to after the curing procedure. However, *S. aureus* remained resistant to all the antibiotics even after plasmid curing.

Antimicrobial activity of the *M. oleifera* leaves and seed oil extracts

All the leaf oil extracts showed inhibitory effects at the tested except on *Proteus* spp. whose growth was not

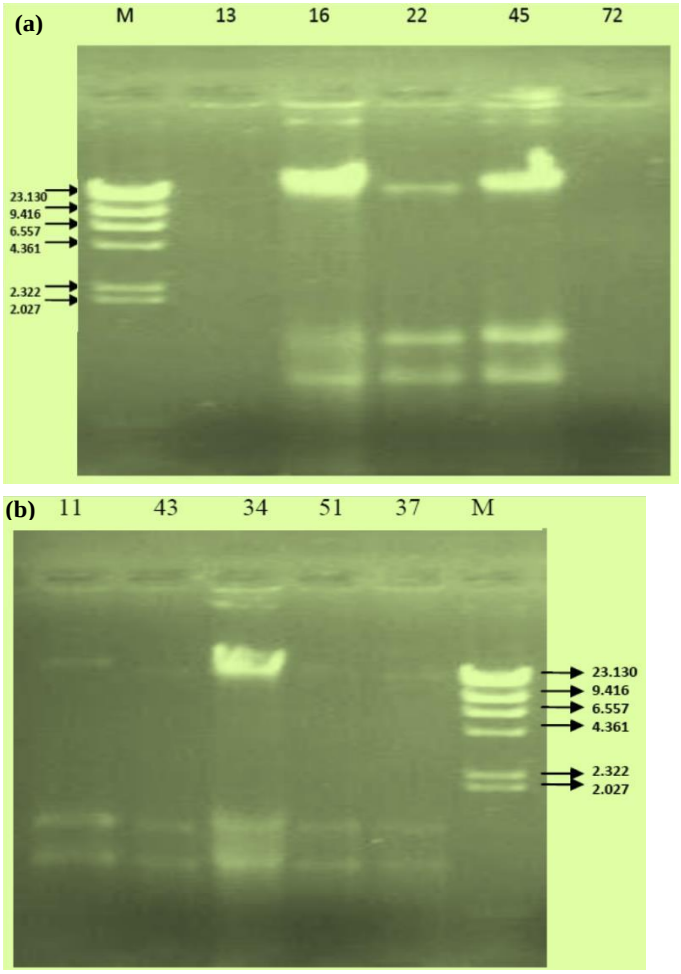

Figure 1. Plasmid profile of the bacterial species isolated from orthopedic wound infection. (a) pDNA from gram negative organisms, lanes (11 = *Proteus spp,* 43 = *Klebsiella spp,* 34 = *Pseudomonas* spp*.,* 51= *E. Coli,* 37= *Klebsiella spp*), (**b**) pDNA from gram positive bacteria, lanes (13, 16, 22, 45 and 72 represent different strains of *S. aureus*). Electrophoresis was done with 2 µl of the plasmid from each sample in 0.8% agarose gel for 1 h at 90 V. The gel was stained with ethidium bromide (0.5 µg/mL) and visualized with UV light (302 nm UV transilluminator), 23 kb marker was used to determine the size of the isolated pDNA.

