African Journal of Microbiology Research

Volume 8 Number 37, 10 September, 2014
ISSN 1996-0808

Academic Journals

ABOUT AJMR

The **African Journal of Microbiology Research (AJMR)** (ISSN 1996-0808) is published Weekly (one volume per year) by Academic Journals.

African Journal of Microbiology Research (AJMR) provides rapid publication (weekly) of articles in all areas of Microbiology such as: Environmental Microbiology, Clinical Microbiology, Immunology, Virology, Bacteriology, Phycology, Mycology and Parasitology, Protozoology, Microbial Ecology, Probiotics and Prebiotics, Molecular Microbiology, Biotechnology, Food Microbiology, Industrial Microbiology, Cell Physiology, Environmental Biotechnology, Genetics, Enzymology, Molecular and Cellular Biology, Plant Pathology, Entomology, Biomedical Sciences, Botany and Plant Sciences, Soil and Environmental Sciences, Zoology, Endocrinology, Toxicology. The Journal welcomes the submission of manuscripts that meet the general criteria of significance and scientific excellence. Papers will be published shortly after acceptance. All articles are peer-reviewed.

Submission of Manuscript

Please read the **Instructions for Authors** before submitting your manuscript. The manuscript files should be given the last name of the first author

Click here to Submit manuscripts online

If you have any difficulty using the online submission system, kindly submit via this email ajmr@academicjournals.org.

With questions or concerns, please contact the Editorial Office at ajmr@academicjournals.org.

Editorial Board

Dr. Hongxiong Guo
STD and HIV/AIDS Control and Prevention,
Jiangsu provincial CDC,
China

Instructions for Author

Electronic submission of manuscripts is strongly encouraged, provided that the text, tables, and figures are included in a single Microsoft Word file (preferably in Arial font).

The **cover letter** should include the corresponding author's full address and telephone/fax numbers and should be in an e-mail message sent to the Editor, with the file, whose name should begin with the first author's surname, as an attachment.

Article Types

Three types of manuscripts may be submitted:

Regular articles: These should describe new and carefully confirmed findings, and experimental procedures should be given in sufficient detail for others to verify the work. The length of a full paper should be the minimum required to describe and interpret the work clearly.

Short Communications: A Short Communication is suitable for recording the results of complete small investigations or giving details of new models or hypotheses, innovative methods, techniques or apparatus. The style of main sections need not conform to that of full-length papers. Short communications are 2 to 4 printed pages (about 6 to 12 manuscript pages) in length.

Reviews: Submissions of reviews and perspectives covering topics of current interest are welcome and encouraged. Reviews should be concise and no longer than 4-6 printed pages (about 12 to 18 manuscript pages). Reviews are also peer-reviewed.

Review Process

All manuscripts are reviewed by an editor and members of the Editorial Board or qualified outside reviewers. Authors cannot nominate reviewers. Only reviewers randomly selected from our database with specialization in the subject area will be contacted to evaluate the manuscripts. The process will be blind review.

Decisions will be made as rapidly as possible, and the Journal strives to return reviewers' comments to authors as fast as possible. The editorial board will re-review manuscripts that are accepted pending revision. It is the goal of the AJMR to publish manuscripts within weeks after submission.

Regular articles

All portions of the manuscript must be typed double-spaced and all pages numbered starting from the title page.

The Title should be a brief phrase describing the contents of the paper. The Title Page should include the authors' full names and affiliations, the name of the corresponding author along with phone, fax and E-mail information. Present addresses of authors should appear as a footnote.

The Abstract should be informative and completely self-explanatory, briefly present the topic, state the scope of the experiments, indicate significant data, and point out major findings and conclusions. The Abstract should be 100 to 200 words in length.. Complete sentences, active verbs, and the third person should be used, and the abstract should be written in the past tense. Standard nomenclature should be used and abbreviations should be avoided. No literature should be cited.

Following the abstract, about 3 to 10 key words that will provide indexing references should be listed.

A list of non-standard **Abbreviations** should be added. In general, non-standard abbreviations should be used only when the full term is very long and used often. Each abbreviation should be spelled out and introduced in parentheses the first time it is used in the text. Only recommended SI units should be used. Authors should use the solidus presentation (mg/ml). Standard abbreviations (such as ATP and DNA) need not be defined.

The Introduction should provide a clear statement of the problem, the relevant literature on the subject, and the proposed approach or solution. It should be understandable to colleagues from a broad range of scientific disciplines.

Materials and methods should be complete enough to allow experiments to be reproduced. However, only truly new procedures should be described in detail; previously published procedures should be cited, and important modifications of published procedures should be mentioned briefly. Capitalize trade names and include the manufacturer's name and address. Subheadings should be used. Methods in general use need not be described in detail.

Results should be presented with clarity and precision. The results should be written in the past tense when describing findings in the authors' experiments. Previously published findings should be written in the present tense. Results should be explained, but largely without referring to the literature. Discussion, speculation and detailed interpretation of data should not be included in the Results but should be put into the Discussion section.

The Discussion should interpret the findings in view of the results obtained in this and in past studies on this topic. State the conclusions in a few sentences at the end of the paper. The Results and Discussion sections can include subheadings, and when appropriate, both sections can be combined.

The Acknowledgments of people, grants, funds, etc should be brief.

Tables should be kept to a minimum and be designed to be as simple as possible. Tables are to be typed double-spaced throughout, including headings and footnotes. Each table should be on a separate page, numbered consecutively in Arabic numerals and supplied with a heading and a legend. Tables should be self-explanatory without reference to the text. The details of the methods used in the experiments should preferably be described in the legend instead of in the text. The same data should not be presented in both table and graph form or repeated in the text.

Figure legends should be typed in numerical order on a separate sheet. Graphics should be prepared using applications capable of generating high resolution GIF, TIFF, JPEG or Powerpoint before pasting in the Microsoft Word manuscript file. Tables should be prepared in Microsoft Word. Use Arabic numerals to designate figures and upper case letters for their parts (Figure 1). Begin each legend with a title and include sufficient description so that the figure is understandable without reading the text of the manuscript. Information given in legends should not be repeated in the text.

References: In the text, a reference identified by means of an author‘s name should be followed by the date of the reference in parentheses. When there are more than two authors, only the first author‘s name should be mentioned, followed by ’et al‘. In the event that an author cited has had two or more works published during the same year, the reference, both in the text and in the reference list, should be identified by a lower case letter like ’a‘ and ’b‘ after the date to distinguish the works.

Examples:

Abayomi (2000), Agindotan et al. (2003), (Kelebeni, 1983), (Usman and Smith, 1992), (Chege, 1998; 1987a,b; Tijani, 1993,1995), (Kumasi et al., 2001)
References should be listed at the end of the paper in alphabetical order. Articles in preparation or articles submitted for publication, unpublished observations, personal communications, etc. should not be included in the reference list but should only be mentioned in the article text (e.g., A. Kingori, University of Nairobi, Kenya, personal communication). Journal names are abbreviated according to Chemical Abstracts. Authors are fully responsible for the accuracy of the references.

Examples:

Chikere CB, Omoni VT and Chikere BO (2008). Distribution of potential nosocomial pathogens in a hospital environment. Afr. J. Biotechnol. 7: 3535-3539.

Moran GJ, Amii RN, Abrahamian FM, Talan DA (2005). Methicillinresistant Staphylococcus aureus in community-acquired skin infections. Emerg. Infect. Dis. 11: 928-930.

Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

Pelczar JR, Harley JP, Klein DA (1993). Microbiology: Concepts and Applications. McGraw-Hill Inc., New York, pp. 591-603.

Short Communications

Short Communications are limited to a maximum of two figures and one table. They should present a complete study that is more limited in scope than is found in full-length papers. The items of manuscript preparation listed above apply to Short Communications with the following differences: (1) Abstracts are limited to 100 words; (2) instead of a separate Materials and Methods section, experimental procedures may be incorporated into Figure Legends and Table footnotes; (3) Results and Discussion should be combined into a single section.
Proofs and Reprints: Electronic proofs will be sent (e-mail attachment) to the corresponding author as a PDF file. Page proofs are considered to be the final version of the manuscript. With the exception of typographical or minor clerical errors, no changes will be made in the manuscript at the proof stage.

Fees and Charges: Authors are required to pay a $550 handling fee. Publication of an article in the African Journal of Microbiology Research is not contingent upon the author's ability to pay the charges. Neither is acceptance to pay the handling fee a guarantee that the paper will be accepted for publication. Authors may still request (in advance) that the editorial office waive some of the handling fee under special circumstances

Submission of a manuscript implies: that the work described has not been published before (except in the form of an abstract or as part of a published lecture, or thesis) that it is not under consideration for publication elsewhere; that if and when the manuscript is accepted for publication, the authors agree to automatic transfer of the copyright to the publisher.

African Journal of Microbiology Research

Table of Content: Volume 8 Number 37, 10 September, 2014

ARTICLES

Methicillin-resistant and methicillin-susceptible *staphylococcus aureus* in allergic Rhinitis patient and healthy individuals: Prevalence, antibiotic susceptibility and Effect on disease severity
Hamed M. Alzoubi, Hani M. Al-Shagahin, Omar SM. Al-Rawashdeh, Mohammed S. Alsbou, Baraah Y. Kasasbeh and Amin A. Aqel

Antimicrobial and phytochemical screening of *Olea europaea* linn. extracts against dental pathogens
Sanjay Kumar, Navneet, Murli Manohar Tiwari and Shiv Shanker Gautam

16s rDNA sequence characterization and homogeneity of *halophilic Bacillus* sp. Isolated from athalosaline lake (Al Jouf, Saudi Arabia)
Maher M. Al-Enazi, A. Z. El-Bahrawy, Bilal Ahmad Tantry and Rahiman Shaik

Microbial and physico-chemical changes in tomato juice subjected to pulsed Electric field treatment
V. Kayalvizhi and Usha Antony

Antimicrobial profile of multidrug-resistant *Staphylococcus* Spp. Isolated from Bovine mastitis cases in the northwest region of Paraná State, Brazil
Noemila Débora Kozerski, Jorge Luiz Pereira De Oliveira, Rodrigo Assunção Moura, Danilo Ratti da Silva, Aliny Fernanda De Oliveira, Priscila Luiza Mello, Renata Olivotto Agostinis, Daniela Dib Gonçalves, Luciana Kazue Otutumi and Lisiane de Almeida Martins

Diversity of yeasts in otchè, a traditional starter used in fermentation of an Opaque sorghum beer "chakpalo"
Yves Kadjogbé Djegui, Adéchola Pierre Polycarpe Kayodé, Raymond A. Atchadé, Emma W. Gachomo, Simeon O. Kotchoni and Joseph Djidjoho Hounhouigan

Evaluation of plant extract in control of dry bubble disease of white button Mushroom caused by *Verticillium fungicola* F.Sp. *fungicola* Preuss (Hassebr)
Narendra Kumar Jatav, R. S. Rana, Jeeva Ram Verma, Shri Kishan Bairwa and G. C. Tiwari

African Journal of Microbiology Research

Table of Content: Volume 8 Number 37, 10 September, 2014

Comparative evaluation of antimicrobial properties and phytochemical Composition of *Artocarpus altilis* leaves using ethanol, n-hexane and water
Mbaeyi-Nwaoha, I. E. and Onwuka, C. P.

Morphological, physiological and pathogenic variability of small-spore *Alternaria* Sp. Causing leaf blight of Solanaceous plants In Algeria
BESSADAT Nabahat, SIMONEAU Philippe, BENICHOU Soumaya, SETTI Benali, Kihal Mabrouk and HENNI Djamel Eddine

academicJournals

Vol. 8(37), pp. 3368-3372, 10 September, 2014
DOI: 10.5897/AJMR2014.6950
Article Number: 3CD25CD47894
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* in allergic rhinitis patient and healthy individuals: Prevalence, antibiotic susceptibility and effect on disease severity

Hamed M. Alzoubi[1]*, Hani M. Al-Shagahin[2], Omar SM. Al-Rawashdeh[3], Mohammed S. Alsbou[4], Baraah Y. Kasasbeh[5] and Amin A. Aqel[1]

[1]Microbiology and Immunology Department, Faculty of Medicine, Mu'tah University, Jordan.
[2]Department of Special Surgery (Ear, Nose, Throat unit), Faculty of Medicine, Mu'tah University, Jordan.
[3]Department of Anatomy and Histology, Faculty of Medicine, Mu'tah University, Jordan.
[4]Department of Pharmacology, Faculty of Medicine, Mu'tah University, Jordan.
[5]Internship program, Alkarak Hospital, Alkarak, Jordan.

Received 7 June, 2014; Accepted 8 September, 2014

Allergic rhinitis is a common condition affecting populations globally. It has been recently suggested to increase the risk of both methicillin resistant and susceptible *Staphylococcus aureus* (MRSA, MSSA) nasal carriage. The aim of this study was to assess the prevalence and antibiogram to MSSA and MRSA among allergic rhinitis patients and healthy individuals, and its effect on disease severity. Nasal swabs were collected from 74 allergic rhinitis patients and 74 healthy individuals. MSSA and MRSA were identified by culture and biochemical methods. Antibiogram was determined by the disc diffusion method. MRSA prevalence was 15% in allergic rhinitis group and 4% among healthy individuals (*P* = 0.024), however there was no significant difference between MSSA nasal carriage among allergic rhinitis (8.1%) and control group (13.5%) (*P* = 0.28). The MRSA carriage was also significantly different between mild (0%) and moderate/severe allergic rhinitis (20%) (*P* = 0.035). MSSA nasal carriage was not significantly different between both groups (*P* = 0.65). Four multidrug-resistant MRSA isolates from allergic rhinitis patients were detected compared to one isolate from healthy individuals. MRSA nasal carriage was higher among allergic rhinitis compared to controls. It was also higher among moderate/severe cases compared to mild cases. This suggests that allergic rhinitis increases the risk for MRSA nasal carriage. MRSA carriage also increases the severity of the disease. Therefore, decolonization of MRSA might be useful in managing severe cases.

Key words: Methicillin resistant *Staphylococcus aureus,* allergic rhinitis, antibiotic susceptibility.

INTRODUCTION

Methicillin resistant *Staphylococcus aureus* (MRSA) became an increasingly important pathogen since its isolation in 1960s (Jevons, 1961). It was initially recognized as a nosocomial pathogen. Nevertheless, since 1990s, there has been an increase in MRSA among population without exposure to health care environment which led to the

recognition of new MRSA strains often referred to as community-associated MRSA (CA- MRSA) (Centers for Disease Control and Prevention, 1999).

Isolates of MRSA are also resistant to penicillin and all other beta lactam antibiotics due to possession of *mec*A gene which codes for a penicillin binding protein (PBP2a) with low affinity to beta-lactams (David and Daum, 2010; Monecke et al., 2011; Pinho et al., 2001). Additionally, the emergence of MRSA isolates with resistance to several other antibiotics (multidrug resistance) represents another major challenge that restricts the available options for the treatment of MRSA infections (DeLeo et al., 2010).

Asymptomatic nasal colonization with MRSA was suggested as a risk factor for the development of subsequent infections such as skin infections and the more fatal necrotizing pneumonia (DeLeo et al., 2010). Risk factors such as children population, recent antibiotic usage and working within healthcare facilities were suggested to increase the risk of MRSA nasal carriage (Costelloe et al., 2011; Kuehnert et al., 2006; Albrich and Harbarth, 2008). However, risk factors for CA-MRSA colonization are not fully outlined and are still being studied (Chih-Jung et al., 2011). In addition, CA-MRSA was shown to infiltrate hospital settings (Otter and French, 2011). Therefore, continuous surveillance is necessary to assess the epidemiology, reservoirs and risk factors for MRSA colonization in the community to efficiently apply infection control and management policies in community and subsequently, in hospitals. Allergic rhinitis was previously reported to increase the risk of *S. aureus* carriage (Shiomori et al., 2000). However, few studies have recently documented increased risk of MRSA nasal carriage among allergic rhinitis patients (Cevik et al., 2014). The scarcity of studies that focused on detecting MRSA among allergic rhinitis patients mandates further exploration of this aspect.

The aim of this study was therefore, to assess the nasal carriage and antibiogram of MRSA and MSSA among allergic rhinitis and its effect on disease severity among patients from Al-Karak province, Jordan. The results of this study might help in guiding the infection control and management plans of probable CA-MRSA infections and its complications among allergic rhinitis patients.

MATERIALS AND METHODS

Study design and data collection

This case - control study was carried out from March until April, 2014, at Al-Karak province in Jordan. The study was approved by the scientific and the ethics committees at the faculty of medicine, University of Mutah, Jordan, number 20147. All participants gave a written informed consent after procedure being fully explained.

Study population

A total of 115 samples were collected from allergic rhinitis patients. Another 115 samples were also collected from non-allergic rhinitis patients who served as a control. A questionnaire was filled in with information on age, sex, education level, profession and the severity score of allergic rhinitis (Bousquet et al., 2008). The questionnaire had also information on illnesses, having a family member who is a healthcare worker and antibiotic consumption over the last 3 months which were considered as exclusion criteria in this study. A total of 41 samples out of the 115 samples were excluded based on those criteria. This brought the study samples to 74 samples from each study group.

Nasal swabs culture, bacterial identification and antimicrobial susceptibility testing of the isolates

This was carried out as previously described elsewhere (Alzoubi et al., 2013). Briefly, the anterior nares of each participant were sampled by rotating a cotton swab three times in the vestibule of both anterior nares. The nasal swabs were then inoculated directly on Mannitol Salt Agar (MSA, BBL Microbiology System, Becton Dickinson Company, MD, U.S.A) and incubated at 35 ± 1°C and examined for growth within 48 h. Each single distinctive morphotype of a mannitol-positive colony was then selected from the MSA plate, subcultured on nutrient agar (BBL Microbiology Systems, Becton Dickinson, Company, MD, U.S.A.) and incubated at 37°C in a humidified incubator overnight for 18 h. Colonies from Nutrient agar were identified as *S. aureus* by Gram's staining, catalase and tube coagulase tests. Identification of the MRSA isolates was performed using 30 µg/ml cefoxitin disc in Mueller-Hinton agar supplemented with NaCl (4% w/v; 0.68 mol/L) according to Clinical and Laboratory Standard Institute (CLSI) guidelines (Clinical and Laboratory Standards Institute, 2012). Antimicrobial susceptibility to fusidic acid, erythromycin, mupirocin, gentamicin, linezolid, teicoplanin, ciprofloxacin, trimethoprim-sulfamethoxazole, tetracyclin, rifampicin, and cefoxitin was performed by disk diffusion that was performed according to the European Committee on Antimicrobial Susceptibility Testing for fusidic acid, and according to the Clinical Laboratory Standards Institute (CLSI) guidelines for the remaining antibiotics (The European Committee on Antimicrobial Susceptibility Testing, 2010; Clinical and Laboratory Standards Institute, 2012). *S. aureus* ATCC 25923 was used as control strain. Discs were purchased from Oxoid, Hampshire, England.

Statistical analysis

The statistical analysis was performed with STATA10 (Stata Corp. 2007. *Stata Statistical Software: Release 10*. College Station, TX: StataCorp LP, USA) to evaluate the significance of results. A *P* value of less than 0.05 was considered as significant using chi-square test.

RESULTS

The prevalence of MRSA nasal carriage in allergic rhinitis patients was about 15% (11/74) and in healthy individuals was 4% (3/74). Table 1 shows the distribution of isolates and statistical analysis. There was a significant difference

*Corresponding author. E-mail: dr_alzoubi@yahoo.com. Tel: (962)-3-238-6287 Ext. 3254. Fax: 962-3-239-7180.

Table 1. Numbers and percentages of MRSA and MSSA nasal carriage and *P* values among healthy individuals (controls) and allergic rhinitis patients.

Population	Controls (no.=74)	Allergic rhinitis (no.=74)	Allergic rhinitis (no.=74)	
			Mild (no.=19)	Moderate/Severe (no.=55)
No. MRSA +ve	3 (4%)	11(15%)	0 (0%)	11 (20%)
P value	0.024	0.024	0.035	0.035
No. MSSA +ve	10 (13.5%)	6 (8%)	2 (10.5%)	4 (7%)
P value	0.28	0.28	0.65	0.65

MRSA, Methicillin resistant *staphylococcus aureus*; MSSA, methicillin sensitive *staphylococcus aureus*, +ve, positive. Significant P value < 0.05.

Table 2. Antibiotic resistance pattern of MRSA and MSSA isolates from allergic rhinitis patients (AR) and healthy individuals (control group).

Pathogen / number	Antibiotic susceptibility (number, %)										
	E	Mup	G	LZD	Teic	Cip	SXT	Tet	Rd	F	Cef
AR-MRSA/11	(7) 63.7	(0) 0	(3) 27.3	(0) 0	(0) 0	(0) 0	(1) 9	(2) 18.2	(0) 0	(5) 45.5	11 100
AR-MSSA/6	(3) 50	(0) 0	(0) 0	(0)0	(0) 0	(0) 0	(0) 0	(1) 17	(0) 0	(2) 33	(0) 0
Control-MRSA/3	(1) 33.3	(0) 0	(0) 0	(0) 0	(0) 0	(1) 33.3	(1) 33.3	(1) 33.3	(0) 0	(2) 66.6	11 100
Control-MSSA/10	(4) 40	(0) 0	(0) 0	(0) 0	(0) 0	(0) 0	(0) 0	(0) 0	(0) 0	(10) 100	(0) 0

E, Erythromycin; Mup, Mupirocin; G, Gentamicin; LZD, Linezolid; Teic, Teicoplanin ; Cip, Ciprofloxacin; SXT, Trimethoprim-sulfamethoxazole; Tet, Tetracycline; Rd, Rifampicin; F, Fusidic acid; Cef, Cefoxitin. MRSA, Methicillin resistant *staphylococcus aureus;* MSSA, Methicillin sensitive *staphylococcus aureus.*

between nasal carriages of MRSA in both groups (p = 0.024). On the other hand, MSSA nasal carriage was about 80% in allergic rhinitis patients and was 13.5% in healthy individuals (Table 1). There was no significant difference between both groups (p = 0.28).

MRSA nasal carriage was significantly different between Mild and moderate/severe allergic rhinitis cases (p = 0.035) as can be seen in Table 1. MSSA nasal carriage was not significantly different among both groups (p = 0.65)

Antibiotic susceptibility of MRSA and MSSA isolates from allergic rhinitis and healthy individuals

The antibiogram of all isolates is shown in Table 2. The highest level of resistance in the 11 MRSA isolates from allergic rhinitis patients was found against erythromycin (63.7%), fusidic acid (45.5%) gentamicin (27.3%) and tetracycline (18.2%) followed by trimethoprim-sulfamethoxazole (9%). MRSA isolates were susceptible to the remaining antibiotic profile. Four MRSA isolates were multidrug resistant. These isolates were resistant to three or more of erythromycin, gentamicin, tetracycline and fusidic acid.

A total of 50, 17 and 33% of MSSA isolates were resistant to erythromycin, tetracycline and fusidic acid respectively. MSSA isolates were sensitive to all remaining antibiotics used in this study.

Within the healthy individuals group, one third of MRSA isolates were resistant to erythromycin, ciprofloxacin, trimethoprim-sulfamethoxazole and tetracycline, while two thirds were resistant to fusidic acid. One MRSA isolate showed multi-resistance pattern to cefoxitin and to four other antibiotics including erythromycin, ciprofloxacin, tetracycline and fusidic acid. On the other hand, 40% of MSSA isolates showed resistance to erythromycin but were sensitive to the remaining antibiotic profile used in this study.

DISCUSSION

Colonization with MRSA is a risk for developing subsequent infections (DeLeo et al., 2010). Risk factors for MRSA nasal carriage in community are still not fully determined and studies are continuing to define these factors (Chih-Jung et al., 2011).

In the current study, higher MRSA nasal carriage rate occurred in allergic rhinitis patients with about 15% carriage rate compared to 4% in healthy individuals group. This is similar to the finding of a recently published paper by Cevik et al. (2014) in Turkey, which revealed higher MRSA carriage rate among allergic rhinitis patients (3.7%) compared to controls (1.5%). The difference in MRSA prevalence among both studies might be explained by the fact that Jordan was shown to have a high prevalence of MRSA in general compared to other Mediterranean and European countries, which was attributed to inefficient

infection prevention and control measures (Borg et al., 2007). This caused increased rates of MRSA isolates in hospitals which could subsequently be infiltrated into community. This was also supported by other two studies from Jordan that found a prevalence of 7.4 (Al-Bakri et al., 2013) and 13.2% (Shehabi et al., 2013) of the community MRSA in Jordan. Other factors such as difference in host factors and difference in the population of the two studies might contribute to the difference in the MRSA prevalence in the current study and that found by Cevik et al. (2014).

The MSSA carriage was not significantly different between both groups of the current study though it was higher in controls (13.5%) compared to allergic rhinitis patients (8.1%).

Among allergic rhinitis patients, 20% of moderate-/severe cases carried MRSA compared to 0% in mild cases. MSSA was found in 7.3% of moderate/severe cases compared to 10.5% in mild cases but was not significantly different. Therefore, MRSA but not MSSA nasal carriage was found to be statistically higher among allergic rhinitis patients than controls, and also higher among moderate/severe allergic rhinitis than mild allergic rhinitis. This finding support the assumption that allergic rhinitis may be a risk factor for MRSA nasal colonization. Taking into consideration how common is allergic rhinitis which was estimated to affect 40-50% of people globally (Bousquet et al., 2008), treatment options should be selected carefully. The possibility of MRSA as the causative agent should be raised when dealing with infections among this category of patients such as sinusitis, otitis media and other body infections. On the other hand, the current study findings suggest that MRSA nasal carriage may be an important factor in exacerbating the severity of symptoms and probably the persistence of chronic sinusitis as has been previously suggested for *S.aureus* (Refaat et al., 2008). Allergic rhinitis results from allergens re-exposure in the nasal membranes such as dust, mite and pollens (Bousquet et al., 2008). Similarly, the MRSA nasal carriage may act as an allergen probably by some of its enterotoxins and this was previously suggested for *S. aureus* (Tang et al., 2011; Rossi and Monasterolo, 2004).

In guinea pigs, it was suggested that persistent allergic rhinitis is mediated by staphylococcus enterotoxin B via stimulating the TH2 response which leads to increased antigen specific IgE production (Tang et al., 2011). Therefore, screening and decolonization of MRSA nasal carriage in allergic rhinitis patient might not only decrease the morbidity and mortality of MRSA associated infections, but it might be recommended as a part of the treatment regime for moderate and severe cases of persistent allergic rhinitis cases, this was also suggested previously for *S. aureus* colonization in general (Refaat et al., 2008). However, further studies might be needed to assess the therapeutic role of MRSA decolonization among moderate/severe allergic rhinitis patients.

The antibiotic sensitivity of MRSA among allergic rhinitis in this study revealed high resistance to erythromycin and fusidic acid followed by gentamicin, tetracycline, and trimethoprim-sulfamethoxazole. There was no resistance for the remaining antibiotics that were used in this study, importantly to teicoplanin and linezolid which is nearly similar to what was found by some studies in Jordan (Al-Bakri et al., 2013). Therefore, it is recommended to avoid using the antibiotics for which MRSA was found to be resistant in the current study for the empirical treatment of MRSA infections such as pneumonia and bacteremia among allergic rhinitis patients.

