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Review

Nosocomial infections in intensive care and medical rehabilitation units, and evaluation of antibiotics prescription

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Although, nosocomial infections (NIs) are considered to be an important cause of mortality and prolonged stay in hospitals, there are only a few studies that are concerned with their prevention and reduction in rehabilitation units. This could be because most NI surveys have been carried out mainly in acute and long-stay in hospitals. As a result, limited information is available on prevention and reduction of NIs in rehabilitation units. The present study is a systematic review that draws attention to the significance of setting up specific means for infection control in hospital units concerned with critical cases, and stresses on the need to adapt to new infection control policies, training of healthcare professionals, educating patients regarding safety measures and fighting nosocomial infections. Furthermore, this study aims to show the obligations of following certain considerations while prescribing antimicrobial therapy so as to maximize the efficacy and reduce the adverse effects of conventional antibiotics. Systematic review survey methods were adopted throughout the study. Results of the study were debatable concerning the question of predominance of Gram positive or negative microorganisms as nosocomial etiological agents. Infection control measures such as continuous surveillance protocols, suitable prevention methods and appropriate therapeutic treatment strategies such as de-escalation and rotational antibiotic therapies might help reduce nosocomial infections. Much attention should be given in hospitals to the environmental conditions including quality of water and air. Patients with a suppressed immune system during their stay in hospitals are susceptible to levels of microorganisms that are considered safe for a healthy community. Hence, this study suggests that special safety levels should be set up regarding the levels of microorganisms in hospitals and these should be much lower than those used for a healthy community.

Key words: Nosocomial infections, rehabilitation units, antibiotics, immunocompromised patients.

INTRODUCTION

Extensive studies have been done on the fundamental aspects of rehabilitation sciences. Several strategies are

under extensive research such as the use of laser therapy and phototherapy (Al-Shenqiti and Oldham, 2009; Al-Shenqiti and Oldham, 2014). Unfortunately, except for discovering new compounds having antifungal properties (Bahashwan, 2011a, b), many other cross-subsidiary aspects are neglected. One of the most neglected aspect is nosocomial infections in rehabilitation units. Acute medical rehabilitation units (MRU) are located in hospitals under the supervision of a physiatrist, a physician who specializes in rehabilitation care. He assesses and monitors the patient's medical and rehab status daily and also gives instructions to the team of rehabilitation professionals, while the acute MRUs provide round-the-clock rehabilitative nursing. The sub-acute MRUs (Subacute Rehab) are less intense and less comprehensive and are supervised only once or twice a week. Nosocomial infections, also called hospital acquired infections or healthcare-associated infection (HAI), are those infections which patients acquire during their stay in a hospital for some other reason other than for which they were admitted (Ducel et al., 2002). It is alarming since the infection was not present in that person when he was admitted. He may show symptoms after he is discharged (Benenson, 1995). Nosocomial infections could also be defined as infections that occur within 48 h of the patient's admission to the hospital or those infections which are acquired within 3 days after discharge or within 1 month of an operation. Occupational infection among the hospital staff are also included (Benenson, 1995). About 10% of the patients admitted to a hospital can be affected due to these infections (Inweregbu et al., 2005). Nosocomial infections are considered as a major obstacle in hospital care and complications in both adults and children (Fanos and Cataldi, 2002).

The main aim of this study was to draw attention to the importance of setting up specific means of infection control in hospital units that are concerned with critical cases. There is need to prepare and adapt to new infection control policies, train healthcare professionals, develop safety measures for patients and educate them on fighting nosocomial infections. The present study also aims to show the absolute necessity of following certain considerations when prescribing antimicrobial therapy so that the prescribed antibiotics can have maximum efficacy and minimum adverse effects.

PREVALENCE OF NOSOCOMIAL INFECTIONS

Several studies have documented and confirmed the high prevalence of nosocomial infections in health care environments and ICUs. Vincent et al. (1995) reported that more than 20% of the patients had ICU-acquired infections (Vincent et al., 1995). In a survey of 827 neonatal ICU patients, Sohn et al (2001) showed that 11.4% patients had 116 NICU-acquired infections (Sohn et al., 2001). The incidence of nosocomial infections varies among different studies but reports suggest high prevalence in ICU patients, the incidence ranging between 6 and 25% in neonatal ICUs. This large variation depends mostly on the weight of the new born child and the treatment conditions (Ferguson and Gill, 1996; Sohn et al., 2001; Babazono et al., 2008). The severity of the matter calls for an urgent need for surveillance at national level and more effective preventive measures to be evolved.

ETIOLOGY OF NOSOCOMIAL INFECTIONS

Pathogenic fungi like various species of *Candida* and *Aspergillus fumigatus* are responsible for nosocomial pneumonia. Being opportunistic, these infections are not so common in immunocompetent patients (Krasinski et al., 1985; Loo et al., 1996; El-Ebiary et al., 1997) but can be seen in organ transplant or immunocompromised, neutropenic patients. Outbreaks of pneumonia have been reported and are likely due to viruses like influenza, parainfluenza, measles, adenoviruses and respiratory viruses. Cases of hospital acquired, ventilator-associated and health-care associated pneumonia are common (American Thoracic Society, Infectious Diseases Society of America, 2005; Mühlemann et al., 2004).

The most common cause of nosocomial infections are Gram-positive bacteria. *Staphylococcus aureus* has been reported to be the predominant pathogen. The other common pathogens associated with nosocomial infections in ICU patients include methicillin-resistant (MRSA) and methicillin-sensitive *S. aureus* (MSSA) (19). Methicillin-resistant *S. aureus* (MRSA) is the cause of up to 60% of nocosomial infections in ICUs (Graffunder and Venezia, 2002; Inweregbu et al., 2005). Vancomycin-resistant *enteroccocus* (VRE) and multidrug-resistant *Acinetobacter* species are also commonly associated bacterial species (National Nosocomial Infections Surveillance System, 2004).

In contrast to previous findings, Ahoyo et al. (2014) showed that 65% of isolated microorganisms from nosocomial infected patients were Gram-negative, while only 30% were Gram-positive (Ahoyo et al., 2014). According to Vincent et al (1995), the infection causing micro-organisms, seen to inhabit ICUs more frequently were Enterobacteriaceae, *S. aureus* and *Pseudomonas aeruginosa* with a percentage of about 30 to 35%, while coagulase-negative staphylococci and fungi were reported to be 19 and 17%, respectively (Vincent et al.,

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1995). Upto 60% cases of *S. aureus* were resistant to methicillin. The most common pathogens in neonatal ICUs were coagulase-negative staphylococci and enterococci as reported by Sohn et al. (2001). Sometime later in 2006, Prashanth and Badrinath (2006) claimed that Acinetobacter species caused nosocomial infections and is of increasing concern in critically ill patients. Also, the risk factors are not well established. The studies reported so far are still not clear about the predominance of the type of microorganisms as nosocomial etiological agents, that is, whether they are Gram positive or negative.

Frequency of nosocomial infections

In a recent study conducted using the National Nosocomial Infections Surveillance System, it was shown that percentage of bloodstream infections was the highest with a percentage that was greater than 39% followed by surgical site infections with a percentage greater than 13%, while pneumonia (PNEUM) and urinary tract infections (UTI) were the lowest with percentages 2.5 and 3.1%, respectively (National Nosocomial Infections Surveillance System, 2004). The percentage infection contribution of the other sites of colonization was only 15.5%. Oskouie et al. (2013) reported that the frequencies of different types of nosocomial infections were as follows: bacteremia (68.9%), urinary tract infections (13.6), lower respiratory tract infections (9.7%) and wound infections (7.8%) (Oskouie et al., 2013).

In ICUs, the frequency of nosocomial infections was about 29.13% of which 30.1% were respiratory tract infections, 39.1% were urinary tract infections and 23.7% were blood stream infections. Other infections were identified to be skin, soft tissue, wound and gastrointestinal tract infections (Shaikh et al., 2008). According to another report, pneumonia at a frequency of 46.9% is the most frequent followed by lower respiratory tract and urinary tract infections at a frequency of around 18%. Bloodstream infections occur at a frequency of 12% (Vincent et al., 1995). On the other hand, Ahoyo et al. (2014) reported that the most recurrent nosocomial infections were UTIs (37.5%) followed by, intravascular catheter-associated (27%) and surgical site infections (19.2%). Lower respiratory tract infections or pneumonia was at 11.7%, while bloodstream infections were at 1.5%, and infections originating otherwise were about 3.1% (Ahoyo et al., 2014).

Sohn et al. (2001) studied 827 neonatal ICU patients in 2001 and reported that 11.4% of these patients had 116 nosocomial ICU-acquired infections of which 53% were bloodstream, 13% were lower respiratory tract and 8.6% were ear-nose-throat or urinary tract infections. Nosocomial infections in a neonatal ICU showed a prevalence of 17.5%. Here, the bloodstream infections, clinical sepsis and pneumonia each showed a frequency of around 5 to 6%. The UTIs and surgical site infections comprised only 0.7%. The rate of infection due to medical interventions were about 14% associated with central intravascular catheters, 16% with total parenteral nutrition, 18.6% with ventilator-associated pneumonia, 13.7% with surgeries and 17.3% with urinary catheters (Su et al., 2007).

Risk factors for nosocomial infections

Several risk factors have been identified for ICU-acquired infections such as long stays in the hospital ICUs, greater than 48 h, mechanical ventilation, trauma, catheterization of blood vessels, and stress ulcer prophylaxis (Vincent et al., 1995). Besides these, use of postnatal steroids and H2-blockers are considered modifiable risk factors for nosocomial infections in neonatal ICUs (Rojas et al., 2005). More recently, Akkoyunlu et al. (2013) highlighted the inclusion of advanced age, long hospital stays, prior infections, transfusion of blood products and prior antibiotic usage as risk factors for nosocomial pneumonia in ICUs (Akkoyunlu et al., 2013). Seasonal viral infections can also be considered as putative risk factors in pediatric nosocomial infections (Mühlemann et al., 2004). It has been demonstrated that the highest risk is due to the pre-formation of urinary catheterization (King et al., 2012). Hence, unnecessary urethral catheterization should be avoided to prevent nosocomial UTIs (Savas et al., 2006).

Nosocomial UTIs constitute 40 to 50% of all hospital acquired infections (Saint and Lipsky, 1999; Merle et al., 2002) and hence are one the most common community acquired infections. These infections are associated with urinary catheters in 80% of the cases (Mulhall et al., 1988). Although, antibiotics are available and used frequently to control UTIs, they are widespread in the human population (Savas et al., 2006). A high incidence of these infections is reported to be caused by *E. coli*, *Enterococcus faecalis* and *Proteous mirabilis* (Chaudhry et al., 2016). Very low birth weights, gestational age and length of central venous lines of neonates especially those undergoing interventions such as mechanical ventilation are at the greatest risk of neonatal nosocomial infections (Pawa et al., 1997; Kasim et al., 2014). Mulu et al. (2013) confirmed that the most likely risk factors for post-operative infections are old age, any underlying disease, extended pre-operative and post-operative stay in hospitals, long surgeries and appendectomy (Mulu et al., 2013). Treating patients with drugs like imipenem, vancomycin and piperacillin-tazobactam can be a cause of imipenem-resistant *P. aeruginosa* infections in hospitalized patients (Onguru et al., 2008). Previous studies have illustrated several putative nosocomial infections risk factors that have been listed in Table 1.

NOSOCOMIAL INFECTIONS IN REHABILITATION ENVIRONMENT

Even though nosocomial infections are important causes

Table 1. Risk factors for putative nosocomial infections.

Active reasons	Passive reasons
Longer lengths of stay (LOS) (> 48 h) in ICU	Advanced age
Mechanical ventilation	Prior infection e.g. viral infections
Diagnosis of trauma	Gestational age
Central venous catheterization	Underlying disease
Pulmonary artery catheterization (PAC)	Very low birth weights (VLBW)
Urinary catheterization	
Stress ulcer prophylaxis	
Longer duration of surgery	
Use of H2-blockers	
Transfusion of blood products	
Prior antibiotic usage	
Length of central venous line of neonates	
Extended preoperative and postoperative hospital stay	
Appendectomy	

Figure 1. Nosocomial infections strategies.

of morbidity and mortality in health care sector along with prolonged stay in hospitals and increasing treatment costs, incompetent (Fanos and Cataldi, 2002) surveys have been carried out to investigate this issue. As a result, scanty information is available regarding rehabilitation sectors in hospitals (Baldo et al., 2002). Only a few studies are available that throw some light on reduction and prevention of NIs in rehabilitation environments. A study conducted in 2003 by Lewis et al. illustrated some modifications for Disease Control and Prevention (CDC) contact precautions applicable to the rehabilitation environment. These were easy to understand, consistent and could be effectively implemented by the hospital staff. These modifications did help in the prevention of nosocomial transmission of epidemiologically important pathogens (Lewis et al., 2003). Later in 2008, Geyik et al. proposed that appropriate interventions, surveillance and constant monitoring are effective in reducing the rate of NIs along with educating the staff on infection control practices (Geyik et al., 2008).

NOCOSOMIAL INFECTION CONTROL

Controlling nosocomial infections may include three strategies: Surveillance, prevention and treatment (Figure 1).

These infections can be prevented to a large extent by instituting careful surveillance of bacterial infections, improving hand hygiene, and limiting antibiotics abuse and invasive procedures (Garner, 1996; Goldman et al.,

1996; Goldmann and Huskins, 1997; Boyce and Pittet, 2002; Cohen et al., 2003; Shiojima et al., 2003; Ledell et al., 2003). The infection control programs should pay particular attention on preventing infections in patients who are at higher risk of infection due to exposure to certain procedures and medical devices (Emori and Gaynes, 1993). According to a study conducted concerning nosocomial infection control (SENIC) measures, it was observed that about 33% of the nosocomial infections could be prevented by taking proper infection control measures like using different surveillance methods and programs designed for prevention and treatment (Haley et al., 1985). Recently, Sonmezer et al. (2016) also suggested that the rate of nosocomial infections can be limited to a large extent by meticulously applying contact measures (Sonmezer et al., 2016).

Surveillance

Surveillance is a continuous process of systematically collecting, analyzing and interpreting all the information related to a patient's health. This process has immense significance while planning, implementing and evaluating the health of patients and timely disseminating all information. The surveillance service basically aims to collect information and make a database for comparing various hospital-acquired infections. It intends to uplift the health of patients by decreasing the rates of nosocomial infections and help in improving clinical practices. The surveillance procedures include the monitoring infections that occur after a surgery, hospital acquired bacteraemia, urinary tract and lower respiratory tract infections. Incidentally, UTI and lower respiratory infections are the second most common source of nosocomial infections. The studies have estimated that two-thirds of bacteraemia is associated with intravascular devices like catheters which are supposed to be the most common source of hospital-acquired bacteraemia (Inweregbu et al., 2005). Establishment of a surveillance system that monitors a device-related infection seems to be the principal strategy. This plan of action can achieve reduction and prevention of nosocomial infections associated with implantable biomaterials (Guggenbichler et al., 2011).

Prevention

A study was conducted to determine prevalence of nosocomial infections in ICU of European hospitals (Vincent et al., 1995) that identified several predisposing factors for nosocomial infections. The factors can be categorized as those related to the following: (i) underlying health status of the patient e.g. old age, malnutrition, alcoholism, heavy smoking, chronic lung diseases, and diabetes (ii) any kind of acute disease or condition e.g. surgery, trauma and burns (iii) invasive procedures like endotracheal or nasal intubation, central venous catheterization, extracorporeal renal support (iv) treatment issues like blood transfusion, recent antimicrobial therapy, immunosuppressive treatments, stress-ulcer prophylaxis, recumbent position, parenteral nutrition and length of stay in hospitals. These infections can be reduced and prevented by smartly regulating use of suitable antiseptics in combination with medical devices (Guggenbichler et al., 2011).

Treatment

There is increasing evidence suggesting that early use of appropriate antibiotics reduces morbidity and mortality. Conventional antibiotics are classified into one of the following four classes according to their mode of action: (i) inhibitors of the fungal cell wall such as penicillin (e.g penicillin V and G), semi-synthetic penicillin (e.g ampicillin, amoxicillin); cephalosporins (e.g cefotaxime, cefradine, ceftazidime); monobactams (e.g aztreonam); carbapenems (e.g meropenem); β- lactamase inhibitors (e.g clavulanate); and glycopeptides (e.g vancomycin). (ii) Inhibitors of fungal cell membranes such as polyenes (e.g nystatin); imidazoles (e.g ketoconazole; and triazoles (e.g fluconazole) (iii) inhibitors of protein synthesis such as aminoglycoside (e.g gentamicin); macrolides (e.g erythromycin); oxaolidinines (e.g erythromycin; ketolides (e.g telithomycin); and streptogramins (e.g synercid. (iv) Inhibitors of nucleic acids such as fluoroquinonolones (e.g ciprofloxacin); nitro-imidazoles (e.g, metronidazole); rifampicin (e.g, sulphonamides) and folate inhibitors.

Appropriate antibiotics are selected for treating a specific infection on the basis of the results of microbiological tests performed on clinical specimens. Some antibiotics have a broad-spectrum effect while others affect either Gram positive or negative bacteria only. It is highly recommended that any drug policy or guidelines should have an objective to stop or at least minimize antibiotic abuse and ease the pressure on a particular antimicrobial agent, rather an alternative drug should be used to prevent resistance in microorganisms (Inweregbu et al., 2005).

Two therapeutic strategies are used frequently for elimination of microbial infections using different antibiotics, de-escalation and rotational antibiotic therapies. De-escalation strategy requires the initiation of broad-spectrum antibiotic therapy at an early stage in patients with suspected sepsis even before diagnosis. On the other hand, rotational antibiotic therapy is a strategy that helps in reducing resistance to a particular antibiotic by its simple withdrawal, or the withdrawal of the whole antibiotic class from the ICU for a short and specified time period. This will stabilize and even decrease the rate of resistance to some extent. There are certain considerations that have to be taken care of while giving

antimicrobial therapy. It is absolutely necessary to acquire an accurate infection diagnosis before prescribing antimicrobial therapy. A clear understanding of the difference between empiric and definitive therapy should be achieved. Instead of using broad spectrum antibiotics straightaway, oral narrow-spectrum antibiotics should be tried first for the shortest duration necessary. Besides reducing drug resistance, this strategy will also be cost effective. It is important to understand the specific characteristics of antimicrobial agents, for instance, their pharmacodynamics and efficacy at the site of infection. Those drug characteristics that influence the efficacy of antibiotics should be taken into account along with the recognition of adverse effects of antimicrobial agents on the host (Leekha et al., 2011).

In the case of a patient suffering from a serious infection, it is critical to rapidly initiate effective antibiotic therapy, followed by de-escalation and, when new information and details are available the therapy should be discontinued promptly. At the same time, antibiotic resistance can be controlled effectively by complementing the therapy with effective infection control measures. The effective implementation of these principles requires the development of multidisciplinary antimicrobial supervising programs that assure adherence (Deresinski et al., 2007). The indiscriminate use of antimicrobials in non-infected patients is dangerous and should be discontinued immediately (Ahoyo et al., 2014). Furthermore, the use of high generation antibiotic and branded prescriptions where there is no requirement, adds to the cost of the therapy (Jose et al., 2016).

The most effective antibiotics against Gram-negative bacteria were found to be imipenem and meropenem (Savas et al., 2006). Antimicrobial agents have several adverse reactions and side effects, one of which is antimicrobial allergy (Leekha et al., 2011). The adverse effects can be direct or indirect. The direct adverse effects include allergy to the drug, its toxicity or its interaction with other drugs and therapeutic failure. Indirect effects, on the other hand, are those adverse effects which can be observed on the commensals and the environmental flora (Leekha et al., 2011). Hence, much attention should be given in hospitals to air, water and other environmental conditions as immuno-compromised patients in hospitals are susceptible to microorganism levels considered safe for healthy people. Torii et al. (2003) emphasized that even low concentrations (10 times lower than safety level) of *Ligionella pneumophila* found in the water used for washing is pathogenic and can cause serious infections in immunocompromised patients (Torii et al., 2003). Furthermore, Legionnaires' disease is estimated to be present in 3 to 15% of community acquired pneumonia and 10 to 50% of nosocomial infections in European countries and the United States (Chang et al., 2011). It has been reported that about 36% of the water samples are contaminated with *L. pneumophila* (Yamamoto et al., 2003; Suzuki et al., 2000).

Reports reveal that *S. aureus* shows a high resistance to methicillin at a frequency of 52.5% but show a low resistance frequency to vancomycin (7.7%). On the other hand, enterococci is highly resistant to vancomycin with a frequency of 67.5%. Among Gram-negative isolates, the highest percentage of resistance is observed against ampicillin (86.4%), tetracycline (77.3%), amoxicillin/ clavulanic acid (72.8%) and trimethoprim/ sulfamethoxazole (64%). In the case of different serotypes of *Salmonella* species, no well-defined differences in antimicrobial susceptibilities were observed. Isolates of *P. aeruginosa* gave an extended beta-lactamase spectrum where most of them were resistant to all beta-lactams (Abd El-Mohdy and Ghanem, 2009), except imipenem. Only 22% were found to be susceptible to gentamicin and ciprofloxacin (Ahoyo et al., 2014). Studies recommend the prescription of the antibiotic imipenem (IMP) for the treatment of *Proteus* spp. infections. In conditions where there is sensitivity to imipenem, amikacin (AK) can be prescribed. Acute *Proteus* infections may require the prescription of imipenem and amikacin together as combined therapy giving a synergistic effect (Bahashwan and El Shafey, 2013).