concentration of 50 µg/mL on all the microorganisms inhibited by the aqueous and ethanolic oil extracts at the tested concentration. The aqueous leaf oil extract had ZI ranging from 3.0 ± 0.0 to 20.3 ± 1.5 mm in *Proteus* spp. and *E. coli* respectively (Table 3). The methanolic leaf oil extract had the highest ZI, 22.7 ± 2.1 mm in *P. aeruginosa.* However, it showed a reduced ZI of 11.0 ± 2.0 mm in *Klebsiella* spp. The ethanolic leaf oil extracts showed the lowest ZI ranging from 3.0 ± 0.0-12.3 ± 1.5 mm in *Proteus spp* and *E. coli* respectively. In all the extracts, all the isolates tested were more susceptible to the methanolic leaf oil extract (Figure 2) while the ethanolic leaf oil extract had the least antimicrobial activity against the tested organisms (Table 3). The antimicrobial activity of the aqueous oil extract, methanolic and ethanolic oil extracts of *M. oleifera* seeds respectively is presented in Table 4. All the seed oil extracts showed inhibitory effects at the concentration of 50 µg/mL. The aqueous oil extract of the seeds had more inhibitory activity with ZI ranging from 15.3 ± 2.2 to 22.0 ± 1.3 mm in *S. aureus* and *E. coli* respectively. This was followed by methanolic seed oil extract with the ZI ranging from 11.0 ± 3.0-18.7 ± 1.5 mm in *S. aureus* and *E. coli* respectively. However, the sensitivity of the isolates to ethanolic seed oil extract was relatively low in most of the isolates tested (Table 4). Three isolates, *Citrobacter* spp., *Proteus* spp and *E. coli* were not sensitive to the ethanolic seed oil extract (Table 4).

Minimal inhibitory concentration of *M. oleifera* leaves and seeds oil extracts

The MIC for the methanolic leaf oil extract was 20 µg/mL, at this concentration, the growth of all the microorganisms were inhibited. The aqueous leaf oil extract had inhibitory effect on *Citrobacter* spp and *E. coli* at a concentration of 20 µg/mL. At a concentration of 30 µg/mL the aqueous leaf oil extract also had inhibitory effect on *S. aureus and Klebsiella* spp*,* but did not have any inhibitory effect on *P. aeruginosa.* A slightly different trend was observed for the seed oil extracts. At the concentration of 20 µg/mL, the aqueous seed oil extract inhibited the growth of all the microorganisms tested except *P. aeruginosa* that was inhibited at 30 µg/mL. At the concentration of 30 µg/mL, the methanolic seed oil extract had inhibitory effect on *Proteus* spp, *Klebsiella* spp and *E. coli* but it showed inhibition on the growth of *S. aureus* and *P. aeruginosa* at concentration of 40 µg/mL. At a higher concentration of 50 µg/mL it inhibited the growth of *Citrobacter* spp. However, for the ethanolic seed oil extract, the MIC was quite high in most of the bacterial species, 60 µg/mL, 40 µg/mL and 50 µg/mL for *S. aureus, Klebsiella* spp*.* and *P. aeruginosa* respectively (Figure 3).

Minimal bactericidal concentration of *M. oleifera* leaves and seeds oil extracts

The aqueous leaf oil extract had the highest bactericidal effect, with concentration as low as 20 µg/mL, all the organisms except *P. aeruginosa* were killed (Figure 4). The methanolic leaf oil extract also had effect on all the tested organisms although at variable concentrations. However *M. oleifera* ethanolic leaf oil extract had no bactericidal activity on half of the tested bacterial species although at a concentration of 40 µg/mL, *Klebsiella* spp. was observed to be killed by the extract. A similar trend was observed for the seed oil extracts (data not shown). The aqueous seed oil extract showed the highest bactericidal effect at concentration of 20 µg/mL. At this concentration, *Citrobacter* species, *Proteus* spp. and *E. coli* were killed while *S. aureus*, *Klebsiella* spp. and *P.*

Table 3. Zones of inhibition of *M. oleifera* leaf oil extracts on different bacterial species.

Organism	Aqueous (Mean ± SD)	Methanolic (Mean ± SD)	Ethanolic (Mean ± SD)
S. aureus	13.2± 2.3	18.0 ± 2.6	9.0 ± 2.4
Citrobacter spp.	16.0 ± 2.0	17.7 ± 4.0	7.7 ± 1.8
Proteus spp.	3.0 ± 0.0	15.0 ± 1.0	3.0 ± 0.0
Klebsiella spp.	13.7 ± 3.5	11.0 ± 2.0	8.3 ± 2.1
E. coli	20.3 ± 1.5	16.3± 2.3	12.3 ± 1.5
P. aeruginosa	16.3 ± 2.5	22.7 ± 2.1	11.0 ± 2.0

Data are presented as mean ±SD as measurement of inhibition zone (mm); Means and standard deviations determined from 3 biological replications; [a]

Table 4. Zones of inhibition of *M. oliefera* seed oil extracts on different bacterial species.