The current study shows that there was a significant nasal carriage of MRSA among allergic rhinitis. The carriage was higher among moderate/severe cases compared to mild cases. This suggests that allergic rhinitis is a risk factor for MRSA nasal carriage. Additionally, MRSA carriage was shown to be associated with increased disease severity therefore, decolonization of MRSA might therefore be useful in managing moderate/severe and resistant cases of allergic rhinitis in line with other management protocols.

Conflict of Interests

The author(s) have not declared any conflict of interest.

ACKNOWLEDGMENTS

We would like to acknowledge the scientific research deanship at Mu'tah university/Jordan for financial support of this work. Acknowledgement is also due to Dr. Asma'a Al-mnayyis for proof reading the paper.

REFERENCES

Al-Bakri A, Al-Hadithi H, Kasabri V, Othman G, Kriegeskorte A, Becker K (2013). The epidemiology and molecular characterization of methicillin-resistant staphylococci sampled from a healthy Jordanian population. Epidemiol. Infect. 141:2384-2391.

Albrich W, Harbarth S (2008). Health-care workers: source, vector, or victim of MRSA? Lancet Infect. Dis. 8:289-301.

Alzoubi H, Aqel A, Abu-Helalah M (2013). Prevalence of Methicillin Resistant *Staphylococcus aureus* Nasal Carriage and Its Antibiogram in Healthcare Workers from South of Jordan. Bull. High Inst. Pub. Health 43:1-12.

Borg M, de Kraker M, Scicluna E, van de Sande-Bruinsma N, Tiemersma E, Monen J (2007). Prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) in invasive isolates from southern and eastern Mediterranean countries. J. Antimicrob. Chemother. 60:1310-1315.

Bousquet J, Khaltaev N, Cruz A, Denburg J, Fokkens W, Togias A (2008). Allergic Rhinitis and its Impact on Asthma (ARIA) 2008 Update (in collaboration with the World Health Organization, GA2LEN and AllerGen). Allergy 63:S8-160

Centers for Disease Control and Prevention (1999). Four pediatric deaths from community-acquired methicillin-resistant *Staphylococcus aureus* - Minnesota and North Dakota, 1997-1999. MMWR Morb Mortal Wkly Rep. 1999; 48:707–10.

Cevik C, Yula E, Yengil E, Ihsan Gulmez M, Akbay E (2014). Identification of nasal bacterial flora profile and carriage rates of methicillin-resistant *Staphylococcus aureus* in patients with allergic rhinitis. Eur. Arch. Otorhinolaryngol. 271:103-107.

Chih-Jung C, Kuang-Hung H, Tzou-Yien L, Kao-Pin H, Po-Yen C, Yhu-Chering H (2011). Factors Associated with Nasal Colonization of Methicillin-Resistant *Staphylococcus aureus* among Healthy Children in Taiwan. J. Clin. Microbiol. 49(1):131-137.

Clinical and Laboratory Standards Institute CLSI (2012). Performance standards for antimicrobial susceptibility testing. CLSI, Wayne, PA, twenty second informational supplement. M100-S22.

Costelloe C, Lovering A ,Montgomery A, Lewis D, McNulty C, Hay A (2011). Effect of antibiotic prescribing in primary care on meticillin-resistant *Staphylococcus aureus* carriage in community-resident adults: a controlled observational study. Int. J. Antimicrob. Agents 39(2):135-41.

David M, Daum R (2010). Community-Associated Methicillin-Resistant *Staphylococcus aureus*: Epidemiology and Clinical Consequences of an Emerging Epidemic. Clin. Microbiol. Rev. 23(3): 616-687.

DeLeo F, Otto M, Kreiswirth B, Chambers H (2010). Community-associated meticillin-resistant *Staphylococcus aureus*. Lancet 375: 1557-1568.

Jevons M (1961). Celbenin-resistant staphylococci. Br. Med. J. 1(5219): 113–114.

Kuehnert M, Kruszon-Moran D, Hill H, McQuillan G, McAllister S, Fosheim G (2006). Prevalence of *Staphylococcus aureus* nasal colonization in the United States, 2001-2002. J. Infect. Dis. 193:172-179.

Monecke S, Coombs G, Shore A, Coleman D, Akpaka P (2011). A field guide to pandemic, epidemic and sporadic clones of methicillin-resistant *Staphylococcus aureus*. PLoS One 6(4): e17936.

Otter J, French G (2011). Community-associated meticillin-resistant *Staphylococcus aureus* strains as a cause of healthcare-associated infection. J. Hosp. Infect. 79:189-193.

Pinho M, deLencaster H, Tomasz A (2001). An acquired and a native penicillin-binding protein cooperate in building the cell wall of drug-resistant staphylococci. Proc. Natl. Acad. Sci. 98:10886-10891.

Refaat M, Ahmed T, Ashour Z, Atia M (2008). Immunological role of nasal *Staphylococcus aureus* carriage in patients with persistent allergic rhinitis. Pan. Afr. Med. J. 1(3):1-5

Rossi R, Monasterolo G (2004). Prevalence of serum IgE antibodies to the *Staphylococcus aureus* enterotoxins (SAE, SEB, SEC, SED, TSST-1) in patients with persistent allergic rhinitis. Int. Arch. Allergy Immunol. 133(3): 261-266.

Shehabi A, Abu-Yousef R, Badran E, Al-Bakri A, Abu-Qatouseh L, Becker K (2013). Major characteristics of *Staphylococcus aureus* colonizing Jordanian infants. Pediatr. Int. 55:300-304.

Shiomori T, Yoshida Si, Miyamoto H, Makishima K (2000). Relationship of nasal carriage of *Staphylococcus aureus* to pathogenesis of perennial allergic rhinitis. J. Allergy Clin. Immunol. 105(3):449-454.

Tang X, Sun R, Hong S, Hu G, Yang Y (2011). Repeated intranasal instillation with *staphylococcal* enterotoxin B induces nasal allergic inflammation in guinea pigs. Am. J. Rhinol. Allergy 25(3):176-181.

The European Committee on Antimicrobial Susceptibility Testing (EUCAST) (2010). Breakpoint tables for interpretations of MICs and zone diameters, Version 1.1, April 2010.[http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Disk_test_documents/EUCAST_breakpoints_v1.1.pdf]. Accessed 1 May 2014.

academicJournals

Vol. 8(37), pp. 3373-3377, 10 September, 2014
DOI: 10.5897/AJMR2014.7024
Article Number: 9B3101E47898
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Antimicrobial and phytochemical screening of *Olea europaea* Linn. extracts against dental pathogens

Sanjay Kumar[1]*, Navneet[1], Murli Manohar Tiwari[2] and Shiv Shanker Gautam[1]

[1]Department of Botany and Microbiology, Gurukul Kangri University, Haridwar, Uttarakhand Pin-249404, India.
[2]Department of Applied Chemistry, Faculty of Engineering and Technology, Gurukul Kangri University, Haridwar, Uttarakhand- 249404, India.

Received 20 July, 2014; Accepted 8 September, 2014

Dental caries is a common disease in human population which has a multifactorial etiology. The present study aimed to investigate the antimicrobial efficacy of various extracts of *Olea europaea* against six bacterial pathogens [Staphylococcus aureus, Streptococcus mutans, Staphylococcus sanguinis, Staphylococcus sobrinus, Staphylococcus salivarius, Lactobacillus acidophilus and one fungi (Candida albicans)]. Plant material was crushed and extracted in petroleum ether, acetone, methanol and aqueous through successive method by using Soxhlet apparatus. The antimicrobial activity of extracts was examined by agar well diffusion method at 200 mg/ml sample concentration and minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) by two fold serial dilution method. Ofloxacin was used as positive control to determine the sensitivity of the strains. The results show that methanol extract was more active than other extracts in its antimicrobial activity. The zone of inhibition exhibited by methanol extract against tested microorganisms ranged between 13.6±0.28 to 20.6±0.28 mm, respectively. Phytoconstituents analysis of plant extract revealed the presence of alkaloids, flavonoids, glycosides, steroids, tannins, terpenoids and saponins. The results validate the traditional uses of *O. europaea* in treatment of dental diseases.

Key words: Antimicrobial activity, agar well diffusion method, dental pathogens, *Olea europaea.*

INTRODUCTION

Human mouth contains a variety of bacteria but few are specifically involved in dental infections. Bacterial invasion causes demineralization and destruction of hard tissues of teeth. The acid production by bacteria causes accumulation of tooth surface, finally producing dental caries. Several bacteria are responsible for dental caries and periodontal infections that is *Streptococcus mutans*, *S. sobrinus*, *Lactobacillus acidophilus*, *Actinomyces* spp., *Nocardia* spp., *Campylobacter*, *Fusobacterium*, *Haemophilus*, *Prevotella*, *Porphyromonas*, *Veillonella* (Kononen et al., 1992, 1994; Marsh, 1992; Schupbach et al., 1995). Some of these organisms produce high level of lactic acid causing fermentation of dietary sugars and are resistant to the adverse effect of low pH (Hardie, 1982).

*Corresponding author. E-mail: sanjay.gku@gmail.com. Tel: +91-9719366637.

According to World Health Organization (WHO) more than 80% of the world's population relies on traditional medicine for their primary healthcare need. Use of herbal medicines in Asia represents a long history of human interactions with the environment. Plants used for traditional medicine contain a wide range of substances that can be used to treat chronic as well as infectious diseases (Diallo et al., 1999). Various plants possess broadest spectrum of synthetic activity and have been the source of many useful compounds (Sofowora, 1982).

Olea europaea (Oleaceae) is commonly known as olive tree. It is a tree bearing silvery green leaves and small white, feathery flowers. It is globally distributed especially in the tropical region. *O. europaea* possess broad spectrum of antimicrobial properties and extensively used to treat various diseases. This herb is used orally for sore throat, kidney problems and backache. Leaf infusions are used else-where as a lotion to treat eye infections or a gargle to relieve sore throat (Ross, 2005).

The objective of present study was to investigate the antimicrobial properties of *O. europaea* to cure dental diseases.

MATERIALS AND METHODS

Plant material

O. europaea was collected from G.B. Pant Herbal Nursery, Herbertpur, Dehradun, Uttarakhand and authenticated at Botanical Survey of India, Northern regional center, Deharadun. Stem and bark are included in this study. The plant materials were shade dried at room temperature and crushed to powder with a help of an electric grinder. The powder was sieved through a 1 mm mesh and was stored in an air-tight container for future use.

Preparation of extract

Plant extracts were prepared by immersing separately 200 g of dried powder in 600 ml of four different solvents that is petroleum ether (PET), acetone (ACE), methanol (MeOH) and aqueous (H_2O) by soxhlet assembly and extracted for 72 h through successive methods (Ahmad et al., 1998). Plant extracts were filtered through Whatman No.1 filter paper and crude extracts obtained by removing solvent in vacuum evaporator at 30-60°C and stored in sterile bottles at 4°C until further use. The yield of PET extract was 5.8 g, ACE extract 6.9 g, MeOH extract 8.6 g and H_2O extract 9.3 g respectively. Extracts were dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 200 mg/ml for agar well diffusion method.

Test microorganisms

The microorganisms were selected for the study on the basis of their clinical pharmaceutical importance as well as for their potential to cause infection in dental region. *S. aureus* (MTCC 1144), *S. mutans* (MTCC 890), *S. sanguinis* (ATCC 10556), *S. sobrinus* (ATCC 33478), *S. salivarius* (MTCC 1938), *L. acidophilus* (MTCC 10307) and *Candida albicans* (MTCC 227) were purchased from IMTECH, Chandigarh and National Chemical Lab (NCL), Pune. *S. aureus*, *S. mutans*, *S. sanguinis*, *S. sobrinus*, *S. salivarius* and *L. acidophilus* were isolated from patient's sample collected from Aggarwal Dental Clinic, Haridwar. The isolated pathogens were identified according to published guidelines (Burneti et al., 1994).

Preparation of inoculum

Stock cultures were maintained at 4°C on slopes of nutrient agar. Slants for experiment were prepared by transferring a loopful culture from stock cultures to test tubes of Mueller-Hinton broth (MHB) for bacteria that were incubated without agitation for 24 h at 37°C.

Antimicrobial activity

The antimicrobial activity of different extracts was determined by agar well diffusion method (Perez et al., 1990). *In vitro* antimicrobial activity was screened by using Mueller Hinton Agar (MHA) medium no. 173 (Hi media Pvt Ltd., Mumbai, India). 0.1 ml of 12-16 h incubated cultures of pathogens were mixed in molten medium and poured in pre-sterilized petri plates. Plates were allowed to solidify for 5-10 min. A cork borer (6 mm diameter) was used to punch wells in medium and filled with extracts of 45 µl of 200 mg/ml final concentration of extracts. DMSO was used as negative control. Efficacies of extracts against pathogens were compared with broad spectrum antibiotic Ofloxacin (positive control). Ofloxacin was dissolved into double distilled water. Plates were incubated at 37°C for 24 h in BOD incubator. At the end of incubation, inhibition zones formed around the well were measured with transparent ruler in millimetre. Each sample was assayed in triplicate and mean values were observed. The antimicrobial activity was interpreted from size of diameter of zone of inhibition measured to the nearest millimeter (mm) as observed from clear zones surrounding the wells.

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

Two-fold serial dilution method (Aboaba et al., 2006) was used to determine the minimum inhibitory concentration (MIC). MeOH extract was diluted double fold (2:2) with nutrient broth in a series of six test tubes. Concentration of 50, 25, 12.5, 6.25, 3.12 and 1.56 mg/ml of crude MeOH extract were prepared separately and dissolved in 1 ml of DMSO. An aliquot of 1 ml of microorganism suspension (1.5×10^6) was inoculated into each tube (Figure 1). Control tubes were inoculated with same quantity of sterile distilled water. All tubes were incubated at 37°C for 24 h. The lowest concentration that did not permit any visible growth when compared with control was considered as the minimum inhibitory concentration. The minimum bacteriocidal concentration was considered as the lowest concentration that could not produce a single bacterial colony. The contents of all tubes that showed no visible growth were cultured on MHA medium incubated at 37°C for 24 h.

Phytochemical screening

The phytochemical analysis of plant extracts were carried out by standard qualitative methods (Trease and Evans, 1987; Scalbert, 1991).

Test for alkaloids

The test solution was acidified with acetic acid and a drop of Mayer's reagent was added. A white precipitate indicated the presence of alkaloid.

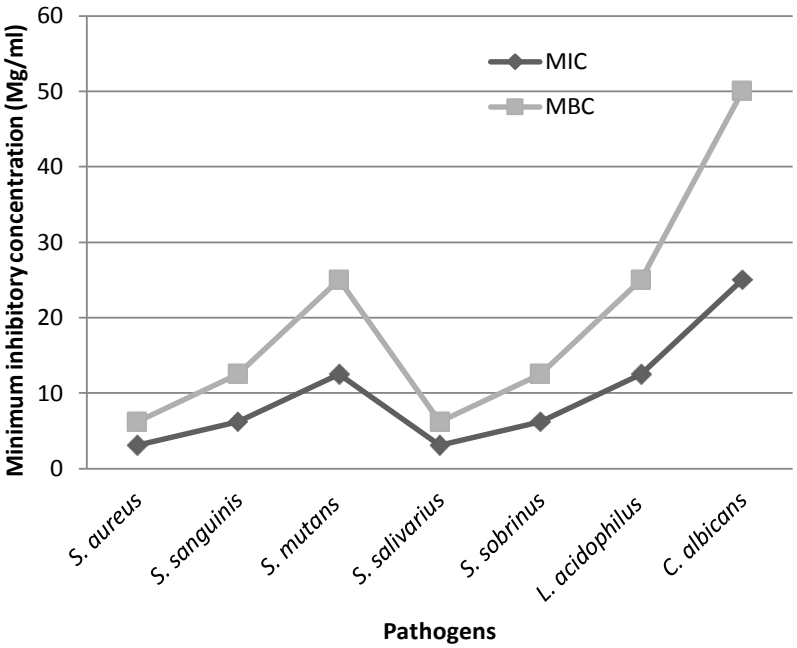

Figure 1. Minimum inhibitory concentrations (MICs) of methanol extract of *O. europaea*. The inhibition is noted at 3.12 mg/ml against S. aureus B) 6.12 mg/ml against *S. sanguinis* and *S. sobrinus*, C) 12.5 mg/ml against *S. mutans* and L. acidophilus and D) 25 mg/ml against *C. albicans*.

Test for flavonoids

On addition of conc. HCl in MeOH extract of the material, a red colour appeared which indicated the presence of flavonoids.

Test for glycosides

The extract was filtered and sugar was removed by fermentation with baker's yeast. The acid was removed by precipitation with $Ba(OH)_2$. The remaining extract contained the glycosides. The hydrolysis of the solution was done with conc. H_2SO_4 and after the hydrolysis the presence of sugar was determined with the help of Fehling's solution.

Test for steroids

The extract was mixed with 3 ml $CHCl_3$ and 2 ml conc. H_2SO_4 was poured from the side of the test tube and the colour of the ring at the junction of two layers was noted. A red colour showed the presence of steroids.

Test for Tannin

Extract was added in 1% ferric chloride and the colour was observed. Bluish black colour appeared which disappeared on addition of dilute H_2SO_4; a yellow brown precipitate showed the presence of tannins.

Test for saponins

Extracts were diluted with water to 20 ml and this was shaken in a graduated cylinder for 15 min. Formation of 1 cm layer of foam indicates the presence of saponins.

RESULTS

The present study shows that medicinal plants possess antimicrobial properties that support their value in herbal medicine for the treatment of dental ailments. The results enumerate the effect of the different extracts of the plant against the pathogens tested at a concentration of 200 mg/ml of plant extracts which shows significant antimicrobial activity against all the pathogens (Table 1). MeOH extract showed the maximum antimicrobial activity against the *S. sobrinus* (20.6±0.28 mm) followed by *L. acidophilus* (20.0±0.50 mm), *S. aureus* (19.0±0.50 mm), *S. sanguinis* (18.3±0.28 mm), *S. salivarius* (17.6±0.57 mm), *S. mutans* (16.6±0.28 mm) and *Candida albicans* (13.6±0.28 mm). MeOH extract showed maximum activity followed by PET, ACE and H_2O extract. The results of MICs and MBCs show that they ranged from 3.12 to 25 mg/ml (Figure 1). *O. europaea* presented similar MICs against *S. sanguinis* and *S. sobrinus* (6.12 mg/ml) respectively. Moreover, MeOH extract of this plant manifested a better MIC against *S. aureus* (3.25 mg/ml) and least MIC recorded against *C. albicans* (25 mg/ml). The phytochemical analysis of plant extract disclosed the presence of alkaloids, flavonoids, glycosides, steroids, tannins, terpenoids and saponins which might be accountable for its antimicrobial potential (Table 2).

Table 1. The inhibition zone diameter of various extracts of *Olea europaea* against dental pathogens.

S/N	Microorganisms	*Diameter of the inhibition zone (mm)				Positive Control (Ofloxacin)
		PET	ACE	MeOH	H_2O	
1	*S. aureus*	10.6±0.28	13.0±0.50	17.6±0.28	14.3±0.28	33.6±0.28
2	*S. aureus* MTCC 1144	11.6±0.28	11.6±0.28	19.0±0.50	14.0±0.50	34.0±0.50
3	*S. mutans*	10.3±0.28	14.3±0.28	16.3±0.76	17.6±0.28	31.6±0.28
4	*S. mutans* MTCC 890	12.0±0.50	14.6±0.28	16.6±0.28	18.6±0.28	32.6±0.57
5	*S. salivarius*	11.3±0.28	13.0±0.50	16.3±0.28	16.6±0.28	29.3±0.28
6	*S.salivarius* MTCC 1938	10.6±0.28	13.6±0.28	17.6±0.57	15.3±0.76	29.6±0.57
7	*S. sanguinis*	9.3±0.28	14.3±0.57	17.0±0.28	15.3±0.28	34.3±0.28
8	*S. sanguinis* ATCC 10556	8.6±0.28	16.0±0.50	18.3±0.28	16.6±0.28	35.6±0.28
9	*S. sobrinus*	11.0±0.50	15.3±0.28	20.0±0.50	18.3±0.57	27.3±0.57
10	*S. sobrinus* ATCC 33478	12.0±0.76	17.0±0.50	20.6±0.28	19.6±0.28	27.6±0.28
11	*L. acidophilus*	12.0±0.50	15.6±0.28	19.3±0.76	16.0±0.50	29.6±0.28
12	*L. acidophilus* MTCC 10307	11.3±0.28	15.3±0.28	20.0±0.50	17.3±0.28	30.0 ±0.50
13	*C. albicans* MTCC 227	7.6±0.57	11.6±0.28	13.6±0.28	9.6±0.28	22.3±0.28

*Zone of inhibition in millimetre (mm) in triplicate expressed as means and standard error of means.

Table 2. The phytochemical screening of crude extracts of *Olea europaea.*

Phytoconstituent	Solvents			
	PET	ACE	MeOH	H_2O
Alkaloids	-	+	+	+
Flavonoids	-	+	+	+
Glycosides	-	+	-	+
Steroids	+	+	+	+
Saponins	-	-	+	+
Tannins	+	+	+	+

+ = Present, - = Absent.

DISCUSSION

Based on results, the antimicrobial potential of *O. europaea* extracts showed broad spectrum activity against selected pathogens. Our results are significantly correlated with other workers regarding this plant. Literature survey showed that major parts of *O. europaea* that is flowers, stems, leaves and fruits had good antimicrobial activity. The fruit extract of *O. europaea* exhibited antibacterial activity against *S. aureus* (18 mm) at concentration 800 µg/ml (Gupta et al., 2008). *O. europaea* aqueous extracts were screened for their antimicrobial activity against six bacteria that is, *B. cereus*, *B. subtilis*, *S. aureus*, *E. coli*, P. aeruginosa, *K. pneumoniae* and two fungi that is, *C. albicans* and *C. neoformans* (Pereira et al., 2007). According to Anesini and Perez (1993), aqueous extract of dried fruit was inactive against *E. coli* and *S. aureus* at a concentration of 62.5 mg/ml. The isolated phenolic components of *O. europaea* showed inhibitory effect against some foodborne pathogens such as *Campylobacter jejuni*, *Helicobacter pylori* and *S. aureus* (Ahmed et al., 2014). Ziad et al. (2011) documented a MIC with ethyl acetate fraction of *O. europaea* at 10 µg/µl for *E. coli* and at 5.5 µg/µl for *K. pneumoniae.*

The phytochemical study showed that the presence of glycosides, alkaloids, flavanoids and amino acids in ethanolic, hydro-alcoholic and aqueous extract (Kaskoos, 2013). Khan et al. (2007) reported that phytoconstituents of flowers of *O. europaea* had shown the presence of flavonoids, steroids, glycoside, tannins and fatty acids. *O. europaea* leave extract showed major antibacterial activity due to the presence of phenolic compounds. The HPLC-DAD analysis of *O. europaea* leaves showed the presence of seven phenolic compounds that is caffeic acid, verbascoside, oleuropein, luteolin 7-O-glucoside, rutin, apigenin 7-O-glucoside and luteolin 4'-O-glucoside (Pereira et al., 2007).

Therefore, the activity observed for *O. europaea* provides a rationale for its use in treatment of dental infection diseases. The presence of phytomedicine in *O. europaea*

would be responsible for the demonstrated antimicrobial activity of the extracts.

Conclusion

O. europaea stems extracts possess a broad spectrum of activity against a panel of microorganisms responsible for the most dental diseases. This study can boost a new possibility for finding novel clinically effective antimicrobial compounds.

Conflict of Interests

The author(s) have not declared any conflict of interest.

ACKNOWLEDGEMENTS

The authors are thankful to the Head of Department of Botany and Microbiology, Gurukul Kangri University, Haridwar for providing necessary laboratory facilities to pursue this research work.

REFERENCES

Aboaba OO, Smith SI, Olude FO (2006). Antibacterial effect of edible plant extract on *Escherichia coli*. Pak. J. Nutr. 5**(**4):325-327.

Ahmad I, Mehmood Z, Mohammad F (1998). Screening of some Indian medicinal plants for their antimicrobial properties. J. Ethnopharmacol. 62:183-193.

Anesini C, Perez C (1993). Screening of plants used in Argentine folk medicine for antimicrobial activity. J. Ethnopharmacol. 39(2):119-128.

Burneti RW, Haber MH, Hackel E, Hanson CA, Keron DF, Lee Lewandrowski E (1994). Clinical Laboratory medicine, Philadelphia US: Williams and Wilkins.

Diallo D, Hveem B, Mahmoud MA, Betge G, Paulsen BS, Maiga A (1999). An Ethanobotanical survey of herbal drugs of Gourma district, Mali. Pharm. Biol. 37:80-91.

Gupta C, Garg AP, Uniyal RC, Kumari A (2008). Antimicrobial activity of some herbal oils against common food-borne pathogens. Afr. J. Microbiol. Res. 2:258-261.

Hardie JM (1982). The microbiology of dental caries. Dental Update 9 (4): 199-00, 202-04, 206-08.

Kaskoos RA (2013). Pharmacognostic Specifications of Leaves of *Olea europaea* Collected from Iraq. Am. J. Phytomed. Clin. Ther. 2(2): 153-160.

Khan MY, Panchal S, Butani A, Kumar V (2007). *Olea europaea*: A phyto-pharmacological review. Pharmacogn. Rev. 1(1):14-18.

Kononen E, Asikainen S, Jousimies-Somer H (1992). The early colonization of gram-negative anaerobic bacteria in edentulous infants. Oral Microbiology and Immunology 7:28-31.

Kononen E, Asikainen S, Saarela M, Karjalainen J, Jousimies-Somerv H (1994). The oral gram-negative anaerobic microflora in young children: Longitudinal changes from edentulous to dentate mouth. Oral Microbiol. Immunol. 9:136-41.

Marsh PD (1992). Microbiological aspects of the chemical control of plaque and gingivitis. J. Dent. Res. 71: 1431-1438.

Pereira AP, Ferreira ICFR, Marcelino F, Valentao P, Andrade PB, Seabra R, Estevinho L, Bento A, Pereira JA (2007). Phenolic compounds and antimicrobial activity of olive (*Olea europaea* L.) leaves. Molecules 12:1153-1162.

Perez C, Pauli M, Bazerque P (1990). An antibiotic assay by the agar well diffusion method. Acta Biol. Med. Exp. 15:113-115.

Ross IA (2005). Medicinal plants of the world, chemical constituents, traditional and modern medicinal uses. Humana Press Totowa, New Jersey. 3:373-81.

Scalbert A (1991). Antimicrobial properties of tannins. Phytochemistry 30:3875-3883.

Schupbach P, Osterwalder V, Guggenheim B (1995). Human root caries: microbiota in plaque covering sound, carious and arrested carious root surfaces. Caries Res. 29:382-395.