Nosocomial infections, especially those associated with ICU reveal that drug resistance rate in bacteria has increased tremendously. A common method by which bacteria develop resistance is by acquiring genetic mutations and transformations. Prescribing antibiotics senselessly leads to the selection of resistant bacteria. The genes that encode drug resistance may get transferred to other bacterial strains. Vancomycin, a broad-spectrum antibiotic, is usually prescribed to treat methicillin-resistant *S. aureus* (MRSA). But unfortunately, use of this antibiotic leads to resistance in enterococci and sometimes in *S. aureus*. Hence, there is an urgent need to very critically scrutinize prescriptions that include antibiotics. Reports reveal that the usage of vancomycin, which was the first line treatment for diarrhea due to *Clostridium difficile* infection, has been discouraged now a days (Inweregbu et al., 2005). Oskouie et al. (2013) reported that the wards that keep neonates had the highest share of nosocomial infections followed by NICU and hematology wards. It was observed that the most common pathogenic organisms were *Staphylococcus* sp. (35%), *Klebsiella* sp. (20.4%), *Serattia* sp. (9.7%), *E. coli* (6.8%) and *Pseudomonas* sp. (5.8%) (Oskouie et al., 2013). Hence, specific measures have to be taken to keep infection under control in critically ill patients. Nosocomial infection rates have to be reduced for which new national infection control policies have to be implemented. All the people who are concerned with healthcare should be educated on patient safety and given adequate training to highlight awareness of the disease and the significance of healthcare. The excessive use and misuse of antimicrobials has to be regulated especially in people who are admitted to hospitals not for

any kind of infection but some other reasons (Ahoyo et al., 2014).

Conclusion and recommendation

A reduction in nosocomial infection, especially in the rehabilitation milieu is of utmost importance. Special attention should be given to immunocompromised patients. The health care sectors should adapt to infection control policies giving adequate and proper training to the hospital staff and other professionals. The patients should also be aware of suitable safety measures. Antimicrobial therapy should be given with care. The antibiotics prescribed must have maximum efficacy and the least adverse effects. Natural products have great therapeutic potential with little side effects. These products and their derivatives are now being highly researched as potential antimicrobial agents. Synergy of the conventional antibiotics with natural products is also investigated to reduce drug doses and increase efficacy.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

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Microbiological study of ventilator associated pneumonia with antimicrobial susceptibility pattern of the isolated strains and its relation to nasal colonization in neonatal intensive care unit

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The aim of this study is to analyze the microbiological profile of ventilator associated pneumonia (VAP) in relation to nasal swabs and prevalence of multi-drug resistant bacteria so as to implement effective treatment and prevention strategies in NICU (Neonatal Intensive Care Unit). One hundred neonates were ventilated for more than 48 h and met the inclusion criteria which were enrolled in the study. All cases were subjected to history taking, thorough clinical examination, Indications of mechanical ventilation, sputum and nasal swabs culture and sensitivity by conventional microbiological methods. As regard to positive sputum culture results, the rate of *Klebsiella pneumoniae* was 16.7% in cases with early onset VAP while it is 39.4% in cases with late onset VAP, A*ceintobacter baumanni, Pseudomonas aeruginosa* and combined *P. aeruginosa* and *K. pneumoniae* was 8.5% each in cases with late onset VAP. As regard, positive nasal swab culture results *K. pneumoniae* which was isolated from 16.7% of all cases, with early onset VAP while it was 23.4% in cases with late onset VAP, *P. aeruginosa*, combined *P. aeruginosa* and *K.* pneumonia was 8.5% each, and *A. baumanni* was 6.4% in cases with late onset VAP. Gram negative bacteria are the most common agents causing VAP in our study. There is a relationship between organisms in nasal swab and VAP. So, nasal screening for colonization may be a valuable tool for de-escalation of empiric therapy targeted to the organism.

Key words: Ventilator associated pneumonia (VAP), nasal swab, antimicrobial sensitivity.

INTRODUCTION

Ventilator associated pneumonia (VAP) is a serious complication related to mechanical ventilation in the neonatal period. VAP is defined as nosocomial pneumonia in mechanically ventilated patients that develops more than 48 h after initiation of mechanical ventilation (MV) (Tripathi et al., 2010). VAP is the most

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common nosocomial infection in neonatal intensive care unit (NICU) (Awasthi et al., 2013).

Diagnosis of a VAP episode requires a combination of clinical, laboratory and radiological criteria. The most prevalent clinical signs associated with VAP refer to changes in the characteristics and volume of respiratory secretions and the appearance of purulent mucus in tracheal aspirate (TA). Other signs include hypo- or hyperthermia and worsening of the respiratory distress (Cernada et al., 2013).

Despite the advancements in antimicrobial regimes, VAP continues to be an important cause of morbidity and mortality. VAP requires a rapid diagnosis and initiation of appropriate antibiotic treatment, as there is adverse effect of inadequate antibiotic treatment on patient's prognosis and the emergence of multidrug-resistant (MDR) pathogen (Jakbrittu et al., 2012)**.**

Specific biomarkers of VAP allowing differentiation of pneumonia from colonization have been extensively studied .The presence of bacterial pathogens will be sensed by specific cytosolic receptors such as Toll-like and Nod-like receptors triggering an inflammatory response (Cernada et al., 2014). Pro-inflammatory cytokines such as IL-1, IL-6, IL-8, IL-10, and TNF-α have been evaluated as markers of VAP, with discordant results **(**Conway et al., 2010)**.**

The aim of this study is to analyze the microbiological profile of VAP in relation to nasal swabs, and prevalence of multi-drug resistant bacteria so as to implement effective treatment and prevention strategies in neonatal intensive care unit (NICUs).

MATERIALS AND METHODS

This prospective observational study was carried out at Tanta University Hospitals, Egypt in Neonatal Intensive Care Unit, Pediatric and Medical Microbiology and Immunology Department on intubated neonates who were admitted to NICU over 1 year (from March 2016 to February 2017).

One hundred (100) cases were enrolled in the study which were divided into 2 groups according to onset of VAP; Early-onset VAP group: 6 patients [2 females (33.3%) and 4 males (66.7%)] and Late-onset VAP group: 94 patients [39 females (41.5%) and 55 males (58.5%)]. One hundred neonates were ventilated for more than 48 h and met the next inclusion criteria which were enrolled in the study.

Inclusion criteria

Inpatient without primary (1ry) underlying pneumonia; with chest x-rays showing newly progressive or persistent infiltrate, consolidation, pneumatocele, worsening of gas exchange as increase FIO_2 requirement, increasing ventilation demand, and increasing oxygenation index, and three of the following: Temperature instability without recognized cause, Leucopenia, leucocytosis, left shift ≥10% or left shift >0.15, new onset of purulent tracheal aspirate or increasing respiratory secretion with increasing suctioning requirement, apnea, tachypnea and retraction, wheezing and rales, changes in heart rates, clinical decision to change antibiotics, elevation in CRP>10 mg/dl.

Exclusion criteria

Neonates requiring intubation less than 48 h and neonatal pneumonia as a cause of intubation.

All cases were subjected to thorough history taking including prenatal, natal and postnatal history coupled with any maternal risk factor, mode of delivery, gestational age and postnatal age, thorough clinical examination on admission, indications of mechanical ventilation**,** sputum culture and sensitivity, nasal swab culture and sensitivity.

Microbiological procedure

Endotracheal aspirate and nasal swabs were collected under complete aseptic precaution for culture and sensitivity .The specimens collected were transported to laboratory within 1 h, All samples were subjected to conventional culture on blood agar, chocolate agar, and MacConkey,s agar medium for identification of the colonies by conventional microbiological and biochemical tests.

Antimicrobial susceptibility of all isolates were determined by the standard Kirby Bauer disc diffusion method according to Clinical and Laboratory Standard Institute Guidelines (CLSI, 2008). Disc diffusion test was performed using Muller Hinton agar (Oxoid, Basingstoke, United Kingdom).

The antimicrobial discs were obtained from Oxoid limited Basingstoke, Hamisphire and England. Antimicrobials were supplied and stored according to the manufacturer's instructions. *Escherichia coli* ATCC 5922, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 25923 were used as control strains. Disc zone diameter was interpreted according to the CLSI (2008) recommendations.

Antibiotics used

Gram negative panel: Imipenem 10 mg, meropenem 10 mg, cefotaxime 30 mg, ciprofloxacin 5 mg, sulfamethoxazole 300 mg, colistin 25 mg, polymyxin 300 mg, piperacillin-tazobactam 85 mg, amikacin 30 mg, levofloxacin 5 mg, ceftazidime 30 mg, gentamycin 10 mg, ampicillin-sulbactam 20 mg and tetracycline 30 mg.

Gram positive panel: Vancomycin 30 mg, erythromycin 15 mg, penicillin G 10 mg, cloramphenicol 30 mg, cefoxitine 30 mg, doxycycline 30 mg, clindamycin 2 µg, amikacin 30 mg, gentamycin 10 mg, ciproflxacin 5 mg, aztronam 30 mg, ceftriaxone 30 mg, cefoperazone 75 mg, cefotaxime 30 mg, cefuroxime sodium 30 mg and sulfamethoxazole 300 mg.

Statistical analysis

The data were statistically analyzed using SPSS software (Statistical Package for the Social Sciences, version 16, SPSS Inc. Chicago, IL, USA).

RESULTS

Demographic data of the studied patients showed that, there was no statistical significant difference between the two groups regarding body weight, gestational age, and sex. However, statistically significant increase in postnatal age in late onset VAP (10.31± 4.41 days) in comparison to early onset VAP (2.83 ± 0.41) was observed (Table 1).

As regard the indications of ventilation in this study,

Table 1. Demographic data of the studied group.

S/N	Demographic Data	Early onset VAP	Late onset VAP	Tests	
				T	P-value
1	Body weight (kg)	2.93±0.84	2.20±1.07	1.628	0.107
2	Gestational age	36.83±3.37	34.04±3.90	1.711	0.090
3	Postnatal age before specimen (In Days)	2.83±0.41	10.31±4.41	4.134	0.000*
4	Sex			X^2/t	P-value
	Female	2(33.3%)	39(41.5%)	0.155	0.694
	Male	4(66.7%)	55(58.5%)		

Table 2. Indications of ventilation in the studied group.

Cause of ventilation	N	Percent age
Apnea	2	2.0
Congenital diaphragmatic hernia	6	5.0
Birth asphyxia	5	4.0
Congenital myopathy	2	2.0
Meningitis	9	9.0
intracranial hemorrhage	2	2.0
Meconium aspiration syndrome (MAS)	12	12.0
pleural effusion	2	2.0
Pneumonia	3	3.0
Respiratory Distress Syndrome (RDS)	57	57.0
Total	100	100.0

Table 3. Sputum culture results in early onset and late onset VAP.

Endotracheal aspirates	Early onset VAP		Late onset VAP		Chi-square	
	N	%	N	%	X^2	P-value
No growth	5	5.00	25	26.6	12.112	<0.001*
Klebsiella pneumonia	1	16.7	37	39.4	1.746	0.186
Acinatobacter baumanni	0	0.0	8	8.5	0.555	0.456
Pseudomonas aeruginosa	0	0.0	8	8.5	0.555	0.456
Staph aureus	0	0.0	4	4.3	0.266	0.606
Proteus	0	0.0	4	4.3	0.266	0.606
Pseudomonas aeruginosa +Klebsiella pneumoniae	0	0.0	8	8.5	0.555	0.456
Total	6	100.0	94	100.0	-	-

Non-significant >0.05, significant <0.05*, high significant <0.001*.

presented in Table 2 are respiratory distress syndrome (RDS) which accounts for 57% of cases, 12% of meconium aspiration syndrome, 9% of meningitis congenital diaphragmatic, 6% of hernia, 2% of congenital myopathy, 2% of intracranial hemorrhage and 2% of pleural effusion.

The results of culture of endotracheal aspirates in this study showed that, the rate of negative sputum culture results was significantly higher in cases with late onset VAP (26.3%) in comparison to early onset VAP where 5% of cases with early onset VAP showed no growth. As regard to positive sputum culture results, the rate of *Klebsiella pneumoniae* was 16.7% in cases with early onset VAP while it is 39.4% in cases with late onset VAP, *Aceintobacter baumanni, P. aeruginosa* and combined *P. aeruginosa* and *K. pneumoniae* was each 8.5% in cases with late onset VAP (Table 3).

As regard to nasal swab culture, the results of the study

Table 4. Nasal swab culture results in early onset and late onset VAP.

Nasal swab culture	Early onset VAP		Late onset VAP		Chi-square	
	N	%	N	%	X^2	P-value
No growth	5	5.00	46	48.9	4.956	0.026*
Klebsiella pneumoniae	1	16.7	22	23.4	0.308	0.579
Acinatobacter baumanni	0	0.0	6	6.4	0.407	0.523
Pseudomonas aeruginosa	0	0.0	8	8.5	0.555	0.456
Staph aureus	0	0.0	2	2.1	0.130	0.718
Proteus	0	0.0	2	2.1	0.130	0.718
Pseudomonas aeruginosa +Klebsiella pneumoniae	0	0.0	8	8.5	0.555	0.456
Total	6	100.0	94	100.0	-	-

Non-significant >0.05, significant <0.05*, high significant <0.001*.

reported that the rate of negative nasal swab culture results was significantly higher in cases with late onset VAP (48.9%) in comparison to early onset VAP where 5% of cases with early onset VAP showed no growth. As regard positive nasal swab culture results, *K. pneumoniae* was isolated from 16.7% of cases with early onset VAP while it was 23.4% in cases with late onset VAP, *pseudomonas*, combined *P. aeruginosa* and *K. pneumoniae* was each 8.5% and *A. baumanni* was 6.4% in cases with late onset VAP (Table 4).

As regard the relationship between endotracheal aspirates and nasal swab cultures results showed that 30% of cases were negative in both cultures while the total number of cases which developed positive endotracheal aspirates culture for *K. pneumoniae* was 38. 23 of them was positive for *Klebsiella* in nasal swab culture, 8 cases developed *A. baumanni* in endotracheal aspirates culture, 6 of them was positive also in nasal swab culture, 8 deveolped *P. aeruginosa* and combined *P. aeruginosa* and *K. pneumoniae* in both sputum and nasal swab cultures (Table 5).

The pattern of antibiotics sensitivity of endotracheal aspirates culture shown in Table 6 where among *K. pneumoniae* strains the highest sensitivity was recorded to Amikacin (13%) followed by Imepenem (6%), among *A. baumanni* (3%) was sensitive to cefoxitim and Polymyxin, while all of the isolated staphylococci (4%) were sensitive to vancomycin.

The pattern of antibiotics sensitivity of the nasal swab culture shown in Table 7 where among *K. pneumoniae* strains highest sensitivity was recorded to Imepenem (6%), among *A. baumanni* 3 strains were sensitive to polymyxin (3%), while 2 strains out of the isolated 4 *S. aureus* strains (2%) were sensitive to vancomycin.

DISCUSSION

VAP is a serious complication related to mechanical ventilation in the neonatal period. VAP is defined as nosocomial pneumonia in mechanically ventilated patients that develops more than 48 h after initiation of mechanical ventilation (MV) (Tripathi et al., 2010).

VAP continues to be the most common Health Care Acquired Infections (HCAIs) in the ICU, making up almost one third of the total HCAIs (Joseph et al., 2010). The pathogenesis of VAP is related to host and treatment related colonization factors. Aspiration of oropharyngeal pathogens and leakage of secretions containing bacteria around the endotracheal tube are principle factors for development of VAP. The progression from colonization to tracheobronchitis to VAP is a dynamic equilibrium (Al-Dorzi et al., 2012).

The aim of this study was to analyze the microbiological of VAP in relation to nasal swabs, risk factors and prevalence of multi-drug resistant bacteria so as to implement effective treatment and preventive strategies in NICU, Tanta University Hospitals, Egypt. The present study showed that regarding body weight, gestational age, and sex there was no significant difference between the 2 groups, while there was statistically significant increase in postnatal age in late onset VAP in comparison to early onset VAP.

As regard the indications of ventilation in this study presented in Table 3, RDS accounts for 57% of cases, 12% of Meconium Aspiration Syndrome, 9% of Meningitis, 6% of Congenital Diaphragmatic Hernia, 2% of Congenital myopathy, 2% of intracranial hemorrhage, and 2% of Pleural Effusion. The results shown were somewhat different from the results of Qazi et al. (2015) who reported RDS in 94%, asphyxia 68%, MAS 30%, Meningitis 22%, pneumonia 20% among cases of ventilated neonates.

The results of endotracheal aspirates culture in this study showed that *K. pneumoniae* was the most common organism in both groups as its rate was (16.7%) in cases with early onset VAP while (39.4%) in cases with late onset VAP, *A. baumanni, P. aeruginosa* and combined *P. aeruginosa and K. pneumoniae* was (8.5%) in cases with late onset VAP. The study performed by Hina et al., (2010) showed that the most common organism associated with VAP is *P. aeruginosa* (43.24%), followed

Table 5. Relationship between endotracheal aspirates culture and nasal swab culture results.

Nasal swab culture		Sputum culture						
		No growth	***Klebsiella pneumoniae***	***Acinatobacter baumanni***	***Pseudomonas aeruginosa***	***Staph aureus***	***Proteus***	***Pseudomonas aeruginosa + Klebsiella pneumoniae***
No growth	N	30	15	2	0	2	2	0
	%	30.0	15.0	2.0	0.0	2.0	2.0	0.0
Klebsiella pneumoniae	N	0	23	0	0	0	0	0
	%	0.0	23.0	0.0	0.0	0.0	0.0	0.0
Aceinetobacter baumanni	N	0	0	6	0	0	0	0
	%	0.0	0.0	6.0	0.0	0.0	0.0	0.0
Pseudomonas aeruginosa	N	0	0	0	8	0	0	0
	%	0.0	0.0	0.0	8.0	0.0	0.0	0.0
staph aureus	N	0	0	0	0	2	0	0
	%	0.0	0.0	0.0	0.0	2.0	0.0	0.0
Proteus	N	0	0	0	0	0	2	0
	%	0.0	0.0	0.0	0.0	0.0	2.0	0.0
Pseudomonas aeruginosa **+** *Klebsiella pneumonia*	N	0	0	0	0	0	0	8
	%	0.0	0.0	0.0	0.0	0.0	0.0	8.0
Total	N	30	38	8	8	4	4	8
	%	30.0	38.0	8.0	8.0	4.0	4.0	8.0
Chi-square	X^2				410.862			
	P-value				<0.001*			

Non-significant >0.05, Significant <0.05*, High significant <0.001*.

by *K. pneumoniae* (18.91%). Also, the overall mortality rate was high in the *Pseudomonas* group (62.5%) (Hina et al., 2010).

In a study performed by Ravi et al. (2015) among the 52 samples collected and processed, 27 (51.92%) showed monomicrobial growth, 20 (38.46%) showed the polymicrobial growth and 5 (9.6%) showed no growth. *A. baumanni* (25.37%) was the most common isolate, followed by *P. aeruginosa* (17.91%), *S. aureus* (17.91%), *K. pneumonia* (10.44%), *E. coli* (8.9%) and *Enterobacter* (8.9%).

In other studies performed by Chastre and Fagon (2002) and Kollef et al. (1993) isolation of *P. aeruginosa* ranges from 15 to 25%. The study of Rocha et al. (2013) showed that *S. aureus* VAP represented 12.5% of the cases with statistical analysis which identified colonization as a risk factor for the development of this infection. This variability may be controlled by the guidelines of infection control followed and different antimicrobial policies in different institutions. Also, culture of endotracheal aspirates showed different bacterial colonization of the proximal airways in most patients in the ICU (Chastre and Fagon, 2002).

Similarly, another factor affecting the results

Table 6. Antibiotics sensitivity according to organisms in endotracheal aspirates culture.

Antibiotic sensitivity		Sputum culture					
		Klebsiella pneumonia	*Acinatobacter baumanni*	*Pseudomonas aeruginosa*	*Staph aureus*	*Proteus*	*Pseudomonas aeruginosa + Klebsiella pneumonia*
Vacomycin	N	0	0	0	4	0	0
	%	0.0	0.0	0.0	4.0	0.0	0.0
Amikacin	N	13	0	0	0	0	0
	%	13.0	0.0	0.0	0.0	0.0	0.0
Cefotaxime	N	0	0	1	0	2	0
	%	0.0	0.0	1.0	0.0	2.0	0.0
Cefoxitin	N	2	3	0	0	0	0
	%	2.0	3.0	0.0	0.0	0.0	0.0
Ceftriaxone	N	0	0	0	0	2	0
	%	0.0	0.0	0.0	0.0	2.0	0.0
Gentamycin	N	2	2	0	0	0	0
	%	2.0	2.0	0.0	0.0	0.0	0.0
Impenem	N	6	0	2	0	0	0
	%	6.0	0.0	2.0	0.0	0.0	0.0
Meropenem	N	2	0	2	0	0	3
	%	2.0	0.0	2.0	0.0	0.0	3.0
Polymyxin	N	1	3	0	0	0	0
	%	1.0	3.0	0.0	0.0	0.0	0.0

(regardless of the bronchoscopic technique used) is the need for rapid processing of specimens for culture, in other to prevent loss of viability of pathogen (Chastre and Fagon, 2002). The results of nasal swab culture results in the rate of *K. pneumoniae* was (16.7%) in cases with early onset VAP while (23.4%) in cases with late onset VAP, *P. aeruginosa* combined *P. aeruginosa and K. pneumoniae* was (8.5%), and *A. baumanni* was (6.4%) in cases with late onset VAP.

This is in agreement with the study of Zhu and Paul (2007) in which the clinical data of 106 critical neonates who were treated with mechanical ventilator between 2003 and 2005 were studied, retrospectively and reported that among the patients in their study, the detection rate of gram negative Bacilli (76.9%) was the highest, followed by gram positive cocci (17.9%) in VAP patients. Also, in a study done by Afjeh et al. (2012), which determine the risk factors and outcomes of (VAP) in the neonatal intensive care unit (NICU), a retrospective cohort study was conducted on 259 patients who were ventilated 48 h and found that, the main pathogens were gram negative bacteria (82.1%) namely *P. aeruginosa*, *K. pneumoniae* and *A. baumanni* which were predominant.