Organism	Aqueous (Mean ± SD)	Methanolic (Mean ± SD)	Ethanolic (Mean ± SD)
S. aureus	15.3± 2.2	11.0± 3.0	8.0± 2.8
Citrobacter spp.	16.0± 2.0	13.7± 1.1	0.0± 0.0
Proteus spp.	17.3± 1.5	13.0± 1.0	0.0± 0.0
Klebsiella spp.	16.7± 2.1	12.0± 1.7	7.0± 1.4
E. coli	22.0± 1.3	18.7± 1.5	0.0± 0.0
P. aeruginosa	19.0± 1.0	13.7± 2.5	9.0± 2.3

Data are presented as mean ±SD as measurement of inhibition zone (mm); Means and standard deviations determined from 3 biological replications.

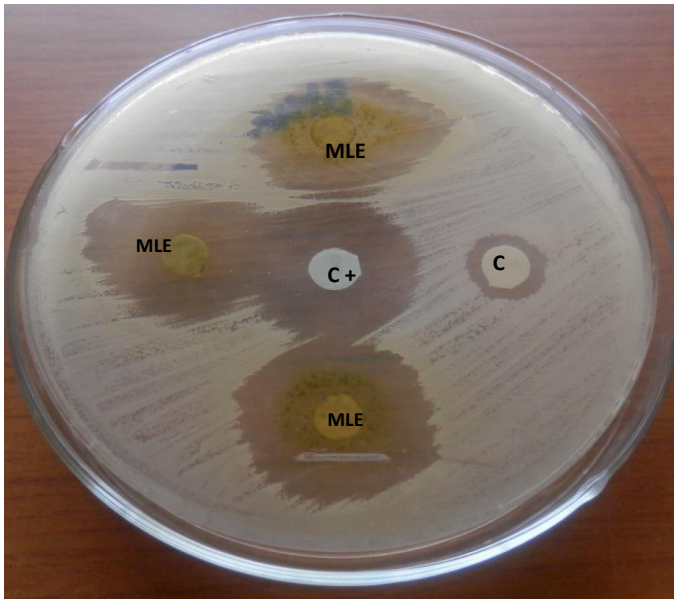

Figure 2. Susceptibility test of the methanolic leaf oil extract on *S. aureus*. The susceptibility tests were done using paper discs diffusion methods. Discs were placed on Muller Hinton Agar (MHA) plates earlier seeded with standardized bacterial suspensions (approximately 1.5×10^6 CFU/mL). C = Paper discs impregnated with 30 µL of a solution of 50 µg/mL of ciprofloxacin and gentamycin were used as control for comparison. MLE= Methanolic leaf extract, C+ = cotrimoxazole, C= gentamycin.

aeruginosa were killed at a concentration of 30 µg/mL. The methanol oil extract had bactericidal effect at 30 µg/mL for *E. coli* and 50 µg/mL for *S. aureus*, *Proteus* species and *P. aeruginosa.* While ethanol seed oil extract had the lowest bactericidal activity with concentration ranging from 50 µg/mL to 70 µg/mL.

Comparison of the MIC and the MBC from different extraction sources

A comparison of the MIC between leaf and seed oil extracts from the three extraction methods showed that the leaf oil extracts from ethanolic, aqueous and methanolic extractions had more inhibitory activity when compared with the seed extracts from the three sources. The MIC of the leaf oil extracts for the three extraction sources were the same as the control. However a different trend was observed for the MBC of the leaf and seed oil extracts from the three extraction methods. The seed and leaf oil extracts from the three sources showed variable MBC at relatively high concentrations. For the ethanol seed oil extract, there was no MBC for *Citrobacter* spp., *Proteus* and *E. coli* even at concentrations as high as 70 µg/mL. No MBC was recorded for *Proteus* spp. for the leaf oil extract as well. A similar trend was observed for the methanolic seed oil extracts. In addition, the methanolic leaf oil extract could not show any observable MBC on *Proteus* spp. For the aqueous seed oil extract, the leaf oil extract had no MBC on *Proteus* and *Pseudomonas* spp.