Sofowora A (1982). Medicinal plants and traditional medicine in Africa. John Wiley and Sons Ltd Nigeria. pp.33-34.

Trease E, Evans WC (1987). Pharmacognosy. Billiare Tindall, London, 13th Edn. pp. 61-62.

Ziad D, Elias A, Rould AM (2011). Antibacterial activity of *Rheum rhaponticum, Olea europaea* and *Viola odorata* on ESBL producing clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae.* Int. J. Pharm. Res. 2(7):1669-1978.

academicJournals

Vol. 8(37), pp. 3378-3381, 10 September, 2014
DOI: 10.5897/AJMR2014.6919
Article Number: 697D04E47900
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

16s rDNA sequence characterization and homogeneity of halophilic *Bacillus* sp. isolated from athalosaline lake (Al Jouf, Saudi Arabia)

Maher M. Al-Enazi[1,2], A. Z. El-Bahrawy[3], Bilal Ahmad Tantry[4] and Rahiman Shaik[5]*

[1]Vice Rector of Academic Affairs, Aljouf Univeristy, Saudi Arabia.
[2]Department of Clinical Laboratory Sciences, College of Applied Medical sciences, Salman Bin Abdulaziz University, Saudi Arabia.
[3]Faculty of Science, biology department , Aljouf University, Saudi Arabia.
[4]Department of Microbiology, College of Medicine, Aljouf University, Saudi Arabia.
[5]Department of Biochemistry, College of Medicine, Aljouf University, Saudi Arabia.

Received 21 May, 2014; Accepted 8 September, 2014

Moderate halophilic bacteria from Dowma al Jandal in extreme north Al Jouf were isolated using cultural-dependent methods. Halophiles from hypersaline environments possess a biotechnological potential. Polymerase chain reaction (PCR) amplification of the 16s rRNA gene and phylogentic analysis were used to identify strains. Culturing was done aerobically in chemically defined media (CDM). Salt concentration (15%) was used at pH 7.2. Universal bacterial primers were used to amplify 16SrDNA from chromosomal DNA isolated from the four distinct colonies. Four moderate halophilic bacterial isolates were analyzed and identified wih 16srDNA sequencing as *Virgibacillus salarius, Bacillus subtilis, Bacillus* sp., and *Virgibacillus marismortui*. Comparison of the 16srDNA sequence alignment to reference sequence data bases showed samples M1, M2, M3 and M4 have 95-99% homology. All of the four isolates had at least 95% similarity to the published sequences implying that they could be species within the described genera.

Key words: Moderate halophiles, hypersaline lake, *Bacillus* sp., 16srDNA sequencing.

INTRODUCTION

Halophiles employ different morphological, physiological, and genetic mechanisms to withstand the environmental conditions in which they live. In recent decades many halophilic microorganisms have been isolated from many athalosaline environments, such as the Dead lake (Arahal et al., 1999), the Great Salt Lake (Waino et al., 2000), the Solar Lake (Cytryn et al., 2000) and the Wadi lake (Weisser and Truper, 1985). Earlier studies reported the significance of halophilic microorganisms in the biogeochemistry of carbon and phosphorus in saline environments (Sánchez-Román et al., 2007) and degradation of organic compounds for potential use in bioremediation studies (Zhao et al., 2009,

*Corresponding author. Email: rahimhi@gmail.com. Tel: +966502450484.

Al-Mailem et al., 2010) and also used as biocontrol agents against certain pathogenic fungi (Sadfi-Zouaoui et al., 2008, Chen et al., 2010). The diversity amongst halophiles among the domains of bacteria, archaea, and eukarya depends on the salinity, temperature, pH and redox conditions of the environments that the organisms are adapted (Oren, 2008). Most of the halophilic and halotolerant micro-organisms represent the domain bacteria that contain moderate rather than extreme halophiles in its phylo-genetic subgroups (Oren, 2002). Kushner (1978) defined the moderate halophiles as organisms growing optimally between 0.5 and 2.5 M salt. Recent studies showed that an extensive research work has been done on isolation and characterization of large number of moderately halo-philic Gram-positive, endospore-forming aquatic isolates in the genus. *Bacillus* have been reported but most of that belongs to marine environments (Yoon et al, 2003; Noguchi et al., 2004; Lee et al., 2006). However, limited studies have been done regarding the microbial species inhabiting athalosaline environments (Lim et al., 2006; Souza et al., 2006). In this study, we characterized the moderate microorganisms using 16S rDNA sequencing techniques and compared the homogeneity with the relevant microorganism by NCBI BLAST analysis.

MATERIALS AND METHODS

Sample collection

Four saline soil samples were collected from Dowma Al Jandal athalosaline lake from random locations along the lake shore at 29° 48′ 41.1″ N, 39° 52′ 5.9″ E. Sample M1, M2 was obtained from loose saline soil. The third and fourth (M3, M4 respectively) was obtained from the lake sediment at 10 cm depth. Soil samples were collected in sterile bags and appropriately labeled and dated. The samples were transported in a cool box with ice packs and transported to the laboratory and stored at 4°C in College of Medicine Aljouf University. Samples were processed within 24 h of collection. Salinity of the saline soil samples was measured using a conductivity meter model KL-1385 (Kelilong Electron Co. Ltd, China).

Sample culturing

Soil sample (5 g) was inoculated into 45 ml on chemically defined medium (CDM). The contents of the CDM per liter were as follows: NaCl (81.0 g), $MgCl_2{\cdot}6H_2O$ (7.0 g), $MgSO_4{\cdot}7H_2O$ (9.6 g),$CaCl_2{\cdot}2H_2O$ (0.36 g), KCl (2.0 g), $NaHCO_3$ (60.0 mg), NaBr (26.0 mg), Ferric chloride (trace), Peptone (5.0 g), yeast extract (10.0 g), glucose (1.0 g), pH 7.2 ± 0.1 at 25°C (Kushner DJ, 1993). All components were added to distilled water and volume was brought up to 1.0 L. The medium was mixed thoroughly and gently heated until dissolved and then autoclaved. After 5 days of incubation, serial dilutions (10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}) of soil sample were made in sterile saline (0.85%). A 100 µL aliquot of each diluted sample was spread on chemically defined medium (CDM) agar (15-20% NaCl w/v) plates. Dilution of each soil sample was analyzed in triplicate. The plates were inverted and incubated for 3 to 7 days at 38°C. Results were recorded as colony forming unit (CFU). Colonies growing on the plates were counted and the density of microorganisms in the original sample was estimated by multiplying the colony count times the dilution.

Light microscopy

On day 7, selected isolated colony samples were characterized by gram-stain and by observed cell morphology (shape, size and color) using a light microscope (Leica DMD108 digital microscope).

Genomic DNA extraction

Genomic DNA was extracted from 5 ml late exponential Phase of culture growth, using Invitrogen Kit (USA) as per manufacture instructions.

PCR amplification and 16s ribosomal RNA sequencing

PCR amplification of the 16S rRNA gene from the purified genomic DNAs, was performed using ABI 9700 thermocycler (Applied Biosystems) using the primer sets, 16S forward primer AGAGTTTGATCHYGGYTYAG and the 16S reverse primer ACGGCTACCTTGTTACGACTT.

Purification of PCR products by gel elution protocol

The purification of the PCR products was done by using inhouse Gel elution kit.

DNA sequencing and analysis

Sequencing was done by using ABI 3730XLS sequencer. The unincorporated dye terminators were removed by using ABI Big dye Terminator kit. The chromatograms were analyzed by Genetool version 1.0. Assembly program is also carried out using the same program. The assembled sequences were analyzed by NCBI BLAST program (hhttp://ncbi.nlm.nih.gov/BLAST) to identify the microorganisms and compared with the published sequences.

RESULTS AND DISCUSSION

The four distinct moderate halophilic bacteria obtained from the saline soil of the Dowma al Jandal Lake design-nated as M1, M2, M3 and M4 were analyzed. The iso-lates were identified as *Virgibacillus salarius, Bacillus subtilis, Bacillus* sp*. and Virgibacillus marismortui.* Men-tioned isolates belong to the phylum of *Firmicutes* and *Bacillaceae* family. The salinity of the Dowma al Jandal athalosaline lake samples varied from a low of 1.3% to a high of 21%. Significant growth was observed after 5-7 days of the incubation, and occurred at 3-15% (W/V), NaCl (optimum 15%), pH 5-9 (optimum 7.2) and tempe-rature 35-40°C (optimum 38°C). All the isolates were Gram positive, strictly aerobic rods, occurring in pairs or small chains, cells were motile, colonies were white to pale yellow and mucoid. And were strongly catalase and oxidase positive.

Alignment and sequencing comparison of samples M1 and M4 show high homology (95-98%) to*Virgibacillus* sp. SK31 and *V. marismortui* TPA3-3 respectively, as shown

Table 1. BLAST Similarity Search Results for M1: Similarity to *Virgibacillus salarius* strain SA-Vb1 by 16S ribosomal RNA gene partial sequence.

Accession	Description	Max score	Total score	Max Identity (%)
NR_041270.1	*Virgibacillus salarius* strain SA-Vb1 16S ribosomal RNA, complete sequence	2372	2372	96
GU397387.1	*Virgibacillus marismortui* strain B33 16S ribosomal RNA gene, partial sequence	2368	2368	96
JN998437.1	*Virgibacillus* sp. SK2 16S ribosomal RNA gene, partial sequence	2366	2366	96
GQ181204.1	*Virgibacillus* sp. Ez223 16S ribosomal RNA gene, partial sequence	2366	2366	96

Table 2. BLAST Similarity Search Results for M2 : Similarity to *Bacillus subtilis* strain H12 by 16S ribosomal RNA gene, partial sequence.

Accession	Description	Max score	Total score	Max Identity (%)
KC441785.1	*Bacillus subtilis* strain H12 16S ribosomal RNA gene, partial sequence	2590	2590	99
JN641292.1	*Geobacillus stearothermophilus* strain DDKRC4 16S ribosomal RNA gene, partial sequence	2588	2588	99
JF411313.1	*Bacillus tequilensis* strain KM34 16S ribosomal RNA gene, partial sequence	2588	2588	99
JF411297.1	*Bacillus tequilensis* strain M60 16S ribosomal RNA gene, partial sequence	2588	2588	99

Table 3. BLAST Similarity Search Results for M3: Similarity *to Bacillus* sp. 2BSG-10NA-12 gene by 16S ribosomal RNA gene, partial sequence.

Accession	Description	Max score	Total score	Max Identity (%)
AB533728.1	*Bacillus* sp. 2BSG-10NA-12 gene for 16S ribosomal RNA, partial sequence	2440	2440	97
AB533758.1	*Bacillus* sp. 2BSG-PDA-4 gene for 16S ribosomal RNA, partial sequence	2435	2435	97
AB533743.1	*Bacillus* sp. 2BSG-10TSA-3 gene for 16S ribosomal RNA, partial sequence	2435	2435	97
GQ407241.1	*Bacillus* sp. DV2-37 16S ribosomal RNA gene, partial sequence	2435	2435	97
JF411333.1	Uncultured *Bacillus* sp. clone GT1-7 16S ribosomal RNA gene, partial sequence	2431	2431	97

in Tables 1 and 4. Samples M2 and M3 demonstrated close relation (97-99%) to *B. subtilis* H12 and Bacillus *sp*. 2BSG-10NA, respectively (Tables 2 and 3). All the isolates had at least 95% and up to 9% similarity to the described sequences within the phylum of *Firmicutes* and which in turn means that the isolates are closely related to genus level and belong to different species. Phylogenetic analysis gives an idea of relationship between the isolates which was made on the basis of 16S rDNA data. Phylogentic interferences made on the basis of 16S rDNA data indicated that the four isolates may be previously undescribed halophilic bacteria. It was the first molecular study conducted by us on virgin athalosaline lake in Dowma al Jandal. These four isolates may be previously undescribed moderate halophilic bacteria which belong to domain bacteria (Oren, 2002). The growth of halophiles mainly depends upon the salt concentration and other factors, like pH and media composition (Oren, 2002). Till date, the MIDI Sherlock microbial identification system does not contain the library for halophilic bacteria. The data and isolates obtained by us can be used to develop the database in MIDI system library for future identification of moderate halophilic bacteria. Furthermore, library can be constructed if the diversity of halophiles are extensively studied throughout the world. Building up a library for halophiles can assist the accurate identification of novel moderate halophiles. Molecular method is the most accurate way to identify the microorganisms than by the

Table 4. BLAST Similarity Search Results for M4: Similarity to *Virgibacillus marismortui* strain TPA3-3 by 16S ribosomal RNA gene, partial sequence.

Accession	Description	Max score	Total score	Max Identity (%)
GU172145.1	*Virgibacillus marismortui* strain TPA3-3 16S ribosomal RNA gene, partial sequence	1877	2440	95
EU435360.1	*Virgibacillus sp*. B1-21 16S ribosomal RNA gene, partial sequence	1871	2435	95
AY505533.1	*Virgibacillus marismortui* strain GSP17 16S ribosomal RNA gene, partial sequence	1871	2435	95
JN624920.1	*Virgibacillus* sp. NBSL35 16S ribosomal RNA gene, partial sequence	1869	2435	95
JF680941.1	*Virgibacillus sp*. IEGM 795 16S ribosomal RNA gene, partial sequence	1868	2431	95

identification of conventional methods, as a matter of fact the later can lead to identification problems.

Conclusion

The extreme environment of the Dowma al Jandal Lake still remains a virgin in exploring of halophiles. The results of this study will be useful to produce novel enzymes that will have potential biotechnological applications in biogeochemistry, bioremediation and can be applied as biocontrol agents. Such a unique environment in this lake provides a scope for further study of prokaryotic diversity.

Conflict of Interests

The author(s) have not declared any conflict of interest.

ACKNOWLEDGEMENTS

This work was made possible by the financial support of Aljouf University, Saudi Arabia, (Research Project No 24/33).

REFERENCES

Al-Mailem DM, Sorkhoh NA, Al-Awadhi H, Eliyas M, Radwan SS (2010). Biodegradation of crude oil and pure hydrocarbons by extreme halophilic Archaea from hypersaline coasts of the Arabian Sea. Extremophiles 14:321-328.

Arahal DR, Marquez MC, Volcani BE, Schleifer KH, Ventosa A (1999). *Bacillus marismortui* sp. nov., a new moderately halophilic species from the Dead Sea. Int. J. Syst. Bacteriol. 49(2):521-530.

Chen L, Wang G, Bu T, Zhang Y, Wang Y, Liu M, Lin X (2010). Phylogenetic analysis and screening of antimicrobial and cytotoxic activities of moderately halophilic bacteria isolated from the Weihai Solar Saltern (China). World J Microbiol. Biotechnol. 26:879-888.

Cytryn E, Minz D, Oremland RS, Cohen Y (2000). Distribution and diversity of Archaea corresponding to the limnological cycle of a hypersaline stratified lake (Solar Lake, Sinai, Egypt). Appl. Environ. Microbiol. 66(8):3269-3276.

Kushner DJ (1993). Growth and nutrition of halophilic bacteria. In: Vreeland RH, Hochstein L (eds), The biology of halophilic bacteria. CRC Press, Boca Raton, FL. pp 87-103

Kushner DJ (1978). Life in high salt and solute concentrations: halophilic bacteria. in Microbial life in extreme environments. ed Kushner D. J. (Academic Press, Ltd. London, United Kingdom). pp. 317-368.

Lee JC, Lim JM, Park DJ, Jeon CO, Li WJ, Kim CJ (2006). *Bacillus seohaeanensis* sp. nov., a halotolerant bacterium that contains L-lysine in its cell wall. Int. J. Syst. Evol. Microbiol. 56:1893–1898.

Lim JM, Jeon CO, Lee SM, Lee JC, Xu LH, Jiang CL, Kim CJ (2006). *Bacillus salarius* sp. nov., a halophilic, spore-forming bacterium isolated from a salt lake in China. Int. J. Syst. Evol. Microbiol. 56:373-377.

Noguchi H, Uchino M, Shida O, Takano K, Nakamura LK, Komagata K (2004). *Bacillus vietnamensis* sp. nov., a moderately halotolerant, aerobic, endospore-forming bacterium isolated from Vietnamese fish sauce. Int. J. Syst. Evol. Microbiol. 54:2117-2120.

Oren A (2002). Diversity Of Halophilic Microorganisms: Environments, Phylogeny, Physiology, And Applications. Ind. Microbiol. Biotechnol. 28:56-63.

Oren A (2008). Microbial life at high salt concentrations: phylogenetic and metabolic diversity. Saline Systems 4(2):13.

Sadfi-Zouaoui N, Essghaier B, Hhajlaoui MR, Fardeau ML, Cayaol JL, Ollivier B, Boudabous A (2008). Ability of moderately halophilic bacteria to control grey mould disease on tomato fruits. J. Phytopathol. 156:42-52.

Sánchez-Román M, Rivadeneyra MA, Vasconcelos C, McKenzie JA (2007). Biomineralization of carbonate and phosphate by moderately halophilic bacteria. FEMS Microbiol. Ecol. 61:273-284.

Souza V, Espinosa-Asuar L, Escalante AE, Eguiarte L, Farmer J, Forney L, Lloret L, Rodriguez-Martínez JM, Soberon X, other authors (2006). An endangered oasis of aquatic microbial diversity in the Chihuahuan desert. Proc. Natl. Acad. Sci. USA 103:6565-6570.

Waino M, Tindall BJ, Ingvorsen K (2000). *Halorhabdus utahensis* gen. nov., sp. nov., an aerobic, extremely halophilic member of the archaea from Great Salt Lake, Utah. Int. J. Syst. Evol. Microbiol. 50: 183-190.

Weisser J, Truper HG (1985). Osmoregulation in a new haloalkaliphilic Bacillus from the Wadi Natrun (Egypt). Syst. Appl. Microbiol. 6:7-11.

Yoon JH, Kim IG, Kang KH, Oh TK, Park YH (2003). *Bacillus marisflavi* sp. nov. and *Bacillus aquimaris* sp. nov., isolated from sea water of a tidal flat of the Yellow Sea in Korea. Int. J. Syst. Evol. Microbiol. 53:1297-1303.

Zhao B, Wang H, Mao X, Li R (2009). Biodegradation of phenanthrene by halophilic bacterial consortium under aerobic conditions. Curr. Microbiol. 58:205-210.

academicJournals

Vol. 8(37), pp. 3382-3391, 10 September, 2014
DOI: 10.5897/AJMR2014.6728
Article Number: 82D8ED747902
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Microbial and physico-chemical changes in tomato juice subjected to pulsed electric field treatment

V. Kayalvizhi and Usha Antony*

Department of Food Technology, Anna University, Chennai 600025, Tamil Nadu, India.

Received 22 February, 2014; Accepted 1 September, 2014

The possibility of microbial reduction by external pulsed electric field (PEF) offers a new application for pulsed power technology. Applying PEF technology to food pasteurization is a promising non-thermal method. One of such application is prevention of microbial growth, an effect that is based on irrreversible permiabilization of cell membranes. The objective of this study was to increase the shelf life of tomato juice using PEF method. It is reported that the microbes present in tomato juice were inactivated by PEF process using co-axial and co-field continuous treatment chambers. The samples were subjected to different electric field intensities (30 and 50 kVcm^{-1}) with the same flow rate (100 mL min^{-1}) and the same pulse number (150 pulses). After treatment, in both co-axial and co-linear chambers, greater reduction in microbial counts were seen at higher intensity of 50 kVcm^{-1}. The co-axial treatment chamber at 150 pulses was more effective than co-linear treatment chamber (1.16 and 0.77 log reduction).

Key words: Pulsed electric field, co-field continuous flow treatment chamber, co-axial continuous flow treatment chamber, tomato juice, microbial growth reduction.

INTRODUCTION

Pulsed electric field (PEF) application is the basic principle of operation in electroporation. Electroporation in cells is the process of destroying the cell membranes through application of high voltage short duration pulses across a liquid (Kishore et al., 2007). Li et al. (2009) have stated that the level of microbial inactivation by high pulsed electric fields is a function of process parameters (electric field strength and electrode-type, total treatment time and temperature, pulse duration and waveform/shapes), media factors (pH, antimicrobial and ionic compounds, conductivity and ionic strength) and microbial entity factors (strain-type and shape, concentration and growth phase of microbes). Previous research has demonstrated that the PEF microbial inactivation depends on two factors, electric field intensity and pulse number. With higher electric field intensity and more pulse number, a better pasterization effect can be obtained (El-Hag et al., 2009). This paper studies PEF microbial inactivation in tomato juice by using co-field and co-axial continuous treat-ment chambers to provide a technical basis for industrial application (Qin et al., 1998).

The mechanisms of microbial inactivation in liquid

*Corresponding author. E-mail: ushaantony@annauniv.edu. Tel: +91 44 22358379. Fax: +91 44 22350299.

.

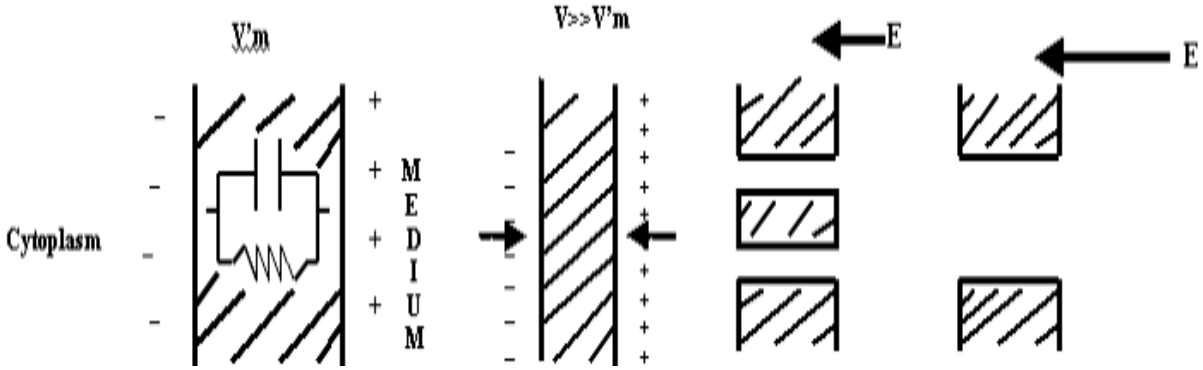

Figure 1. Dielectric Breakdown of Cell Membrane (Zimmermann. 1986).

foods (Raso and Heinz, 2006; Huang and Wang, 2009; U.S FDA,2009), and the various treatment chambers available (Gaouda et al., 2010; Min et al., 2007) have been discussed. In our earlier study, we reported reduction of microbes in tomato juice using static and co-axial PEF treatment at same intensities (Sathyanathan et al., 2012). The present simulation study was carried out on samples using two dfferent continuous treatment chamber (co-axial and co-linear). Continuos treatment chambers have the advantage of treating larger volumes. Major components in the prototype include a high voltage repetitive pulse generator 220 V/100 kV with maximum stored energy of 490 J, charging capacitor of 50 nF and the wave forming network that has resistance of 22 Ω and tail forming resistance of 1200 Ω.

Microbial inactivation mechanisms

The two theories that explain the mechanism of breakdown of cell membrane are Dielectric Rupture Theory and Electroporation Theory.

Dielectric rupture theory

The cell membrane is considered as a capacitor filled with dielectric material whose dielectric constant of the order of two. Most foods have a dielectric constant in the range of 60 to 80 (Raso and Heinz, 2006). As a result, free charges accumulate at both membrane surfaces which is shown in Figure 1 (Zimmermann, 1986). Exposure of the cell membrane to an electric field leads to an increase in transmembrane potential (TMP) (Huang and Wang, 2009). The increase in TMP leads to a reduction in the membrane thickness. The TMP of the cell membrane is given as:

$$U(t) = 1.5rE\cos\theta \qquad (1)$$

Where, U (t) Transmembrane potential (V); r Radius of the cell (mm); E Applied electric field strength (Vmm^{-1}); θ Angle between a given membrane site and the field direction (degrees).

Electroporation theory

Electroporation occurs in a cell which when exposed to the high-voltage electric fields temporarily destabilizes the lipid bi-layer and proteins of the cell membrane. In a cell membrane, protein channels, pores and pumps are present. The opening and closing of many channels constituted by proteins is dependent on TMP. When PEF is applied, many voltage sensitive protein channels will open. Protein channels, once open, will experience current much larger than the current normally experienced by the protein channels during metabolic activities. As a result, protein channels are irreversibly denatured by joule heating. Thus, electroporation in the cell membrane occurs both in the protein channels and in the lipid bi-layer resulting in the inactivation of the cell (Min et al., 2007).

During electroporation, lipid bi-layer suffers from any one of the following possible fates on the membrane namely (Figure 2):

1. A slight increase in membrane conductance.
2. Mechanical rupture of the cell wall or any structural changes in the membrane.
3. Reversible electrical breakdown resulting in complete discharge of the membrane.
4. Irreversible electrical breakdown when the intensity of the electric field is raised (Sundarrajan et al., 2008).

PEF microbial inactivation system

The block diagram of PEF bacteria inactivation system is shown in Figure 3. The continuous treatment chamber adopts co-linear electrode and co-axial electrode respectively and the sample is exposed to exponentially

.

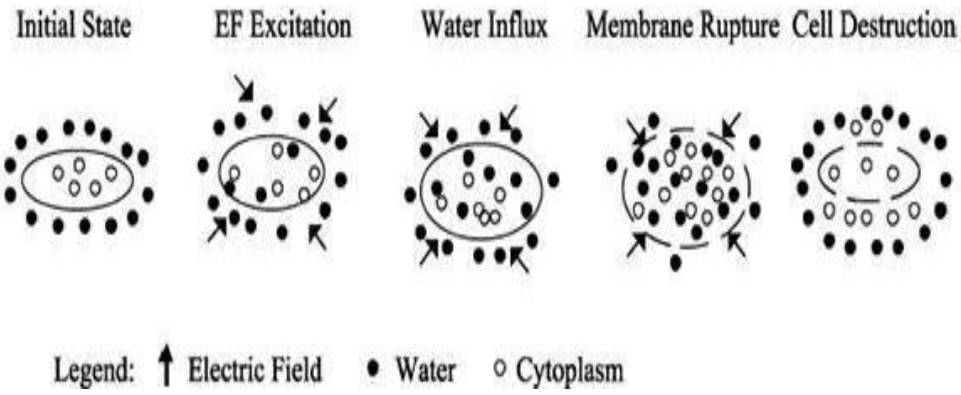

Figure 2. Electroporation of cell membrane (Wu et al., 2004).