As regard the Antibiotics sensitivity of sputum culture the results of this study showed that *K. pneumonia* strains recorded highest sensitivity to Amikacin (13%) followed by Imepenem (6%), among *A. baumanni* (3%) which was sensitive to cefoxitim and Polymyxin. In the study of

Table 7. Antibiotics sensitivity according to organisms in nasal swab culture.

Antibiotic sensitivity		Nasal swab culture					
		Klebsiella pneumoniae	*Aceinetobacter baumanni*	*Pseudomonas aeruginosa*	*Staph aureus*	*Proteus*	*Pseudomonas aeruginosa +Klebsiella pneumonia*
Vacomycin	N	0	0	0	2	0	0
	%	0.0	0.0	0.0	2.0	0.0	0.0
Amikacin	N	3	0	0	0	0	0
	%	3.0	0.0	0.0	0.0	0.0	0.0
Cefotaxime	N	0	0	1	0	0	0
	%	0.0	0.0	1.0	0.0	0.0	0.0
Cefoxitin	N	0	1	0	0	0	0
	%	0.0	1.0	0.0	0.0	0.0	0.0
Ceftriaxone	N	0	0	0	0	2	0
	%	0.0	0.0	0.0	0.0	2.0	0.0
Gentamycin	N	0	2	0	0	0	0
	%	0.0	2.0	0.0	0.0	0.0	0.0
Impenem	N	6	0	2	0	0	0
	%	6.0	0.0	2.0	0.0	0.0	0.0
Meropenem	N	2	0	2	0	0	3
	%	2.0	0.0	2.0	0.0	0.0	3.0
Polymyxin	N	0	3	0	0	0	0
	%	0.0	3.0	0.0	0.0	0.0	0.0
Resistant	N	12	0	3	0	0	5
	%	12.0	0.0	3.0	0.0	0.0	5.0
Total	N	23	6	8	2	2	8
	%	23.0	6.0	8.0	2.0	2.0	8.0

Abdulrahman (2005), *K*. *pneumoniae* strains showed sensitivity to aztereonam and imipinem, were 9 (100%), 6 (75%) to ciprofloxacin, 4 (44.4%) to sulbactam-ampicillin and amoxicillin-clavulinic acid, 2 (25%) to cefuroxime. 2 (22.2%) to amikacin and gentamicin, 1 (11.1%) to trimethoprim-sulphamethoxazole and cefotaxime, and 9 (100%) resistant to both ampicillin and cefoxitin.

In this study, all of the isolated *staphylococci* (4%) were sensitive to vancomycins which were resistant to all used antibiotics. This pattern of sensitivity to staphylococcal strains was to some extent different from the study of Abdulrahman (2005), who reported that *S. aureus* isolates sensitive to rifampin and vancomycin were 15 (100%), 11 (73.3%) to sulbactam-ampicillin, 10 (66.7%) to ciprofloxacin, 9 (60%) to imipinem and

amoxicillin-clavulinic acid, 8 (53.3%) to tetracycline, 7 (46.7%) to cefuroxime and trimethoprim, 6 (40%) to ampicillin, while 2 (13.3%) were resistant to gentamicin; Methicillin-resistant Staphylococcus aureus (MRSA) represented 40%.

Conclusion

Gram negative bacteria are the most common agents causing VAP in our study. There is a relationship between organisms in nasal swab and VAP. So, Nasal screening for colonization may be a valuable tool for de-escalation of empiric therapy.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Antimicrobial activity of selected plant species and antibiotic drugs against *Escherichia coli* O157:H7

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Recent research has focused on natural plant products as alternative for disease control in both developed and developing countries. Medicinal plants can be a possible source for new potent antimicrobial agents to which pathogenic strains are not resistant. The present study was carried out to determine the *in vitro* antimicrobial activity of 14 plant species namely; *Allium sativum*, *Aloe vera*, *Bryophyllum pinnatum*, *Cassia ocidentalis*, *Citrus sinensis*, *Euphorbia hirta*, *Mangifera indica*, *Myristica fragrans*, *Ocimium gratissimum*, *Piper guineese, Psidium guajava*, *Spermacoce verticilata*, *Vernonia amygdalina* and *Zingiber officinale* and 3 antibiotic drugs namely; ampicillin, ciprofloxacin and streptomycin on *Escherichia coli* O157:H7 isolated from human clinical sample. The extracts of the plant species were prepared by cold percolation method using ethanol and water as solvents. Phytochemical analyses of the extracts of the different plant species were determined using standard methods. Agar well diffusion method was used to evaluate the antimicrobial sensitivity test of the plant extracts and that of antibiotic drugs at different concentrations ranging from 31.25 to 500 mg/ml. The minimum inhibitory concentration (MIC) of the antimicrobial agents against *Escherichia coli* O157:H7 was also conducted. Phytochemical analyses of the plant species revealed the presence of bioactivity principle such as alkaloids, balsam, cardiac glucoside, flavonoids, phenols, resins, saponins, tannins, terpenes and steroids. The results showed that all the antimicrobial agents exhibited inhibitory effects against the growth of the bacterial isolate at various degrees. Among the plant species employed in the study, the ethanolic and water extracts of *P. guajava* showed the highest inhibitory effect against the bacterium with growth inhibition mean zone diameters of 29.9 and 26.0 mm respectively at 500 mg/ml. Following *P. guagava* in order of inhibitory effect against *E. coli* O157:H7 are ethanolic extracts *A. sativum, Z. officinale, V. amygdalina and M. indica* with mean zones of inhibition of 21.2, 20.8, 20.3 and 19.9 mm respectively at 500 mg/ml. The results also revealed that of the three antibiotic drugs used in the study, ciprofloxacin exhibited the highest inhibitory effect against the organism with zone of inhibition of 38.6 mm, followed by streptomycin 30.2 mm, while ampicilin had the least 22.3 mm. The MIC results reveal that some of the plant species showed similar inhibitory effect against the bacterium, while the MIC results of the rest of the plants varied from one another. The *in vitro* study of the antimicrobial activity of the extracts of the various plant species and that of the antibiotic drugs against *E. coli* O157:H7 has demonstrated that certain folk medicine can be as effective as modern medicine in combating pathogenic microorganisms.

Key words: *In vitro,* antimicrobial activity, plant species and antibiotic drugs, *Escherichia coli* O157:H7.

INTRODUCTION

Many works have been done which aim at knowing the different antimicrobial and phytochemical constituents of medicinal plants and using them for the treatment of microbial infections (both topical and systemic applications) as possible alternatives to chemically synthetic drugs to which many infectious microorganisms have become resistant (Akinpelu and Onakoya, 2006). Edeoga et al. (2005) reported that the pace of development of new antimicrobial drugs has slowed down; while the prevalence of resistance (especially multiple resistances) has increased astronomically. The increase in number of antibiotic resistant bacteria is no longer matched by expansion in the arsenal of agents available to treat infections. Literature reports and ethnobotanical records suggest that plants are the sleeping giant of pharmaceutical industry and they may provide natural source of antimicrobial drugs that will provide novel or lead compounds that may be employed in controlling some infections globally (Akinpelu and Onakoya, 2006; Cragg and Newmam, 2013; Gairola et al., 2014; Gordon et al., 2013; David et al., 2015).

The medicinal value of plants lies in archetypal plant constituents that produce a definite physiological action on the human body. The most important of these bioactive compounds of plants are alkaloids, cardiac glycosides, flavonoids, tannins, saponin, phenolic compounds, steroids and terpenes (Sofowora, 1993; Edeoga et al., 2005; Ojo et al., 2014; Omonkhua et al., 2015). Many phytochemical compounds have been shown to be bioactive, that is they exhibit remarkable biological activity in other living organism (Harborne et al., 1975; Hartog et al., 1993; Jin-Hyung et al., 2011). Many workers have demonstrated the antidiarrhoeal and antimicrobial activities of phytochemical compounds such as tannins (Mukherjee et al., 1998), flavonoids (Galvez et al., 1993; Ekuadzi et al., 2014), alkaloids (Gricilda and Molly, 2001), saponins, sterols and terpenes (Otshudi et al., 2000) containing plant extracts. The phytochemical research based on ethno-pharmacological information is generally considered as effective approach in the discovery of new antimicrobial agents from higher plants ((Erodgrul, 2002; Kloucek et al., 2005; Veeramuthu et al., 2006). In general, most of the plants used in folk medicine have not been screened for their antimicrobial activity (Kubmarawa et al., 2007).

Escherichia coli O157:H7 is an emerging cause of food borne illness such as haemorrhagic colitis (bloody diarrhoea), haemolytic-uremic syndrome (HUS) (leads to kidney failure) and thrombotic thrombocytopenic purpure (TTP) (leads to cardiac and neurological manifestations) (Doyle and Padhye; 1989; Center for Disease Control and Prevention (CDC), 2003). It was first recognized as a cause of illness in 1982 during an outbreak of severe bloody diarrhoea. The outbreak was traced to contaminated hamburgers (Riley et al., 1983). Since then, there have been many reports throughout the world describing the severe disease associated with this organism (Karch, 1996). This strain of *E. coli* produces powerful toxins (Belongia et al., 1993). It grows slowly at refrigeration temperature and can survive under acid environment (Thomas et al., 1995). No specific therapy has been proved in patients with *E. coli* O157:H7. Some studies have suggested that HUS is likely to develop in patients treated with antibiotics (Pavia et al., 1990).

As early as 1980's Karch et al. (1986) performed an elegant *in vitro* experiment in which they added trimethoprim-sulfamethoxazole to cultures of *E. coli* O157:H7 and found that these drugs increased the release of Shiga toxin by the bacterium. These findings have since been extended to other enterohaemorrhagic strains of *E. coli* and other antibiotics. The findings have also raised the possibility that antibiotic treatment of *E. coli* O157:H7 infections might actually increase the risk of the haemolytic uraemic syndrome (CDC, 2003). Wong et al. (2000) provided data that validate this concern. Specifically, children who received antibiotics (trimethoprim-sulfamethazole or β-lactams) for diarrhoea caused by *E. coli* O157:H7 had a significant higher risk of the haemolytic-uraemic syndrome than those who did not receive antibiotics. The association was strong and independent of confounding variables, such as objective indices of the severity of illness. In 1996 an outbreak of infection with these organisms in Japan was associated with the consumption of white-radish sprouts (Wachsmuth et al., 1997). Subsequent analysis of risk factors for the haemolytic-uraemic syndrome revealed that antibiotics prevented the disease. In a Japanese study, the majority of patients received antibiotics, so the effect of no treatment could not be compared with that of treatment (Wong et al., 2000; Proulx et al., 1992).

Even though *E. coli* O157:H7 is widely considered sensitive to multiple classes of antibiotics (Griffin and Tauxe, 1991), strains showing multiple resistance to streptomycin, tetracycline and sulphisoxazole have been described in the USA (Swerdlow et al., 1992; Kim et al., 1994). Mawak and Ashamu (2006) also reported that out of the 8 antibiotics tested against *E. coli* O157:H7, only 4 namely; ampicillin, ciprofloxacin, gentamycin and to ease stomach cramps and stomach acidity in newborns (Sofowora, 1993) and gastrointestinal disorders (Hugo

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and Russel, 1997). ofloxacin inhibited the growth of the organism. The emerging resistance of *E. coli* O157:H7 and other enterohemorrhagic *E. coli* (EHEC) to antibiotics may have both epidemiologic and clinical implications. Resistant strains might have a selective advantage over other fecal flora of cattle to which antimicrobial agents are administered in feed or for therapeutic purposes, thereby increasing the frequency with which EHEC can be found in food of bovine origin (Dupont et al., 1996). Many factors influence the transmission of a gastrointestinal infection with *E. coli* O157:H7 to the haemolytic-uraemic syndrome (Lothar, 2000). The data of Wong et al. (2000) support the theory that antibiotics have an important role in this progression.

According to Lothar (2000) much has been learned since the publication of the 1983 report linking the haemolytic uraemic syndrome to gastrointestinal infection with *E. coli* O157:H7, but much work still needs to be done to devise specific therapies to halt this progression. One way to prevent antibiotics resistance of pathogenic species is by using new compounds that are not based on existing synthetic antimicrobial agents (Rogas et al., 2006). Medicinal plants might represent an alternative treatment in both severe and non-severe cases of infectious diseases and can also be a possible source for new potent antibiotics to which pathogenic strains are not resistant (Rogas et al., 2006).

Plants under study

Fourteen plant species used to determine antimicrobial activity against *E. coli* O157:H7 isolate are described as follows:

1. Garlic (*Allium sativum*) belongs to the family Liliaceae and is commonly known as garlic (David, 1997). The use of garlic in history goes back to thousands of years for treatment of numerous conditions throughout the world; garlic has generally been used for the treatment of diarrhoea, dysentery and many other ill health conditions (Murray, 1995). Garlic can be used as an antimicrobial agent, for immune enhancement and for cancer prevention (Rees et al., 1993; Lawson, 1998). Garlic has also been known to be capable of a broad spectrum antibiotic activity in inhibiting the growth of both gram positive and gram negative bacteria (Ross et al., 2001). In vitro, garlic powder induced inhibitory effect on the growth of *E. coli* O157:H7 with MIC value 10000 mg/liter (Eman and Hoda, 2008).
2. Indian aloe (*Aloe vera*) is a member of the family Liliaceae. It is related to onion, garlic and asparagus and has been noted to possess keratolic action (Hutchison et al., 2004). This is the action of removing damaged skin, and replacing it with new cells. It also allows the free flow of blood through the veins and arteries, clearing them of small blood clots (Erique, 1988). The same author reported that this plant has been proved to stop the destructive action of many bacteria such as *Salmonella* and *Staphylococcus* that produce pus. It also combats *E. coli, Streptococcus faecalis* as well as being effective against yeast (*Candida albicans*).
3. Life plant (*Bryophyllum pinnatum*) belongs to the family Crassulaceae. The crushed leaves or juice expressed from them are warmed as a poultice with shea-butter or palm oil and rubbed on abscesses or other inflammatory condition. It is also used for the treatment of arthritis and also used as anti-diarrhoeal plants (Iwu, 1993).
4. Stinking weed (*Cassia occidentalis*) belongs to the family Leguminosae. It is cosmopolitan in distribution (Akobundu, 1998). The plant has an unpleasant odour; however it is desirable because of its medicinal virtues. The documented properties and actions of this plant include: Antibacterial, antifungal, antiparasitic, antiseptic and insecticidal (George and Roger, 1998).
5. Sweet orange (*Citrus sinensis*) belongs to family Rutaceae and originated in tropical and sub-tropical Southeast Asia (Bangbose, 1980). The plants are large shrubs or small trees with spiny shoots. It is commonly known as sweet orange. The leaves, stems and bark of *C. sinensis* have high medicinal value and are used in treating viral, protozoan and bacterial infections (Sofowora, 1993).
6. Asthma herb (*Euphorbia hirta*) belongs to the family Euphorbiaceae. It is a creeping plant with very small leaves and branched stems. The plant is popularly known as Australian asthma herb. It is used to treat asthma and respiratory tract inflammation. It is also used
7. Mango (*Mangifera indica*) belongs to the family Anacardiaceae, which consists of about sixty genera and six hundred species, which are mainly tropical trees and shrubs (Trease and Evans, 1989). It was also observed that aqueous leaf extract of *M. indica* inhibited the growth of *E. coli* and some other pathogenic bacteria (Akinpelu and Onakoya, 2006).
8. Nutmeg (*Myristica fragrans*) belongs to the family Myristicaceae. Nutmeg is a common household remedy for diarrhoea (Shidore et al., 1985). It has been also reported that nutmeg may be of value in the treatment of refractory diarrhoea in some patients (Shafran et al., 1996).
9. Bush tea (*Ocimum gratissimum*) belongs to the family lamiaceae. It is a stout aromatic herb with its flower arranged in loose racemes (Trease and Evans, 1989). Apart from its flavouring purpose, its use as a medicinal plant is well documented. It is a folk remedy for many diseases such as fever, diarrhoea, dysentery, stomach ache, headache and cough (Tella, 1986).
10. Brown pepper (*Piper guineense*) belongs to the family Piperaceae. It is a native to South-Western India. It is cultivated in tropical regions around the world, and praised as a spice and a medicine since ancient times

(Garland, 1984). They are used to treat nausea, stomach ache or lack of appetite. It is antiseptic and antibacterial and is effective in reducing fever (Duke, 1985).

11. Guava (*Psidium guajava*) belongs to the family Myrtaceae (Gill, 1988). The plant is used in folk medicine to treat fever, diarrhoea and as tonic in psychiatry (Iwu, 1993). The methanolic extracts of *P. guajava* were also shown to possess antibacterial effect on *Bacillus subtilis*, *Stapylococcus aureus*, *E. coli* and *Pseudomonas aeruginosa* (Abdelrahim, 2002). Clinical studies on plant drugs from leaves of *P. guajava* on some volunteers with gastrointestinal ailments were found to be effective (Olajide et al., 1999). Bark and leaf extracts of *P. guajava* is also used for diarrhoea, stomach ache and diabetes (Tanaka et al., 1992). In several studies, guava showed significant antibacterial activity against such common diarrhoea-causing bacteria as *S. aureus, Shigella dysentriae, Salmonella typhi*, *Escherichia coli* and *P. aeuroginosa* (Lozoya et al., 2002). Human clinical trials have also indicated the effectiveness of guava in treating diarrhoea in adults and infants (Tona, 1999; Lin, 2002).

12. Button weed (*Spermacoce verticillata*) belongs to family Rubiaceace. It is commonly referred to as shrubby false button weed (Burkill, 2000). It is used in treating acute diarrhoea and other gastrointestinal tract infections by the oral use of the leaf extracts (Burkill, 2000).

13. Bitter leaf (*Vernonia amygdalina*) commonly known as bitter leaf belongs to family Vernoniaceae. The water extracts serves as tonic drink for the prevention of certain illness (Kokwaro, 2000). The bitter taste is due to anti-nutritional factors such as alkaloids, saponins, tannins and glycosides (Tanaka et al., 1992). It possesses antimicrobial activities against organisms such as *S. dysenteriae, S. aureus*, *Streptococcus pyogenes* and *E. coli* (Nwokedi et al., 2003).

14. Ginger (*Zingiber officinale*) belongs to the family Zingiberaceae. Ginger is the common name for this plant. Ginger has analgesic, sedative, antipyretic and antibacterial properties (O'Hara et al., 1998).

Although the antimicrobial activity of the plant species investigated in this study have been well documented, there is still dearth of information as regarding the antimicrobial activity of these plants against *E. coli* O157:H7. The development of drug resistance in human pathogens against commonly used antibiotics has necessitated a search for new antimicrobial substances from other sources including plants that can aid in treating infection associated with *E. coli* O157:H7.

MATERIALS AND METHODS

Source of plant materials and antibiotic drugs

All plants were obtained from Jos North Local Government Area of Plateau State, Nigeria and authenticated in the Department of Plant Science and Technology, Faculty of Natural Sciences, University of Jos, Nigeria. The antibiotic drugs were purchased from a pharmaceutical shop.

Preparation of plant extracts

Plant extracts were prepared by cold percolation method described by Akinpelu and Onakoya (2006). The various test plant species were well dried under the shade and then ground into fine powder using an electrical blender. A portion of 250 g each of the plants powder was separately soaked in 300 ml of 95% ethanol and another portion in 300 ml of sterile distilled water in glass containers and then covered with their lids. The plants soaked in ethanol were kept at room temperature those soaked in water were kept at refrigeration temperature (to prevent spoilage) for the period of 7 days to permit full extraction of the active ingredients or the chemical components. The fluids were then filtered using whatman No 1 filter paper into beakers. The extracts were obtained by oven drying the filtrate at 50°C and then kept in refrigerator pending analysis.

Phytochemical screening of the medicinal plant extracts

The phytochemical screening of the ethanolic extracts of the plant parts mentioned above was carried out in order to elucidate the chemical constituents such as alkaloids, balsam, cardiac glycosides, flavonoids, phenols, saponins, tannins, terpenes and steroids, responsible for their antimicrobial and therapeutic activities. The plant extracts were screened for the presence of these agents using standard qualitative procedures described by Trease and Evans (1989) and Sofowora (1993).

Source of microorganism

The culture of *E. coli* O157:H7 was obtained from the Microbiology Unit of the Department of Plant Science, University of Jos, Nigeria. The organism was isolated from human stool sample. The bacterial culture was maintained on nutrient agar slant and kept in refrigerator prior to use.

Sensitivity test

Before carrying out the antimicrobial tests, five grams (5 g) of each of the extracts was weighed separately and dissolved in 10 ml of sterile distilled water to produce a solution of 500 mg/ml. A serial doubling dilution was then carried out for each of the solutions to obtain concentrations of 250, 125, 62.5 and 31.25 mg/ml (Taura and Oyeyi, 2009). The same concentration levels were prepared using antibiotic drugs.

The antimicrobial activity of each of the plant extracts was determined using agar well diffusion method (Irobi et al., 1996; Akande and Hayashi, 1998). The bacterial isolates were subcultured three times in fresh Tryptone Soya Broth (TSB) in order to obtain a more vigorous population. The stocks were incubated at 37°C for 24 h. A 0.5 ml of the standardized portion of the new culture was aseptically transferred into Petri dishes containing nutrient agar and left for about 20 min to allow the microorganisms fix on the medium. Wells where extracts were to be introduced into the plates were carefully marked using sterile cork borer (6 mm diameter) and small drops of extract of various concentrations (500, 250, 125, 62.5 and 31.25 mg/ml) were added into the wells. A well

Table 1. Phytochemical analysis of the plant species.