Reduction of viable bacterial counts by the extracts

The time of kill estimates the time it took the extracts to kill the bacterial pathogens, hence showing a progressive reduction in the number of colonies that survive over a given time frame. The time of kill assayed at a maximum time interval of 0 to 5 h showed a reduction of the *Klebsiella* spp. from 8.2 to 2.3 CFU/mL by the methanolic leaf oil extract and 9.0 to 4.8 CFU/mL by the aqueous leaf oil extract (Figure 5). For *S. aureus,* the colonies reduced from 9.4 to 3.2 CFU/mL with the methanolic seed oil extract and 9.6 to 6.4 CFU/mL with the aqueous seed oil extracts at 0 to 5 h (Figure 5).

DISCUSSION

Microorganisms such as bacteria, fungi and other parasites are common pathogens associated with wound infections (Sani et al., 2012). However, the resistance of these microbial agents to conventional antibiotics has

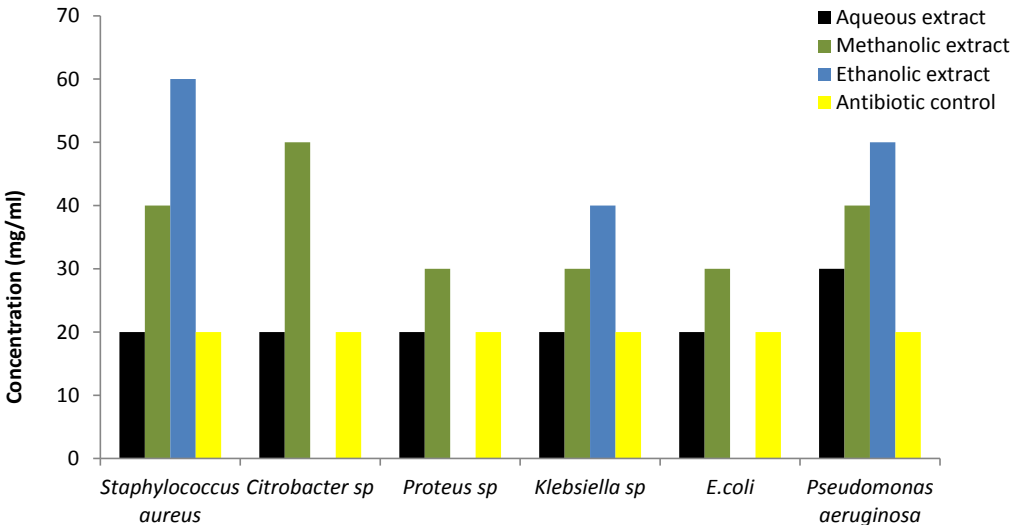

Figure 3. The minimal inhibitory concentration of *M. oleifera* seed oil extracts against orthopedic wound infection pathogens. Black bars indicate the MIC of *M. oleifera* aqueous seed oil extracts, green bars indicate the MICs of the methanolic seed oil extract, blue bars show ethanolic seed oil extracts and the yellow bars are the antibiotic control.