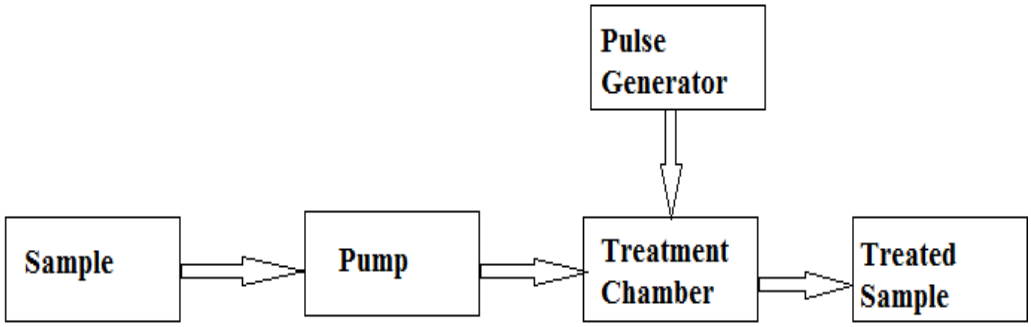

Figure 3. Block diagram of PEF bacteria inactivation system

decaying electric field.

MATERIALS AND METHODS

Design of treatment chamber and its electric field analysis

In this study, co-axial treatment chamber and the co-linear treatment chamber models were taken and simulation studies were carried out. The treatment chamber was made with aluminum and teflon as electrode material and insulation material. The relative permittivity of aluminum is 1. The inner high voltage electrode has the radius of 4 cm and the outer ground electrodes has the radius of 4 cm; in between these electrodes, a gap of 2 cm is used as the flow path of liquid food. This insulator gives the support to the positioning of the electrodes and gives the smooth flow of the liquid food inside the treatment chamber. The electrical current flows perpendicular to food flow in co-axial treatment chamber and parallel to food flow in co-linear chamber (Min et al., 2007). The modified co-axial and co-linear treatment chambers widely used in industrial applications is shown in Figure 4 (Sale and Hamilton, 1967).

Simulation of co-axial and co-linear treatment chamber

Here co-axial and co-linear continuous flow treatment chambers have been modeled for simulation studies using a software package Maxwell 2D simulator (ANSOFT). The ANSOFT model for co-axial and co-linear treatment chamber is as shown in Figure 5. The primary objective of the simulation is to ensure uniform field distribution across the effective treatment region. Finite element method analysis is used to measure field distribution inside the treatment chamber's effective treatment region. The problem definition of simulation studies on co-axial and co-linear treatment chambers are given below.

Problem definition

Problem statement: Analysis of electrostatic field distribution in the effective treatment region.
Domain: The field domain is enclosed in a boundary. All space outside this boundary is excluded from field problem domain. This is an interior problem.
Type of problem: Electrostatic problem as liquid foods are usually negligibly magnetizable.
Governing equation: This electrostatic field simulator solves for the electric potential Φ, in the Laplace equation, $\nabla^2\Phi=0$.
Plane: Axis symmetry (RZ Plane) has been used for this simulation.
Boundary condition: Balloon boundary condition has been used for this simulation.

The following design aspects have been considered during the design of PEF treatment chambers.

.

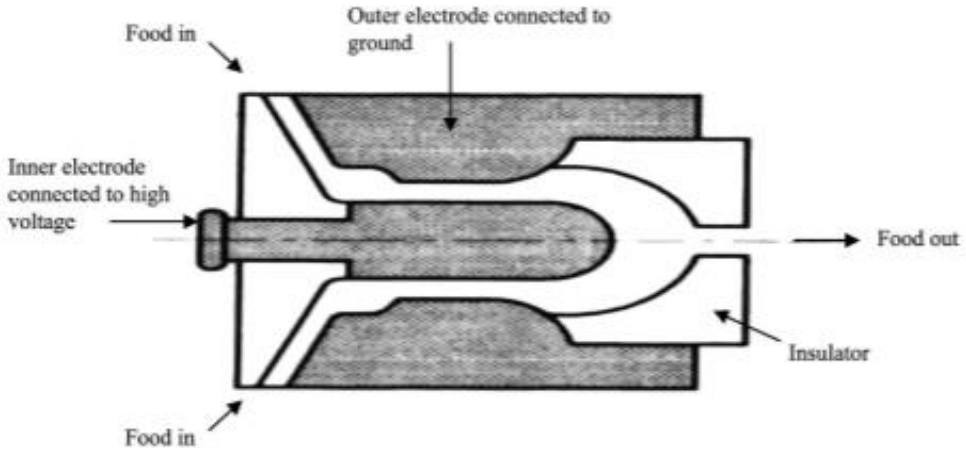

Figure 4. Diagram of the co-axial treatment chamber (Min et al. 2007).

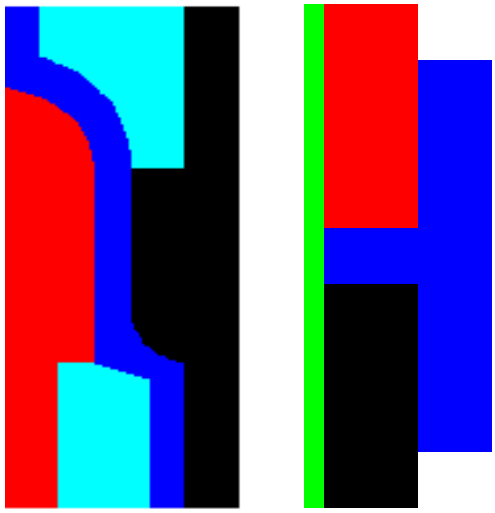

Figure 5. ANSOFT model for co-axial and co-field treatment chambers.

Electrode material

During the PEF treatment, electrochemical reactions can occur and they may result in partial electrolysis of the solution, corrosion of the electrode and introduction of particles of the electrode material in the liquid. These phenomena should be minimized because they could provoke microbial inactivation and over estimate microbial inactivation by PEF. The electrodes can be made up of stainless steel and electro-chemically inert materials such as gold, platinum, carbon and metal oxides.

Electric field strength

Dielectric breakdown of food occurs, when the strength of applied electric fields exceeds the electric field strength of the food product treated in the treatment chamber. This breakdown mechanism of the food is generally characterized as causing damage on the electrode surfaces in the form of pits, a result of arching and increased pressure, leading to treatment chamber explosions and evolution of gas bubbles.

Electrode gap

Electric field strength in a treatment zone of a PEF chamber is inversely proportional to the cross sectional area of the fluid flow and to the gap distance between the two electrodes. The electrode gap is chosen such that the treatment area has uniform field to ensure all fluids have undergone the field.

Insulator

In continuous PEF treatment system, insulators are used to ensure the separation of electrodes and to provide the smooth flow of liquid between the electrodes. Plexi glass is the most commonly used insulator in the PEF treatment chamber system.

Uniform field distribution

Distribution of the field strength depends on the characteristics of the treatment chamber, basically shape of electrodes, gap of the chamber, gas impurities in electric materials. The treatment chamber should be designed to provide uniform electric field strength, such that the actual applied field strength does not exceed the dielectric strength of the fluid foods under the treatment. The probability of dielectric breakdown in foods can be reduced by the use of a smooth electrode surface to minimize electron emission and the use of round electrode edges to prevent electric field enhancement near sharp edges.

PEF treatment

The Marx's generator circuit and the complete experimental setup for the continuous flow PEF treatment of tomato juice processing is shown in Figures 8 and 9. The tomato juice was prepared fresh as described in the earlier study (Sathyanathan et al., 2012). Marx's generator circuit was used to produce impulse voltages of 1.3/45 µs

.

Table 1. Applied field and pulses for PEF treatment of tomato juice,

Liquid food	Type of treatment chamber	Applied field (kV/cm)	Pulse number	Duration (seconds)
Tomato juice	Co-axial	30	150	110
	Co-axial	50	150	240
	Co-axial	50	300	240
	Co-linear	30	150	110
	Co-linear	50	150	240

as shown in the Figure 10. Tomato juice was treated with optimized test voltages and pulse rate as shown in the Table 1. The control and treated samples were stored in autoclaved, wide-mouthed, screw-capped glass bottles 75% fill, at room temperature (25±3°C). The experiment was repeated twice.

Analysis of shelf life

Chemical and microbial tests on control and treated samples were carried out in the Food Safety Laboratory of National Agro Foundation, Anna University Campus, Taramani to analyze shelf life of the tomato juice. These tests were carried on the first day and seventh day of PEF treatment. The PEF treatments were carried out on two separate batches of tomato juice on two different days and all chemical, nutritional and microbial analyses were done in duplicates. All chemical and reagents used were of analytical grade.

Chemical and nutritional analysis

The chemical tests were done to analyze whether the treated food had tolerable levels of chemical properties for human consumption as given by U.S FDA (2009). pH, °Brix and acidity were tested. pH value of tomato juice was measured by using AP-1plus pH meter (Susima Technologies, Chennai) and the °Brix value of tomato juice was measured by °Brix meter (Arico India hand refractrometer). pH and acidity value of liquid food for human consumption should be less than 5 and 0.4%, respectively. The titratable acidity was estimated in tomato juice filtrate by titration with 0.1 N sodium hydroxide to the end point with phenolphthalein indicator. Further, vitamin C content of the juice was tested by the reduction of the blue dye 2, 6 dichlorophenolindophenol by ascorbic acid (AOAC, 2000).

Microbial analysis

Both control and treated samples were analyzed for microbes present by suitable serial dilution and pour plate method using standard plate count agar (Hi-Media Ltd, Mumbai). Plates were incubated at 37°C for 48 h. The colony counts were carried out using a digital colony counter (Lapiz colony counter). The total bacterial counts were carried out on the same day and seventh day (Harrigan and McCance, 1998).

RESULTS AND DISCUSSION

Simulation results

From the simulation study, it was clearly seen that there were no flux lines inside the conductors (field inside the conductor is always zero) and the electricfield lines start from the electrode with positive charge and terminate at the electrode with zero potential. The electric field is not constant due to fringing effect and it gets equally distributed in between the electrodes. Equal stress distribution between the contacts is due to equal contact gap distance between the electrodes in the effective treatment region as shown in Figure 6. The intensity of the electric field is maximum near the high voltage electrode and it decreases when the distance increases as shown in Figure 7. The uniform electric field distribution is achieved across the effective treatment region of the chamber (Schroder et al., 2009)

Chemical test results

There was no significant difference ($p < 0.05$) in the chemical properties of tomato juice with respect to pH, acidity, °Brix values in both control and treated samples in co-axial and co-linear continuous treatment chambers (Table 2). The PEF treatment and ionic strength are responsible for electroporation and compression of the cell membrane, where as pH of the medium affects the cytoplasm when the electroporation is complete. U.S FDA reported that, depending on the microorganism present in the liquid medium, acidic pH enhanced microbial inactivation. No mention has been made on what microorganisms were affected or what range of pH was used (U.S FDA, 2009). Vitamin C content also did not change significantly in all the treated samples. In both treatment chambers, only 0.9 and 1.0% reduction of vitamin C for the applied field of 30kVcm^{-1} and only 2.0 and 4.0% reduction for the applied field of 50 kVcm^{-1} was observed, which is in accordance with the reports of Zhang et al. (2003). Thus, the chemical properties and vitamin C content did not vary much when fresh tomato juice was subjected to PEF in both co-axial and co-linear continuous treatment chambers.

Microbial test results

Both PEF treatments in co-axial and co-linear chambers

.

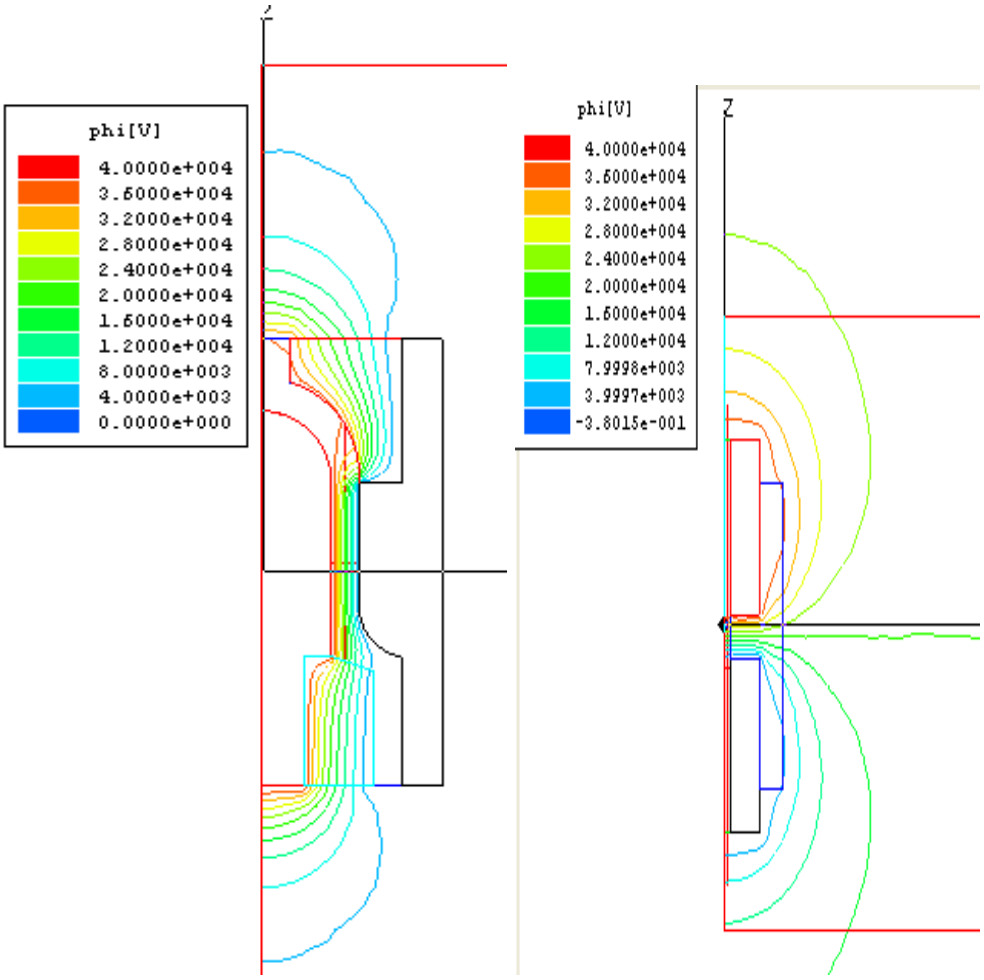

Figure 6. Phi Plot for the co-axial and co-field treatment chambers.

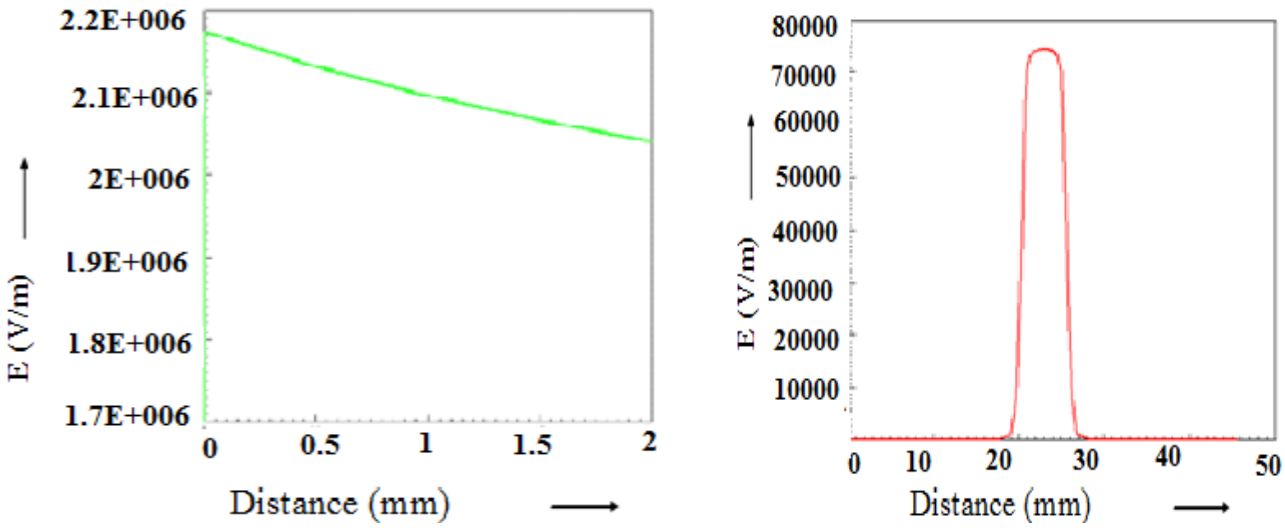

Figure 7. Field plot for the co-axial and co-field treatment chambers.

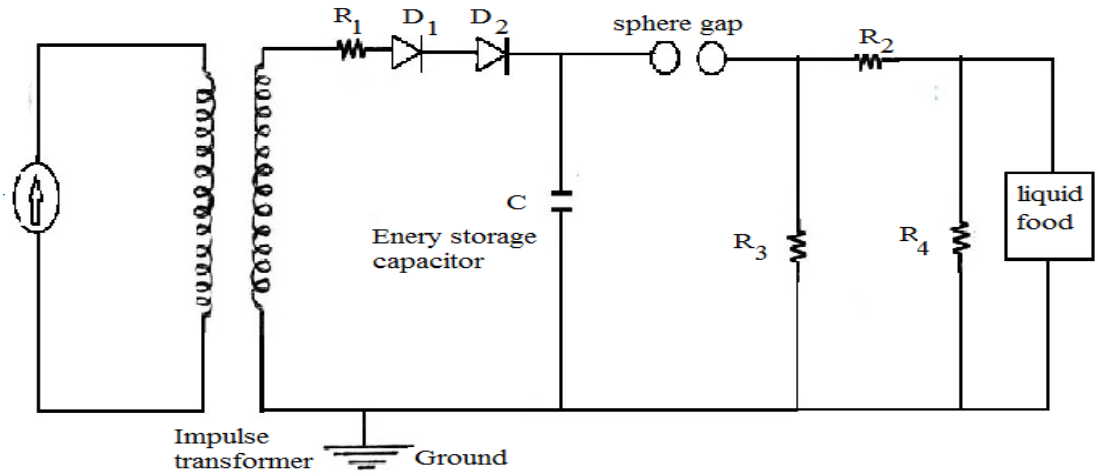

Figure 8. Circuit for producing impulse voltage wave.

Figure 9. Complete experimental setup for single stage impulse generator.

caused significant reduction in microbial content (Table 3). While 30 kVcm^{-1}, 150 pulses/110 s resulted in 0.82 and 0.35 log cfumL^{-1} reduction of microbes for first day and there was 0.87 and 0.37 log cfumL^{-1} reduction for seventh day in co-axial and co-linear chamber, respectively (Figure 11). Higher field strength of 50 kVcm^{-1}, 150 pulses/110 s showed 1.09 and 0.66 log cfu mL^{-1} microbial reduction for first day and 1.16 and 0.77 log cfu mL^{-1} reduction for seventh day in co-axial and co-linear chambers, respectively (Figure 12). It is thus clearly seen

.

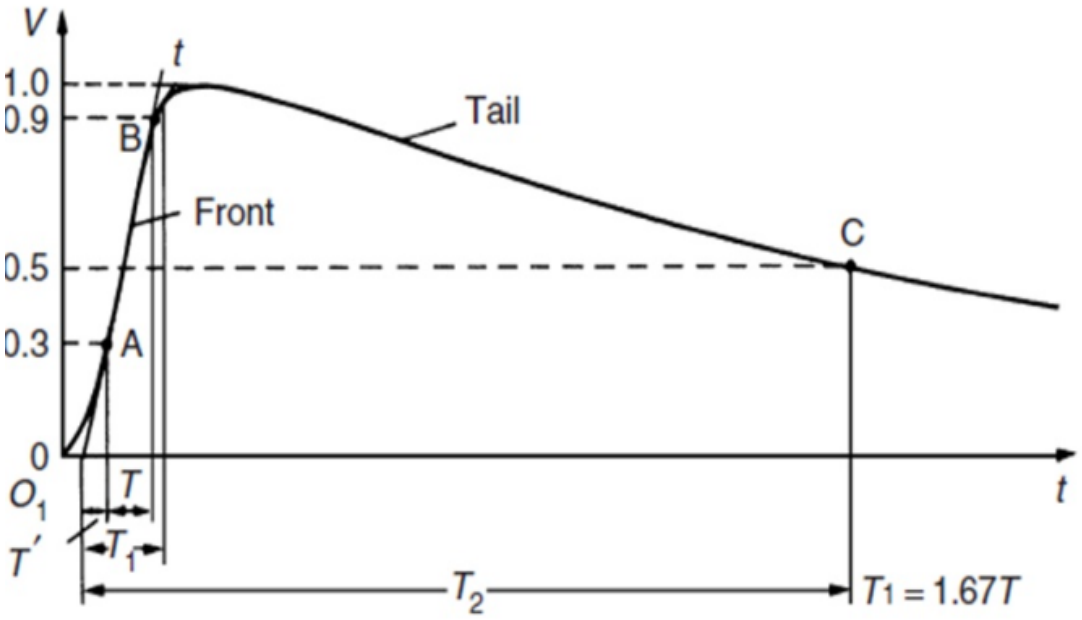

Figure 10. Test impulse wave form.

Table 2. Chemical changes in tomato juice after PEF treatment in co-axial and co-linear continuous chambers.

Treatment chamber	Sample	pH		Acidity* (%)		°Brix		Vitamin C* (%)	
		1st Day	7th Day	1st Day	7th Day	1st Day	7th Day	1st Day	7th Day
Untreated co-axial	Control	4.47±0.33	4.47±0.13	0.19±0.1.	0.16±0.4	6±0.13	6±0.16	15.22±0.71	14.56±0.04
	30kv, 150 Pulses	4.22±0.02	4.47±0.33	0.19±0.7	0.16±0.2	6±0.15	6±0.13	15.16±0.04	14.44±0.42
	50kv, 150 Pulses	4.20±0.02	4.71±0.03	0.19±0.2	0.17±0.2	6±0.13	6±0.14	15.16±0.04	14.83±0.19
	50kv, 300 Pulses	4.17±0.03	4.47±0.35	0.19±0.1	0.16±0.1	6±0.12	6±0.12	15.08±0.57	14.80±0.09
Co-linear	30kv, 150 Pulses	4.36±0.08	4.39±0.07	0.28±0.3	0.28±0.3	6±0.13	6±0.13	15.18±0.07	14.84±0.03
	50kv, 150 Pulses	4.33±0.09	4.41±0.94	0.28±0.1	0.28±0.2	6±0.12	6±0.11	15.18±0.01	14.56±0.01

Acidity* - Equivalent to % citric acid; Vitamin C*: Equivalent to % ascorbic acid.

Table 3. Microbial counts on first day after PEF treatment in co-axial and co-linear continuous treatment chambers.

Type of treatment chamber	Applied field (kV/cm)	Pulse number	Microbial count Log cfu mL^{-1}	Microbial count reduction Log cfu mL^{-1}
Control	Untreated	Nil	4.09	0.00
Co-axial	30	150	3.27	0.82
Co-axial	50	150	3.00	1.09
Co-linear	30	150	3.74	0.35
Co-linear	50	150	3.43	0.66

that higher microbial reduction was achieved by using co-axial treatment chamber at 50 $kVcm^{-1}$, 150 pulses. Inhibition of microbial growth and better shelf life extension can also be achieved at 50 $kVcm^{-1}$, 300 pulses in co-axial treatment chamber without much affecting the nutritional value of tomato juice (Table 4).

.

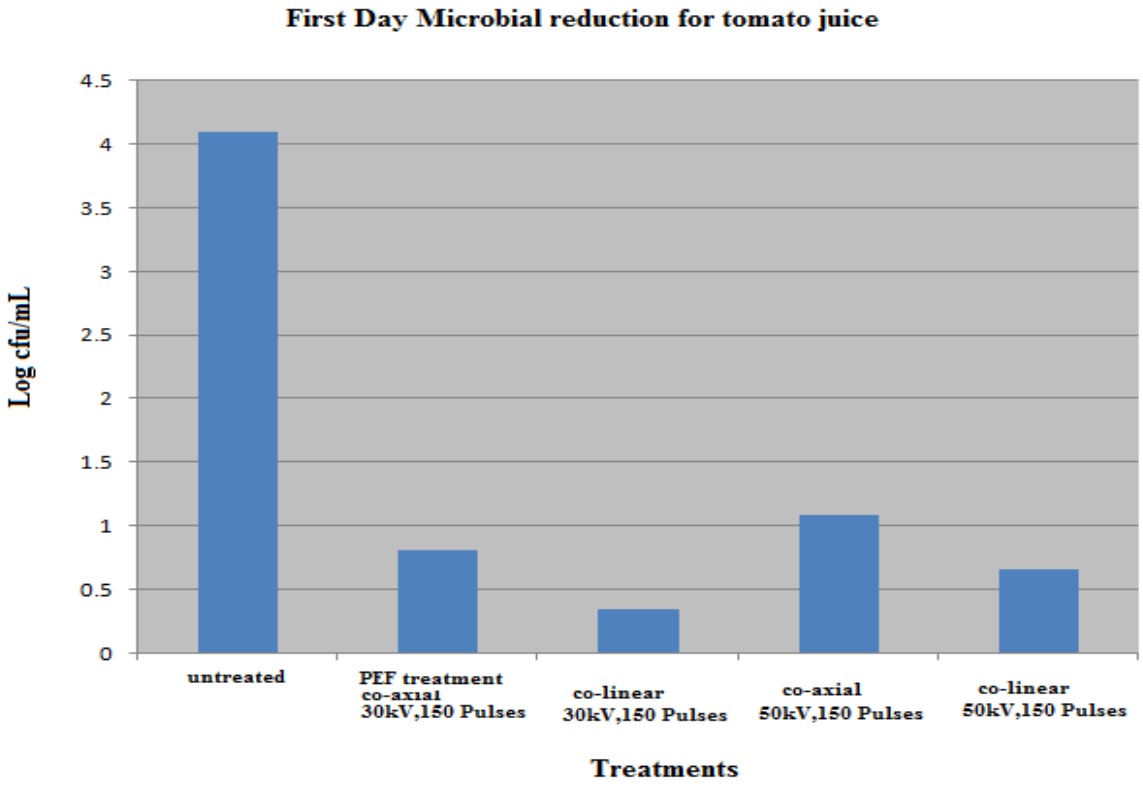

Figure 11. Microbial reduction in tomato juice on first day PEF treatment.