Biochemical components	Plant species													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Alkaloids	++	+	+	+	+	+	++	+	+	++	++	+	++	++
Flavonoids	++	+	+	+	++	+	++	++	++	++	++	+	++	++
Tannins	++	+	+	+	++	-	++	+	+	++	+	+	+	++
Saponins	+	+	+	+	+	+	+	+	+	+	+	+	++	+
Cardiac glucoside	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Terpenes and Steroids	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Balsam	+	-	+	+	+	+	+	+	+	+	+	+	+	+
Resins	+	+	+	+	++	+	++	+	+	++	+	+	+	+
Phenols	+	+	+	+	+	+	+	++	+	+	+	+	+	+

+ + = appreciable amounts, + = trace amounts, - absent; 1 = *A. sativum*, 2 = *A.vera*, 3 = *B. pinnatum*, 4 = *C. ocidentalis*, 5= *C. sinensis*, 6 = *E. hirta*, 7 = *M. indica*, 8 = *M. fragrans*, 9 = *O. gratissimum*, 10 = *P. guajava*, 11 = *P. guineese*, 12= *S. verticilata* 13 = *V. amygdalina,* 14 = *Z. officinale.*

was also made at the central portion of the agar medium and drops of sterile distilled water and or 95% ethanol were placed therein to serve as controls. The plates were incubated at 37°C and the zones of inhibition were measured after 24 h. The growth inhibitory effects of the plant extracts were compared with that of 3 standard antibiotics, namely; ampicillin, ciprofloxacin, and streptomycin at the same concentrations as the plant species. The presence of zones of inhibition was regarded as the evidence of antimicrobial action. The zones of inhibition were measured with a ruler at right angles across the zones to find the average diameter in millimeters.

Determination of minimum inhibitory concentration (MIC)

The MIC was determined for the plant species that showed inhibitory effect against the test organism. The macro broth dilution method (utilizing nutrient broth) was used for the determination of MIC. Serial doubling dilutions of the plant extracts and the standard antibiotics were prepared according to the method described by Taura and Oyeyi (2009) in test tubes containing 2 ml of broth to arrive at concentrations of 125 mg/ml, 62.25 mg/ml, 31.25 mg/ml, etc. A drop of the standardized inoculum corresponding to a 1:100 dilution of the overnight broth culture of the organism was then introduced into each of the tubes. The tubes were then incubated at 37°C overnight after which they were examined for signs of turbidity, which would indicate the growth of the test organism. Control tubes were also included alongside with the incubated tubes (for easy reading) as demonstrated for antibiotics by Norrel and Kessley (1997). The controls were prepared as follows:
A = Positive (growth) controls - seeded tube but no extract
B = Negative (sterility) - Unseeded tube with extract
C = Negative (sterility) - Unseeded tube without extract

Statistical analysis

Each test was replicated twice for each plant's extract and antibiotic at various concentrations. The data obtained from the study were subjected to statistical analysis using analysis of variance (ANOVA) and the least significant difference (LSD) was used to test whether there was a significant between the means or not. Statistical package employed was IBM SPSS software, version 22.

RESULTS

Table 1 shows the phytochemical profile of the ethanolic extracts of the twenty plant species. The results showed that alkaloids, balsam, flavonoids, saponins, cardiac glycosides, phenols, tannins, terpenes and steroids were present in the crude extracts of the plant species. However, two of the plants (*A. vera* and *E. hirta*) did not possess all the chemical compounds, while the rest of the plants contained all. It was observed also that some of the plant species contained these compounds in appreciable amounts and others had them in trace amounts.

The antimicrobial activity of ethanolic and water extracts of 14 plant species and of 3 commonly used antibiotic drugs on human isolate of *E. coli* O157:H7 are presented in Tables 2 and 3 respectively. All the 14 plants showed inhibitory effect against the growth of the bacterial isolate at various degrees (Table 2). The results also reveal that among these 14 plants species, the ethanolic and water extracts of *P. guajava* exhibited the highest inhibitory effect against bacterial isolate with growth inhibition mean zone diameters of 29.9 and 26.0 mm respectively at a high concentration of 500mg/ml. These were closely followed by the ethanolic extracts of *A. sativum, Z. officinale, V. amygdalina* and *M. indica* with mean zones of inhibition of 21.2, 20.8, 20.3 and 19.9 mm respectively at 500 mg/ml concentration. The results also show that the ethanolic and water extracts of *A. sativum, M. indica, P. guajava, V. amygdalina* and *Z. officinale* inhibited the growth of the test organism at all the concentrations employed in this study. It was observed that the ethanolic extracts of *P. guineese, S. verticillata C. sinensis,* and water extract of *M. fragrans* also showed promising activity against the test bacterium by producing moderate mean zones of inhibition of 16.9, 15.3 15.0 and

Table 2. Antimicrobial effect of the different concentrations of water and ethanolic extracts of the different plant species on *E. coli* O157:H7.

Plant species	Solvent	Diameter of zone of inhibition (mm) at various concentrations (mg/ml)				
		500	250	125	62.5	31.25
Allium sativium	E	21.2	16.4	10.6	6.9	3.7
	W	17.5	13.6	7.6	4.3	2.1
Aloe vera	E	6.0	2.6	0.0	0.0	0.0
	W	5.6	2.2	0.0	0.0	0.0
Bryophyllum pinnatum	E	5.7	2.4	0.0	0.0	0.0
	W	3.6	1.3	0.0	0.0	0.0
Cassia occidentalis	E	7.1	4.4	1.7	0.0	0.0
	W	5.8	3.1	0.0	0.0	0.0
Citrus sinensis	E	15.0	6.3	2.5	0.0	0.0
	W	9.8	4.9	1.2	0.0	0.0
Euphorbia hirta	E	4.5	2.3	0.0	0.0	0.0
	W	0.0	0.0	0.0	0.0	0.0
Mangifera indica	E	19.9	13.9	7.0	4.5	2.3
	W	14.6	9.4	5.7	3.1	1.8
Myristica fragrans	E	8.4	5.3	2.1	0.0	0.0
	W	15.8	11.2	6.1	2.6	0.0
Ocimum gratissimum	E	8.7	4.6	1.9	0.0	0.0
	W	6.2	3.7	0.0	0.0	0.0
Piper guineese	E	16.9	11.6	5.8	2.7	0.0
	W	12.0	5.9	2.5	0.0	0.0
Psidium guajava	E	29.9	21.8	16.4	10.4	5.6
	W	26.0	17.6	11.8	6.6	3.4
Spermacoce verticillata	E	15.3	9.8	5.5	2.1	0.0
	W	10.0	5.3	2.2	0.0	0.0
Vernonia amygdalina	E	20.3	14.7	8.8	5.3	2.0
	W	15.6	10.5	6.8	3.4	1.6
Zingiber officinale	E	20.8	16.8	10.3	6.5	3.2
	W	17.1	13.3	7.2	3.9	1.9

E = Ethanolic extract of plant species; W = Water extract of plant species.

15.8 mm, respectively at 500 mglml. On the other hand, the crude extracts of 4 plant species namely; *A. vera, B. pinnatum, C. occidentalis* and *O. gratissimum* exhibited relatively minor effect on the growth of *E. coli* O157:H7 isolate with zone diameters ranging between 5.7 and 8.7 mm for their ethanolic extracts and 3.6 and 6.2 mm for their water extracts at 500 mg/m.

The results also show that both ethanolic and water extracts of *A. vera* exhibited analogous activity on the test organism with mean zones of inhibition of 6.0 and 5.6 mm respectively at 500 mg/ml. The ethanolic extract of *E. hirta* showed only slight activity on the organism with mean zones of inhibition of 2.3 and 4.5 mm at 250 and 500 mg/ml, respectively, while the water extract of the same plant showed no activity at all the concentrations (Table 2).

When the results of the activity of water (aqueous) and ethanolic extracts of the plant species against the test organism were compared statistically, it was observed that there was a significant difference ($P<0.05$) between the aqueous and ethanolic extracts with respect to the degree of their inhibitory effect, with the latter having higher inhibition than the former. The results in Table 2 also indicate that the plant extracts that inhibited the growth of the test organism decreased in effectiveness as the extract concentration decreases. Thus at a lowest concentration of 31.25 mg/ml most of the plant extracts showed little or no activity against the test organism. At higher concentrations of 250 and 500 mg/ml most of the plants that exhibited activity had better zones of inhibition.

Table 3 shows the sensitivity of *E. coli* O157:H7 to 3 different standard antibiotic drugs. The results reveal that 3 of the antibiotics, namely, ciprofloxacin, streptomycin

Table 3. Antimicrobial effect of different concentrations of standard antibiotics on *E. coli* O157:H7.

Antibiotic	Diameter of zone of inhibition (mm) at various concentrations (mg/ml)				
	500	250	125	62.5	31.25
Ampicillin	22.3	15.2	9.8	6.8	2.2
Ciprofloxacin	38.6	29.6	22.5	17.9	12.0
Streptomycin	30.2	23.0	16.7	11.3	6.3

and ampicillin showed inhibitory effects against the test organism with zones of inhibition of 38.6, 30.2 and 22.3 mm respectively at 500 mg/ml. Statistical analysis of the results in Table 3 indicated that there was significant difference between the zones of inhibition exhibited by some of the antibiotics against the test organism with regard to various concentrations.

On comparing the results of the antimicrobial tests of the plant drugs and that of the standard antibiotic drugs against the test organism (Tables 2 and 3), it was observed that only one of the standard antibiotic drug, ciprofloxacin that inhibited the growth of the test organism more than all the plant drugs at all the concentrations employed in this study. Comparison of the results in Tables 2 and 3 also reveal that extracts of *P. guajava* showed antimicrobial activity against the test organism that is comparable to that of streptomycin. In the same manner, extracts of *A. sativum, M. inidca*, *V. amygdalina,* and *Z. officinale* exhibited activity against the test organism almost similar to ampicillin.

The minimum inhibitory concentration (MIC) of the plant extracts and that of the antibiotic drugs that showed activity against the test organism was determined and the results are presented in Tables 4 and 5 respectively. The MIC results in Table 4 reveal that some of the plant species exhibit equal or similar MIC on the test organism, while the MIC results of the rest of the plants varied from one another. Out of the 14 plant species that their MICs were determined in this study, the ethanolic and water extracts of *P. guajava* had the lowest MIC values of 0.25 and 0.5 mg/ml respectively. These were followed by the MIC results of *A. sativum*, *M. inidca*, *V. amygdalina* and *Z. officinale*, which manifested equal but also low MICs of 1 and 2 mg/ml for their ethanolic and water extracts respectively. The results in Table 4 also reveal that the ethanolic and water extract of *C. sinensis, M. fragrams*, *P. guineese* and *S. verticillata* presented mordrate MIC values ranging between 4 and 8 mg/ml. On the other hand, the extracts of *A. vera*, *B. pinnatum*, *E. hirta, C. occidentalis* and *O. gratissimum* presented high MIC values that varied between 16 and 62.5 mg/ml. The MIC results of the plant extracts show that ethanolic extracts of most of the plant species exhibited a lower MIC values than their water extracts (Table 4).The results in Table 5 show that ciprofloxacin had the lowest MIC value (0.125 mg/ml). This was followed by streptomycin (0.25 mg/ml), while ampicillin had the highest MIC value of 1 mg/ml.

A comparison between the MIC results of the plant extracts with those of the standard antibiotics reveal that the standard antibiotic (ciprofloxacin) had a lower MIC value than the MIC values of the plant species (Table 4 and 5). The results show that the ethanolic extracts of *P. guajava* presented the same MIC value with that of streptomycin (0.25 mg/ml) against *E. coli* O157:H7. However, the ethanolic and water extracts of *P. guajava* manifested better MICs against the test organism than ampicillin (1 mg/ml). The results also show that that ethanolic extracts of *A. sativum, Z. officinale, V. amygdalina and M. indica* manifested the same MIC (1 mg/ml) with standard antibiotics ampicillin against the test organism.

DISCUSSION

The results of the phytochemical screening obtained in this study may have been affected by the type of solvent used for extraction. Only ethanolic extracts of the plant species were used to determine the type of chemical compounds present in them. Certain plant–based drugs have presented high yield of extractable chemical substances in their water extract (Rojas et al., 2006). This shows that water, and not always alcohol, can serve as the best solvent for extracting certain chemical compounds from some plants. Another factor that could have affected the type of chemical compounds present in the plant species is the source of the plants. It is known that environmental stress and mineral deficiency (e.g. boron shortage) affect phenolic anabolism and tannins present in the plants (Harbone, 1993). The presence of the phytochemical compounds in appreciable quantities in most of the plant species suggests that such could be useful in curing infections associated with *E. coli* O157:H7 most especially those that have antibacterial medicinal potentials. For example, Jin-Hyung et al. (2011) reported that flavonoid (phloretin) inhibited *E. coli* O157:H7 biofilm formation and ameliorates colon inflammation in rats.

Recent research has focused on natural plant products as alternatives for disease control in both developing and developing countries (Tona et al., 1998; Keita et al.,

Table 4. Minimum inhibitory concentration (MIC) of the ethanolic and water extracts of the plant species on *E. coli* O157: H7.

Plant species	Solvent	MIC (mg/ml)											
		125	62.5	31.25	16	8	4	2	1	0.5	0.25	0.125	0.0625
A. sativum	E	-	-	-	-	-	-	-	-	+	+	+	+
	W	-	-	-	-	-	-	-	+	+	+	+	+
A. vera	E	-	-	-	+	+	+	+	+	+	+	+	+
	W	-	-	-	+	+	+	+	+	+	+	+	+
B. pinnatum	E	-	-	-	+	+	+	+	+	+	+	+	+
	W	-	-	+	+	+	+	+	+	+	+	+	+
C. occidentalis	E	-	-	-	-	+	+	+	+	+	+	+	+
	W	-	-	-	+	+	+	+	+	+	+	+	+
C. sinensis	E	-	-	-	-	-	-	+	+	+	+	+	+
	W	-	-	-	-	-	+	+	+	+	+	+	+
E. hirta	E	-	-	+	+	+	+	+	+	+	+	+	+
	W	*	*	*	*	*	*	*	*	*	*	*	*
M. fragrans	E	-	-	-	-	-	+	+	+	+	+	+	+
	W	-	-	-	-	-	-	+	+	+	+	+	+
M. indica	E	-	-	-	-	-	-	-	-	+	+	+	+
	W	-	-	-	-	-	-	-	+	+	+	+	+
O. gratissimum	E	-	-	-	-	+	+	+	+	+	+	+	+
	W	-	-	-	+	+	+	+	+	+	+	+	+
P. guineese	E	-	-	-	-	-	-	+	+	+	+	+	+
	W	-	-	-	-	-	+	+	+	+	+	+	+
P. guajava	E	-	-	-	-	-	-	-	-	-	-	+	+
	W	-	-	-	-	-	-	-	-	-	+	+	+
S. verticillata	E	-	-	-	-	-	-	+	+	+	+	+	+
	W	-	-	-	-	-	+	+	+	+	+	+	+
V. amygdalinca	E	-	-	-	-	-	-	-	-	+	+	+	+
	W	-	-	-	-	-	-	-	+	+	+	+	+
Z. officinale	E	-	-	-	-	-	-	-	-	+	+	+	+
	W	-	-	-	-	-	-	-	+	+	+	+	+

* = Not tested (since it did not show activity); - = No bacterial growth (Clear broth); + = Bacterial growth (Cloudy broth); W = Water extract; E = Ethanolic extract.

Table 5. Minimum inhibitory concentration (MIC) of the standard antibiotic drugs on *E. coli* O157: H7.

Antibiotic	MIC (mg/ml)											
	125	62.5	31.25	16	8	4	2	1	0.5	0.25	0.125	0.0625
Ampicillin	-	-	-	-	-	-	-	-	+	+	+	+
Ciprofloxacin	-	-	-	-	-	-	-	-	-	-	-	+
Streptomycin	-	-	-	-	-	-	-	-	-	-	+	+

- = No bacterial growth (clear broth); + = Bacterial growth (cloudy broth).

2000; Todar, 2005). The significance of the inhibitory effects of crude extracts of various plant species against *E. coli* O157:H7 have been demonstrated by the results from this study. Among the plant extracts tested in this work, those of the leaves of *P. guajava* (guava) showed the most remarkable inhibitory effect against the test organism (*E. coli* O157:H7). The largest zones of inhibition caused by the extracts of leaves of *P. guajava* against the test organism further supported the effective use of guava to control diarrhoea, dysentery and gastro-

enteritis (Arima, 2003). In addition guava is known to be rich in phytochemical compounds such as flavonoids, phenols, terpenes, and essential oils. Much of guava's therapeutic activity is attributed to these compounds (Holetz, 2002). Following *P. guajava* in order of inhibitory effects against *E. coli* O157:H7 are *A. sativum, Z. officinale*, *V. amygdalina* and *M. indica.* Phytochemical constituents such as alkaloids, flavonoids, saponins, terpenes and steroids are some of the major compounds present in these plants and they are known to exhibit antimicrobial properties. Thus, the plants also showed high antimicrobial properties against the bacterial isolate. The present finding confirms the scientific studies carried out on some of these plant species as having effectiveness in inhibiting the growth of certain bacterial isolates *in vitro* (Ali, 1994; Tona, 1999; Nwokedi et al., 2003; Akinpelu and Onakoya, 2006; Okorondu et al., 2006). The present study also agrees with work of Eman and Hoda (2008) that reported that garlic powder was able to induce strong inhibitory effect on the growth of *E. coli* O157:H7.

It is interesting to note that *P. guajava, A. sativum, Z. officinale, V. amygdalina* and *M. indica* were the only plant species that exerted inhibitory effect on the test bacterial isolate at all the concentrations considered in this research. This suggests that the compounds responsible for the antibacterial activity were present in appreciable quantity at all the concentrations as to have been able to inhibit the growth of the test organism. The extracts of *C. senensis, P. guineese, S. verticillata and M. fragrans* also showed promising activities against the test microorganism by producing moderate zones of inhibition on the culture plates. The results obtained from this study thus compliment earlier reports by Soforowa (1993), Burkill (2000) and Shafran et al. (1996) that justify that some of these plants showed antimicrobial activity against some pathogenic microorganisms associated with diarrhoea.

The results of this investigation also reveal that some of the plant species, namely, *A. vera, B. pinnatum, C. occidentalis, E. hirta and O. gratissimum* exerted very minimal antimicrobial effect on the test organism. The relatively low inhibitory activity exhibited by these plants extracts against the test organism is surprising as this contradicts previous reports (Tella, 1986; Erique, 1988; Iwu, 1993; George and Roger, 1998). These reports showed that these plants have strong antimicrobial activities against both gram-positive and gram-negative bacteria. The reduced effectiveness of these plant extracts observed in this study may be due to low concentration of phytochemical constituents or that the method of extraction did not yield high concentrations of the chemicals that could have had reasonable effects on the test organism.

The findings of the present study showed that the ethanolic extracts of all the plant species exhibited a higher degree of antimicrobial activity when compared to the water extracts with the exception of *A. vera* and *M. fragrans*. The higher susceptibility of the test bacterial isolate to ethanolic extracts of most of the plant species is not surprising as previous studies have reported ethanol to be a better solvent than water for extracting secondary metabolites (which are inhibitory to microorganisms) from most plants (Olukoya et al., 1993; Okorondu et al., 2006). Caceres et al. (1993) tested guava leaf extract obtained with three solvents of different polarities (n-hexane, acetone and ethanol). They discovered that ethanolic extract of the plant was most efficient against the pathogenic enterobacteria tested. The parity in the activity and spectrum of extracts as a result of the nature of solvents lend more weight to the findings of Obi and Onuoha (2000). These workers reported high recovery of alkaloids and essential oils with ethanol than with water. Thus, it could be that some of the active principles responsible for medicinal property of the plants may not be extractable using only water as solvent. The higher inhibitory activity exhibited by ethanolic extracts than the water extracts by most of the plant species also correlated with the preparations of medicinal plants by traditional medical practitioners (TMP) who use rum and liquor to extract the active plant compounds. Furthermore, the reason why the ethanolic extracts of most of the plant drugs exerted more antibacterial effect against *E. coli* O157:H7 isolate than their aqueous (water) extracts could be due to the fact that the antibacterial activity of the plants seemed to depend on their polar constituents with the ethanolic extracts being more polar than the aqueous extracts (Sofowora, 1993).

Both water and ethanol were found to be suitable solvents for the extraction of bioactive agents from *A. vera.* Their ethanolic and water appeared seems to exert similar antimicrobial effect on the test organism. This suggests that the polar constituents of the ethanolic and water extracts of this plant are similar in activity (Sofowora, 1993). It is interesting to note that the use of water extract of nutmeg (*M. fragrans*) for anti-diarrhoeal activity, normally practiced in India folk medicine (Shidore et al., 1985) is confirmed in this study, as the water extract of the plant seems to show more activity against the test organism than the ethanolic extract.

The results of this study show that only the ethanolic extract of the leaf of *E. hirta* was slightly effective against the *E. coli* O157:H7 isolate. The water extract of the same plant showed no activity at all. This may be attributed to the fact that ethanolic extract contained a small quantity of the bioactive agents which had little effect on the organism. Another reason could be that the polar constituents that could have exerted effect on the organism were not present in the water extract (Sofowora, 1993) that was why no inhibition was encountered.

It was observed that the test plants that showed activity

against the *E. coli* O157:H7 isolate became more effective in inhibiting the organism as the concentration of the plant extracts increased. This suggests that the antibacterial activity of the crude plant extracts appeared to be dosage dependent. Hence, the concentrated decoction may be an effective therapy against diarrhoegenic agents. This finding may be useful in dosage administration. However, it reflects the problem of drug administration by traditional healers in which the dosage of the unrefined herbal preparations is often very small to make any meaningful impact or too large, which may be harmful to the body system (Nwokedi et al., 2003). Meanwhile, it may be pertinent to continue cooperation with traditional healers to regulate and standardize the dosage of herbal medicines they administer to the patients that patronize them.