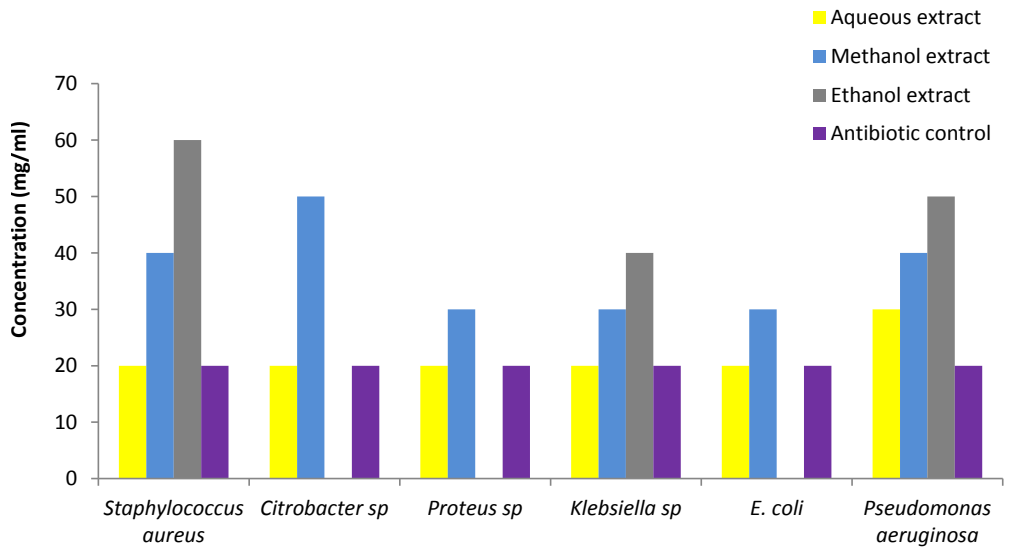

Figure 4. The MBC of *M. oleifera* leaf oil extract: yellow bars represent the MBC of the aqueous leaf oil extract, blue bars indicate the methanol leaf oil extract, ash bars represent the ethanolic leaf oil extract while the purple bars are the antibiotic controls.

become very alarming. In the present study, an evaluation of the antibiotic susceptibility pattern of microorganisms isolated from orthopaedic wounds and also the antimicrobial potential of *M. oleifera* leaf and seed oil extracts against orthopaedic wound pathogens were tested. The results from our biochemical analysis showed that gram negative organisms such as *Citrobacter* spp., *Proteus* spp., *Klebsiella* spp., *E. coli* and *P. aeruginosa* were the predominant organisms present in the infected wounds. However, *S. aureus* was the only gram positive organism identified in the samples. This observation corroborates with the findings from other work where *S. aureus* was reported as the predominant gram positive bacterial isolate in wound infections (Samuel et al., 2010; Magiorakos et al., 2012). The antibiogram of the isolates screened in this study showed that over 90% of the bacterial species showed high level of resistance to most of the tested antibiotics (cloxacillin, erythromycin, amoxycilin, augmentin and tetracycline). However, gentamicin and cotrimoxazole having resistance levels of 70.4 and 75.6% respectively were the antibiotics with the least resistance profiles. The high