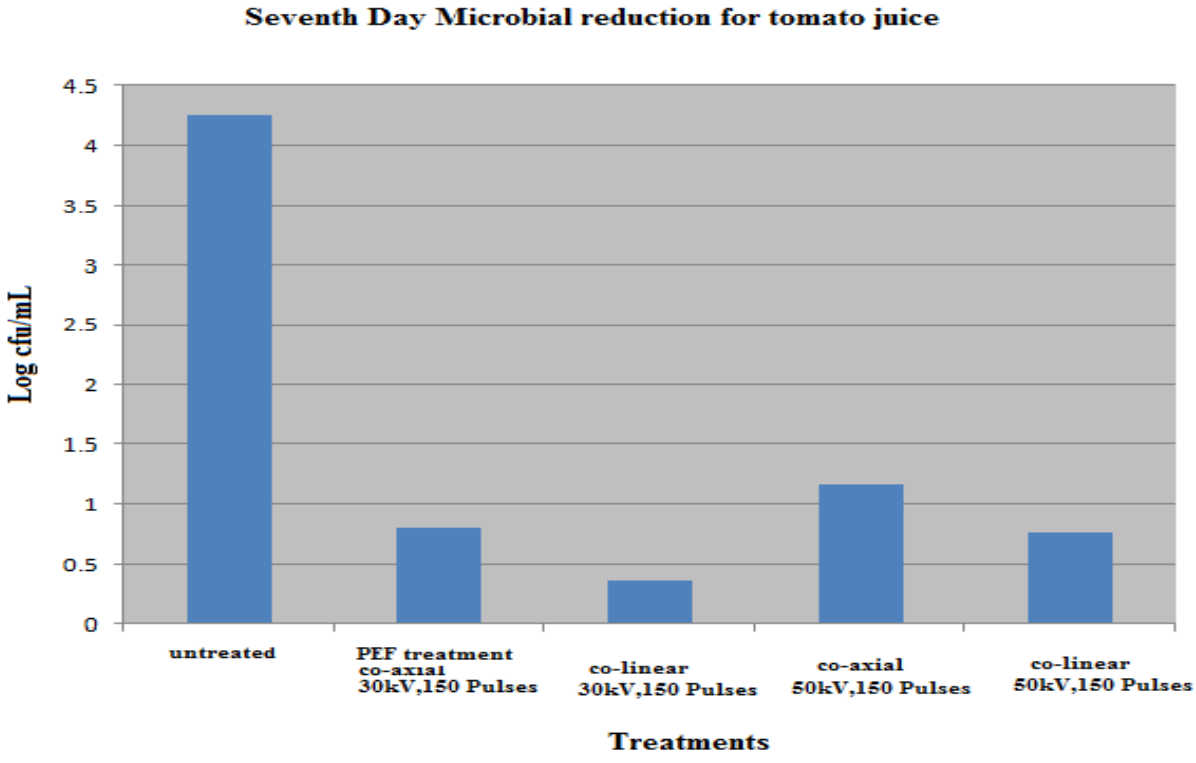

Figure 12. Microbial reduction in tomato juice on seventh day of PEF treatment.

.

Table 4. Microbial reduction on the seventh day after PEF treatment in co-axial and co-linear continuous treatment chamber.

Type of treatment chamber	Applied field (kV/cm)	Pulse number	Microbial count Log cfu mL^{-1}	Microbial count reduction Log cfu mL^{-1}
Control	Untreated	Nil	4.26	0.00
Co-axial	30	150	3.39	0.87
Co-axial	50	150	3.10	1.16
Co-linear	30	150	3.89	0.37
Co-linear	50	150	3.49	0.77

Conclusion

PEF treatments of tomato juice samples in co-axial and co-linear continuous treatment chambers at 30 $kVcm^{-1}$,150 pulses and 50 kV/cm,150 pulses were performed. Chemical properties- pH, acidity, °Brix value maintained were almost the same in both co-axial and co-linear treatment chambers and vitamin C showed little change for all treated samples. Our results indicate that shelf life extension of the tomato juice at room temperature (25±3°C) for seven days is possible in both co-axial and co-linear continuous treatment chamber. However, better shelf life was achieved by higher electric field with higher number of pulses (50 kV/cm, 150) in co-axial treatment chamber. Further extension of shelf life will be possible by storage under refrigirated conditions after PEF treatment.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

We gratefully acknowledge Division of High Voltage Engnieering, Anna University, Chennai http://www.annauniv.edu/HighVoltage/index.php and Food Safety and Standards Division, “National Agro Foundation”, Centre for Biotechnology, Taramani, Chennai for the provision of laboratory facilities.

REFERENCES

AOAC (2000). Official Methods of Analysis: Method No.27.1.18A, 15th ed. Association of Official Analytical Chemists, Washington, DC.

El-Hag AH, Otunola A, Jayaram SH, Anderson WA (2009). Reduction of microbial growth in milk by pulsed electric fields: IEEE Explore, 446-478.

Gaouda AM, Ayman H, El-Hag, Jayaram SH (2010). Detection of discharge activities during pulsed electric field food processing: IEEE: 16-22.

Harrigan WF, McCance ME (1998). Laboratory Method in Food and Dairy Microbiology. Academic Press, London. ISBN 0123260434

Huang K, Wang J (2009). Design of Pulsed Electric field Chambers for liquid food pasteurization process: A review. J. Food Eng. 95(2):227-239.

Kishore NK, Emani SS, Maiti TK, Bisht GS (2007). Studies on pulsed electric field applications for food sterilization: IEEE Second International Conference on Industrial and Information Systems, ICIIS2007:8-11.

Li J, Wei X, Xu X, Wang Y (2009). Bacteria inactivation by PEF with co-axial treatment chamber and Tube plate Treatment chamber: IEEE Proceedings of the 9th International Conference on Properties and Applications of Dielectric Materials: Vol 978:4244-4368.

Min S, Evrendilek GA, Zhang HQ (2007). Pulsed Electric Fields: Processing System: Microbial and enzyme inhibition, and shelf life extension of foods. IEEE: 59-73.

Qin BL, Barbosa-C´anovas GV, Swanson BG, Pedrow PD, Olsen RG, (1998). Inactivating microorganisms using a pulsed electric field continuous treatment system. IEEE Trans. Ind. Appl. 34(1):43-50.

Qin BL, Zhang Q, Barbosa-Canovas GV, Swanson BG, Pedrow PD (1995). Pulsed electric field treatment chamber design for liquid food pasteurization using the finite element method: Trans. ASAE 38(2):557-565.

Raso J, Heinz V (2006). Pulsed electric fields technology for the Food industry:Springer Science Business Media, LLC. pp. 1-16.

Sale AJH, Hamilton WA (1967). Effects of high electric fields on microorganisms:Biochim. Biophys. Acta. 148:781-788.

Sathyanathan T, Kayalvizhi V, Gowri Sree V, Sundararajan R (2012). Microbial growth reduction in tomato juice by pulsed electricfield with co-axial treatment chamber: IEEE10th International Conference on the Properties and Applications of Dielectric Materials.

Schroder S, Buckow R, Knoerzer K (2009). Numerical simulation of pulsed electric fields (PEF) processing for chamber design and optimization: Seventh International Conference on CSIRO: 153-161.

Sundarrajan R, Campbell D, Harper J, XiaoF, Ma R, Otto K (2008). Characterization of fruit juices treated with electrical pulses: 2008 Annual report Conference on Electrical Insulation Dielectric Phenomena, IEEE Explore: 925-929.

US FDA (2009). Kinetics of microbial inactivation for alternative food processing technologies - Pulsed Electric Fields. Posted on the website of the U. S. Food and Drug Administration: Center for Food Safety and Applied Nutrition.

Wu TF, Tseng SY**,** Hung JC (2004). Generation of pulsed electric fields for processing microbes. IEEE Trans. Plasma Sci. 32:1551-1562.

Zimmermann U (1986). Electrical breakdown, electropermeabilization and electrofusion. Rev. Physiol. Biochem. Pharmacol.105:175-256

Zhang QH, Min S, Jin ZT (2003), Commercial scale pulsed electric field on tomato juice. J. Agric. Food Chem. 51(11)3338-44.

.

academicJournals

Vol. 8(37), pp. 3392-3397, 10 September, 2014
DOI: 10.5897/AJMR2014.6856
Article Number: 8C1CC5847905
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Antimicrobial profile of multidrug-resistant *Staphylococcus* spp. isolated from bovine mastitis cases in the northwest region of Paraná State, Brazil

Noemila Débora Kozerski[2], Jorge Luiz Pereira De Oliveira[1,2], Rodrigo Assunção Moura[1], Danilo Ratti da Silva[2], Aliny Fernanda De Oliveira[2], Priscila Luiza Mello[1], Renata Olivotto Agostinis[2], Daniela Dib Gonçalves[1], Luciana Kazue Otutumi[1] and Lisiane de Almeida Martins[1]*

[1]Department of Veterinary Preventive Medicine, Paranaense University (UNIPAR), Paraná State, Brazil.
[2]School of Veterinary Medicine, Paranaense University (UNIPAR), Paraná State, Brazil.

Received 25 April, 2014; Accepted 22 August, 2014

Bovine mastitis is the main cause of economic losses in dairy industry. The aim of this study was to determine the prevalence and antibiotic-resistance profile of *Staphylococcus aureus* and coagulase-negative *Staphylococcus* spp. isolated from bovine mastitis cases in the municipality of Altônia, northwest region of Paraná State, Brazil. Two hundred and forty-eight dairy cows were examined for the presence of mastitis. Milk samples from mastitis cases were collected and analyzed for the presence of *Staphylococcus* spp. according to the National Mastitis Council (NMC). The antimicrobial susceptibility of the isolates was also evaluated. The results suggest the presence of methicillin-resistant strains in high frequency (20.40%). Hence, the determination of antimicrobial resistance profile is essential to control mastitis in dairy cows. The use of antibiogram may also help to reduce the emergence of multidrug-resistant strains, since the choice of antibiotics will not be based on empirical methodology.

Key words: *Staphylococcus aureus*, coagulase-negative staphylococci, multidrug resistance, methicillin-resistance *Staphylococcus aureus*.

INTRODUCTION

Bovine mastitis is the main cause of economic losses in dairy industry due to reduced milk yield and increased expenditure on treatment of affected cows (Schroeder, 2012; Kateete et al., 2013). Among the bacterial species capable of causing this disease, *Staphylococcus aureus* and coagulase-negative *Staphylococcus* spp. (CoNS) are the major etiological agents in many countries (Moon et al., 2007; Santos et al., 2007; Sawant et al., 2009; Taponen and Pyörälä, 2009; Guimarães et al., 2012; Jamali et al., 2014; Supré et al., 2014). Despite implementing control measures, it is difficult to eradicate mastitis in dairy cows because many strains are resistant to the antimicrobial drugs currently used (Güler et al., 2005; Jamali et al., 2014).

The bovine mastitis may be treated with a variety of antimicrobial classes, including penicillins, tetracyclines,

*Corresponding author. E-mail: lisiane.almeida.martins@gmail.com.

lincosamides, macrolides and fluoroquinolones. However, the practice of incorporating antibiotics into feeds to prevent infectious diseases and the inadequate use of antibiotics in therapy allowed the emergence of multi-drug-resistant (MDR) strains (Jamali et al., 2014).

The isolation of MDR *S. aureus* and CoNS, including methicillin-resistant isolates, from bovine mastitis cases in Brazil and other countries has been reported with relatively high frequency (Zanette et al., 2010; Ferreira et al., 2007; Guimarães et al., 2012). Hence, the characterization of the antibiotic-resistance profile of these bacteria is crucial for a correct antibiotic choice and an effective mastitis treatment. The aim of this study was to determine the prevalence and antibiotic-resistance profile of *S. aureus* and CoNS isolated from bovine mastitis cases in the municipality of Altônia, northwest region of Paraná State, Brazil.

MATERIALS AND METHODS

Sample collection

The survey was conducted with 248 dairy cows from Holsteins, Jersey and Girolando breeds, belonging to 24 properties located in the municipality of Altônia, northwestern region of Paraná state, Brazil. The selection of a representative number of animals to study was performed from a list of 82 properties, totaling of 840 animals, using BioEstat program 5.0 (Ayres, 2007).

All the milk samples were collected from July 2009 to December 2010. The dairy cows were examined for the presence of mastitis both clinically and by the use of screening test California Mastitis Test (CMT). Milk samples were collected only from dairy cows with positive CMT. Approximately 30 mL of milk were collected from each mammary quarter in sterile flasks of 50 mL, according to the National Mastitis Council (NMC, 2004). The samples were kept at 4.0°C until processing at the Laboratory of Veterinary Preventive Medicine and Public Health at Paranaense University (UNIPAR). The microbiological analysis was performed using milk pools from the four mammary quarter samples of the same animal.

Bacterial isolation and identification

The milk samples were plated on blood agar and incubated at 37°C for 24 h for colony isolation. After this period, the isolates were identified according to the National Mastitis Council (NMC, 2004).

Antimicrobial susceptibility

The antimicrobial susceptibility tests were performed according to CLSI (2013). The disc diffusion method was performed using the following antibiotics: gentamicin (10 µg); ampicillin (10 µg), oxacilin (1 µg), penicillin (10 UI); cephalothin (30 µg), ceftiofur (30 µg); enrofloxacin (5 µg); trimethoprim-sulfamethoxazole (25 µg), clindamycin (2 µg), erythromycin (15 µg) and tetracycline (30 µg). The antibiotics were selected according to the classes available for mastitis treatment in Brazil.

RESULTS

Among the 248 animals studied, 82 (33.0%) were positive for mastitis on CMT. Of these, 54/82 (65.00%) animals confirmed the presence of mastitis on the microbiological analysis of milk samples. The overall prevalence of mastitis among all the animals studied was 21.80% (54/248 animals). The microbiological tests identified 59 different isolates among 54 milk samples. In three samples, it was possible to identify two or more isolates from different bacterial species. The presence of a yeast isolate was found in one milk sample.

The most prevalent bacteria was *Staphylococcus aureus,* representing 29 out of 59 (49.10%) isolates, followed by CoNS with 14 (23.70%) isolates; *Streptococcus dysgalactiae* 3 (5.10%); coagulase-positive/VP-negative *Staphylococcus* spp. 3 (5.10%); *Corynebacterium* sp. 3 (5.10%); *Enterococcus faecalis* 2 (3.40%); and *Trueperella pyogenes*, *Enterococcus* sp., *Streptococcus* sp., *Streptococcus acidomihimus* and yeasts with one (1.70%) isolate each.

The antimicrobial susceptibility tests were performed only with the most common isolates of *S. aureus*, CoNS, *S. dysgalactiae* and coagulase-positive/VP-negative *Staphylococcus* spp. because of its importance in the mastitis epidemiology in Brazil. The most effective antibiotics were enrofloxacin with 93.85% of sensitivity, followed by streptomycin with 91.85% and gentamicin with 87.75%. The less effective antibiotics were penicillin with 67.35% of resistance, followed by ampicillin and tetracycline with 63.25 and 51.05%, respectively (Table 1). The antimicrobial susceptibility among each pathogen isolated is listed in Tables 2 and 3.

The analysis of oxacilin-resistance profile was 20.40% (10/49 isolates). Among the CoNS isolates, 35.70% (5/14 isolates) were oxacillin-resistant while 17.25% (5/29 isolates) of *S. aureus* isolates were-oxacillin-resistant. No oxacilin-resistance was observed among *S. dysgalactiae* and coagulase-positive/VP-negative *Staphylococcus* spp. isolates analyzed. All the oxacilin resistant isolates showed resistance to the other beta-lactams tested, including the cephalosporins (Table 4).

This study identified 12 (24.50%) isolates with multi-drug resistance profile. These strains were identified as *S. aureus* (7 isolates) and CoNS (5 isolates). The most effective antimicrobials against these bacteria were streptomycin and enrofloxacin, as compared to other antimicrobials tested (Table 4). Two MDR isolates showed resistance to all antibiotics tested.

DISCUSSION

The study was carried out to determine the antimicrobial resistance profile of *S. aureus* and CoNS strains isolated from milk samples from cows with bovine mastitis in the municipality of Altônia, northwest region of Paraná state, Brazil.

The prevalence of bovine mastitis was also assessed and revealed that 21.80% animals examined had

Table 1. Antimicrobial resistance profile of all isolates obtained from mastitis cases studied.

Antimicrobials	Resistance profile					
	Sensitive		Intermediate		Resistant	
	%	n	%	n	%	n
Penicillin	32.65	16	0.00	0	67.35	33
Ampicillin	34.70	17	2.05	1	63.25	31
Oxacilin	75.50	37	2.05	1	22.45	11
Streptomycin	91.85	45	0.00	0	8.15	4
Gentamicin	87.75	43	4.10	2	8.15	4
Cephalothin	79.60	39	0.00	0	20.40	10
Ceftiofur	77.55	38	0.00	0	22.45	11
Tetracycline	40.80	20	8.15	4	51.05	25
Enrofloxacin	93.85	46	2.05	1	4.10	2
Erythromycin	73.45	36	14.30	7	12.25	6
Clindamycin	73.45	36	4.10	2	22.45	11
Sulfa	65.30	32	6.10	3	28.60	14
Average	68.90%		3.55%		27.55%	

Table 2. Antimicrobial resistance profile of CoNS and coagulase-positive/VP-negative *Staphylococcus* spp. obtained from mastitis cases studied.

Antibiotic	*Staphylococcus* spp. negative coagulase						*Staphylococcus spp.* PC/NVP					
	Sensitive		Intermediate		Resistant		Sensitive		Intermediate		Resistant	
	%	n	%	n	%	n	%	n	%	n	%	n
Penicillin	42.85	6	0.00	0	57.15	8	33.50	1	0.00	0	66.50	2
Ampicillin	64.30	9	0.00	0	35.70	5	33.50	1	0.00	0	66.50	2
Oxacilin	64.30	9	0.00	0	35.70	5	100	3	0.00	0	0.00	0
Streptomycin	92.85	13	0.00	0	7.15	1	100	3	0.00	0	0.00	0
Gentamycin	78.55	11	7.15	1	14.30	2	100	3	0.00	0	0.00	0
Cephalothin	78.55	11	14.30	2	7.15	1	100	3	0.00	0	0.00	0
Ceftiofur	85.70	12	7.15	1	7.15	1	100	3	0.00	0	0.00	0
Tetracycline	35.70	5	7.15	1	57.15	8	66.50	2	33.50	1	0.00	0
Enrofloxacin	85.70	12	7.15	1	7.15	1	100	3	0.00	0	0.00	0
Erythromycin	64.30	9	14.30	2	21.40	3	100	3	0.00	0	0.00	0
Clindamycin	64.30	9	0.00	0	35.70	5	100	3	0.00	0	0.00	0
Sulfa	57.15	8	7.15	1	35.70	5	66.50	2	33.50	1	0.00	0

infection in their udders. This finding closely agrees with those found by other authors worldwide. (Salih et al., 2011; Tolosa et al., 2009 Piepers et al., 2007; Mello et al., 2012; Oliveira et al., 2009, 2011). Among the isolates analyzed in this study, *S. aureus*, CoNS, *S. dysgalactiae* and coagulase-positive/VP-negative *Staphylococcus* spp. were the most frequent, corroborating with the results found in other studies (Zanette et al., 2010; Ferreira et al., 2007 and Piepers et al., 2007; Silva et al., 2014).

The presence of mastitis in dairy cows requires the use of antibiotics to treat the animals. However, the inappropriate use of these drugs over the years allowed the emergence of multi-drug resistant bacteria (Silveira-Filho et al., 2014; Gaze et al., 2008; Thomson et al., 2008; Krause and Hendrick, 2011). This study showed that 27.55% of isolates were antibiotic-resistant among the *S. aureus,* CoNS, *S. dysgalactiae* and coagulase-positive/VP-negative *Staphylococcus* spp. isolated from bovine mastitis. Similar results were also found in different regions of the world (Szweda et al., 2014; Güller et al., 2005, Sawant et al., 2009).

The highest resistance rates were detected for the β-lactam antibiotics penicillin (67.35%) and ampicillin (63.25%). These results were expected because these

Table 3. Antimicrobial resistance profile of *Staphylococcus aureus* and *Streptococcus dysgalactiae* obtained from mastitis cases studied.

Antibiotic	*Staphylococcus. aureus*						*Streptococcus dysgalactiae*					
	Sensitive		Intermediate		Resistant		Sensitive		Intermediate		Resistant	
	%	n	%	n	%	n	%	n	%	n	%	n
Penicillin	24.10	7	0.00	0	75.90	22	0.00	0	66.50	2	33.50	1
Ampicillin	38.00	11	0.00	0	62.00	18	33.50	1	66.50	2	0.00	0
Oxacilin	79.30	23	3.45	1	17.25	5	0.00	0	100	3	0.00	0
Streptomycin	89.65	26	0.00	0	10.35	3	0.00	0	100	3	0.00	0
Gentamycin	89.65	26	3.45	1	6.90	2	0.00	0	100	3	0.00	0
Cephalothin	89.65	26	3.45	1	6.90	2	0.00	0	100	3	0.00	0
Ceftiofur	96.55	28	0.00	0	3.45	1	0.00	0	66.50	2	33.50	1
Tetracycline	41.35	12	3.45	1	55.20	16	33.33	1	33.33	1	33.33	1
Enrofloxacin	96.55	28	0.00	0	3.45	1	0.00	0	100	3	0.00	0
Erythromycin	72.40	21	17.25	5	10.35	3	0.00	0	100	3	0.00	0
Clindamycin	79.30	23	3.45	1	17.25	5	33.33	1	33.33	1	33.33	1
Sulfa	72.40	21	3.45	1	24.15	7	33.33	1	33.33	1	33.33	1

Table 4. Antimicrobial resistance profile of MDR *S. aureus* and CoNS isolated in this study.

Strain	Type	PEN	AMP	OXA	SXT	GEN	CEFA	CTF	TET	ENO	ERI	CLI	SUT
1	CoNS	R	R	R	S	I	R	R	R	I	R	R	R
6	CoNS	R	R	R	S	S	R	R	R	S	I	R	R
40	CoNS	R	R	R	R	R	R	R	R	R	R	R	R
48	CoNS	R	R	R	S	S	S	S	R	S	I	R	I
78B	CoNS	R	R	R	S	R	R	R	R	S	R	R	R
4	*S. aureus*	R	R	R	R	S	R	R	R	S	I	R	R
7	*S. aureus*	R	R	R	R	S	R	R	R	S	S	S	S
9B	*S. aureus*	R	R	I	S	S	S	S	R	S	S	R	R
19	*S. aureus*	R	R	R	S	I	R	R	R	S	S	S	S
24	*S. aureus*	R	R	S	S	S	S	S	R	S	R	S	R
44	*S. aureus*	R	R	R	S	R	R	R	R	S	R	R	R
63	*S. aureus*	R	R	R	R	R	R	R	R	R	R	R	R
Resistance (%)		100	100	83.30	33.30	33.30	75.00	75.00	100	16.65	50.00	75.00	75.00

PEN - Penicillin; AMP - Ampicillin; OXA - oxacilin; SXT - streptomycin; GEN - gentamicin; CEFA - cephalothin; CTF -ceftiofur; TET - tetracycline; ENO - enrofloxacin; ERI - erythromycin; CLI - clindamycin; SUT - trimethoprim-sulfamethoxazole

two antibiotics are widely used in mastitis treatment (McDougall et al., 2008; Guimarães et al., 2012; Güller et al., 2005). According to Vintov et al. (2003), the resistance to penicillin and other beta-lactams in bovines may vary from 10 to 70% depending on the region studied. Another antibiotic with high resistance rate was tetracycline with 55.05%. Similar results were found in Brazil, Guimarães et al. (2012) showed a resistance rate of 33.3% among Gram-positive strains isolated from bovines.

The most effective antibiotics were enrofloxacin, streptomycin and gentamicin. This high sensitivity was also demonstrated in other studies (Kaszanyitzky et al., 2004; Byarugaba, 2004). Despite these data, Freitas et al. (2005) reported high resistance level to gentamicin in CoNS isolated in regions where this antibiotic was used. These diverging results may occur due to farming conditions, handling and drug therapy programs to which animals are subjected.

According to Magiorakos et al. (2012), bacteria that exhibit resistance to three or more classes of antibiotics can be classified as multidrug-resistant (MDR) strains. In this study, it was possible to detect *S. aureus* (7 isolates) and CoNS (5 isolates) with these characteristics. Among these, 10 isolates showed resistance to oxacilin. It is important to emphasize that all oxacilin-resistant isolates were also resistant to other beta-lactams tested. According to Livermore et al. (2001), this resistance profile indicates the presence of methicillin-resistant *S. aureus* (MRSA) and

methicillin-resistant coagulase-negative staphylococci (MRCoNS).

The prevalence of MRSA and MRCoNS (20.40%) in this study was higher than those found in other studies. Vanderhaeghen et al. (2010) detected methicillin-resistant staphylococci (MRS) in 9.3% of clinical and subclinical mastitis cases in Belgian cows. Variations in findings may be attributable to different sample sizes and study locations.

The presence of MRSA and MRCoNS can complicate mastitis treatment because the resistance mechanism of these bacteria confers resistance to almost all types of β-lactam antibiotics available (Sawant et al., 2009). In addition to this, infections by MRS require rapid and accurate diagnosis for elimination at an early stage, because these strains can cause severe damage to infected sites and may be widespread in the environment (Lee et al., 2001, 2004). In most routine microbiological settings, the detection of methicillin resistance among staphylococcal isolates is based on phenotypic assays such as the disk diffusion test and MIC determination (Moon et al., 2007). Despite these data, the presence of MRS strains in this study need to be considered with caution, as molecular tests for *mecA* gene was not performed.

In addition to these data, presence of MRS strains among dairy cattle may represent a risk to human health due to risk of consuming contaminated food, mainly raw milk (Lee, 2003; Olivier et al., 2009).

Conclusion

The prevalence of MDR isolates causing mastitis was of concern. Hence, the determination of antimicrobial resistance profile is essential to control mastitis in dairy cows. The use of antibiogram may help to reduce the dissemination of MDR strains, since the choice of antibiotics will not be based on empirical therapy. More epidemiological and molecular studies are necessary to determine the current role of MDR strains in mastitis. These studies may help in the development of control programs to prevent the spread of these microorganisms in the environment.

Conflict of Interests

The authors have not declared any conflict of interests.

REFERENCES

Ayres M (2007). BioEstat: aplicações estatísticas nas áreas das ciências biomédicas. Belém: Universidade Federal do Pará. 364 p.

Byarugaba DK (2004). A view on antimicrobial resistance in developing countries and responsible risk factors. Int. J. Antimicrob Agents 24:105-110.

CLSI (2013). Performance standards for antimicrobial susceptibility testing; twenty-third informational supplement. CLSI document M100-S23. Clinical and Laboratory Standards Institute, Wayne, PA.

Ferreira JL, Lins JLHA, Cavalcante TV, Macedo NA, Borjas A (2007). Prevalência e etiologia da mastite bovina no município de Teresina, Piauí. Ciênc. Anim. Bras. 8:261-266.

Freitas MFL, Pinheiro-Junior JW, Stamford TLM, Rebelo A, Silva DR, Silveira-Filho VM, Santos FGB, De Sena MJ, Mota RA (2005). Perfil de sensibilidade antimicrobiana in vitro de *Staphylococcus* coagulase positivos isolados de leite de vacas com mastite no agreste do estado de Pernambuco. Arq. Inst. Biol. 72:171-177.

Gaze W, O'Neill C, Wellington E, Hawkey P (2008). Antibiotic resistance in the environment, with particular reference to MRSA. Adv. Appl. Microbiol. 63: 249- 280.

Guimarães G, França, CA, Krug FS, Peixoto RM, Krewer CC, Lazzari, AM, Costa MM (2012). Phenotypic characterization, biofilm production and antimicrobial resistance of *Staphylococcus* spp. isolates from cattle and buffaloes mastitis. Pesq. Vet. Bras. 32:1219-1224.