The sensitivity test of *E. coli* O157:H7 to different standard antibiotics that served as positive control showed that all the three (ciprofloxacin, streptomycin and ampicillin) were active against the organism. The findings of this study are in agreement with former studies by Akinpelu and Onakoya (2006) and Mawak and Ashamu (2006) in which streptomycin and ampicillin acted against certain bacterial isolates including *E. coli* O157:H7. In addition, Antai and Anozie (1987) observed that ciprofloxacin inhibited the growth of many serotypes of pathogenic *E. coli.*

On comparing the results of the antimicrobial activity of the extract of the plant species with those of the standard antibiotics, it was observed that none of the plant extracts was more active against *E. coli* O157:H7 than ciprofloxaxin. However, the extracts of some of the plant species (A*. sativum, M. indica P. guajava, V. amygdalina* and *Z. officinale*) presented antimicrobial activity comparable to that of other standard antibiotics (streptomycin and ampicillin). This finding agrees with the work of Gnan and Demello (1999) who compared the effects of the extracts made of guava leaves and fruits at a concentration of 6.5 mg/ml upon test organism to those of conventional antibiotics (chloramphenicol, cefoxitin and metaxotin) and found that the results were comparable. Akinpelu and Onakoya (2006) revealed also that extracts of *P. guajava* compared favourably with a standard antibiotic (streptomycin) when tested against certain gastrointestinal organisms. In another study Rojas et al. (2006) reported that the water extract of *Jacaranda mimosifolia* and *Piper pulchrum* showed a higher activity than standard antibiotic drug (gentamycin sulphate) against *Bacillus cereus*. In addition, Irobi et al. (1996) found that leaf extracts of *P. guajava* were more efficient than oxytetracycline for treating acute diarrhoea in humans. The present findings and the reports of previous workers have confirmed the fact that some of the herbal preparations used by the traditional healers actually possess medicinal potency similar to standard antibiotics. Many of the herbal remedies of old have since been adopted and adapted by conventional Western allopathic medicine, simply due to the fact that they are effective. Thus the inestimable value of medicinal plants to health care systems in the world has increasingly become appreciated (Egunyomi, 2015). In some remote communities in Colombia, traditional healers claim that their medicine is cheaper and more effective than modern medicine (Rojas et al., 2006). Rojas et al. (2006) also reported that patients of rural communities who rely mostly on traditional medicine claimed to have a reduced risk to get infectious diseases from resistant pathogen than people in urban areas treated with synthetic antibiotics. However, if they are treated in a hospital the chance of contracting a nosocomial infection may increase. Thus, one way to prevent antibiotic resistance of pathogenic species according to Akinpelu and Onakoya (2006) is by using new compounds that are not based on existing synthetic antimicrobial agents. Drug resistance has been a source of grave concern in clinical practice (Antai and Anozie, 1987). It is regrettable to note in a typical Nigerian setting, the escalation of antibiotic resistance of pathogenic organisms due to the uncontrolled use of antibiotics and the common practice of self-medication. This suggests that the Nigerian Society for Microbiologists and the Nigerian Medical Association should embark on a nationwide programme of public enlightenment through the mass media, on the dangers of antibiotic abuse.

A close examination of Tables 4 and 5 reveals that, apart from the standard antibiotic drug (ciproflaxacin) which had a lower MIC value than all the extracts of the plant species, the other two standard antibiotics (streptomycin and ampicillin) had equal MIC values with the extracts from some of the plant species. The low MIC value exhibited by ciproflaxacin could be attributed to the fact that it is among the latest antibiotic drugs not yet exposed to bacterial resistance. Thus, *E. coli* O157:H7 was found to be highly sensitive to the drug. Among the plant species, *P. guajava, A. sativum, Z. officinale, V. amygdalina and M. indica* could be regarded as the first choice plants whole extracts for inhibiting the growth of *E. coli* O157:H7, since they compared favourably with standard antibiotics. It was also observed that the extracts of four plants, namely *C. senensis, M. fragrans, P. guineese and S. verticillata* showed relatively low MIC values against the test organism. This suggests that the four plants could also serve as alternative sources of herbal medicines for treating infection caused by the pathogen. Again, the extracts of plants such as *A. vera, B. pinnatum, C. occidentalis, O. gratissimum* and *E. hirta* may not be effective for the treatment of the infection associated with the test organism, as they exhibited high MIC values against the test organism. Generally, the ethanolic extracts of most of the plant species had lower MIC values than the water extracts of the same plants. This shows that the chance to find antimicrobial agents

was more apparent in ethanol than in water extracts of the same plants.

The results of the antimicrobial activity and that of the MIC of the various the crude extracts of the plant species revealed that out of the 14 plants species examined, only 9 inhibited the growth of *E. coli* O157:H7 substantially, with *P. guajava* showing the best antimicrobial activity. The inhibition of *E. coli* O157:H7 *in vitro* by the crude extracts of these plants species, points to the fact that they were actually fortified with bioactive principles. The crude extracts of some of the plant species also compared favourably with antibiotic drugs by exhibiting similar antimicrobial activity. Furthermore, as the extracts of the plant species contain phytochemical constituents, it therefore suggests that they could be important source of antimicrobial drugs which could be beneficial in curbing the spread of infectious disease associated with *E. coli* O157:H7.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Comparative assessment of Ni and As(III) mediated alterations in diazotrophic cyanobacteria, *Anabaena doliolum* and *Anabaena* sp. PCC7120

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The comparative effects of nickel (Ni^{2+}) and arsenite (As(III)) on two diazotrophic cyanobacterial species were investigated in terms of photosynthetic attributes. Both metals demonstrated inhibitory effects on growth, pigments (chl a and phycocyanin) and photosystem II (PS II) photochemistry. However As(III) exerted severe effects as compared to Ni reflected by (1) reduced growth (2) significant inhibition of chl a and phycocyanin, (3) reduction in maximum photochemical efficiency of PSII and (4) depleted plastoquinone pool, thus suggesting it as more toxic. Moreover, comparative analysis of two species also demonstrated interspecies variation in terms of stress adaptive strategies reflected through higher sensitivity of *Anabaena doliolum* over *Anabaena* PCC7120. Thus the study recommends application of *A.* PCC7120 as biofertilizer in Ni and As(III) contaminated paddy fields.

Key words: *Anabaena* sp. PCC7120, *Anabaena doliolum*, Nickel, As(III), maximal photochemical yield (Fv/Fm).

INTRODUCTION

Anthropogenic activities have altered the global biogeochemistry due to release of metals in recent years (Bhagat et al., 2016). Not only aquatic ecosystem but soil organisms are also negatively affected by metal contamination. Effect of elevated metal input on soil organism is reflected in form of reduced species diversity, abundance and biomass and changes in microbe mediated processes (Bengtsson and Tranvik, 1989; Giller et al., 1998; Vig et al., 2003). Although few metals hold prime importance for all living organisms due to their key role in basic life processes like photosynthesis and respiration, their elevated concentration in cells causes either their inappropriate binding to metal binding sites of enzymes or undesirable redox reactions thus causing lethal effects (Waldron et al., 2009a, b, 2010).

Nickel is one such metal that plays a vital role in the cellular physiology of living organism (Poonkothai and Vijaywathi, 2012). It is coordinated by proteins either

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directly or through tetrapyrrole ring of coenzyme F_{430} which coordinates a nickel atom in methyl-coenzyme M reductase (Ragsdale, 2003). Statistical data revealed that nickel emission from natural and anthropogenic sources are 2.9-56.8×10^3 and 33.1-194.2×10^3 t $year^{-1}$, respectively (Tercier-Waeber and Taillefert, 2008). Some anthropogenic sources that causes elevated Ni level into environment are energy supplying power stations (coal burning power plants, petroleum combustion and nuclear power stations), mining and associated activities, disposal of NiCd batteries, chemical industries (planting, metal finishing, pigment production, cement manufacturing) (Poonkothai and Vijaywathi, 2012; Nnorom and Osibanjo, 2009).

Apart from them, heavy metals are the non-degradable elements that occur naturally in biosphere. In past few years, their accumulation in environment as a result of their increased utilization in industrial activities such as in mining processes has raised a global concern (Huertas et al., 2014). Arsenic is a toxic metalloid and present in two biologically active forms arsenate (As^V) and arsenite (As^{III}). Arsenate is analogous to phosphate thus replaces phosphate from essential biochemical reactions such as glycolysis and oxidative phosphorylation causing toxic effects (Tawfik and Viola, 2011; Nriagu and Jerome, 2000). However arsenite is reported to bind dithiols, forming dithiols thus disrupting protein functions and producing reactive oxygen species (ROS) (Liu et al., 2002; Meng et al., 2004; Wysocki et al., 2001). Use of arsenic as herbicides, insecticides, rodenticides, food preservatives and byproduct of used fosil fuel are major anthropogenic activities that are challenging the environment (Flora et al., 1995).

Diazotrophic cyanobacteria are the only group of prokaryotes proficient in performing oxygenic photosynthesis and N_2-fixation, thus contributing significantly to global photosynthetic biomass production and biofertilizer (Dadheech, 2010). Being an essential component of cyanobacterial ureases and hydrogenases Ni is required at low concentration (Huertas et al., 2014), however at higher concentrations it causes inhibition of pigments (chlorophyll, phycocyanin and carotenoids), enzyme activities (nitrate reductase and glutamine synthatase) and loss of electrolyte (Na^+ and K^+) (Rai et al., 1985, 1986, 1990; Martínez-Ruiz and Martínez-Jerónimo, 2015).

Similarly, arsenic is also reported to inhibit chlorophyll biosynthesis, photosynthetic pigments and Rubisco and generates oxidative stress through ROS generation thus damaging lipids, proteins and nucleic acids (Tantry et al., 2015; Srivastava et al., 2009; Pandey et al., 2012). Effect of arsenite and nickel on *Anabaena* spp. have been studied however no reports exists regarding comparative study of arsenite and Ni on different cyanobacterial strains. *Anabaena* spp. commonly found in tropical conditions have different geographical isolates (sps.) and displays niche specificity. This study is the first to provide comparative effect of Ni and As(III) on *Anabaena* sps. (*Anabaena doliolum* and *Anabaena* sp. PCC7120) in terms of (1) growth behavior, (2) photosynthetic pigments, (3) and chlorophyll fluorescence. Attempts have been made to verify these results statistically. Present study is important in the sense that the results would provide important information regarding the cyanobacteria's ability to tolerate arsenic and nickel.

MATERIALS AND METHODS

Organism and growth condition

Anabaena spp., *Anabaena* PCC7120 and *A. doliolum* were cultivated photoautotrophically under sterile condition in BG-11 medium (Supplementary Table 1) (N_2-fixing condition) buffered with Tris/HCl at 25 ± 2°C under day light fluorescent tubes emitting 72 µmol photon m^{-2} s^{-1} PAR (photosynthetically active radiation) light intensity with a photoperiod of 14:10 h at pH 7.5. The cultures were shaken manually 2 to 3 times daily for aeration.

Mode and source of stress application

Nickel stress was applied as $NiCl_2$ at concentrations 0 to 32 µM and arsenite stress was applied as sodium meta arsenite at concentrations 0 to 80 mM. Sodium meta arsenite and nickel chloride autoclaved separately and calculated amount were added directly into the sterilized medium to achieve the desired concentration and working standards were obtained by further dilutions.

Measurement of survival

Exponentially growing cells of *Anabaena* PCC7120 and *A. doliolum* treated with their respective concentrations were collected at four time points (1, 7, 10 and 15 days). Cells never exposed to nickel and arsenite were used as control. Growth was estimated by measuring the OD (optical density) of the culture at 750 nm in a UV–VIS spectrophotometer (Systronics, India) up to 16th day.

Pigments

Chlorophyll a, carotenoid and phycocyanin were measured as per the method of Bennett and Bogorad (Bennett and Bogorad, 1973), by taking the absorbance at 663, 480 and 645 nm respectively. The extinction coefficient of chl a at 665 nm in absolute methanol is 74 .5 ml/mg-cm (Mackinney, 1941).

Measurement of chlorophyll fluorescence

Chl fluorescence in dark- and light-adapted control as well as treated cultures was measured using a PAM 2500 Chl fluorometer (WALZ GmbH, Effeltrich, Germany). The fluorometer was connected to a computer by the data acquisition system (PAMWIN, Walz, Germany). Prior to each measurement, the culture was dark-adapted for 30 min (Guo et al., 2006). The minimal fluorescence yield of the dark-adapted state (F_0) was measured by the modulated light which was too low to induce significant

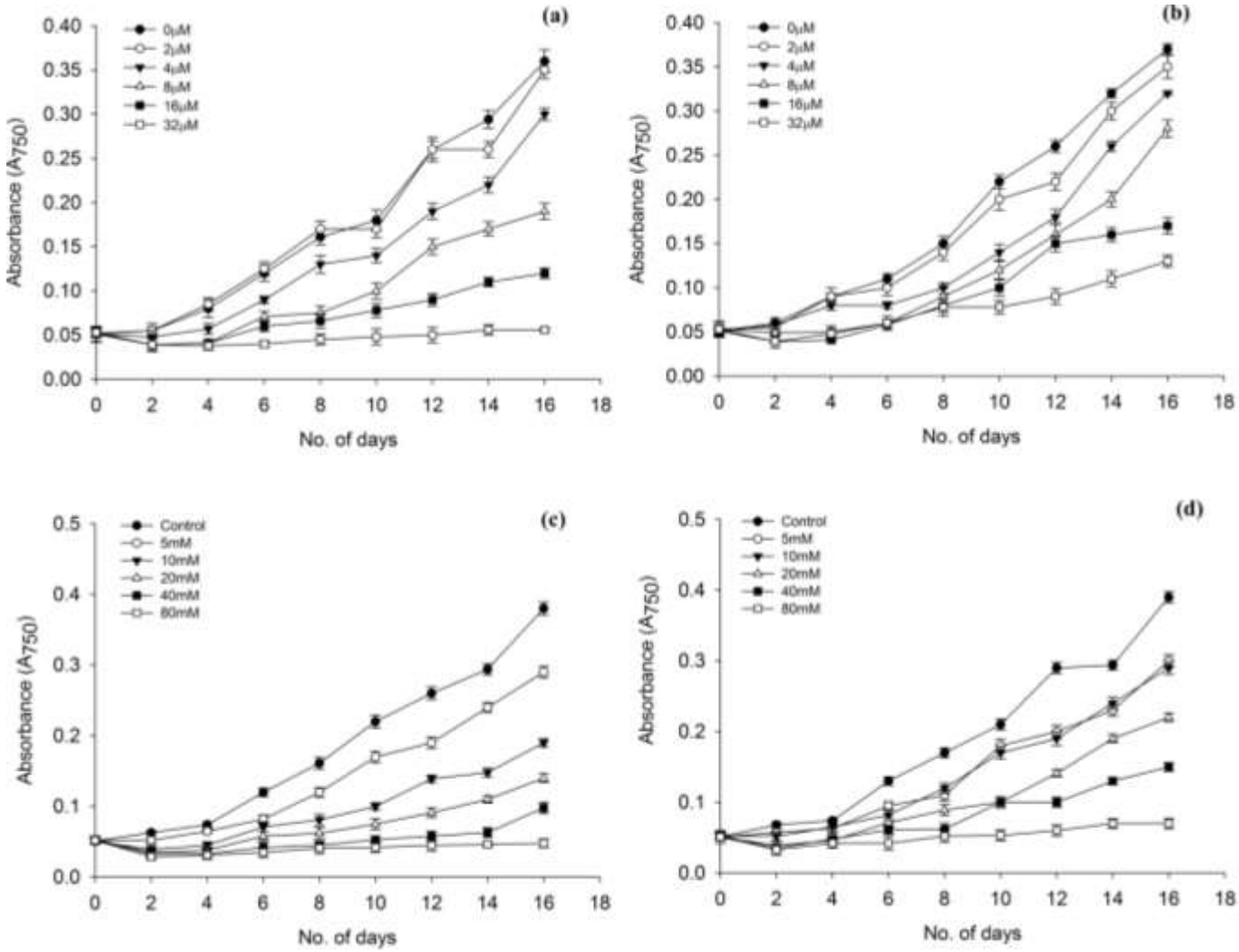

Figure 1. Population growth curves of (a) *A. doliolum* (b) *Anabaena* sp. PCC7120 exposed to different concentrations of Ni^{2+} (c) *A. doliolum* (d) *A.* sp. PCC7120 exposed to different concentrations of As(III). Mean values for three bioassays with three replicates ± standard deviation bars.

physiological changes in the plant, and was recorded after dark adaptation. Subsequently, a saturating pulse was given to measure the maximal fluorescence yield of the dark-adapted state (Fm) (Qin et al., 2006). The maximal photochemical quantum efficiency of PSII (Fv/Fm) was determined after a 20-min dark acclimation period in selected cultures. Other calculated fluorescence parameters was the pastoquinone pool (Fv/2) (Bolhar-Nordenkampf et al., 1989).

Statistical analysis

Each treatment consisted of three replicates; the results presented are mean values. Each experiment was repeated five or six time; results from a representative experiment are presented. The results were statistically analyzed by one-way ANOVA and the Duncan's new multiple range test (DMRT) to determine the significant difference among group means. A p value ≤0.05 was considered statistically significant (SPSS for Windows, version 20.0).

RESULTS

Measurement of growth and survival

The present study deals with assessment of comparative toxic effects of Ni and arsenite over two strains of *Anabaena* viz. *A. doliolum* and *Anabaena* sp. PCC7120. Being a vital component of paddy fields diazotrophic cyanobacteria have always fascinated researchers from all over the world. Figure 1a and b shows the growth trends for *A. doliolum* and *Anabaena* sp. PCC7120 respectively exposed to various concentrations of Ni; as displayed in the figure the cell density was inhibited significantly by all of the tested Ni concentrations except Ni (2 µM). Similarly, Figure 1c and d represents growth

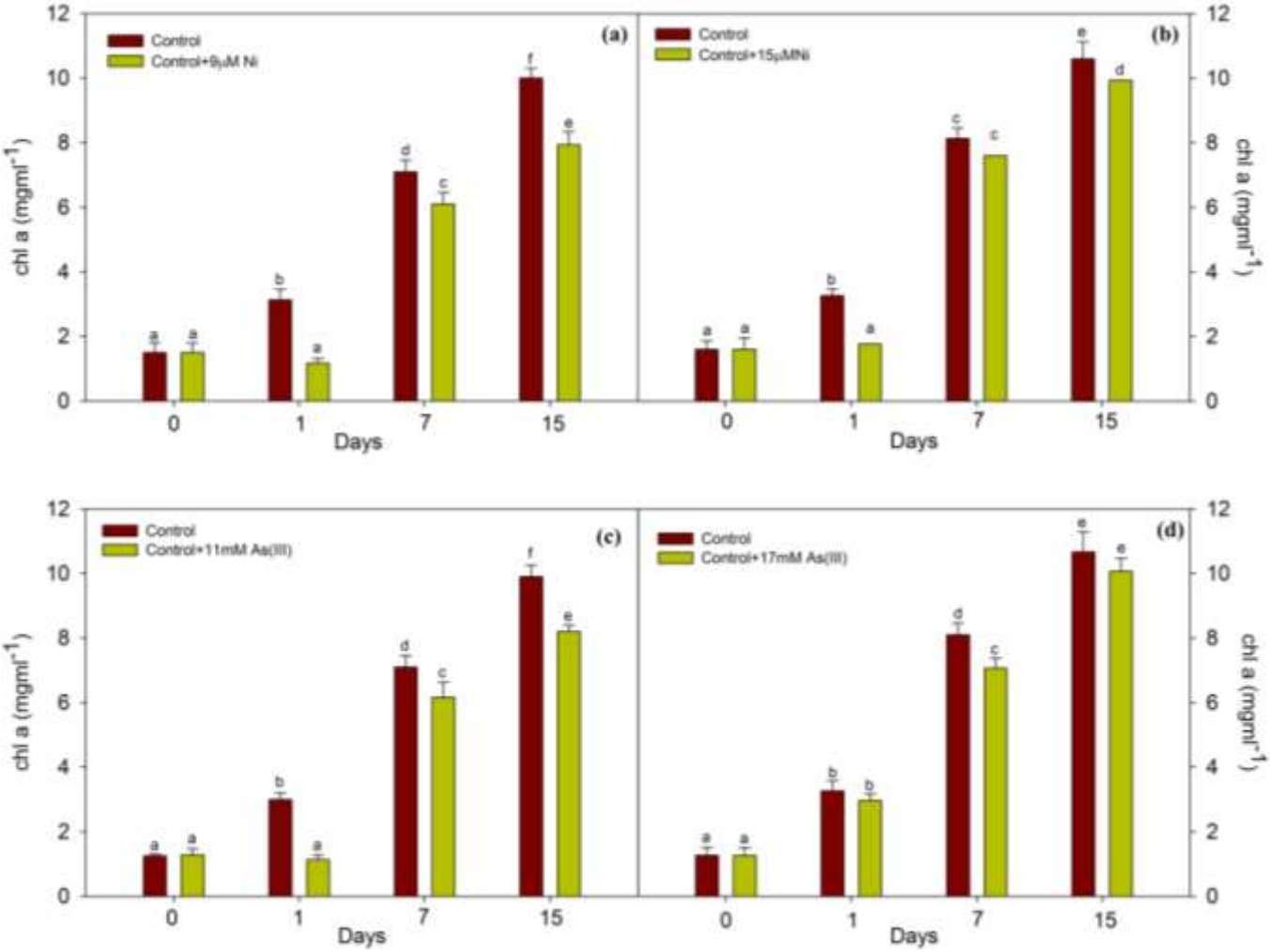

Figure 2. Effect on chlorophyll a content of (a) *A. doliolum* (b) *Anabaena* sp. PCC7120 exposed to different concentrations of Ni^{2+} (c) *A. doliolum* (d) *A.* sp. PCC7120 exposed to different concentrations of As(III). Mean values for three bioassays with three replicates ± standard deviation bars.

pattern for *A. doliolum* and *Anabaena* sp. PCC7120 respectively exposed to arsenite. However under arsenite treatment all the concentrations were inhibitory. Moreover *A. doliolum* appears to be more sensitive as compared to *Anabaena* sp. PCC7120 (Figure 1a to c). The average IC_{50} determined for Ni was 9 and 15 µM and for arsenite 11 and 17 mM respectively for *A. doliolum* and *Anabaena* sp. PCC7120.