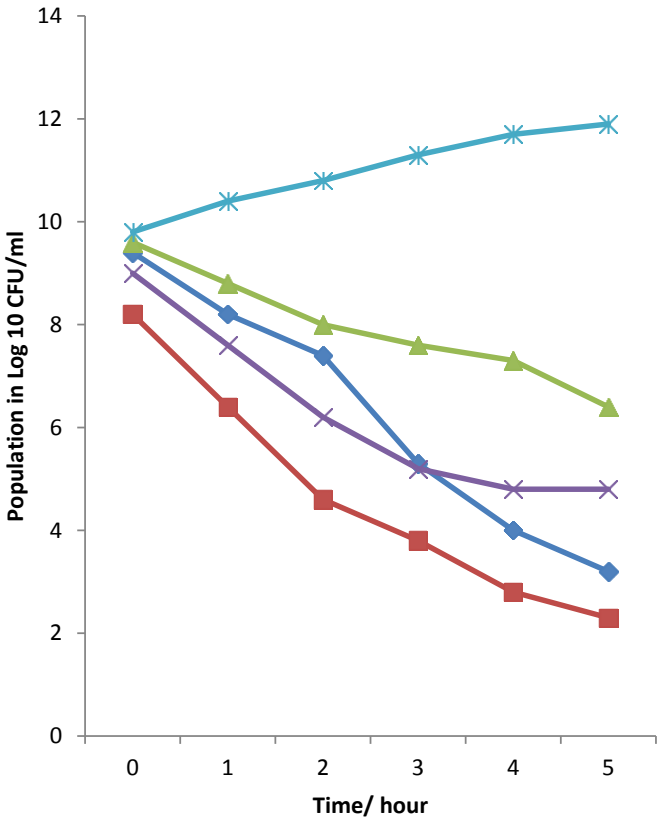

Figure 5. Time of kill of the aqueous and methanolic oil extracts on *S. aureus* and *Klebsiella* spp. The blue line represents the time of kill of the methanolic leaf oil extracts on *S. aureus*, the red line represents the time of kill for the *Klebsiella* spp., the green line represents the time of kill of the methanolic seed oil extract on *S. aureus,* the purple line represents the time of kill of the aqueous seed oil extracts on *Klebsiella* spp., while the light blue one represents the antibiotic control (cotrimazole). The time of kill was measured as the reduction in population of viable cells per hour.

level of resistance observed among the tested bacterial isolates in this study could be as a result of indiscriminate use of the tested antibiotics. It could also be due to the transfer of resistant genes among organisms as has been reported in microbial communities like biofilms (Andhoga et al., 2002). Although the resistance observed in gentamicin and cotrimoxazole are relatively high, the two antibiotics proved to be the candidates of choice for treatment of orthopaedic wound infections in this study. Other studies have also reported that gentamicin was the most effective antibiotic against most isolates of gram negative organisms isolated from wound infection (Rathi et al., 2006). The antimicrobial activity of the aqueous, methanolic and ethanolic leave oil extracts of *M. oleifera* against individual isolates of the bacteria revealed that the zones of inhibition (ZI) of the extracts varied from one bacterial species to the other. Methanolic leaf oil extract showed appreciable antibacterial activity on the orthopedic wound organisms, an indication of its high anti-bacterial potential and effectiveness in the treatment of wound infections. Evaluation of the wound healing property of aqueous oil extracts of leaves of *M. oleifera* on male Swiss albino mice showed a significant increase in wound closure rate, skin breaking strength, granuloma breaking strength, granuloma dry weight and decrease in scar area (Srinivasan et al., 2001). However, in our study, the aqueous and ethanolic leaf oil extracts showed no activity against *Proteus* spp., with both extracts showing no ZI against the organism. The variations in the activity of the different leaf oil extracts against individual organisms might be as a result of the differences in the concentration and the amount of phytochemical constituents of each extract. These differences in the phytochemical constituents might have been caused by the difference in polarity of the solvents used in the extraction process (Faizi et al., 1995). Several studies have characterised the chemical compounds present in *M. oleifera* plant leaves (Fuglie, 1999). Two nitrile glycosides from the ethanolic extracts of *M. oleifera* leaf and three mustard oil glycosides have been identified (Fuglie, 1999). Although the ethanolic and aqueous leaf oil extracts showed a considerable reduction in the growth of the tested organisms, the methanolic leaf oil extract showed a broader ZI on all the organisms, which could suggest that the method of extraction has a huge effect on the efficacy of the extracts. Additionally, it is also possible that due to the different methods of extraction employed in this work, each extract could possibly have a different biochemical pathway during metabolism in the organisms. For the seed oil extracts, the activity of the individual extracts (ethanolic, methanolic and aqueous) varied amongst the tested organisms. A comparison of the leaf and seed oil extracts showed that the methanolic and ethanolic leaf oil extracts showed relatively higher activity than their corresponding counterparts from the seed oil extracts. A possible explanation for this could be due to the presence of more bioactive phytochemicals in the methanolic and ethanolic leaf oil extracts when compared to the seed extracts. It could also be that the phytochemicals in the two extracts were more soluble due to the polarity of the solvents. Furthermore, the methanol and ethanol have probably eluted more bioactive components from the leaf than in the seed. In contrast to this result, a higher activity of ethanolic leaf oil extract of *M. oleifera* and a relatively lower activity of the aqueous oil extract was observed at a concentration of 100 µg/mL (Shanholtzer et al., 1992). The reason for this discrepancy in results could not be explained. However, the MIC of the extracts showed that ethanolic leaf oil extract had the lowest activity on the organisms but showed the highest MBC values. MBC values have been shown to be consistently higher than MIC values probably because higher concentrations of antibiotics may be required to kill bacterial organisms than that required to inhibit their growth. The MIC values for the seed oil extracts followed a similar trend with the leaf extracts.