Güler L, Ok U, Gündüz K, Gülcü Y, Hadimli HH (2005). Antimicrobial susceptibility and coagulase gene typing of *Staphylococcus aureus* isolated from bovine clinical mastitis cases in Turkey. J. Dairy Sci. 88:3149-54.

Jamali H, Radmehr B, Ismail S (2014). Short communication: Prevalence and antibiotic resistance of *Staphylococcus aureus* isolated from bovine clinical mastitis. J. Dairy Sci. 97:2226-30.

Kaszanyitzky EJ, Eyed Z, Jánosi S, Keseru J, Szabó I, Veres Z, Somogyi P (2004). *Staphylococci* isolated from animals and food with phenotypically reduced susceptibility to beta-lactamase-resistant beta-lactam antibiotics. Acta. Vet. Hung. 52:7-17.

Kateete DP, Kabugo U, Baluku H, Nyakarahuka L, Kyobe S, Okee M, Najjuka CF, Joloba ML (2013). Prevalence and antimicrobial susceptibility patterns of bacteria from milkmen and cows with clinical mastitis in and around Kampala, Uganda. PLoS One 7:8 (5):e63413.

Krause DO, Hendrick S (2011) Zoonotic Pathogens in the Food Chain. CABI, Oxfordshire, UK. Available: http://www.ssu.ac.ir/fileadmin/templates/fa/daneshkadaha/daneshkadebehdasht/begh/ebook/Zoonotic_Pathogens_in_the_Food_Chain.pdf . Accessed 2012 July.

Lee JH (2003). Methicillin (oxacillin)-resistant *Staphylococcus aureus* strains isolated from major food animals and their poten- tial transmission to humans. Appl. Environ. Microbiol. 69:6489-6494.

Lee JH, Jeong JM, Park YH, Choi SS, Kim YH, Chae JS, Moon JS, Kim S, Eo SK (2004). Evaluation of the methicillin-resistant *Staphylococcus aureus* (MRSA)-screen latex agglutination test for detection of MRSA of animal origin. J. Clin. Microbiol. 42:2780-2782.

Lee, JH, Suh JT, Kim YS, Lenz W, Bierbaum G, Schaal KP (2001). Typing and antimicrobial susceptibilities of methicillin-resistant *Staphylococcus aureus* (MRSA) strains isolated in a hospital in Korea. J. Korean Med. Sci. 16:381-385.

Livermore DM, Winstanley TG, Shannon KP (2001). Interpretative reading: recognizing the unusual and inferring resistance mechanisms from resistance phenotypes. J. Antimicrob. Chemother. 48:87-102.

Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, Harbarth S, Hindler JF, Kahlmeter G, Olsson- Liljequist B, Paterson DL, Rice LB, Stelling J, Struelens M, Vatopoulos A, Weber JT, Monnet DL (2012). Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin. Microbiol. Infect. 18:268-81.

McDougall S, Parker KI, Heuer C, Compton CW. (2008). A review of prevention and control of heifer mastitis via non antibiotic strategies. Vet. Microbiol.134:177-185.

Mello PL, Mello PL, Agostinis RO, Barzon EM, Colombo RB, Silva AV, Martins, LA (2012). Prevalência da mastite subclínica e associação dos agentes etiológicos com a contagem de células somáticas de vacas leiteiras da região Sudoeste do Paraná. Vet. e Zootec. 19: 513-521.

Moon JS, Lee AR, Kang HM, Lee ES, Kim MN, Paik YH, Park YH, Joo YS, Koo HC (2007). Phenotypic and genetic antibiogram of methicillin-resistant staphylococci isolated from bovine mastitis in Korea. J. Dairy Sci. 90:1176-85.

National Mastitis Council (NMC) (2004). Microbiological procedures for

the diagnosis of udder infection. 3rd ed Arlington, VA: National Mastitis Council Inc.
Oliveira AA, Melo CB, Azevedo HC (2009). Diagnóstico e determinação microbiológica da mastite em rebanhos bovinos leiteiros nos tabuleiros costeiros de Sergipe. Ciênc. Anim. Bras. 10:226-230.
Oliveira CMC, Sousa MGS, Silva NS, Mendonça CL, Silveira JAS, Oaigen RP, Andrade SJT, Barbosa JD (2011). Prevalência e etiologia da mastite bovina na bacia leiteira de Rondon do Pará, estado do Pará. Pesq. Vet. Bras. 31:104-110.
Oliver SP, Boor KJ, Murphy SC, Murinda SE (2009). Food safety hazards associated with consumption of raw milk. Foodborne Pathog. Dis. 6:793-806.
Piepers S, De Meulemeester L, de Kruif A, Opsomer G, Barkema HW, De Vliegher S (2007). Prevalence and distribution of mastitis pathogens in subclinically infected dairy cows in Flanders, Belgium. J. Dairy Res. 74:478-483.
Salih MD, Junnaid AU, Tambulual FM, Magaji AA, Tafaku S (2011). Prevalence of bovine mastitis in lactating cow in same selected commercial dairy farms in Sokota Metropolis. Appl. Sci. Res. 2:290-294.
Santos EMP, Brito MAVP, Lange C, Brito JRF, Cerqueira MMOP (2007). *Streptococcus* e gêneros relacionados como agentes etiológicos de mastite bovina. Acta Sci. Vet. 35:17-27.
Sawant AA, Gillespie BE, Oliver SP (2009). Antimicrobial susceptibility of coagulase-negative Staphylococcus species isolated from bovine milk. Vet. Microbiol. 134:73-81.
Schroeder J (2012). Bovine Mastitis and Milking Management. North Dakota State University. Available: www.ag.ndsu.edu/pubs/ansci/dairy/as1129.pdf. Accessed 2013 March.
Silva NC, Guimarães FF, de P Manzi M, Gómez-Sanz E, Gómez P, Araújo-Júnior JP, Langoni H, Rall VL, Torres C (2014). Characterization of methicillin-resistant coagulase-negative staphylococci in milk from cows with mastitis in Brazil. Antonie Van Leeuwenhoek 106(2):227-33.
Silveira-Filho VM, Luz IS, Campos AP, Silva WM, Barros MP, Medeiros ES, Freitas MF, Mota RA, Sena MJ, Leal-Balbino TC (2014) Antibiotic resistance and molecular analysis of *Staphylococcus aureus* isolated from cow's milk and dairy products in northeast Brazil. J. Food Prot. 77(4):583-591.
Supré K, Lommelen K, De Meulemeester L (2014). Antimicrobial susceptibility and distribution of inhibition zone diameters of bovine mastitis pathogens in Flanders, Belgium. Vet. Microbiol. 171(3-4):374-381.
Szweda P, Schielmann M, Frankowska A, Kot B, Zalewska M (2014). Antibiotic Resistance in *Staphylococcus aureus* Strains isolated from cows with mastitis in Eastern Poland and analysis of susceptibility of resistant strains to alternative nonantibiotic agents: Lysostaphin, Nisin and Polymyxin B. J. Vet. Med. Sci. 76:355-62.
Taponen S, Pyörälä S (2009). Coagulase-negative staphylococci as cause of bovine mastitis- not so different from *Staphylococcus aureus*? Vet. Microbiol. 134(1-2):29-36.
Thomson K, Rantala M, Hautala M, Pyorala S, Kaartinen L (2008) Cross- sectional prospective survey to study indication-based usage of antimicrobials in animals: results of use in cattle. BMC Vet. Res. 4: 15.
Tolosa T, Geberetsadik Z, Rrgassa F (2009). Bovine mastitis and its associated risk factor in lactating cow in Wolayta Sodo, Southern Ethiopia. Anim. Health Prod. 57(4):311-319.
Vanderhaeghen W, Hermans K, Haesebrouck F, Butaye P (2010) Methicillin-resistant Staphylococcus aureus (MRSA) in food production animals. Epidemiol. Infect. 138(5):606-25.
Vintov, J, Aarestrup FM, Zinn CE, Olsen JE (2003). Association between phage types and antimicrobial resistance among bovine *Staphylococcus aureus* from 10 countries. Vet. Microbiol. 95:133-147.
Zanette E, Scapin D, Rossi EM (2010). Suscetibilidade antimicrobiana de *Staphylococcus aureus* isolados de amostras de leite de bovinos com suspeita de mastite. Unoesc & Ciência - ACBS. 1:65-70.

academicJournals

Vol. 8(37), pp. 3398-3404, 10 September, 2014
DOI: 10.5897/AJMR2014.6868
Article Number: F3BD57847909
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Diversity of yeasts in otchè, a traditional starter used in fermentation of an opaque sorghum beer "chakpalo"

Yves Kadjogbé Djegui[1], Adéchola Pierre Polycarpe Kayodé[1]*, Raymond A. Atchadé[1], Emma W. Gachomo[2], Simeon O. Kotchoni[2] and Joseph Djidjoho Hounhouigan[1]

[1]Département de Nutrition et Sciences Alimentaires, Faculté des Sciences Agronomiques, Université d'Abomey-Calavi, 01 BP 526 Cotonou, Bénin.
[2]Department of Biology and Center for Computational and Integrative Biology, Rutgers University, 315 Penn St., Camden, NJ 08102, USA.

Received 29 April, 2014; Accepted 1 September, 2014

Sorghum beer is a significant diet component of millions of poor rural communities in sub-Saharan Africa. In this study, we identified and biochemically characterized yeast strains isolated from otchè, a traditional starter used in fermentation of chakpalo, an opaque sorghum beer in Benin. 12 samples of otchè were collected from 12 different commercial processing sites. The mean values of pH, titratable acidity, dry matter content and refractive index of the starters analyzed were 3.37, 0.17 (% as lactic acid), 7.15 and 7.0%, respectively. The mean yeast count per sample was 8.72 log cfu/ml. Based on phenotypical and biochemical characterization (carbon and nitrogen assimilation) profile, 50 yeast strains were identified and found to belong to five genera and ten species. *Saccharomyces cerevisiae* was found to be the most predominant yeasts species of otchè.

Key words: Sorghum, beer, chakpalo, otchè, yeast, *Saccharomyces cerevisiae*.

INTRODUCTION

Opaque beers are mostly prepared from Guinea corn (*Sorghum bicolor*) and sometimes from other cereals such as millet and maize (generally used as adjunct or substitutes) (Kayode et al., 2005). Opaque beers are popular alcoholic beverage in the northern Guinea Savannah region of West Africa. They are known as tchoukoutou and chakpalo in Benin, dolo in Burkina-Faso, pito in Ghana and burukutu or otika in Nigeria (Kayodé et al., 2005; Odunfa, 1985). Largely consumed by the poorest rural community in these regions, the beverage plays a significant role in the diet of millions of the consumers (Kühle et al., 2001; Jespersen, 2003). The nutritional attributes of several commercial sorghum beers have been reported to contain significant amount of protein, ash, carbohydrate, iron (Fe) and zinc (Zn) (Novellie and De Schaepdrijver, 1986). This justifies a renewed interest for the beverage by the community. It is generally viewed as a significant source of dietary nutrients, considering the large quantity of daily consumption by the people (Briggs et al., 2004).

*Corresponding author. E-mail: polykap@yahoo.fr. Tel: +229 97870734.

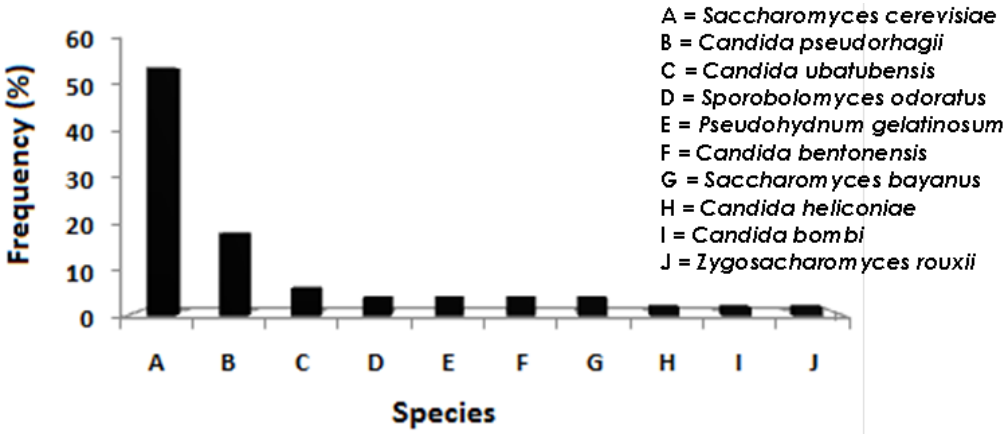

Figure 1. Frequency distribution of yeast species involved in otchè.

In Benin, the beverage is produced by women through a three-phases based process: malting, mashing and fermentation (Pattison et al., 1998; Kayode et al., 2012). The fermentation step is the most important step of the process. The fermentation aspect of the process has several beneficial effects, which include reduced loss of raw materials, improvement of protein quality and carbohydrate digestibility, improved bioavailability of micronutrients and elimination of toxic and anti-nutritional factors such as cyanogenic glycosides (Sanni and Lönner, 1993; Iwuoha and Eke, 1996; Padmaja, 1995; Sindhu and Khetarpaul, 2001). The success of the fermentation depends, among other factors, on the quality of the fermentation starter. Traditional starters of African opaque beers have been reported to contain mainly yeasts and lactic acid bacteria (Van der Aa Kühle et al., 2001; Demuyakor and Ohta, 1991; Sefa-Deheh et al., 1999; Sanni and Lönner, 1993). In addition, the yeast strains have been reported to be involved in several different types of beverage processes (Zulu et al., 1997; Torner et al., 1992; Gadaga et al., 1999). To date, no study on “otchè”, the traditional starter of chakpalo, has been carried out. Therefore, it is necessary to assess physicochemical, biochemical and microbial characteristics of this traditional starter.

In order to establish the microbial diversity population of otchè, we carried out in this study a detail microbial screening of different geographically collected otchè from Benin and further determined their physicochemical properties, fermentation ability and carbon assimilation sources.

MATERIALS AND METHODS

Sample collection

Twelve samples of otchè were collected from twelve commercial processing sites in the center regions of Benin (Figure 1). The processors (beverage processing personnel; one per site) were selected based on their well-established brewing traditional skills. The samples were collected and kept in sterile screw-capped bottles, packed in insulated iceboxes, transported to the laboratory and immediately screened for microbial diversity population (Hounhouigan et al., 1993).

Physicochemical property analysis of ochè

In order to characterize the physicochemical properties of the starter, we first determined the dried matter of the samples using the AACC method (AACC, 1984). The pH was determined using a digital pH meter (HI 8418; Hanna instruments, Limena, Italy) calibrated with buffers at pH 4.0 and 7.0 (WTW, Weilheim, Germany). The acidity titration, expressed as lactic acid, was carried out using the method described by Nout et al. (1989). The refractive index was measured using a refractometer (Sopelem 9596, France).

Determination of yeast population density

To quantitatively determine the microbial community, duplicate samples of “otchè” (10 ml) were diluted in 90 ml sterile peptone physiological saline solution (5 g peptone, 8.5 g NaCl and 1000 ml distilled water, pH = 7.0) and homogenized with a Stomacher lab-blender (type 400, London, UK). Decimal dilutions were plated as described previously (Hounhouigan et al., 1993). Total counts of yeast population were determined as described previously (Hounhouigan et al., 1993).

Identification of yeast strains

The identification of microbial (yeast) strains was performed according to the current guidelines (Yarrow, 1998; Kurtzman et al., 2011). The isolates from twelve representative collection sites were purified using successive sub-culturing method on oxytetracycline glucose yeast agar (OGYA, CM0545, Basingstoke Hampshire, England), where oxytetracycline was used as selection marker. Additionally, microscopic observation was carried out to ascertain the identification of microbial strains. The isolates were then tested for fermentation ability on sucrose, lactose, glucose and raffinose. They were also tested for nitrogen assimilation properties on selected nitrogen sources such as nitrate, ethylamine, L-lysine, cadaverine and creatine. The assimilation of carbon sources was performed using API 20 C AUX strips (BioMérieux, Lyon, France) according to the manufacturer’s instructions. The Diazonium Blue B

Table 1. Physico-chemical characteristics and yeasts content of the starter of chakpalo.

Samples origin	pH	Titratable acidity (% lact. acid)	Dry matter (%)	Refractive index	Yeasts (log cfu/g)
Bantè (n= 3)	3.58 ± 0.08b*	0.07 ± 0.02a	9.68 ± 0.76b	11 ± 1b	8.76 ± 1.1a
Dassa (n= 3)	3.32 ± 0.14a	0.12 ± 0.02a	5.34 ± 0.99a	5 ± 0a	9.41 ± 0.48a
Glazoué (n= 3)	3.34 ± 0.18a	0.09 ± 0.03a	9.48 ± 1.77b	5 ± 1a	9.21 ± 0.07a
Savè (n = 3)	3.24 ± 0.15a	0.41 ± 0.35b	4.08 ± 0.68a	5 ± 0a	7.51 ± 0.06b
Mean	3.37 ± 0.18	0,17 ± 0,22	7.15 ± 2.74	7 ± 3	8.72± 0.92
[a]CV (%)	4.4	92.6	40.0	46.2	9.8

[a]Coefficient of variation.*Values with the same letter in the same column are not significantly different (P < 0.05).

(DBB) reaction, a test to differentiate between ascomycetous and basidiomycetous yeasts, was performed as described by Kurtzman et al. (2011).

Statistical data analysis

For statistical data analysis, mean values and standard deviation are reported. The data were analyzed using the SPSS 11.0 statistical program. The on-line available software (http:www.cbs.knaw.nl) of Central bureau voor Schimmelcutures, Utrecht, the Netherlands was used for identification of yeasts.

RESULTS

Properties of otchè and number of yeast

Table 1 shows physico-chemical characteristics of a number of yeasts found in otchè collected from different sampling sites. The mean pH value of the samples analyzed was 3.37 ± 0.18. Data analysis showed that there is no significant difference between samples from Dassa, Glazoué and Savè. Samples collected from Bantè had the highest ($p<0.05$) pH value. The average titratable acidity value of otchè was 0.17 (% as lactic acid). The acidity of otchè collected from Savè was significantly ($p<0.05$) higher than those of the samples collected from the other sites. The average dry matter of the traditional starter used for the fermentation of chakpalo was 7.15%. The average refractive index was 7.0 (Table 1). The lowest values of the refractive index were obtained from samples collected from Dassa, Glazoué and Savè, while the highest was from samples collected from Bantè.

The mean yeast count per traditional starter was 8.72 $\log_{10}$ cfu/ml. There was no significant ($p>0.05$) difference between the yeast content of otchè from Dassa, Bantè and Glazoué (Table 1). But the yeast content of otchè from Savè is significantly ($p<0.05$) lower than those from other sites.

Phenotypic characteristics of yeast isolates

All the 50 yeast strains were subjected to microscopic observation to further confirm the microbial identity before carrying out the fermentation and assimilation tests. According to Table 2, only a few isolates could ferment lactose (4%) and raffinose (28%) whereas the majority of the strains fermented glucose (98%) and sucrose (88%). Most of the isolates assimilated ethylamine (82%), nitrate (80%), creatine (80%), L-Lysine (76%), cadaverine (66%). The Diazonium Blue B test revealed that 10% of the isolates were basidiomycetous, whereas 90% were ascomycetous (Table 2). On the basis of their fermentation profile and their nitrogen assimilation pattern, the yeast strains were grouped into 25 distinct clusters based on their biochemical characteristics. 32% of the yeast strains were in the first cluster, 6% were in the second cluster, while the remaining strains were diversely grouped into the 23 other clusters as shown in Table 2.

Assimilation of carbon compounds by the yeast isolates

Based on the carbon utilization, thirty three assimilation profiles were distinguished. All yeasts assimilated glucose (100%), the majority of strains assimilated galactose (94%), saccharose (90%) and maltose (88%). A relative majority of the strains assimilated D-raffinose (68%) and palatinose (54%). Fourty eight percent assimilated D-trehalose and N-aceltyl-glucopyranoside, 44% assimilated L-arabinose and potassium 2-cétogluconate, 40% assimilated Cycloheximide (Actidione), Lactic Acid and D-mannitol; 38% assimilated Methyl-αD-glucopyranoside D-Xylose and D-ribose; 36% assimilated D-Melesitose; 34% assimilated Glycerol, 32% assimilated D-cellobiose and 28% assimilated D-sorbose. A relatively small number of yeasts assimilated L-sorbose (18%), Glucosamine (18%), D-Melibiose (12%) and Rhamnose (12%). None of the yeasts tested assimilated levulinic acid, potassium gluconate, sodium glucuronate, erythriotol, inositol and D-lactose (Table 3).

Identification of yeasts isolates

The yeast strains identified were found to belong to five genera and ten species of yeasts (Figure 1). These were: *Saccharomyces cerevisiae* (54%), *Candida pseudorhagii*

Table 2. Phenotypic characters of yeasts isolated from chakpalo.

Cluster	Microbial isolates number	Fermentation ability				Assimilation of nitrogen source					DBB test
		Glu[1]	Lac	Suc	Raf	Nit	Eth	Lys	Cad	Ctr	
I	2, 4, 6, 8, 11, 14, 19, 24, 29, 30, 32, 33, 34, 35, 36, 48	+	-	+	-	+	+	+	+	+	_
II	41, 44, 47	+	-	+	+	+	+	+	+	+	_
III	9, 37	+	-	+	-	-	-	-	-	+	-
IV	10, 31	+	+	+	-	+	+	+	+	+	-
V	13, 39	+	-	+	+	+	+	+	+	+	+
VI	15, 17	+	-	+	+	+	+	+	-	+	-
VII	16, 21	+	-	-	-	+	+	+	+	+	-
VIII	18, 20	+	-	+	-	+	+	-	-	-	-
IX	22, 27	+	-	+	-	+	+	+	-	+	-
X	38, 40	+	-	+	-	+	+	-	-	+	-
XI	1	+	-	-	-	+	+	+	-	+	-
XII	3	+	-	+	-	-	-	-	+	+	-
XIII	5	+	-	+	-	-	-	-	-	-	-
XIV	7	+	-	+	-	-	-	+	+	+	-
XV	12	+	-	-	+	+	+	+	+	+	+
XVI	23	+	-	+	+	+	-	+	+	-	-
XVII	25	+	-	+	+	-	+	+	-	+	-
XVIII	26	-	-	+	+	-	+	+	+	-	-
XIX	28	+	-	+	-	-	+	-	-	+	-
XX	42	+	-	+	+	+	+	-	+	-	+
XXI	43	+	-	+	+	-	-	-	-	-	+
XXII	45	+	-	-	-	+	+	+	+	+	-
XXIII	46	+	-	+	+	+	+	-	-	-	-
XXIV	49	+	-	+	-	+	-	-	+	-	-
XXV	50	+	-	-	-	-	-	-	-	-	-
Frequency (%)		98	4	88	28	80	82	76	66	80	10

[1]Glu = glucose, Lac = lactose, Suc = Sucrose, Raf = rafinose, Nit = nitrate, Eth = ethylamine, Lys = L-lysine, Cad = cadaverine, Crt = creatine DBB = diazonium Blue B. (+) Fermented or assimilated; (-) did not ferment or did not assimilate. Frequency (%): represents the ratio of isolates that were able to ferment each sugar or to assimilate nitrogen source or DBB. How it is calculated: for each sugar or nitrogen source or DBB, the number of + was divided by the total number of isolates (50).

(18%), *Candida ubatubensis* (6%), *Candida bentonensis* (4%), *Saccharomyces bayanus* (4 %), *Pseudohydnum gelatinosum* (4%), *Sporobolomyces odoratus* (4%), *Candida heliconiae* (2%), *Candida bombi* (2%) and *Zygosacharomyces rouxii* (2%). *Saccharomyces cerevisiae* was found to be the dominant yeast species in the traditional starter otchè.

DISCUSSION

The highest mean values of pH and refractive index were noticed in samples collected from Bantè. In the same way, the titratable acidity of otchè collected from Savè was significantly ($p<0.05$) higher than that of the samples collected from the other sites. Also, dry matter value varies from a site to another one. As observed in other studies (Demuyakor and Ohta, 1991; Owuama, 1999), traditional starters can easily be distinguished by their specific physicochemical such as pH, dry matter, titratable acidity and refractive index.

The yeast concentration of otchè was relatively lower than that reported in tchoukoutou, an opaque sorghum beer of Benin (Kayodé et al., 2011). Distribution frequencies of microbial species vary according to the localities and ingredients used for the production of African beers (Faparusi et al., 1973; Nout, 1980; Odunfa, 1985; Demuyakor and Ohta, 1991; Sanni and Lönner, 1993; Sefa-Dedeh et al., 1999).