Pigments

Figure 2a and b displays effect on chl a content following Ni stress in *A. doliolum* and *Anabaena* sp. PCC7120 respectively and Figure 2c and d shows chl a content following As(III) treatment in *A. doliolum* and *Anabaena* sp. PCC7120 respectively. It clearly demonstrates that both Ni and As caused more pronounced inhibition of chl a content in *A. doliolum* as compared to *Anabaena* sp. PCC7120. *A. doliolum* exhibited significant decrease in chl a content at all days of treatment however in *Anabaena* sp. PCC7120 significant decrease was observed only at 7th day of As(III) treatment and 1st and 15th day of Ni treatment as measured by Duncan's test (DMRT). Figure 3a and b demonstrates effect of Ni on carotenoid content in *A. doliolum* and *A.* PCC7120 respectively and Figure 3c and d displays As(III) mediated alterations on carotenoid content in *A. doliolum* and *A.* PCC7120 respectively. Significant increase was found in *A. doliolum* at 1, 7 days of Ni treatment and at 1 and 15 days of As(III) treatment, however in *A.* PCC7120 at 1st day of Ni treatment and 1 and 7 day of As(III) treatment, carotenoid content was significantly increased. Similar to chl a, significant decrease in phycocyanin

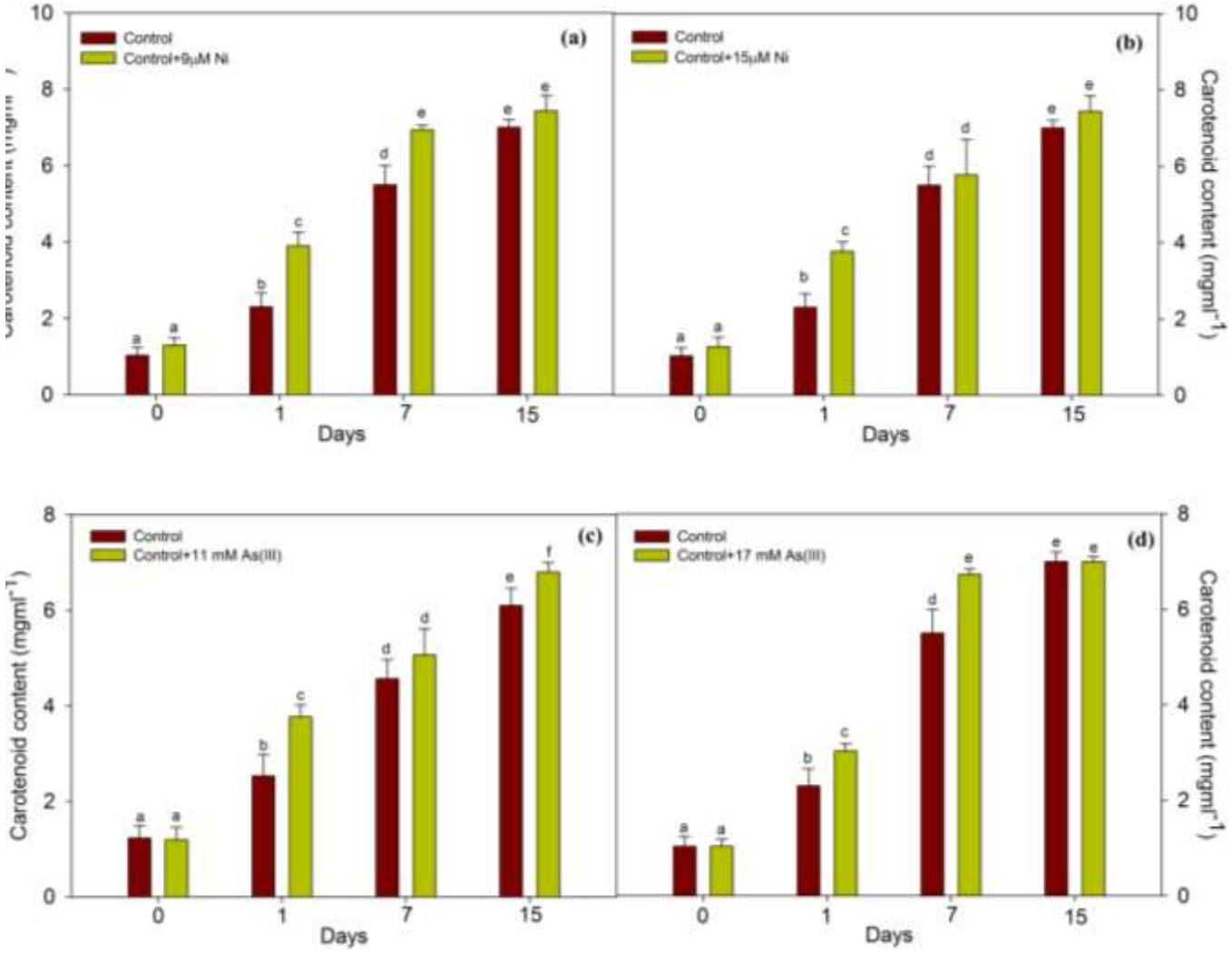

Figure 3. Effect on carotenoid content of (a) *A. doliolum* (b) *Anabaena* sp. PCC7120 exposed to different concentrations of Ni^{2+} (c) *A. doliolum* (d) *Anabaena* sp. PCC7120 exposed to different concentrations of As(III). Mean values for three bioassays with three replicates ± standard deviation bars.

content was noticed at all days of treatment under both Ni and As(III) stress in both species, however among both stresses As(III) caused more pronounced inhibition in both species (Figure 4a to d).

Chlorophyll fluorescence

The test metals were found to reduce maximal quantum yield in a concentration-dependent manner, which was more pronounced in *A. doliolum* following As(III) treatment (Figure 5). Figure 6 presents the impact of the test metals on plastoquinone pool (Fv/2) of *A. doliolum* and *A.* sp. PCC7120 after 24 h of Ni and As (III) treatment.

DISCUSSION

Growth behavior studies suggested sensitivity of *A. doliolum* over *A.* sp. PCC7120. This finds support from the studies of Singh et al. (2015), they found that *A. doliolum* is more sensitive as compared to *Anabaena* sp. PCC7120 under cadmium stress. Similarly, Agrawal et al. (2014) found following trend of tolerant behavior *A.* L31> *Anabaena* sp. PCC7120>*A. doliolum* under butachlor stress among three closely related species of *Anabaena*. This further attested the tolerant behavior *A. doliolum* over *Anabaena* sp. PCC7120 thus suggesting the presence of separate strategies to combat stress even within species. Further requirement of high concentration of As(III) as compared to Ni may be attributed to ability of

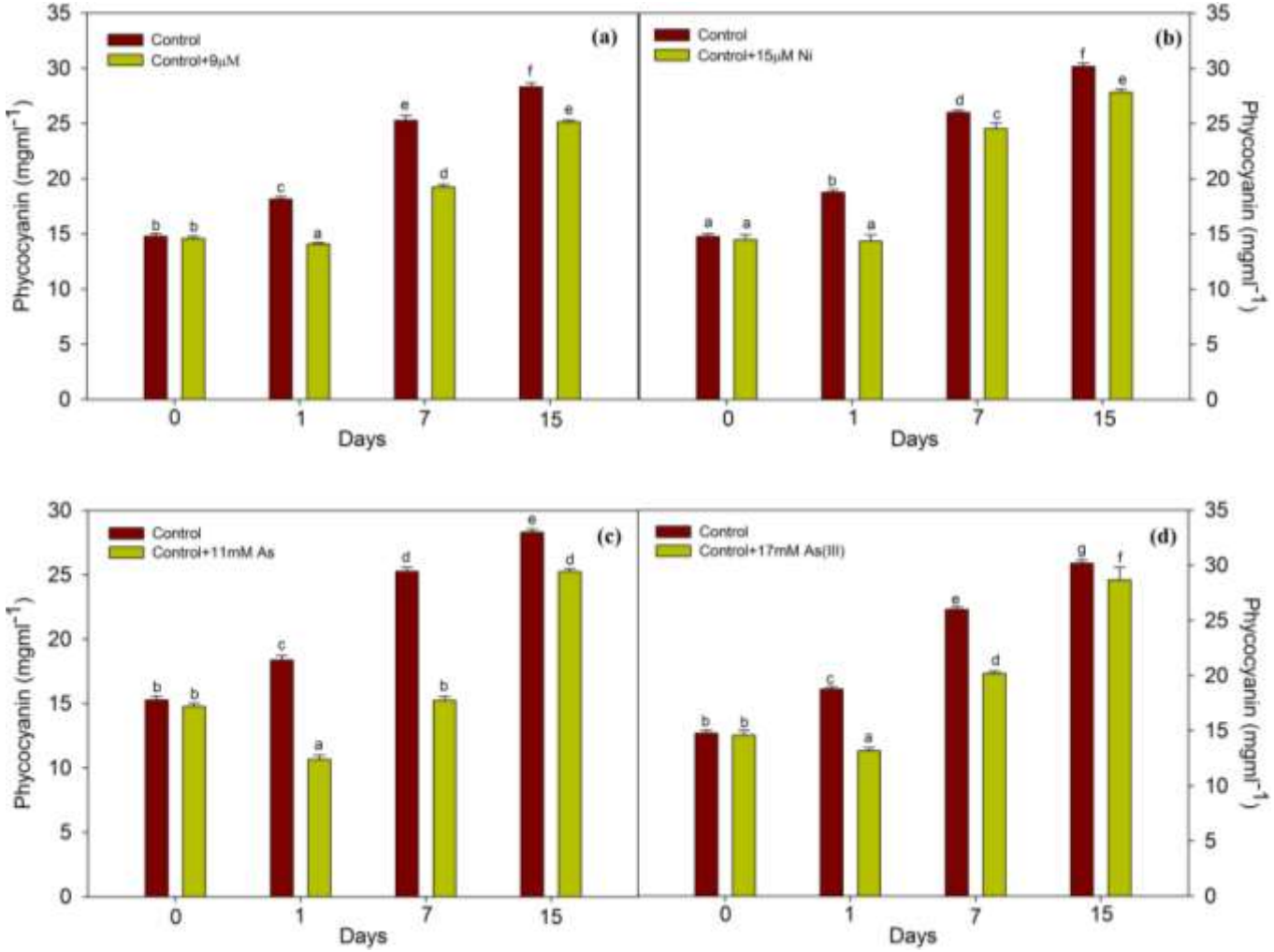

Figure 4. Effect on phycocyanin content of (a) *A. doliolum* (b) *Anabaena* sp. PCC7120 exposed to different concentrations of Ni^{2+} (c) *A. doliolum* (d) *Anabaena* sp. PCC7120 exposed to different concentrations of As(III). Mean values for three bioassays with three replicates ± standard deviation bars.

Anabaena to accumulate high concentrations of As(III). Significant reductions in the photosynthetic pigments chl a and phycocyanin whereas significant increment in carotenoid content was found in both the species. Ni is known to affect the active site of O_2-evolving complex to which it interacts, thus causing depletion of 2 extrinsic polypeptides resulting in diminished e^- transport activity (Boisvert et al., 2007). However As(III) mediated chl a content inhibition may be attributed to inhibition of ϭ-aminolevulinic acid dehydrogenase, a key enzyme of chlorophyll biosynthetic pathway (Shrivastava et al., 2009). Other metals are also known to produce similar decrease in chl a content. For example, Carfagna et al. (2013), found decrease in chl a in a green alga, *Chlorella sorokiniana* under Cd/Pb stress.

Carotenoid content was significantly increased in *A. doliolum* at 1 and 7 days of Ni treatment and at 1 and 15 days of As(III) treatment, however in *A.* PCC7120 at 1st day of Ni treatment and 1 and 7 day of As(III) treatment. Carotenoids are known to be major players of antioxidant response against ROS and found to be increased under metal stress (Yu et al., 2015). Significant decrement in phycocyanin content was noticed at all days of treatment under both Ni and As(III) stress in both species. Phycocyanin is located on exterior side of thylakoid membrane and thus possibly toxicant exposure is prolonged causing severe inhibition as compared to chl a. This observation finds support from work of Pandey et al. (2012) they observed significant reduction in phycocyanin content under As(V) stress.

Maximum photochemical efficiency of PSII (efficiency at which light absorbed by PSII is used for

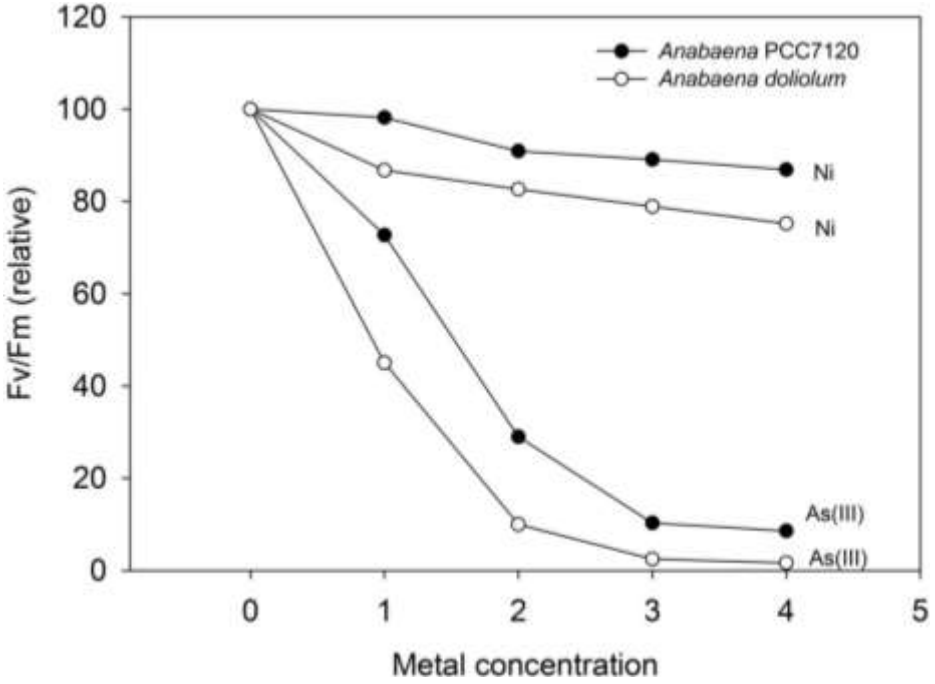

Figure 5. Ni and As (III) induced reduction in maximum quantum yield of *A. doliolum* and Anabaena sp. PCC7120 24 h of treatment, Fv/Fm (100%) for *A. doliolum* = 0.242±0.0003, Fv/Fm (100%) for *Anabaena* sp. PCC7120=0.275±0.0007. On the X-axis, metal concentrations (0, 1, 2, 3, 4, 5 and 6) represent, respectively, 0, 2, 4, 8, 16 and 32 µM for Ni and 0, 5, 10, 20, 40 and 80 mM for As(III).

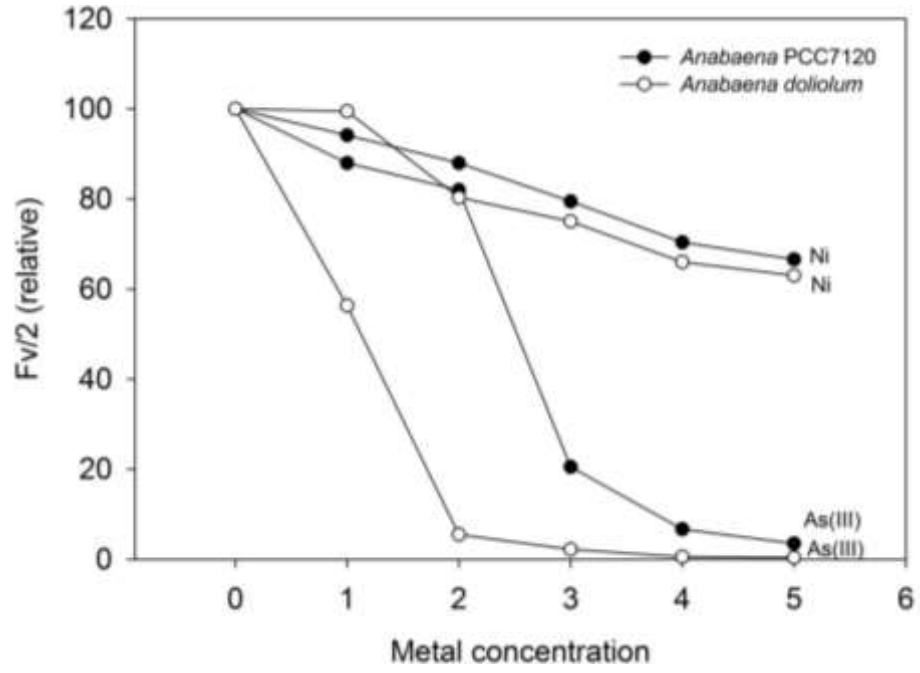

Figure 6. Effects of the test metals on the platoquinone pool of both cyanobacterium after 24 h of treatment. Fv/2 (100%) for *A. doliolum* = 0.181±0.0004, Fv/2(100%) for *Anabaena* sp. PCC7120 = 0.341±0.0003. On the X-axis, metal concentrations (0, 1, 2, 3, 4, 5 and 6) represent, respectively, 0, 2, 4, 8, 16 and 32 µM for Ni and 0, 5, 10, 20, 40 and 80 mM for As(III).

photochemistry when all reaction centers are open) of both test cyanobacteria following treatment with different concentrations of Ni and As(III) after 24 h was recorded. It was found to be affected significantly. The findings of our study are supported by Rahman et al. (2011). The ratio of Fv/Fm is considered as a stress indicator and designates the potential yield of the photochemical reaction (Björkman and Demmig, 1987). Fv/Fm remains high under control condition following irradiation because Q_A is in oxidized state due to transfer of electrons to NADP and finally to CO_2 via Q_B, the plastoquinone pool, and PSI. However under stress condition Fv/Fm may decrease because reoxidation of Q_A is restricted as a result of decrease or partial block of electron transport from PS II to PSI. A noteworthy decrease in the plastoquinone pool as represented by the Fv/2 ratio (Figure 6) could be one of the possible causes for the reduced quantum yield under metal stress.

In summary, among both test cyanobacterium *A. doliolum* appeared as a sensitive strain towards Ni as well as As(III) exposure at low concentrations which are toxicologically and environmen-tally relevant. Both metals significantly inhibited the population growth, pigment content (chl a, phycocyanin) and maximal photochemical efficiency of PSII, which was found to be more pronounced in *A. doliolum* (Figure 6). However increase in carotenoid content was found thus suggesting onset of defense mechanism. Thus present study suggests *Anabaena* sp. PCC7120 as more efficient candidate to be used as biofertilizer as compared to *A. doliolum* and needs to be further investigated. Further studies exploring effect on nitrogen fixing abilities and antioxidative defence system of both test cyanobacteria is ongoing so as to present a holistic view demonstrating integrative effect as well as help in unveiling the tolerance mechanism.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Supplementary Table 1. Detailed composition of modified BG11 stock solution and 1X medium.

Macronutrient	**g/100 ml (Stock solution)**	**ml/litre**
$MgSO_4$	7.5	1.0
K_2HPO_4	4.0	1.0
$CaCl_2$	3.6	1.0
Citric acid with Ferrous ammonium citrate	0.6	1.0
EDTA	0.1	1.0
Na_2CO_3	2.0	1.0
Micronutrient	**Mg/100 ml**	**ml/litre**
H_3BO_3	286.0	1.0
$MnCl_2.4H_2O$	181.0	1.0
$ZnSO_4.7H_2O$	22.2	1.0
$Na_2MoO_4.5H_2O$	39.0	1.0
$CuSO_4.5H_2O$	7.9	1.0
$Co(NO_3)_2.6H_2O$	0.04	1.0

K_2HPO_4, EDTA and Ferrous ammonium citrate were autoclaved separately and added to the cold sterilized culture medium. The pH of the medium was maintained at 7.5. To avoid any alteration in pH, the medium was buffered with 0.5 g HEPES buffer.

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Full Length Research Paper

Efficient production of second generation ethanol and xylitol by yeasts from Amazonian beetles (Coleoptera) and their galleries

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Beetles of the Passalidae family live and feed on decaying wood and their guts are richly colonized by yeasts. The goal of this research was to prospect xylolytic yeasts with potential for the production of second-generation bioethanol. Therefore, 83 specimens of beetles belonging to the Passalidae and the Scarabaeidae families were collected in the Amazonian rainforest in Itacoatiara - AM, Brazil. Morphological differences of the beetles were identified and 25 chosen specimens were dissected. Yeasts from galleries inhabited by beetles and from insect guts were isolated. Isolates were previously selected through tolerance tests for temperature, ethanol and xylose assimilation capacity. Those isolates were then submitted to a panel of conditions related to ethanol production. The ethanol production reached 24.70 g.L^{-1} and the xylitol production reached 21.66 g.L^{-1}. One of the isolates with a promising profile was identified as *Spathaspora roraimanensis* and six as *Spathaspora passalidarum*. Three isolates showed to be more promising and, curiously, all came from the gut of the species *Popilius marginatus* (Percheron, 1835). In plate testing, however, the isolates obtained from galleries showed a greater capacity to assimilate xylose. As reported in this field of study, no isolate tolerated all conditions tested. Wild isolates with this profile may be used for testing larger-scale ethanol production, genetic engineering, or evolutionary techniques.

Key words: Beetles, bioethanol, *Popilius marginatus*, xylose.

INTRODUCTION

Fuels from renewable resources are becoming progressively important in times of increasing environmental concern. In the specific case of bioethanol production, researchers have sought to use plant biomass as the raw material (Brat et al., 2009).

Usually, production of bioethanol is a biological process in which sugars such as glucose, fructose and sucrose are converted into cellular energy by microbial

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fermentation and thus produce ethanol and carbon dioxide as metabolic residues (Tao et al., 2011). When production occurs from easily accessible sugars, it is referred to as first generation (1G) production, whereas, if lignocellulosic materials such as agricultural residues, forest materials and dedicated crops are used, they are called second-generation (2G) production (Joelsson et al., 2016).