Plasmid profiling of the isolates showed the presence of plasmids in all the gram negative organisms tested. These plasmids may be responsible for the observed resistance in the tested isolates as a reasonable number of the gram negative organisms lost their resistance after curing. Most bacterial plasmids harbour antibiotic resistant genes some of which are acquired through horizontal gene transfer. Interestingly, 60% of the gram positive organisms tested in this study had plasmids and they were resistant to the tested antibiotics. However, 40% of the gram positive organisms (*S. aureus*) had no plasmids but they still showed resistance to most of the tested antibiotics. The presence of resistance in the *Staphylococcus* strains that had no plasmids could suggest that antibiotic resistance in *Staphylococcus* spp. and perhaps other bacteria could be of both chromosomal and plasmid origin. From this study, it has been demonstrated that the application of extracts from natural plants such as *M. oleifera* for the treatment of wound infections can be a promising alternative to conventional antibiotics. The methanolic leaf and aqueous seed oil extracts showed the highest antimicrobial activity when compared to the other extracts. The potential of using these extracts for the treatment of wound infection should be fully harnessed.

CONFLICTS OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

The authors are grateful to the technical staff of the Department of Pharmacology, Faculty of Pharmaceutical Sciences and the Department of Microbiology, Faculty of Biological Sciences, University of Nigeria Nsukka.

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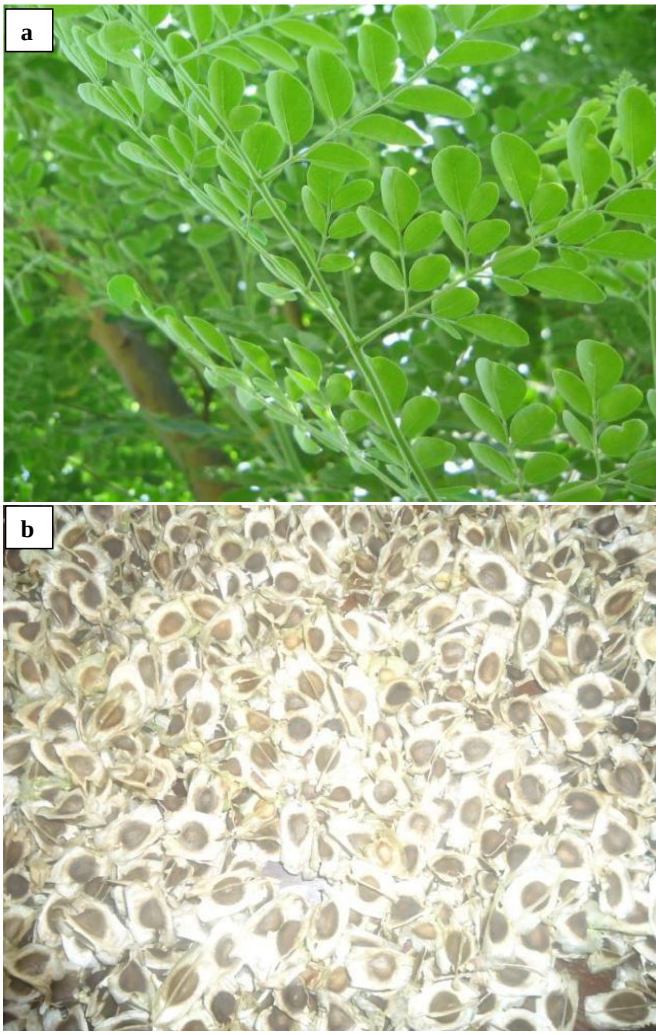

Supplementary Figure 1. Pictures of *M. Oleifera* plants. The plant samples obtained from Dekina Local Government Area of Kogi State Nigeria and identified at the Department of Botany, University of Nigeria, Nsukka (a) *M. oleifera* leaves, (b) *M. oleifera* seeds.


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