Fermentation and assimilation tests showed that the majority of yeasts assimilated glucose and sucrose whereas only a few strains could ferment lactose and

Table 3. Assimilation profiles of yeasts isolated from Chakpalo.

	a*	b	c	d	e	f	g	h	I	j	k	l	m	n	o	p	q	r	s	t	u	v	w	x	y	z	za	zb	zc	zd	ze	zf	zg	Total (%)
*GAL	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	94
ACT	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	-	-	+	+	-	40
SAC	+	+	+	+	-	-	+	+	+	+	+	+	+	+	-	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	90
NAG	+	-	-	+	-	-	-	-	+	+	+	-	-	-	-	+	+	-	-	-	-	-	+	+	-	-	-	-	-	-	-	+	+	48
LAT	+	-	-	-	-	-	-	-	+	+	+	-	-	-	-	+	-	-	-	-	+	-	+	+	+	-	+	+	+	-	-	-	+	40
ARA	+	-	-	+	-	-	-	-	+	+	+	-	-	-	-	-	-	+	-	-	-	-	+	+	+	-	-	-	-	+	+	-	+	44
CEL	+	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	+	32
RAF	+	-	+	+	-	-	+	-	+	+	+	-	+	-	-	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	68
MAL	+	+	+	+	+	+	+	-	+	+	-	-	+	+	+	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	88
TRE	+	-	-	+	-	-	+	-	+	+	+	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	+	-	+	+	48
2KG	+	-	-	+	-	-	-	-	+	+	+	-	-	-	-	+	-	-	-	-	-	-	+	+	+	-	-	-	-	+	-	+	+	44
MDG	+	-	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+	-	-	+	-	-	+	38
MAN	+	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	+	+	-	-	-	-	+	-	+	+	40
LAC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
INO	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
SOR	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	28
XYL	+	-	-	+	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	+	-	+	38
RIB	+	-	-	+	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	+	38
GLY	+	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	+	34
RHA	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	+	-	-	-	-	-	+	12
PLE	+	-	+	+	-	-	-	+	-	+	+	-	-	+	+	-	-	-	-	-	+	-	+	+	+	+	+	-	-	+	-	-	+	54
ERY	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
MEL	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	+	+	-	-	-	+	-	-	-	+	12
GRT	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
MLZ	+	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+	+	-	+	-	-	+	-	-	+	36
GNT	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
LVT	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
GLU	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100
SBE	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	18
GLN	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	18
ESC	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	+	24
Nb of isolates (%)	16	14	4	8	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	

a* = 2, 6, 11, 14, 29, 18, 20, 30; b = 32,34,35, 36, 31, 43, 42; c = 38, 40; d = 16,21,15,17; e = 3; f = 33; g = 49; h = 12; i = 37; j = 10 ; k = 5; l = 41; m = 47; n = 48; o = 46; p = 44; q = 1; r = 45; s = 50; t = 22; u = 24; v = 13; w = 25; x = 27; y = 4; z= 9; za = 23; zb = 39; zc = 7; zd = 8; ze = 28; zf = 26; zg = 19; GAL = D- Galactose, ACT = Cycloheximide (Actidione), SAC = D- Saccharose, NAG = N- Acety-Glocusamine, LAT = Lactic Acid, ARA = L-arabinose, CEL = D-cellobiose, RAF = D-raffinose, MAL = D-maltose, TRE = D-trehalose, 2KG = Potassium 2-cétogluconate, MDG = Methyl-αD-glucopyranoside, MAN = D-mannitol, LAC = D-lactose, INO = Inositol, SOR = D-sorbose, XYL= D-Xylose, RIB = D-ribose, GLY= Glycerol, RHA = Rhamnose, PLE = Palatinose, ERY = Erythritol, MEL = D-Melibiose, GRT = Sodium glucuronate, MLZ = D-Melesitose, GNT = Potassium Gluconate, LVT = Acide levulinique, GLU = D-Glucose, SBE = L-sorbose, GLN= Glucosamine, ESC = Sulfate of ammonium.

raffinose. These results were quite different from previous data by Sanni and Lönner (1993) on 49 yeasts isolates from Nigerian traditional beers known as burukutu, pito, sekete, agadagidi and palm win. The authors reported that yeasts isolated from these beers fermented glucose (77.55%), galactose (46.94%), maltose (40.82%), sucrose (44.89%) and melibiose (4.08%), but none of them fermented lactose. In this study, *S. cerevisiae* appeared to be the most predominant microbial strain in otchè, the starter used for the fermentation of Chakpalo, the Benin-opaque sorghum beer. This microbial identification data is in agreement with our previously reported findings of the prevalence of *S. cerevisiae* in tchoukoutou, another sorghum beer from Benin (Kayode et al., 2011, Greppi et al., 2013). Similarly, Konlani et al. (1996) reported a prevalence of 55-90% of *S. cerevisiae* in opaque sorghum beer from Togo and Burkina-Faso. Glover et al. (2005) also identified 72% of 247 isolates as *S. cerevisiae* based on their assimilation profiles. This comparative data indicates that the prevalence of *S. cerevisiae* in the starter is lower than in the beer itself. According to Vaughan-Martini and Martini (1998), an isolate is characterized as *S. cerevisiae* if such microbial strain could assimilate glucose, sucrose, maltose, trehalose, raffinose and ethanol. In our study, it has been noticed that many isolates could not assimilate all these sugars. However, we were able to characterize our isolated strains as *S. cereviciae* strains on the basis of additional biochemical characterization. Van der Aa Kühle et al. (2001) and Demuyakor and Ohta (1991) also identified many isolates from Ghanaian and Burkina-Faso sorghum beers as *S. cerevisiae*, even though these microorganisms had a very different carbon assimilation profile from the taxonomical key proposed by Vaughan-Martini and Martini (1998). Our results are in agreement with these findings.

Conclusion

This study suggests that *S. cerevisiae* is the predominant microbial species in the traditional starter used for the fermentation of chakpalo. Moreover, we suggest a more robust microbial identification approach based on genome fingerprinting techniques for the characterization of traditional starters used in the fermentation of opaque sorghum beers in Africa. The current study highlights the acidity profile, dry matter of otchè, a traditional starter for the fermentation of Chakpalo, the Benin-opaque sorghum beer.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

Financial support provided by Aires-Sud Project Grant No. 7190, IRD, France, is gratefully acknowledged.

REFERENCES

AACC (1984). Approved methods of the American Association of cereal chemistry. American Association of Cereal Chemistry, St. Paul, MN.

Briggs DE, Boulton CA, Brookers PA, Stevens R (2004). Native African beers. In Brewing: Science and practice. Woodhead publishing Ltd, Cambridge, UK. pp. 589-605.

Demuyakor B, Ohta Y (1991). Characteristics of pito yeasts from Ghana. Food Microbiol. 8:183-193.

Faparusi SI, Olofinboba FO, Ekundayo JA (1973).The microbiology of burukutu beer. Z. Allg. Mikrobiol. 13:563-568.

Gadaga TH, Mutukumira AN, Narvhus JA, Feresu SB (1999). A review of traditional fermented foods and beverages of Zimbabwe. Int. J. Food Microbiol. 53:1-11.

Glover RLK, Abaidoo RC, Jackobsen M, Jespersen L (2005). Biodiversity of Saccharomyces cerevisae isolated from a survey of pito production sites in various parts of Ghana. Syst. Appl. Microbiol. 28:755-761.

Greppi A, Rantsiou K, Padonou W , Hounhouigan J, Jespersen J, Jakobsen M, Cocolin M (2013).Determination of yeast diversity in ogi, mawè, gowé and tchoukoutou by using culture-dependent and independent methods. Int. J. Food Microbiol. 165:84-88.

Hounhouigan DJ, Nout MJR, Nago CM, Houben JH, Rombouts FM (1993). Microbiological changes in mawe during natural fermentation. World J. Microbiol. Biotechnolog. 10:410-413.

Iwuoha CI, Eke OS (1996). Nigerian indigenous fermented foods: their traditional process operation,inherent problems, improvements and current status. Food Res. Int. 29:527-540.

Jespersen L (2003). Occurrence and taxonomic characteristics of strains of Saccharomyces cerevisiae predominant in African indigenous fermented foods and beverages. FEMS Yeast Res. 3:191-200.

Kayode APP, Vieira-Dalodé G, Linnemann AR, Kotchoni SO, Hounhouigan AJD, van Boekel MAJS, Nout MJR (2011). Diversity of yeasts involved in the fermentation of tchoukoutou, an opaque sorghum beer from Benin. Afr. J. Microbiol. Res. 5 (18): 2737-2742.

Kayode APP, Adegbidi A, Linnemann AR, Nout MJR, Hounhouigan DJ (2005). Quality of farmer's varieties of sorghum and derived foods as perceived by consumers in Benin. Ecol. Food Nutr. 44:271-294.

Kayode APP, Deh DC, Baba-Moussa L, Kotchoni SO, Hounhouigan JD (2012). Stabilisation and preservation of probiotic properties of the traditional starter of African opaque sorghum beers. Afr. J. Biotechnol. 11 (30):7725-7730.

Konlani S, Delgenes JP, Moletta R, Traore A, Doh A (1996). Isolation and physiological characteristics of yeasts involved in sorghum beer production. Food Biotechnol. 10: 29-40.

Kühle AVA, Jesperen L, Glover RLK, Diawara B, Jakobsen M (2001). Identification and characterization of Saccharomyces cerevisiae strains isolated from West African sorghum beer. Yeast 18: 1069 - 1079.

Kurtzman CP, Fell JW, Boekhout T, Robert V (2011). Methods for isolation, phenotypic characterization and maintenance of yeasts. In: Kurtzman CP, Fell JW & Boekhout T (eds) The Yeasts, a Taxonomic Study, 5th edn. Elsevier. pp. 87-110.

Nout MJR (1980). Microbiological aspects of the traditional manufacture of Busaa, a Kenyan opaque maize beer. Chem. Mikrobiol. Technol. Lebensm. 6: 137-142.

Nout MJR, Rombouts FM, Havelaar A (1989). Effect of accelerated natural lactic fermentation of infant food ingredients on some pathogenic micro-organisms. Int. J. Food Microbiol. 8:351-361.

Novellie L and De Schaepdrijver P (1986). Modern developments in traditional African beers. Prog. Ind. Microbiol. 23:74-157.

Odunfa SA (1985). African fermented foods. In Wood, BJB. (ed.), Microbiol. of Fermented Foods. Elvisier Applied Science, London, UK. pp.167-195.

Owuama CI (1999). Brewing beer with sorghum. J. Inst. Brew. 105:23-34.

Padmaja G (1995). Cyanide detoxification in cassava for food and feed uses. Crit. Rev. Food Sci. Nutr. 35:299-339.

Pattison TL, Geornaras I, von Holy A (1998). Microbial populations associated with commercially produced South African sorghum beer as determined by conventional and Petrifilm plating. Int. J. Food

Microbiol. 43:115 -122.
Sanni AI, Lönner C (1993). Identification of yeast isolated from Nigerian traditional alcoholic beverages. Food Microbiol. 10:517-523.
Sefa-Dedeh S, Sanni AI, Tetteh G, Sakyi-Dawson E (1999). Yeasts in the traditional brewing of pito in Ghana. World J. Microbiol. 15:593-597.
Sindhu SC, Khetarpaul N (2001). Probiotic fermentation of indigenous food mixture: effects on antinutrients and digestibility of starch and protein. J. Food Comp. Anal. 14:601-609.
Torner MJ, Martinez-Anaya MA, Antna B, Benedito de Barber C (1992). Headspace flavor compounds produced by yeasts and lactobacilli during fermentation of prferments and bread doughs. Int. J. Food Microbiol. 15: 145 -152.
Vaughan-Martini A, Martini A (1998). Saccharomyces Meyen ex Reess. In Kurtzman CP, Fell JW (eds.) TheYeasts, A Taxonomic study. Elsevier Science: Amsterdamj. pp. 358 -371.
Yarrow D (1998). Methods for the isolation, maintenance and identification of yeasts. In: Kurtzman CP & Fell JW (eds) The Yeasts. A Taxonomic Study, 4th edn. Elsevier, 418 Amsterdam. pp. 77-100.
Zulu RM, Dillon VM, Owens JD (1997). Munkoyo beverage, a traditional Zambian fermented maize gruel using Rhynchosia root as amylase source. Int. J. Food Microbiol. 34:249-258.

academicJournals

Vol. 8(37), pp. 3405-3408, 10 September, 2014
DOI: 10.5897/AJMR2014.6869
Article Number: A884C2047923
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Evaluation of plant extract in control of dry bubble disease of white button mushroom caused by *Verticillium fungicola* f.sp. *fungicola* Preuss (Hassebr)

Narendra Kumar Jatav[1]*, R. S. Rana[2], Jeeva Ram Verma[3], Shri Kishan Bairwa[4] and G. C. Tiwari[5]

[1]Plant Pathology, Parmanand Degree Collage Gajsinghpur Sriganganagar ,335024 Affiliated to Swami Keshawa Nand Rajasthan Agricultural University ,Bikaner Rajasthan India.
[2]Plant Pathology CCSHAU Hisar Haryana India.
[3]Plant Pathology Jodhpur Agricultural University, Rajasthan India.
[4]Plant Pathology Agricultural Research Station Sri Ganganagar Swami Keshawa Nand Rajasthan Agricultural University ,Bikaner Rajasthan India.
[5]Plant Breeding Genetics, Parmanand Degree Collage Gajsinghpur Sriganganagar ,335024 Affiliated to Swami Keshawanand Rajasthan Agricultural University ,Bikaner Rajasthan India.

Received 30 April, 2014; Accepted 13 June, 2014

Dry bubble disease induced by *Verticillium fungicola* has been observed as an important disease of white button mushroom (*Agaricus bisporus*) in India. The symptoms produced on differentiated fruit body are localized light brown depressed spots. The adjacent spots coalesce together and form irregular blotches. If the pathogen infection is established before differentiation, sclerodermoid fruiting bodies appear on casing surface. Five plant extracts tested by poisoned food technique inhibited the growth of both host and pathogen. But their cumulative effect was found to improve the mushroom yield. *Azadirachta indica* (Neem) effectively controlled the pathogen and increased the yield of white button mushroom by 16.52% compared to control.

Key words: *Agaricus bisporus, Verticillium fungicola,* dry bubble, wheat straw compost, compost, long method of composting, short method of composting, *Azadirachta indica*, *Parthenium parthefolium.*

INTRODUCTION

Agaricus bisporus (white button mushroom) is the most widely accepted food among the consumers. It is cultivated all around the world contributing nearly 40% of total world production of mushroom (Flegg, 1992). In India, white button mushroom is being cultivated in majority of the states under both seasonal and controlled conditions.

*Corresponding author. E-mail: drnarendrakumarjatav@gmail.com.

Its annual production capacity is approximately 42,500 tons (Dandge, 2012). Haryana has become one of the leading states in white button mushroom production with 5312 tons/annum (Tiwari, 2004). Mushroom production is adversely affected by many biotic and abiotic factors. Among the several biotic factors associated with yield reduc-tion, fungal diseases significantly affect the mush-room production.

The most common fungal diseases of white button mushroom are cobweb, dry/wet bubble, false truffle and green mould (Sharma, 1995). Out of these diseases, dry bubble caused by *Verticillium fungicola* is prevalent in all mushroom growing areas and has 25-50% incidence (Sharma, 1995). Dry bubble disease of white button mushroom causing brown spots was reported for the first time by Malthouse (1901). He found a species of *Verticillium* associated with the disease. Two types of symptoms were observed. Initially fungal growth appeared on the casing soil which later spread and turned grayish yellow. After that light brown superficial spots appeared on the caps which finally coalesced to become large brown blotches. This disease is transmitted by contaminated casing soil (Kumar et al. 2014), growers and splash of water (Fekete, 1967; Cross and Jacobs, 1969). Mushrooms infected by *V. fungicola* shows typical thickening of stem, resulting in onion shaped fruiting bodies. However, the symptoms vary for the stage of development at which the infection takes place. When mushrooms are infected at an early stage, symptoms appear as small undifferentiated masses of tissue up to 2 cm diameter. Fruiting bodies are not properly formed and caps are partially differentiated. When infected at a later stage, the stipes are distorted and have tilted caps. Infected mushroom show the presence of grey white mycelial growth and become discoloured and dry but do not rot. They show small pimple like outgrowth or brown grey spots (1-2 cm diameter) on the surface. Such spots often have a yellow or bluish grey halo around them.

In Haryana, the white button mushroom is being cultivated on compost prepared by long method in low cost mushroom houses under seasonal conditions. These factors coupled with poor sanitation and persis-tence of *V. fungicola* in soil set a serious threat to the future of mushroom cultivation in Haryana.

MATERIALS AND METHODS

Effect of different plant extracts against *V. fungicola* and *Agaricus bisporus in vitro*

Fresh 100 g of neem (*Azadirachta indica*), congress grass (*Parthenium parthenifolium*), water hycinth (*Eichchornia crassipes*), patak rai (*Clerodendron enerni*), and amerbel (*Cuscuta cernua*), were homogenized in an electric blender with 100 ml of distilled sterilized water. The homogenate after passing through double layer of muslin cloth was centrifuged at 1000 rpm for 10 min at room temperature. The supernatant was sterilized by mili- pore membrane filter (0.22 µm), and it was considered as 100% aqueous extract of the botanical origin. Further, it was diluted to 50% with distilled sterilized water. Two mililiters of each concentration of each plant extracts were added to 20 ml of sterilized potato dextrose agar medium in Petri-plate. Mycelial agar disc of 5 mm diameter *V. fungicola* were placed in each Petri-plate and incubated at 20±1°C for 14 days. The medium without any plant extract served as control.

Observation

Observations were recorded for radial growth of pathogen.

$$\text{Percent Inhibition} = \frac{C - T}{C} \times 100$$

C = Diameter of colony in the control; T=Diameter of the colony in the treatment.

Effect of different plants on the development of dry bubble disease *in vivo*

Dried leaves of six plants viz., leaves of neem (*Azadirachta indica*), congress grass (*Parthenium parthenifolium*), water hycinth (*Eichchornia crassipes*), patak rai (*Clerodendron enerni*), and amerbel (*Cuscuta cernua*) (Table 1) were mixed individually with compost material on 16th day of composting. Later on, at the end of composting, it was artificially inoculated with *V. fungicola* and spawned with (M-140) white button mushroom and the yield was compared with the control (inoculated with pathogen without botanicals). The four replications of each treatment were kept. Ratio was 0.1% of plant material used to compost substrate.

RESULTS AND DISCUSSION

Effect of different plant extracts on *V. fungicola*

Many scientists have advocated the use of ecofriendly biopesticides for management of various plant pathogens. The use of plant extracts against mushroom pathogens have not been exploited much. Control of pathogens provided by chemicals is the most effective (Jatav et al. 2014), but residual toxicity in mushroom and fungicidal resistance are of great concern. In the present study (Table 2), five plants extracts used inhibited the growth of *V. fungicola* from 0-19.40% at tested concentration. At 50% concentration neem leaf extracts exhibited maximum inhibition (19.40%) and *Clerodendron enermi* (Patak rai) did not show inhibition at all. Antifungal properties of Neem against moulds of mushroom have been reported by Grewal and Grewal (1988). Sharma and Jandaik (1994) also observed that *Azadirachta indica, Eucalyptus, Tegetus erectus* and garlic extract inhibited the growth of various weed fungi of *A. bisporus.* Bhalla (1998), Gea et al. (2011) and Szumigaj et al. (2012) tried neem, onion, garlic and *eucalyptus* extracts at 25, 50 and 100% concentration against *V. fungicola* and found neem giving 28.0% inhibition followed by onion, garlic and *eucalyptus,* respectively.

Effect of different plant extracts on *Agaricus bisporus in vitro*

All the five plant extracts tried *in vitro* against *V. fungicola*

Table 1. Botanicals used test their efficacy against *V. fungicola.*

Name of the plant	Botanical name	Plant part used
Neem	*Azadirachta indica*	Leaves
Congress grass	*Parthenium parthenifolium*	Leaves
Water hyacinth	*Eichchornia crassipes*	Leaves
Patak rai	*Clerodendron ener*	Leaves
Amar bel	*Cuscuta cernua*	No Leaves

Table 2. Effect of different plant extracts on mycelial growth of *Verticillium fungicola in vitro.*

Plant extract	Concentration (%)			
	Colony diameter (mm)		Per cent growth inhibition	
	25	50	25	50
Azadirachta indica	80.91	72.54	10.10	19.40
Parthenium parthenifolium	85.41	80.66	5.10	10.34
Cuscuta cernua	87.84	85.41	2.40	5.11
Eichchornia crassipes	89.10	88.20	1.00	2.00
Clerodendron enermi	90.00	90.00	0.00	0.00
Control	90.00	90.00	0.00	0.00
CD at 5%	0.45	1.65	1.13	2.64

Table 3. Effect of different plant extracts on mycelial growth of *Agaricus bisporus in vitro.*

Plant extract	Concentration (%)			
	Colony diameter (mm)		Per cent growth inhibition	
	25	50	25	50
Azadirachta indica	83.61	76.14	7.10	15.40
Parthenium parthenifolium	87.14	84.24	3.17	6.40
Cuscuta cernua	89.00	88.74	1.11	1.40
Eichchornia crassipes	90.00	90.00	0.00	0.00
Clerodendron enermi	90.00	90.00	0.00	0.00
Control	90.00	90.00	0.00	0.00
CD at 5%	1.83	1.26	1.88	2.25

were also screened against *A. bisporus.* All these had toxic effect on *A. bisporus* corresponding to the *V. fungicola*. Neem (Table 3) inhibited the growth of host fungus by 15.40% followed by *Parthenium parthenifolium* (6.40%), *Cucuta* spp (1.40%) and *Clerodendron* sp.(0.00 %) at 50% concentration. These results are in agreement with those of Bhalla (1998) and Szumigaj et al. (2012) who observed that the maximum inhibition was recorded by Onion (31.2%) followed by neem (28.0 %), garlic (23.2%) and *Eucalyptus* (12.0 %) respectively at 100% concentration.

Effect of different plants on yield of white button mushroom *in vivo*

Five botanicals tried *in vitro* were also tested in *in vivo* condition for the management of dry bubble disease of *A. bisporus.* Plant extracts which were inhibitory to *V. fungicola* and *A. bisporus* behave differently in *in vivo* condition (Table 4) . All the plants improved the yield significantly.*Cuscuta* spp., *Eichchornia* and *Clerodendron* increased yield insignificantly. This indicated that plants have no toxic effects on mushroom mycelia in compost in field conditions that is perhaps because of selectivity of compost. The present studies indicate that plant material in general controlled the mushroom pathogen and can be integrated with chemicals to achieve the best results. Bhalla (1998) recorded similar results while investigating management of dry bubble disease of *A. bisporus*. Singh and Singh (2005), Gea et al. (2011) and Szumigaj et al. (2012) also advocated the integrated use of chemicals and botanicals.

Table 4. Effect of different plant material on dry bubble disease of *Agaricus bisporus in vivo.*

Plant material (0.5%)	Spawn run (days)		Pin head initiation/first picking (days)		Yield (kg/100 kg compost)		Per cent increase in yield over control	
	2004-2005	2005-2006	2004-2005	2005-2006	2004-2005	2005-2006	2004-2005	2005-2006
Azadirachta indica	14	13	21/26	20/24	5.99	5.40	16.52	14.81
Parthenium parthenifolium	16	15	24/28	22/26	5.44	5.00	10.00	8.00
Cuscuta cernua	18	17	26/30	23/27	5.12	4.70	2.34	2.12
Eichchornia crassipes	20	19	27/31	25/29	5.10	4.68	1.96	1.70
Clerodendron enermi	21	20	29/33	27/31	5.02	4.61	0.39	0.21
Control	23	22	30/35	29/33	5.00	4.60		
CD at 5%					0.27	0.23		

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

I feel great pride and privilege to express my sincere gratitude and profound regards to my Major Advisor, Dr. R.S. Rana, Professor, Department of Plant Pathology, CCS Haryana Agricultural University, Hisar, for providing scholary, constructive guidance and innovative ideas throughout this scientific pursuit. I acknowledge that without suggestions, immense interest and affectionate behaviour, it would not have been possible for me to complete this research. The present work bears the impression for concrete suggestions, careful and reasoned criticism and meticulous attention to every detail.

REFERENCES

Bhalla P (1998). Studies on dry bubble *Verticillium fungicola* disease of white button mushroom *Agaricus bisporus.* M.Sc. Thesis, CCSHAU, Hisar.

Cross MJ, Jacobs L (1969). Some observations on the biology of the spores of *Verticillium malthousei.* Mush. Science 17: 239-244.

Dandge V S (2012). Studies on som fungi in button mushroom in Akola(Amanatpur). Recent Res. Sci. Technol. 4(10):28-30.

Fekete K (1967). Morphology, biology and control of *Verticillium malthousci* a parasite of cultivated mushroom. Phytopathology 59(1):1-32.

Flegg PB (1992). Future strategies for mushroom production. Mushroom Res. 1(1):15-18

Gea FJ, Santosh M, Dianez F, Tello JC, Navarro MJ (2011). Effectiveness of compost tea from spent mushroom substrate on dry bubble (*Vericillium fungicola*). Proc of the 7th Int. Conf. Mushroom Biol. Mushroom Prod. 2: 190-195.

Grewal PS, Grewal SK (1988). Selective fungicidal properties of some plant extracts to mushroom weed moulds. Phytopathol. Med. 27:112-114.

Jatav NK, Rana RS, Verma JR, Bairwa SKV (2014). Chemical control of dry bubble disease induced by *Verticillium fungicola* [Preuss] Hassebr on white button mushroom *Agaricus bisporous*. Afr. J. Microbiol. Res. 9(21):2202-2207.

Kumar N, Mishra AB, Bharadwaj MC (2014). Effect of *Verticillium fungicola* (Preuss) Hassebr inoculation in casing soil and conidial spray on white button mushroom *Agaricus bisporous*. Afr. J. Agric. Res. 9(14):1141-1143.

Malthouse GT(1901). A mushroom disease. Tran.. Ed. Field National Microscopic Soc. Part-III 9:182.

Sharma SR (1995). Management of mushroom diseases. In: Ad. in Horti .(ed Chaddha, K.L.) 13:195-238.

Sharma VP, Jandaik CL(1994). Effect of some plant materials in controlling different moulds in *A. bisporus* (Lange) Imbach. Indian J. Mycol. Plant Pathol. 24 (3): 183-185.

Singh M, Singh RD (2005). Management of mushroom pathogens through botanicals. Indian Phytopathol. 58 (2): 189-193.

Szumigaj TJ, Ullinski Z, Slusarski C (2012). Assesment of of effectiveness of biological agent in the control of fungal diseases of the white button mushroom. Progress Plant Prot. 52 (2):963-968.

Tiwari RP (2004). Now in the lime light. The Hindu Survey of Indian Agriculture. p.132.

academicJournals

Vol. 8(37), pp. 3409-3421, 10 September, 2014
DOI: 10.5897/AJMR2014.6930
Article Number: DE486A247925
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Comparative evaluation of antimicrobial properties and phytochemical composition of *Artocarpus altilis* leaves using ethanol, n-hexane and water

Mbaeyi-Nwaoha, I. E.* and Onwuka, C. P.

Department of Food Science and Technology, University of Nigeria, Nsukka, Enugu State, Nigeria.

Received 30 May, 2014; Accepted 1 September, 2014

This study was undertaken to describe the comparative evaluation of phytochemical constituents and antimicrobial activity of *Artocarpus altilis* leaves in three different solvents (ethanol, n-hexane and water). Qualitative and quantitative phytochemical analysis carried out confirmed the presence of alkaloids, tannins, flavonoids, steroids, reducing sugar, saponins, soluble carbohydrate, hydrogen cyanide and glycoside in all the extracts. The antimicrobial activity was tested using two different methods: Agar well diffusion method and agar dilution method against seven bacterial and three fungal strains. The results showed that antibacterial and antifungal activities were more effective using the agar dilution method with the ethanol extract having the highest inhibitory activity against the microorganisms as compared to the other solvents used (n-hexane and water). At concentrations of 10, 9, 8, 7 and 6 mg/ml, ethanol extract showed total inhibitory activity against all the test microorganisms used. N-hexane extract showed inhibitory activity against all the microorganisms up to a concentration of 8 mg/ml, while the aqueous extract had no activity against the microorganisms at all the concentrations tested (10 to 1 mg/ml). Under the agar diffusion method, the microorganisms were tested at concentrations of 100, 50, 25, 12.5 and 6.25 mg/ml and the method showed very minimal inhibition against all the microorganisms tested with only the ethanol extract showing a little degree of inhibition with an MIC of 3.98 mg/ml against *B. subtilis*. The other solvents (n-hexane and water) showed no activity against the test microorganisms at all the concentrations tested. Anti-nutrient analysis revealed the presence of phytate, oxalate, tannin and cyanide in all the extracts.

Key words: Agar diffusion method, anti-nutrient, *Artocarpus altilis* leaves, phytochemical constituents.