Saccharomyces cerevisiae is the preferred microorganism for ethanol production, due to its capacity to grow into simple sugars such as disaccharides. However, wild strains of *S. cerevisiae* are unable to ferment D-xylose, which is one of the major polysaccharide constituents of lignocellulosic biomass. Also, D-xylose is the second most abundant sugar in the cell walls of plants and lignocellulosic biomass is of interest as a substrate for production of ethanol, xylitol and other microbial products (Carvalho et al., 2002; Hahn-Hägerdal et al., 2006; Doran-Peterson et al., 2008; Junyapate et al., 2014).

Although hydrolysis of plant biomass, which breaks hemicellulose and exposes cellulose to an enzymatic attack, is important to the production of 2G ethanol and it produces sugar mixtures rich in glucose and xylose, fermentation inhibitors are generated by the chemical pretreatment. Acetic acid, furfural and hydroxymethylfurfural (HMF) are considered key components among many inhibitors that are formed during pretreatment. According to Slininger et al. (2016), in order to advance the 2G ethanol process of production, research and procedures are required to allow evolution of yeast strains. It is necessary to work on the capability of surviving and functioning efficiently using both hexose and pentose sugars in the presence of such inhibitory compounds. Thus, an industrial strain, with high metabolic potential for xylose fermentation and good inhibitor tolerance would be potentially useful for industrial bioethanol production (Li et al., 2015).

Different approaches in genetic engineering have been used to allow fermentation of D-xylose by wild strains of *S. cerevisiae*, but growth and productivity rates are significantly lower in this sugar compared to glucose, and therefore the process is not industrially competitive (Hahn-Hägerdal et al., 2007; Brat et al., 2009). Therefore, there is strong pressure to improve the economic viability of 2G ethanol production, thus motivating researchers to explore alternatives beyond conventional *Saccharomyces* species (Radecka et al., 2015). Recently Slininger et al. (2016) used a wild strain of *Pichia stipitis* (NRRL Y-7124) with promising capacity for pentose fermentation and reported good results using evolutionary techniques to obtain more robust variants.

Alternatively, D-xylose can be converted into the polyol xylitol ($C_5H_{12}O_5$), which is an important chemical product and with higher financial value than ethanol. Xylitol is extensively used in food and pharmaceutical industries as sweetener (Guo et al., 2013; Li et al., 2013; Sena et al., 2016; Zhang et al., 2016).

Xylose-fermenting yeasts are commonly found into the digestive tract and/or feeding tubes of many xylophagous insects, suggesting an association with wood digestion (Suh et al., 2003, 2006). In fact, the gut of beetles and other insects is considered a hotspot of yeast diversity (Suh and Blackwell, 2004; Boekhout, 2005; Rivera et al., 2009; Cadete et al., 2009, 2012; Urbina et al., 2013; Gouliamova et al., 2015; Cadete et al., 2015, 2016).

In this context, it was hypothesized that the almost unexplored biodiversity of the Amazonian rainforest near Itacoatiara – AM, Brazil, could provide us yeast isolates from beetle guts and galleries in tree trunks with potential for 2G ethanol production.

MATERIALS AND METHODS

Collection of beetles and deposition in arthropod collection

Adult beetles (Passalidae) were collected in natural habitats due to their association with decomposing wood. Beetles were collected at two sites in the Amazon forest: Campus II of the Federal University of Amazonas (UFAM), kilometer 260 of Rodovia AM-010 (S03°05.654' W058°27.464'), and in the Sol Nascente community (S03°01.045' W058°28.830'), both in the municipality of Itacoatiara - AM.

The beetles were collected individually with aid of sterile tweezers, deposited in sterile Petri plates and brought to the Mycology Laboratory of the Institute of Exact Sciences and Technology (ICET) at the Federal University of Amazonas (UFAM). The insects were transported alive with bark fragments collected from where they were found. A sampling of insects would be destroyed by the dissection process, so control specimens were collected from the same gallery and at the same time as the beetles that would be dissected (Suh and Blackwell, 2004). The entomologist Dr. Claudio Ruy Vasconcelos da Fonseca identified the specimens of beetles, and control specimens were deposited into the collection of the National Institute of Amazonian Research (INPA). A summary of the methods used to carry out the selection procedures was presented in Figure 1.

Isolation of yeasts from the intestinal tract of beetles

The beetles were kept in sterile Petri plates for three days, without feeding, before euthanasia (in 0.56% KCl solution) and dissection. According to Suh and Blackwell (2004), keeping specimens without food assists in eliminating some contaminating organisms that may eventually be isolated from their gut. The insects were submitted to a surface disinfection by submersion in 70% ethanol (5 min), bleach (5 min) and sterile water (10 min) prior to removal of their guts. Sterile water (100 µl) was seeded in acidified yeast malt YM extract agar (composition/L: 5 g peptone, 3 g yeast extract, 3 g malt extract, 10 g dextrose, 15 g agar, 1000 mL distilled water, supplemented with 0.1 g chloramphenicol; pH adjusted to 3.5 with hydrochloric acid), as a negative control (Suh and Blackwell, 2004).

Guts were removed aseptically with the aid of a stereomicroscope in a biological safety cabinet. After dissection, the large gut of the insects was cut into three parts with a sterile scalpel and crushed with a clamp into sterile microtubes containing 100 µL of saline solution, and this was spread on acidified YM agar (modified from Ravella et al., 2011). The cultures were incubated at 30°C for five days. After that, individual colonies with yeast morphology were taken from and purified by at least two successive

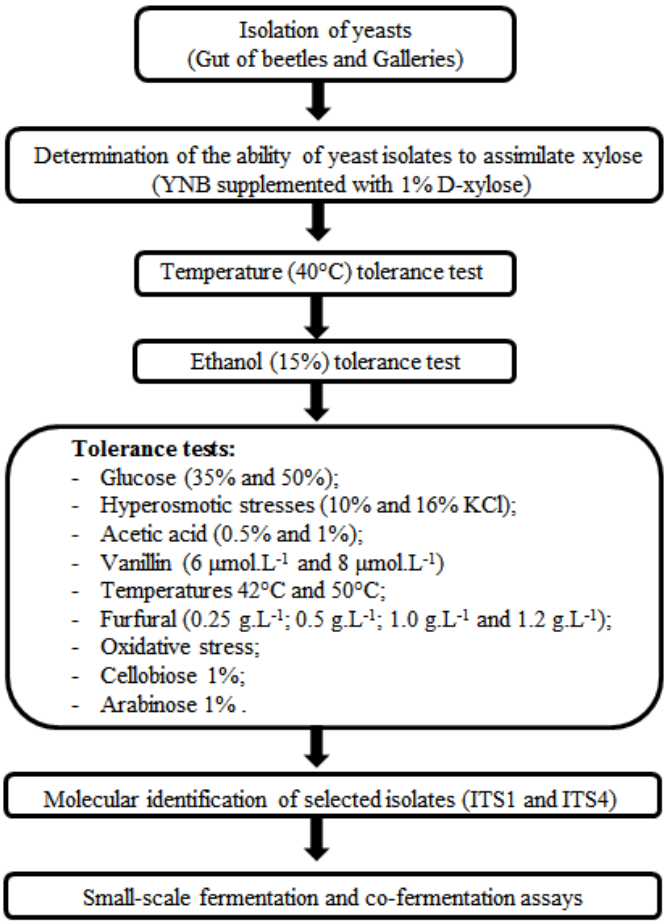

Figure 1. Flowchart showing the approach for the isolation of yeasts from beetles and their galleries and screening for ethanol and xylitol producers.

replications in YM agar. From each culture, the square root of the total colonies was calculated. That number was the number of colonies isolated for later identification.

Determination of the ability of yeast isolates to assimilate xylose

To determine the ability to utilize xylose, the isolates were seeded in yeast nitrogen-based medium (YNB) supplemented with 1% D-xylose per puncture and incubated at 30°C for 72 h. As a positive control, the same strains were inoculated in YNB supplemented with 1% glucose (Tanahashi et al., 2010). After growth, the growth rate was evaluated by measuring the diameter of the colonies considering a horizontal axis and its perpendicular axis, after the arithmetic mean was obtained of the measurements for each isolate, according to Golinski et al. (2008).

Tolerance tests

Tolerance tests evaluated the growth performance of yeast isolates under some typical stress conditions in fermentations for production of 2G ethanol. The conditions tested respectively were: Glucose (35% and 50%), hyperosmotic stresses (10 and 16% KCl), acetic acid (0.5 and 1%), temperatures of 42 and 50°C, vanillin (6 and 8 µmol.L^{-1}), furfural (0.25, 0.5, 1.0 and 1.2 g.L^{-1}) and oxidative stress. Also concentrations of cellobiose 1% and arabinose 1% (Ali and Khan, 2014; Li et al., 2015) were tested. For this, the strains were previously cultured overnight in YEPD agar medium (composition/L: 10 g yeast extract, 20 g peptone, 20 g dextrose, and 20 g agar, 1000 mL distilled water) and washed twice with sterile water. The optical density was adjusted (OD_{600}=1.0), dilution of the suspension (10^{-1}, 10^{-2} and 10^{-3}) was carried out, and for oxidative stress and temperature (42 and 50°C), the density was adjusted to OD_{600} = 2.0. 4 µL of each diluted suspension in each solid medium was applied. Except for the acetic acid test, where the plates remained incubated for four weeks at 30°C, and against furfural, with ten day incubation, the remaining tests were performed with 48-hour incubation at 30°C.

To determine the resistance to oxidative stress, the cells were mixed with 20 mL of YEPD agar (cooled to approximately 50°C) and immediately plated. Thereafter, sterile filter paper (0.5 mm diameter) was placed in the center of each plate with 6 µL of hydrogen peroxide 30% (H_2O_2), followed by incubation of two days at 30°C. The diameters of the growth inhibition zones (in mm) were recorded (Li et al., 2015).

Molecular identification of selected isolates

The molecular method for yeast identification was based on the amplification and sequence analysis of rDNA internal transcribed spacer (ITS) (Caggia et al., 2001). Prior to extraction, the yeasts were grown in a YEPD medium and incubated for 24 h at 30°C. For the extraction of the genomic DNA with a pipette tip, an isolated colony was resuspended in 1 mL of sterile water in microtube that was centrifuged for 1 min at 10,000 to 12,000 rpm. Then the supernatant was removed. After that, 100 µL of InstaGene™ Matrix (under continuous stirring) was then added to the pellet and incubated at 56°C for 30 min. The suspension was homogenized in a vortex for 10 s and the tubes incubated in boiling water (100°C) for 8 min, with further vortexing for 10 s and centrifuging at 10,000 to 12,000 rpm for 3 min. 2.5 µL of the supernatant result per 50 µL of the PCR reaction was used. The DNA sample was stored at -20°C, according to da Silva et al. (2012).

The primers used to amplify the rDNA ITS region were ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). The amplification reaction was performed in 50 µL (final volume) containing 1 µL of each primer, 25 µL of TopTaq® Master Mix Kit (Qiagen), 21 µL of miliQ water and 2 µL of genomic DNA (sample) in a thermocycler. Samples were sent to Macrogen (Rockville, USA) for sequencing. The PCR product sequences were compared to the ITS regions deposited in GenBank (http://www.ncbi.nlm.nih.gov) and the similarity compared using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) (modified Tao et al., 2011).

Small-scale fermentation and co-fermentation assays

The fermentation tests were performed in 125 mL Erlenmeyer flasks in culture medium containing yeast extract (5 g.L^{-1}); peptone (5 g.L^{-1}); NH_4Cl (2 g.L^{-1}); KH_2PO_4 (1 g.L^{-1}); $MgSO_47H_2O$ (0.3 g.L^{-1}) and glucose and/or xylose under stirring at 120 rpm for 70 h. The strains were previously cultured in YP medium with 2% xylose, under stirring at 120 rpm, at 28°C. From the pre-inoculum, dilutions were performed so that the initial optical density $(OD)_{600nm}$ of all strains was equal to 1. Each isolate was inoculated in fermentation liquid medium (4% xylose), resulting in a final volume of 50 mL. The Erlenmeyer flasks were incubated in a horizontal shaker at 28°C for 70 h and at 120 rpm. At intervals of 0, 3, 6, 9, 12, 20, 30, 40, 50, 60 and 70 h, aliquots of 200 µL were withdrawn to evaluate OD_{600nm},

Table 1. Species of beetles used for the isolation of yeasts.

Family	Genus	Species	Specimens
Passalidae	Passalus	*Passalus latifrons* (Percheron, 1841)	11
Passalidae	Passalus	*Passalus interruptus* (Lin, 1758)	4
Passalidae	Veturius	*Veturius platyrhinus* (Westwood, 1845)	1
Passalidae	Popilius	*Popilius marginatus* (Percheron, 1835)	5
Passalidae	Passalus	*Passalus punctiger* (Lep & Serv. 1825)	1
Passalidae	Passalus	*Passalus convexus* (Dalman, 1817)	1
Passalidae	Veturius	*Veturius transversus* (Dalman, 1837)	1
Scarabaeidae	Cetoniinae sp	Unidentified species	1

the cell viability by serial dilution and an aliquot of 800 µL was centrifuged at 10,000 rpm for three minutes. The supernatant was filtered through a 0.2 µm membrane and subsequently frozen for analysis of xylose, xylitol, glycerol and ethanol levels through high performance liquid chromatography (HPLC). For the co-fermentation assays, glucose (2%) was added.

RESULTS

In this research, 83 specimens of beetles were collected from two different sites in the Amazon forest. Due to the easily observable morphological differences, 24 specimens of beetles were dissected and identified as belonging to the Passalidae family and one to the Scarabaeidae family (Table 1). From the intestinal contents of these insects and swabs rotated inside the log galleries they inhabited, 380 and 412 isolates suggestive of yeasts were obtained, totaling 792 isolates. The obtained isolates were then subjected to screening tests to verify their potential for production of 2G ethanol.

Screening of yeast strains for bioethanol production

The 792 isolates obtained were initially submitted to a screening to verify xylose assimilation capacity. Only twelve isolates, all from the intestinal contents of the insects, did not present growth in the medium containing xylose as the only carbon source. Considering the size of the colonies (see Materials and methods), the isolates obtained from galleries had higher xylolytic capacity when compared to the isolates obtained from the guts of the beetles (t test; $p<0.001$) (Figure 2).

The 780 strains that assimilated xylose were submitted to a temperature tolerance test of 42°C for 48 h. In this condition, 73 isolates showed growth. These 73 isolates were then tested against 15% ethanol. Sixteen strains showed growth after 48 h of incubation at 30°C.

The 16 selected strains were subsequently submitted to tolerance tests to evaluate the growth performance under typical stress conditions in fermentations. Growth on the plate containing 1% acetic acid was not observed. The G7-1.4 isolate showed growth on the plate containing 0.5% acetic acid after 22 days of incubation. There was growing in the plates with supplementation of furfural 0.25g.L^{-1}. The isolates were categorized according to the growth observed in the plates as: Absence of growth (-), slight growth (+), moderate growth (++) and intense growth (+++) (Table 2).

The degree of resistance to oxidative stress was demonstrated by the diameter of the inhibition halo (mm). The experiment was performed in triplicate and expressed as the mean halo diameter. Four isolates (P22-1.2, P22-1.3, P22-2.19 and P22-2.20) were not inhibited by hydrogen peroxide (H_2O_2) in the tested condition (Table 2). Under the conditions used here, of the 16 isolates pre-selected and tested against this panel, three, P16-1.1; P21-1.7 and P21-2.1, showed superior tolerance (Table 2).

Molecular identification of the selected isolates

Considering the results obtained in tests for xylose assimilation capacity, temperature tolerance for 42ºC and tolerance for 15% of ethanol, 16 isolates were submitted to molecular identification. The P8-2.12 isolate showed high homology (98%) to *Spathaspora roraimanensis* XMD23.2 (JN099269.1); the P16-1.1, G13-2.1, G13-3.8, G14-1.8, G14-2.2 and G18-3.7 isolates showed high homology (99%) with *Spathaspora passalidarum* ATCC MYA-4345 (NR_111397.1). It was possible to obtain genera identification through the sequencing of isolates P19-1.1, P21-1.7, P21-2.1, P22-3.18 (*Candida* sp.) and P22-1.2, P22-1.3, P22-2.19, P22-2.20 (*Schwanniomyces* sp.). The G7-1.4 isolate has a good sequence, but low similarity (96%) with *Candida jeffriesii*, being able to be a new species (Table 3).

Small-scale fermentation tests

The 16 isolates selected were submitted to small-scale fermentations initially in media containing 4% xylose as the only carbon source. Isolates P22-1.2, P22-1.3 and P22-2.19 did not produce ethanol. After 30 h of

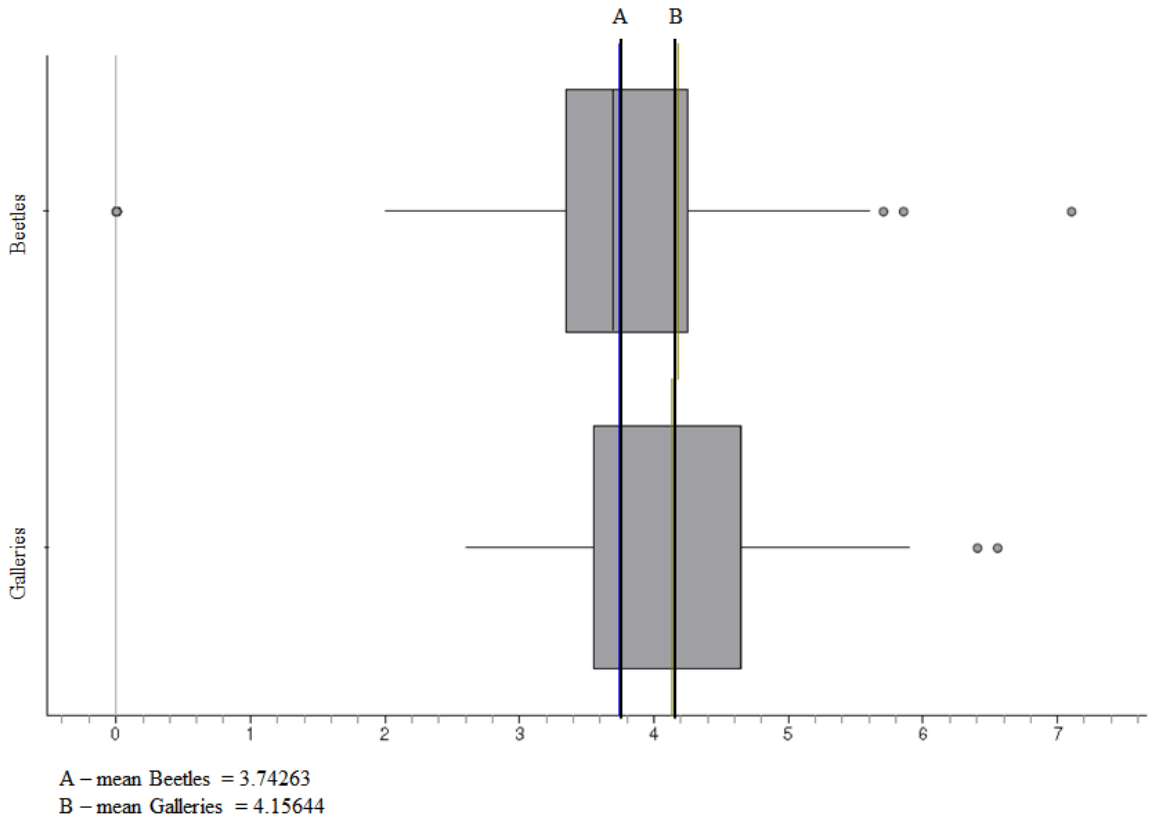

Figure 2. Difference between the dimension of the colonies (considering the mean of two perpendicular measures from the largest diameter) of yeasts isolated from the intestinal contents of beetles compared to yeasts isolated from inhabited galleries (** *t* test: p <0.001), when cultivated in nitrogen-based agar with 1% of D-xylose as the sole carbon source.

fermentation, isolates P16-1.1, G13-2.1, G13-3.8, G14-1.8, G14-2.2 and G18-3.7 showed a peak of ethanol production with approximately 85% fermentation efficiency (Figure 3), demonstrating considerable capacity to convert xylose into ethanol. The production of xylitol exceeded the yields of ethanol in the yeasts P8-2.12 (12.25 $g.L^{-1}$), P19-1.1 (21.66 $g.L^{-1}$), P21-1.7 (19.20 $g.L^{-1}$), P21-2.1 (19.14 $g.L^{-1}$) P22-2.20 (17.57 $g.L^{-1}$), P22-3.18 (22.10 $g.L^{-1}$) and G7-1.4 (20.01 $g.L^{-1}$) (Table 4). Ethanol or xylitol were the main products of xylose metabolism.

The results of the fermentative parameters ($Y_{P/S}$ = ethanol or xylitol yield; Q_P = ethanol or xylitol productivity; η% = etanol or xylitol fermentation efficiency; Y% = xylose consumption) relative to the comparison between fermentation in media containing only 4% xylose and co-fermentation in media with 4% xylose and 2% glucose are shown in Table 4. These results were calculated according to the fermentation time (maximum ethanol or xylitol production time). During the co-fermentation process, it was observed that glucose depletion occurred rapidly, that yeasts simultaneously consumed xylose and that, at the end of 70 h of fermentation, and xylose had not been totally consumed.

DISCUSSION

Ascomycetic yeasts that both ferment and assimilate xylose have been associated with insects that feed on decaying wood (Young et al., 2010; Ravella et al., 2011; Tao et al., 2011). Here the xylose assimilation capacity of 792 yeasts isolated from the gut of beetles and their galleries were tested. Only 12 did not show growth in the medium containing xylose as the sole carbon source. Interestingly, a significantly higher xylolytic capacity was observed in the isolates from galleries compared to those obtained from the intestinal contents of the beetles (Figure 2). This is preliminary data, however, to our knowledge there is no mention of this in the literature, which may be of interest in future native yeast screenings for bioethanol production.

Considering stress conditions involved in the fermentation processes for the production of 2G ethanol (Costa et al., 2014; Li et al., 2015), the sixteen isolates that showed tolerance to ethanol and were selected had different degrees of tolerance (Table 2), but none of those tolerated all the conditions imposed. This information is corroborated by Li et al. (2015), where none of the five strains of *S. cerevisiae* tolerated all

Table 2. Cultivation tests of sixteen isolates selected on a panel of conditions frequently observed in the production of second-generation ethanol.

Strains	Glucose 35%	Glucose 50%	Cellobiose 1%	KCl 16%	KCl 10%	Acetic acid 1%	Acetic acid 0.5%	42°C (OD_{600} 1.0)	50°C (OD_{600} 1.0)	42°C (OD_{600} 2.0)	50°C (OD_{600} 2.0)	Arabinose 1%	Vanillin 6 µmol L^{-1}	Vanillin 8 µmol L^{-1}	Furfural 1.2 gL^{-1}	Furfural 1.0 gL^{-1}	Furfural 0.5g L^{-1}	Furfural 0.25 gL^{-1}	H_2O_2 (mm)
P8-2.12	-	-	+++	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	37.34
P16-1.1	++	+++	+++	-	++	-	-	-	-	+	-	+	+++	++	-	-	-	++	17
P19-1.1	++	+++	+	-	++	-	-	-	-	-	-	+	+++	++	-	-	-	++	21.67
P21-1.7	++	+++	+	-	+	-	-	-	-	+	-	+	+++	+++	-	-	-	++	24.34
P21-2.1	++	+++	+++	-	+	-	-	-	-	++	-	+	+++	+++	-	-	-	++	21
P22-1.2	+++	+++	+	-	+	-	-	-	-	-	-	-	+++	++	-	-	-	-	0
P22-1.3	+++	+++	++	-	+	-	-	-	-	-	-	+	+++	++	-	-	-	-	0
P22-2.19	+++	++	+	-	+	-	-	-	-	+	-	-	+++	++	-	-	-	-	0
P22-2.20	+++	+++	++	-	++	-	-	-	-	-	-	+	+++	++	-	-	-	-	0
P22-3.18	++	+++	+++	-	++	-	-	-	-	-	-	+	+++	+++	-	-	-	+++	23.67
G7-1.4	++	++	+++	-	+	-	+	-	-	-	-	-	++	+	-	-	-	+	22.67
G13-2.1	+	++	+++	-	-	-	-	-	-	-	-	+	++	-	-	-	-	++	28.67
G13-3.8	-	+	+++	-	-	-	-	-	-	-	-	+	++	-	-	-	-	++	28.67
G14-1.8	-	+	+++	-	-	-	-	-	-	-	-	+	++	-	-	-	-	++	27
G14-2.2	-	-	+++	-	-	-	-	-	-	-	-	+	++	-	-	-	-	++	29.67
G18-3.7	-	-	+++	-	-	-	-	-	-	-	-	+	++	-	-	-	-	++	29.67

- = absence of growth; + = slight growth; ++ = moderate growth; +++ = intense growth. OD = optical density, mm = millimeters (for detailed information see materials and methods).

Table 3. Molecular identity of yeasts isolated by rDNA sequencing.

Isolate code	Organisms	Origin	Identification (%)	Genbank identification number/access number	rDNA[a]
P8-2.12	*Spathaspora roraimanensis*	Beetle gut	98	JN099269.1	ITS1/ITS4
P16-1.1	*Spathaspora passalidarum*	Beetle gut	99	NR_111397.1	ITS1/ITS4
P19-1.1	*Candida* sp.	Beetle gut	84	JQ901890.1; JQ647915.1	ITS1/ITS4
P21-1.7	*Candida* sp.	Beetle gut	90	JQ647915.1; JQ901890.1; FN4241041	ITS1/ITS4
P21-2.1	*Candida* sp.	Beetle gut	93	JQ647915.1; JQ901890.1; JX448364.1	ITS1/ITS4
P22-1.2	*Schwanniomyces* sp.	Beetle gut	99	JQ425347.1; HQ115736.1; AJ586527.1; EF198011.1; JQ425390.1	ITS1/ITS4
P22-1.3	*Schwanniomyces* sp.	Beetle gut	99	HQ115736.1; JQ425347.1; EF198011.1; AJ586527.1; JQ425390.1	ITS1/ITS4
P22-2.19	*Schwanniomyces* sp.	Beetle gut	99	HQ115736.1; JQ425347.1; EF198011.1; JQ425390.1	ITS1/ITS4
P22-2.20	*Schwanniomyces* sp.	Beetle gut	99	JQ425390.1; EF198011.1; AJ586527.1; JQ425347.1; JQ425390.1; LN875174.1	ITS1/ITS4
P22-3.18	*Candida* sp.	Beetle gut	99	FN424204.1; NR_137087.1; JF916546.1	ITS1/ITS4
G7-1.4[b]	*Candida jeffriesii*	Gallery	96	NR_111398.1	ITS1/ITS4
G13-2.1	*Spathaspora passalidarum*	Gallery	99	NR_111397.1	ITS1/ITS4

Table 3. Contd.

G13-3.8	*Spathaspora passalidarum*	Gallery	99	NR_111397.1	ITS1/ITS4
G14-1.8	*Spathaspora passalidarum*	Gallery	99	NR_111397.1	ITS1/ITS4
G14-2.2	*Spathaspora passalidarum*	Gallery	99	NR_111397.1	ITS1/ITS4
G18-3.7	*Spathaspora passalidarum*	Gallery	99	NR_111397.1	ITS1/ITS4

[a]Region of the rDNA gene used for identification. [b]Good sequence; may also represent a new species.

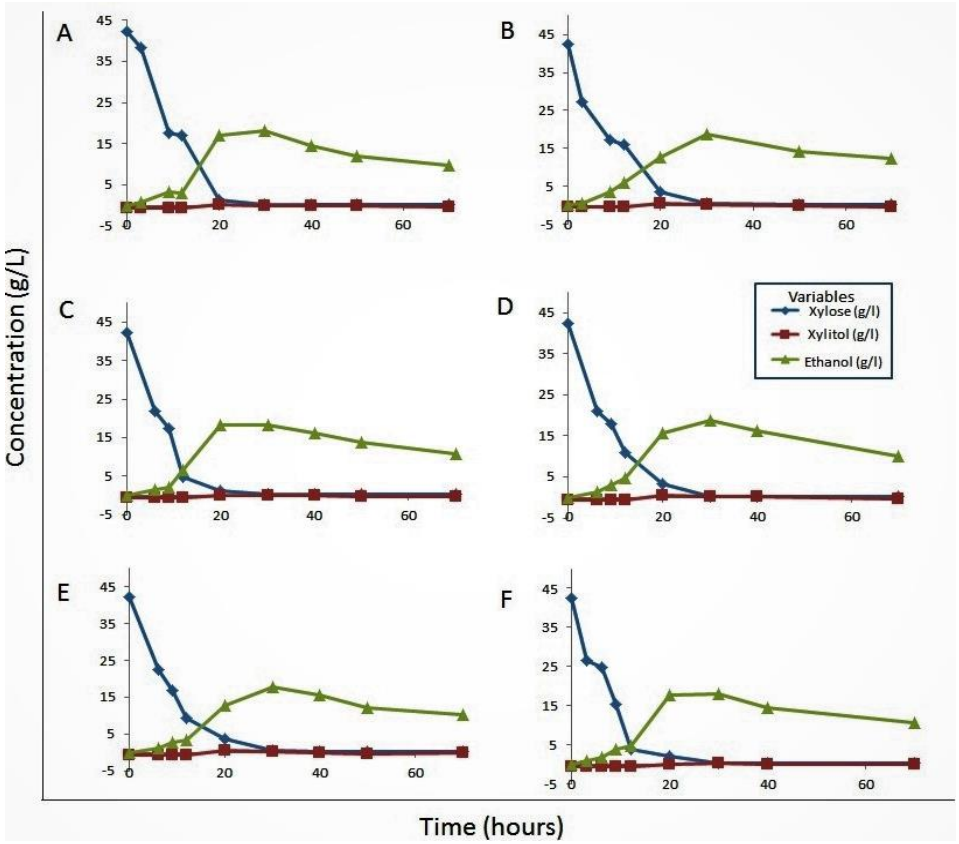

Figure 3. Fermentation kinetics in media containing 4% of xylose as a carbon source at 30°C and 120 rpm, where (A) P16-1.1 ethanol production peak - 18.04 $g.L^{-1}$, (B) G13-2.1 ethanol production peak - 18.85$g.L^{-1}$, (C) G13-3.8 ethanol production peak - 18.40$g.L^{-1}$, (D) G14-1.8 ethanol production peak - 18.68$g.L^{-1}$, (E) G14 -2.2 ethanol production peak - 17.85$g.L^{-1}$ and (F) G18-3.7 ethanol production peak - 17.92 $g.L^{-1}$.

Table 4. Fermentation parameters - comparison between fermentation in media with 4% of xylose and co-fermentation in medium with xylose 4% and glucose 2%.

Strains	Xylose concentration (%)	Glucose concentration (%)	Ethanol (g.L-1)	Xylitol (g.L-1)	$Y^1_{P/S}$ (g/g)	$Y^2_{P/S}$ (g/g)	Q^1_P (g/l.h)	Q^2_P (g/l.h)	η^1 (%)	η^2 (%)	Y (%)	Peak of production (hours)
P8-2.12	4	0	2.09	12.25	-	0.39	-	0.64	-	43.19	76.49	20
	4	2	6.51	10.59	-	0.23	-	0.15	-	24.90	99.62	70
P16-1.1	4	0	18.04	ND	0.43	-	0.60	-	82.25		99.19	30
	4	2	22.87	ND	0.47	-	0.76	-	82.25		99.35	30
P19-1.1	4	0	1.52	21.66	-	0.59	-	0.56	-	64.47	88.80	40
	4	2	5.02	16.76	-	0.13	-	0.87	-	14.58	75.51	20
P21-1.7	4	0	2.04	19.20	-	0.53	-	0.49	-	57.80	88.12	40
	4	2	7.28	11.80	-	0.26	-	0.18	-	28.72	95.71	70
P21-2.1	4	0	5.22	19.14	-	0.59	-	0.66	-	64.46	78.78	30
	4	2	6.03	17.99	-	0.43	-	0.37	-	46.40	88.81	50
P22-2.19	4	0	ND	13.60	-	0.44	-	0.20	-	47.96	76.15	70
	4	2	ND	ND	-	-	-	-	-	1.07	63.62	70
P22-2.20	4	0	ND	17.57	-	0.59	-	0.36	-	64.27	72.71	50
	4	2	ND	ND	-	-	-	-	-	8.04	18.06	70
P22-3.18	4	0	ND	22.10	-	0.54	-	0.32	-	58.65	99.57	70
	4	2	7.15	13.39	-	0.32	-	0.20	-	35.42	87.51	70
G7-1.4	4	0	1.94	20.01	-	0.58	-	0.69	-	62.89	84.28	30
	4	2	3.31	17.40	-	0.37	-	0.26	-	40.20	99.25	70
G13-2.1	4	0	18.85	ND	0.45	-	0.63	-	85.94	-	99.18	30
	4	2	23.81	ND	0.49	-	0.79	-	93.84	-	99.03	30
G13-3.8	4	0	18.40	ND	0.44	-	0.61	-	83.91	-	99.15	30
	4	2	23.69	ND	0.49	-	0.79	-	93.30	-	99.10	30
G14-1.8	4	0	18.68	ND	0.44	-	0.62	-	85.03	-	99.32	30
	4	2	24.70	ND	0.51	-	0.82	-	97.46	-	98.93	30
G14-2.2	4	0	17.85	ND	0.42	-	0.59	-	81.63	-	98.87	30
	4	2	23.95	ND	0.49	-	0.80	-	94.57	-	98.86	30
G18-3.7	4	0	17.92	ND	0.43	-	0.59	-	81.74	-	99.11	30
	4	2	24.26	ND	0.50	-	0.81	-	95.44	-	99.20	30

$Y^1_{P/S}$ = ethanol yield, $Y^2_{P/S}$ (g/g) = xylitol yield, Q^1_P = ethanol productivity, Q^2_P = xylitol productivity, η^1 % = ethanol fermentation efficiency, η^2 % = xylitol fermentation efficiency, Y% = xylose consumption, ND = not detected.

stress conditions imposed, and that seems to be a common condition for wild strains (Slininger et al., 2015).

The production of toxic compounds during pretreatment of lignocellulosic biomass negatively affects yeast growth and fermentation capacity, but removal of these compounds greatly increases the cost of ethanol production. Dubey et al. (2016) reaffirm the importance of these toxic compounds and, according to these authors, strains with greater tolerance to furfural and 5-hydroxymethyl furfural (5-HMF) would make the production of lignocellulosic ethanol economically viable. In the cited research, these authors described a wild strain with higher fermentation performance in the presence of 0.5 $g.L^{-1}$ of furfural and 7.6 $g.L^{-1}$ of 5-HMF compared to an industrial strain and a laboratory strain, demonstrating interest in searching for wild isolates with the appropriate profile.

Kumari and Pramanik (2012) selected mutant yeasts that showed good tolerance to high temperatures and ethanol. They reported that both growth and ethanol production processes in xylose fermenting yeasts were strongly inhibited at an initial vanillin concentration of 1.0 $g.L^{-1}$. Considering vanillin, furfural and acetic acid, the acetic acid showed the least toxic effect in all strains evaluated. This differed from our results, in which growth was not observed on the plate containing 1% acetic acid, except in the G7-1.4 isolate with 0.5% acetic acid. Eleven of the isolates showed growth against 0.2 $g.L^{-1}$furfural and all the isolates were to some degree inhibited by furfural at concentrations of 1.2, 1.0 and 0.5$g.L^{-1}$. Regarding supplementation with vanillin, only the isolates P8-2.12, G13-2.1, G13-3.8, G14-1.8, G14-2.2 and G18-3.7 did not show growth on the plates with 8 µmol L^{-1} vanillin (Table 2).

Molecular identification was done by the amplification of the regions ITS1 and ITS4 which are recommended universal primers for fungi identifications (Trost et al., 2004). The G7-1.4 isolate was potentially a yeast species not previously described from the *Candida* genus. *Candida* species have not been extensively reported as fermentative yeasts for industrial utilization such as the production of bioethanol nor in the production of other useful organic compounds except as causal agents of human diseases (Ebabhi et al., 2013). The yeasts that were identified as belonging to the *Candida* genus (Table 3) also did not produce ethanol, but they were capable of producing xylitol.

Morais et al. (2013) reported for the first time the conversion of D-xylose to ethanol by the yeasts *Schwanniomyces polymorphus* and *Wickerhamomyces pijperi*. In this study, isolates that were identified as *Schwanniomyces* sp. did not produced ethanol, but produced xylitol instead.

The isolated species found here differed from other studies, such as in Rivera et al. (2009). These authors isolated 403 yeasts from beetles (Coleoptera: Dendroctonus). The yeasts were isolated from the gut, ovaries, eggs and feces of insects collected from pines at 34 sites in Mexico, Guatemala and the USA. The yeasts were related to three genera: *Candida* species (*C. ernobii*, *C. piceae*, *C. membranifaciens*, *C. lessepsii*, *C. arabinofermentans* and *C. oregonensis*), *Pichia* spp. (*P. americana*, *P. guilliermondii*, *P. scolyti*, *P. mexicana*, *P. glucozyma* and *P. canadensis*) and *Kurashia* spp. (*K. capsulata* and *K. cf. molischiana*). Studies of the association between beetles and galleries conducted in China to investigate the fungal community of Chinese white pine beetles (*Dendroctonus armandi* Tsai and Li, Scolytidae) revealed that yeasts of the genus *Candida* predominated both in the insect and in its galleries (Hu et al., 2015).

In our research, isolates with high homology with the strain *S. passalidarum* obtained higher yields of ethanol in media containing 4% of xylose. Previous research (Cadete et al., 2009; Hou, 2012) has shown that all species of the *Spathaspora* clade isolated from decomposing wood trunks or insects associated with this substrate have converted xylose into ethanol more efficiently than the species of reference *Pichia stipitis*.

Cadete et al. (2016) classified some species of *Spathaspora* as ethanol producers and xylitol producers, according to the main product of xylose metabolism. In their research, ethanol was the main product for *S. passalidarum*. Among the xylitol producers, the *S. roraimanensis* species had higher production, with this being the physiological characteristic associated with the biochemical activity of xylose reductase (XR). The ethanol producers such as *S. passalidarum* revealed XR activities with both NADH and NADPH as cofactors. Xylitol producers had strictly NADPH-dependent XR activity. Considering this, in our research, the isolates P16-1.1, G13-2.1, G13-3.8, G14-1.8, G14-2.2 and G18-3.7 can be considered ethanol producers and the isolates P8-2.12, P19-1.1, P21-1.7, P21-2.1, P22-2.20, P22-3.18 and G7-1.4 can be considered xylitol producers.

According to Hou (2012), under aerobic conditions, glucose and xylose consumption occur simultaneously, which does not occur under anaerobic conditions, where xylose consumption begins after glucose depletion. It suggests that *S. passalidarum* may use different xylose transport systems under anaerobic and aerobic conditions.

Cadete et al. (2016), under severe oxygen limitation conditions, obtained ethanol production above 20 $g.L^{-1}$ for *S. passalidarum*. Compared with this study, the isolates P16-1.1 (18.04 $g.L^{-1}$), G13-2.1 (18.85 $g.L^{-1}$), G13-3.8 (18.40 $g.L^{-1}$), G14-1.8 (18.68 $g.L^{-1}$), G14-2.2 (17.85 $g.L^{-1}$) and G18-3.7 (17.92 $g.L^{-1}$) were efficient in ethanol production (Figure 3) and better results was obtained when co-fermentation was carried out (up to 24.7 $g.L^{-1}$). The peak of production was 30 h for both fermentation and co-fermentation. The fermentation efficiency (η%) for these isolates varied between 81 and 85.94%. During co-fermentation, a variation of 89-95.44% was obtained

(Table 4), which seems promising. Considering the results of Cadete et al. (2012), who, with *S. passalidarum* in a medium with D-xylose (50 $g.L^{-1}$) as the exclusive carbon source, obtained the maximum ethanol production in 24 h at a temperature of 30°C, with ethanol production ranging from 15 to 18 $g.L^{-1}$ (Q_P = 0.6 to 0.75 $g.L.h^{-1}$) and fermentation efficiency of approximately 70%. Khoja et al. (2015) found that the optimum yield was achieved in 34°C for *Zymomonas mobilis* with the bioethanol yield being 8.0% (v/v) with a fermentation efficiency of 88.96%. These authors observed that *S. cerevisiae* was suitable for low temperature process while *Z. mobilis* could be used in regions having an elevated-temperature process. In another study, Chibuzor et al. (2016) examined the production of bioethanol from cassava peels and found that the combination of *Rhizopus nigricans*, *Spirogyra africana* and *S. cereviceae* could be suitable for ethanol production, where they obtained the highest ethanol yield of 14.46 g/cm^3 and a concentration of 38% (v/v).

Strains with this profile, as recently reported by Slininger et al. (2015), may help techniques to force the evolution of these wild strains. These authors describe a significant improvement of the strain *Scheffersomyce stipitis* NRRL Y-7124, both for the quantitative ethanol production (55-60 $g.L^{-1}$ in the modified strain against 40-45 $g.L^{-1}$ in the parent strain) and for tolerance to toxic agents.

Regarding the xylitol producers, isolate P8-2.12 - *S. roraimanensis* was not efficient when compared to that reported by Cadete et al. (2016) for the same species. Our isolate produced 12.25 $g.L^{-1}$ of xylitol. The *S. roraimanensis* studied by these authors produced 27.4 $g.L^{-1}$. However, isolates P19-1.1, P21-1.7, P21-2.1, P22-2.19, P22-2.20, P22-3.18 and G7-1.4 showed good xylitol production with 21.66, 19.20, 19.14, 13.60, 17.57, 22.10 and 20.01 $g.L^{-1}$ respectively, but during the co-fermentation test good xylitol production was not obtained, in some cases not having any xylitol production (Table 4). Ping et al. (2013) determined the production of xylitol by *Candida tropicalis* using non-detoxified corn hemicellulose hydrolyzate, obtaining a maximum concentration of 38.8 $g.L^{-1}$ of xylitol. Among the species studied by Junyapate et al. (2014), *C. tropicalis* produced the highest concentration of xylitol (7.2 $g.L^{-1}$). Cadete et al. (2015) evaluated the production of xylitol from 50 $g.L^{-1}$ of xylose from five strains of the species *Cyberlindnera xylosilytica sp*. The maximum production of xylitol was reached in 72 h, which was practically the same among the isolates, with a mean production of 33.02 $g.L^{-1}$. It is considerably higher than the profile of the isolates of the study.

Three isolates (P16-1.1, P21-1.7 and P21-2.1) tolerant to several stress conditions were obtained which are normally encountered in the process of obtaining second-generation ethanol. Good comparative ethanol production from xylose was observed, with apparent potential for larger scale trials or tools for altering these wild strains.

These three isolates, with better performance, were curiously all isolated from the gut of the beetle *Popilius marginatus* (Percheron, 1835). Isolate P16-1.1, identified as *S. passalidarum* had, in particular, considerable potential for subsequent research. This study adds some information and possibly relevant isolates in the search for economically viable production of 2G ethanol, thus reaffirming the interest in the sampling of natural environments to obtain wild strains for bioethanol production. Furthermore, the results suggest that yeast isolated from insect galleries have a higher xylolytic capacity than yeasts isolated from the gut of beetles.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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