INTRODUCTION

Over the years, emerging and re-emerging infections and spread of deadly, drug-resistant strains of organisms pose a challenge on the global public health for their treatment. Bacterial resistance to antibiotics and anti-microbials over time has been a major therapeutic problem and the rate at which new antibiotics are now being produced is reducing (Russell, 2002). There is a continuous and urgent need to discover new antimicrobial compounds

*Corresponding author. E-mail: miphie2003@yahoo.co.uk or ifeoma.mbaeyi-nwaoha@unn.edu.ng.

with diverse chemical structure and novel mechanism of activities for new and re-emerging infectious disease. Therefore, researchers are increasingly looking for new leads to develop better drug against microbial infections. In recent years, secondary plant metabolites (phytochemicals), previously with unknown pharmacological activities, have been extensively investigated as a source of medical agents (Krishnaraju et al., 2005). These natural products can provide unique elements of molecular diversity and biological functionality, which is indispensable for novel drug discovery (Nisbet and Moore, 1997). Thus, it is anticipated that phytochemicals with adequate antibacterial efficacy can be used for the treatment of bacterial infections and illness ranging from sore eyes to sciatica. These plants have the capacity to produce a large number of organic chemicals so called secondary metabolites; which are divided into different categories based on their mechanism of function like chemotherapeutic, bacteriostatic, bactericidal and antimicrobial agent. The World Health Organization also has recognized the importance of traditional medicine and has been active in creating strategies, guidelines and standards for botanical medicines in the search for novel antimicrobial agents in ensuring the safety of man and also reducing losses of food products to microbial spoilage. The widespread use of this plant species in medicine and more recent empirical evidence suggest validity in its value in traditional medicine and potential for modern drug discovery (McIntoch and Manchew, 1993). Recent research in Indonesia has reported the patenting of phytochemicals isolated from leaf tissue of breadfruit trees for the prevention of stroke and cardiovascular diseases (Sagita, 2009).

Breadfruit (*Artocarpus altilis*), is a large, round, starchy fruit produced by a tree. It is a species of flowering tree in the Mulberry family of about 50 general and over 1000 species and was introduced into Venezuela in 1789 as a food for the slaves (Rincon, 2004). *A. altilis* is a long-lived, tropical, evergreen tree and has been cultivated in the Pacific Island for nearly three millennia. The breadfruit is principally grown as a source of carbohydrates and is an important component of agro-forestry systems. Unlike herbaceous starch crops harvested for their vegetative storage tissues, breadfruit is a tree grown for its fruit. In addition to producing abundant, nutritious, tasty fruits, this multipurpose tree provides medicine, construction materials, insect repellent and animal feed (Ragone, 2006). Over thousands of years of cultivation, humans have selected hundreds of cultivars, many of which are seedless and are vegetatively propagated. Seedless cultivars have no seeds but rather contain tiny aborted ovules (Deivanaz and Subhash, 2010). The loss of fertility in breadfruit is due to triploidy (2n=3x=84) (Ragone, 2001). The term ‘breadfruit’ is derived from the Greek words artos (bread) and karpos (fruit). The fruit when cooked or baked effuses a fragrance that is reminiscent of fresh baked bread hence the name (Natural Standard, 2013). It is a common plant in and around town occasionally planted as an ornamental plant in parks and gardens.

Breadfruit has long been a traditional starch crop through Oceania (Melanesia, Micronesia and Polynesia). The prominence of seedless cultivars increases as one travels from New Guinea eastward through Melanesia (where seeded cultivars are common) into Western Polynesia (where few seeded and seedless cultivars are prevalent) and into Eastern Polynesia (where virtually all cultivars are seedless triploids with identical zymotypes). The greatest diversity of seedless cultivars occurs in the eastern Polynesian islands (Society Islands and Marquesas) and Pohnpei and Chuuk in Micronesia (Ragone, 1991). Breadfruit trees grow to a height of about 85 ft (26 m) and bears fruits at seven years and continues to produce for 30 to 40 years, it grows best where annual rainfall is between 1,500 and 3,000 mm (McCoy, 2010). The leaves are thick, large alternate, coriaceous, ovate to oblong, up to 50 cm long, wide and acuminate and are deeply cut into pinnate lobes. All parts of the tree are rich in milky, gummy latex. The fruit is seedless with the surface marked with polygonal faces (Stuartxchange, 2012). The fruits are usually variable in shape, size and surface texture. They are round, oval or oblong ranging from 9 to 20 cm (3.6 – 8 in) wide and more than 30 cm (12 in). The tough skin is composed of five to seven-sided disks. The skin texture varies from smoothly to slightly bumpy or spiny. The colour is light green, yellowish-green or yellow when mature, although one unusual variety has pinkish or orange-brown skin. Research has shown that breadfruit (*A. altilis)* possess antibacterial, anti-inflammatory, antidiabetic, antioxidant, and immunomodulatory properties (Kumbhani, 2010). The crop is considered a carbohydrate food source. It is high in starch, and also in fair amounts of vitamin B and C. Studies has also shown presence of anti-nutritional factors such as oxalates, phytic acid, tannin, trypsin indicator and haemagglutin (Oladunjoye et al., 2010).

Therefore, this research work is aimed at evaluating the effects of the phytochemical and antimicrobial components of the seedless breadfruit (*A. altilis*) leaf extracts on some selected microorganisms. The specific objectives of this study include: to produce extracts from the seedless breadfruit leaf using three different extracts (ethanol, hexane and water); to determine the proximate composition and phytochemicals of seedless breadfruit leaf as well as the extracts, and; to evaluate the antimicrobial properties of these leaf extracts on the selected test organisms.

MATERIALS AND METHODS

Plant material

Fresh seedless breadfruit leaves were obtained from a compound in Ihe-akpu Awka village in Nsukka, Enugu state, Nigeria. The plant was identified by the botanist in Herbarium section, Department of

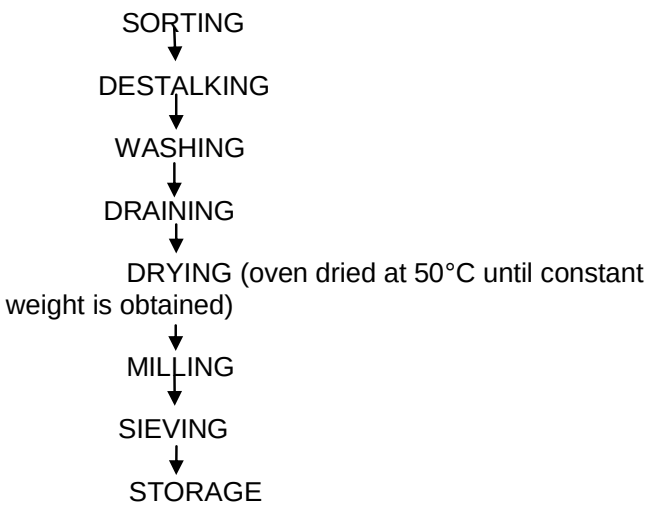

Figure 1. Flowchart for sample preparation.

Plant Science and Biotechnology, University of Nigeria, Nsukka, Enugu State.

Test organisms for antimicrobial activities/reagents

Test organisms used for the evaluation of the antimicrobial microbial potency of the leaves were obtained from Pharmaceutical Microbiology Laboratory of the Department of Pharmacy, University of Nigeria, Nsukka. All reagents used were all of analytical quality.

Sample preparation

The fresh seedless breadfruit leaves were sorted, destalked and washed thoroughly in water several times until they become clean and free from debris. The leaves were then drained, spread on trays and oven-dried till the weight became constant. After drying, the dried leaves were milled and then finally stored in an air tight container ready to use (Figure 1).

Extraction of antimicrobial compounds in seedless breadfruit leaves powder

The extraction was carried out using three solvents namely; water (used as control), hexane and ethanol. Cold maceration extraction as described by Anowi et al. (2012) was carried out. One (1) kg of the dried leaf powder was soaked in 2000 ml of analytical ethanol and was allowed to stand for 48 h with intermittent shaking. The suspension was filtered with filter paper and the filtrate (extract) was exposed for evaporation. After complete evaporation of the solvent, the crude ethanolic extract of the plant was recovered and stored at 4°C until used.

Fractionation of the crude extract

Fractionation of the crude extract was carried out adopting the method described by Shahia et al. (2001). Then, 200 g of ethanol crude extract was mixed with 400 g of silica gel until the extract was no longer sticky. The mixture was transferred into a 300 ml quick fit round bottom flask. 1000 ml of n-hexane was added into the bottle and shaken for about 10 min. The fraction was filtered and the mixture was further rinsed three times with 200 ml of the solvent (n-hexane) after which the filtrate became colorless. The filtrate was then oven dried at 40°C.

The supernatant rotavapour was used for 10 min at 40°C. After drying, the supernatant was transferred into the 300 ml round bottom flask and treated (fractionated) with 1000 ml of absolute ethanol and also shaken for about 10 min after which it was filtered and the mixture further treated three times with 200 ml of the absolute ethanol. The filtrate was collected and also oven-dried at 40°C. The supernatant was collected and oven-dried at 40°C for about 10 min after which it was transferred into the round bottom flask and then treated with 1000 ml of aqueous solution for about 10 min amidst shaking at intervals. The mixture was filtered and the mixture was further treated three times with 200 ml of aqueous solution. The filtrate was then collected and oven-dried at 40°C. The three different fractions (ethanol, n-hexane, and aqueous fraction) were obtained for evaluation of the antimicrobial activity.

Determination of phytochemicals in seedless breadfruit leaves powder and extracts

Qualitative phytochemical screening of seedless breadfruit leaves powder and its hexane, ethanol, and water soluble extracts was done. Phytochemical screening procedures to be used were adopted from Oloyed (2005). This analysis determined the biologically active compounds that contribute to the flavor, colour and other characteristics of seedless breadfruit leaves.

Test for alkaloids

About 0.2 g of each of the samples was boiled with 5 ml of 2% hydrochloric acid on a steam bath for 5 min. The mixtures were allowed to cool and filtered and the filtrates were shared in equal proportions into 3 test tubes and labeled A, B, C. One (1) ml portion of the filtrate was treated with 2 drops of the following reagents, respectively. With Dragendroff's reagent, a red precipitate was shown. With Meyer's reagent, a creamy white colored precipitate indicated the presence of alkaloid (Oloyed, 2005).

Test for flavonoids

About 0.5 g of each of the samples were introduced into 10 ml of ethyl acetate and heated in boiling water for 1 min. The mixtures were then filtered. Four (4) ml of the filtrates was shaken with 1 ml dilute ammonia solution and kept. Formulation of a yellow color in the presence of dilute ammonia solution indicated the presence of flavonoids (Oloyed, 2005).

Test for steroids

Approximately 9 ml of ethanol was added into the sample and refluxed for few minutes and then filtered. The filtrate was concentrated to 2.5 ml in a boiling bath, allowed to stand for 1 h and the waxy matter was filtered off. One (1) ml of concentrated sulphuric acid was carefully added to 0.5 ml of the chloroform extracted in a test tube to form a lower layer. A reddish brown interface showed the presence of steroids (Oloyed, 2005).

Test for saponins

About 0.1 g of each of the samples was boiled with 5 ml of distilled

water for 5 min, the obtained mixtures were filtered while still hot and the filtrates were then used for the following tests. To 1 ml of the filtrates, 2 drops of olive oil was added, the mixtures were shaken and observed for the formation of emulsion. Then, 1 ml of each filtrate was diluted with 4 ml of distilled water. The mixture was vigorously shaken and then observed on a stand for stable froth (Oloyed, 2005).

Test for tannins

Into 2 g of each of the samples was added 5 ml of 45% ethanol and boiled for 5 min. The mixtures were cooled and filtered. Then, 3 drops of lead subacetate was added into 1 ml of each filtrate. A gelatinous precipitate was observed indicating the presence of tannins. 0.5 ml of bromine water was added into another 1 ml each of the filtrates. A pale brown precipitate was observed indicating the presence of tannins (Oloyed, 2005).

Test for glycosides

Two grams (2 g) of each of the samples was mixed with 30 ml of distilled water and they were heated for 5 min on a water bath, filtered and used as follows: into 5 ml of the filtrates was added 0.2 ml of Fehling solution A and Fehling solution B until they turn alkaline and heated in a water bath for 2 min. A light blue coloration was observed (instead of brick red precipitate) which indicates the presence of glycosides (Oloyed, 2005).

Test for carbohydrates (Molisch test)

About 0.1 g of each of the sample was boiled with 2 ml of distilled water and filtered. To the filtrates, few drops of naphthol solution in ethanol (Molisch's reagent) were added. Concentrated sulphuric acid in a Pasteur pipette was then gently poured down the side of each of the test tubes to form a lower layer. A purple interfacial ring indicated the presence of carbohydrates (Oloyed, 2005).

Test for reducing sugar

About 0.5 g each portion was dissolved in distilled water and filtered. The filtrate was heated with 5 ml of equal volume of Fehling's solution A and B. Formation of a red precipitate of cuprous oxide was an indication of reducing sugar (Oloyed, 2005).

Quantitative phytochemical analysis of seedless breadfruit leaves powder and its extracts

The quantitative phytochemical analysis was carried out according to the method of Harborne (I973).

Determination of steroids

One gram (1 g) of each of the samples was weighed out and macerated with 20 ml of ethanol. The mixtures were filtered and 2 ml each of the filtrates were pipetted into test tubes. Then, 2 ml of colored reagent was then added into the test tubes and they were then allowed to stand for 30 min and the absorbance was measured at 550 nm.

Determination of flavonoids

One gram (1 g) of each of the sample was measured out and macerated with 20 ml of ethyl. The mixture was filtered and then 5 ml of the filtrate was measured into test tubes. To each test tube containing the filtrate, 5 ml of dilute ammonia was added and the mixture was shaken. The upper layers were collected and absorbance was measured at 490 nm.

Determination of saponin

One gram (1 g) each of the samples was weighed out and macerated with 10 ml of petroleum ether. The suspension was decanted into a beaker and another 10 ml of petroleum ether was added. The supernatant was decanted and mixed with the first. The mixture was evaporated to dryness and 6 ml of ethanol was added. Approximately 2 ml of the mixture was pipetted into a test tube and the mixture was allowed to stand for 30 min, then the absorbance was read at 550 nm.

Determination of alkaloid

About 1 g of the samples were weighed out and macerated with 20 ml of 20% H_2SO_4 in ethanol (1:1). The mixture was filtered and 1 ml of the filtrate was pipetted into test tubes. Five (5) ml of 40% H_2SO_4 and the content of the test tube were mixed properly. The mixture was allowed to stand for 3 h and the absorbance measured at 568 nm.

Determination of soluble carbohydrate

Approximately 1 g each of the samples were weighed out and macerated with 50 ml of distilled water. The mixture was filtered and 1 ml of the filtrate was pipetted into test tubes and 2 ml of saturated picric acid was added. The absorbance was then measured at 530 nm.

Determination of glycosides

About 1 g of the sample was weighed out and 2.5 ml of 15% lead acetate was added and the mixture was filtered. Two (2) ml of chloroform was added to the filtrate and the mixture was shaken vigorously. The lower layer was collected and evaporated to dryness. 3 ml of glacial acetic acid was added and the 0.1 ml of 5% ferric chloride and 0.25 ml concentrated H_2SO_4 were added and the mixture was shaken. The mixture was then left to stand in the dark for 2 h and absorbance was measured at 530 nm.

Determination of reducing sugar

0.5 g of the sample was treated with 10.0 ml of 80% ethyl alcohol. In 3.0 ml of the alcoholic extract, 3.0 ml of 3,5-dinitro-salicyclic acid (DNSA) reagent was added. The mixture was heated for five (5) minutes in a boiling water bath. After the colour had developed, 1.0 ml of 40% Rochelle salt was added when the content of tube was still warm. The tubes were then cooled by spraying with cold water. Absorbance was read and recorded at 515 nm.

Determination of anti-nutrients in seedless breadfruit leaves powder and crude extracts

Determination of tannin

The tannin content of the seedless breadfruit leaves powder and its extracts were determined by the method of Pearson (1976). One

gram of the test sample was weighed out into a conical flask and 10 ml of water was added. The mixture was shaken at 5 min interval for 30 min and filtered to get the extract. About 2.5 ml of the supernatant was transferred into a 50 ml flask. 1 ml of Follin-Denis reagent was then added in to the flask followed by 2.5 ml of saturated Na_2CO_3 solution. The absorbance was read at 720 nm spectrophotometrically after 90 min and incubated at room temperature.

$$\text{Tannin (\%)} = \frac{\text{An x C x 100}}{\text{As}} \times \frac{\text{Vf}}{\text{Va}}$$

Where, Aa = absorbance of test sample; As = absorbance of standard solution; C = Concentration of standard solution; W = Weight of sample used; Vf = Total volume of extract; Va = Volume of extract analyzed.

Determination of phytate

Phytate content was determined according to the method of Pearson (1976). Approximately 0.5 g of sample was weighed out into a 500 ml flat bottom flask, placed in a shaker and extracted with 100 ml of 2.4% hydrochloric acid for one hour at 25°C. After this, the mixture was decanted and filtered. 5 ml of the filtrate was diluted to 25 ml with distilled water and 10 ml of it was collected into a flask and 15 ml of 0.1 M sodium chloride was added to it. The mixture was passed through No.1 Whatman filter paper to elute inorganic phosphorus and 15 ml of 0.7 M sodium chloride was to elute phytate. The absorbance was read at 520 nm.

Determination of oxalate

The determination of oxalates was carried out by the titration method of AOAC (2010). Two grams (2 g) of sample was suspended in a mixture of 190 ml of distilled water and 10 ml of HCl in a 250 ml volumetric flask and digested for one hour at 100°C, cooled and made up to 100 ml with distilled water. The digest was then filtered through Whatman No. 1 Filter paper using a suction pump. A duplicate proportion of 125 ml of the filtrate was measured into 250 ml beaker and four (4) drops of methyl red indicator added into each beaker. Concentrated NH_4OH or NH_3 solution was added drop wise until the test solution changed from its salmon pink color to a faint yellow colour (pH 4 - 4.5). Each portion was heated up to 90°C and 10 ml of 5% $CaCl_3$ was added while being stirred constantly. After heating, it was cooled and left over night at 5°C. The supernatant was decanted and the precipitate completely distilled in 10 ml of 20% (v/v) H_2SO_4 solution. At this point, the filtrate resulting from digestion of 2 g of the sample was combined and made up to 300 ml. Aliquots (125 ml) of the filtrate was heated until near boiling and then titrated against 0.05 M standard $KMnO_4$ solution to a faint pink color. Oxalic acid content was calculated using the formula:

$$\text{Oxalate (mg/100 g)} = \frac{\text{T x (Vme) (Df) x } 10^5}{\text{ME x Mf}}$$

Where; T= Volume of $KMnO_4$; Vme = Volume – mass equivalent (1 ml of 0.05 M $KMnO_4$ solution is equivalent to 0.00225 g anhydrous oxalic acid); Df = The dilution factor; ME = molar equivalent of $KMnO_4$ in oxalic acid ($KMnO_4$ redox reaction is 5); Mf = mass of sample used.

Determination of cyanide

Cyanide content was determined according to the method of AOAC (2010). Five grams of the sample was weighed into a conical flask and 50 ml of distilled water was added to it and the solution was allowed to stand overnight and then filtered. 1 ml of the sample filtrate was measured into a test tube and 4 ml of alkaline picrate was added and it was allowed to stand for 5 min. The absorbance was read at 490 nm. The reading was taken with the reagent blank at zero.

Determination of antimicrobial properties of seedless breadfruit leaves powder

The antimicrobial properties of the seedless breadfruit leaves powder were determined using two different methods: Agar Well Diffusion technique described by Wan et al. (1998) and Agar Dilution technique as described by Wiegand et al. (2008).

Test microorganisms used

The test microorganisms used for the evaluation of the antimicrobial potency of the leaf extracts were clinical isolates of *Bacillus subtilis, Klebsiella pneumonia, Shigella dysenteriae, Proteous mirabellis, Staphyloccocus aureus, Echerichia coli, Salmonella typhi, Pseudomonas aeruginosa, Candida albicans* and *Aspergillus niger*

Standardization of the test organisms

The test microorganisms used were standardized using 0.5% MacFaland Turbid Equivalent. A 1% v/v solution of sulphuric acid was prepared by adding 1 ml of concentrated sulphuric acid to 99 ml of water and 1% w/v solution of barium chloride was prepared by dissolving 0.5 MacFarland equivalent standard prepared by adding 0.6 ml of barium chloride solution to 99.4 ml of sulphuric acid solution.

Preparation of nutrient agar

The media was prepared following the manufacturer's instruction (Oxoid, 1982). 28 g of dehydrated nutrient agar powder was suspended in 1000 ml of distilled water and was allowed to soak. The suspension was then dissolved by heating in a water bath at 100°C. Then, 20 ml volume each of the molten agar was dispensed into bijou bottles and autoclaved at 121°C for 15 min. The sterile molten nutrient agar was allowed to cool to 40°C.

Preparation of sabouraud dextrose agar (SDA)

The media were prepared according to the manufacturer's instructions. SDA powder (63 g) was suspended in 1000 ml of distilled and allowed to soak. The suspension was boiled to melt at 100°C in a water bath. The molten agar was sterilized in an autoclave at 121°C for 15 min. It was then allowed to cool to 40°C according to Oxoid (1982).

Preparation of agar slants

A 2.8 g quantity of nutrient agar and 3.8 g of Mueller Hinton agar was each dissolved in 100 ml of distilled water by heating over flame. 5 ml volume each of the molten agar was then dispensed into macAnthony bottles and sterilized at 121°C for 15 min. The sterile molten agar was kept in a slanting position to gel.

Preparation of culture

A 3 ml volume of sterile nutrient broth was prepared and the test

Table 1. Qualitative phytochemical composition of seedless breadfruit leaves using ethanol, n-hexane and water extracts.

Phytochemicals	Red sug	Sap	Alk	Sol CHO	Steroid	HCN	Gly	Flav
Water extract	+	++	++	++	++	+	++	+++
Ethanol	++	++	++	++	+	+	++	++
n-hexane	++	++	++	+	+	+	++	++
SBLP	++	++	++	++	++	+	++	+++

Key; + = present in low concentration; ++ = present in moderate concentration; +++ = present in high concentration; Red sug = Reducing sugar; Sap = Saponin; Alk = Alkaloid; Sol CHO = Soluble carbohydrate; HCN = Hydrogen cyanide; Gly = Glycoside; Flav = Flavonoid; SBLP = Seedless breadfruit leaves powder.

organism was inoculated into the nutrient broth aseptically under flame at 37°C for 24 h.

Agar well diffusion technique

The molten agar on cooling to about 40-47°C after sterilization was aseptically poured into sterile plates. Then, 0.1 ml of sterile water and 0.1 ml of the diluents was transferred into the center of a sterile Petri-dish. Also, 20 ml of the sterile molten agar was then added and the dish was swirled to mix its content. The plates were allowed to stand for 30 min so that solidification can take place. Six holes were then bored on each plate using a 6 mm diameter cork borer. Each hole was for a concentration of the breadfruit leave extract. The holes were labeled 1 to 6 to representing 100, 50, 25, 12, 6.25 and 3.125 mg/ml concentration of the three breadfruit leave extracts, respectively. Four drops of each concentration was dropped in corresponding holes and the plates were then kept undisturbed for 15 min so as to allow the extracts to diffuse properly and dry to a considerable level before incubation. The measurements (in millilitres) of the zone of inhibitions of the extracts against the test organisms were taken and recorded.

Determination of minimal inhibitory concentration (MIC) using agar dilution technique

The MIC of the extracts of the extracts was determined using agar dilution technique. A 50 g of the extract was dissolved in 10 ml of sterile water, thus obtaining 50 mg/ml stock solution, using the arithmetic method of dilution:

$C_1V_1 = C_2V_2$,

Where; C_1= initial concentration; C_2= final concentration; V_1= initial volume; V_2= final volume.

Concentrations (10, 9, 8, 7, 6, 5, 4, 3, 2 and 1 mg/ml) were obtained from the stock by seeding (4.0, 3.6, 3.2, 2.8, 2.4, 2.0, 1.6, 1.2, 0.8 and 0.4 ml) aliquots of the stock in their corresponding volumes (16, 16.4, 16.8, 17.2, 17.6, 18.0, 18.4, 18.8, 19.2 and 19.6 ml) of pre-sterilized Mueller Hinton agar at a temperature of 40°C. The media were then poured into sterile Petri dishes and allowed to solidify. The surfaces of the media were allowed to dry and streaked with 24 h old cultures of the test microorganisms. The plates were later incubated in an incubator at 37°C for 24 h, observations were made and it was further incubated for another 24 h, after which they were examined for the presence or absence of growth. The MIC was taken as the lowest concentration that prevented microbial growth.

Data analysis and experimental design

The proximate and anti-nutrient composition was analyzed using one-way analysis of variance (ANOVA) based on completely randomized design; mean separation will by Duncan's New Multiple Range Test. The antimicrobial testing was statistically analyzed using a 3-factorial or split plot in completely randomized design (CRD).

RESULTS

Phytochemical composition of seedless breadfruit leaves powder (SBLP) using ethanol, n-hexane and water extracts

Qualitative and quantitative phytochemical composition of seedless breadfruit leaves powder and ethanol, n-hexane and water extracts are shown in Tables 1 and 2. The result confirmed the presence of reducing sugar, saponins, alkaloids, soluble carbohydrate, steroid, hydrogen cyanide, glycosides, flavonoids and tannins (Tables 1 and 2, respectively).

The seedless breadfruit leaves had the highest amount of reducing sugar (2.15±0.005 mg/100g) while the n-hexane extract contained the highest amount of reducing sugar out of the three extracts. Ethanol extract was observed to contain 1.77 ± 0.00 mg/100g of reducing and finally the water extract had the least reducing sugar value of 1.45 ± 0.00 mg/100g.

The amount of saponin was observed to be the highest in the seedless breadfruit leaves with a value of 2.37 ± 0.01 mg/100g. Water extract had the least amount of saponin among all the extracts with a value of 2.20±0.005 mg/100g. The ethanol extract had a value of 2.21±0.00 mg/100g of saponin while the n-hexane extract had a value of 2.22±0.00 mg/100g.

Seedless breadfruit leaves had the highest amount of alkaloid with a value of 3.35±0.02 mg/100g. The ethanol extract had the highest alkaloid content (2.99±0.05 mg/100 g) out of the three extracts used. n-hexane extract contained 2.52±0.00 mg/100g while the water extract contained 2.12±0.00 mg/100 mg.

The steroid content of the seedless breadfruit leaves

Table 2. Quantitative phytochemical composition of seedless breadfruit leaves (SBLP) using water, n-hexane and ethanol extracts.

Phyt (mg/100 g)	RS	Sap	Alk	Sol CH	Ster	HCN	Gly	Flav
Water	1.45 ^{d}±0.00	2.20^{b}±0.01	2.12^{d}±0.00	0.79^{b}±0.01	1.92^{b}±0.00	0.05^{b}±0.01	2.37^{b}±0.00	3.11^{d}±0.01
Eth	1.77^{c}±0.00	2.21^{b}±0.00	2.99^{b}±0.00	0.69^{d}±0.01	1.72 ^{d}±0.00	0.04^{b}±0.01	2.32 ^{c}±0.01	3.79^{b}±0.01
n-H	1.98^{b}±0.00	2.22^{b}±0.00	2.52^{c}±0.00	0.71^{c}±0.00	1.89^{c}±0.01	0.04^{ab}±0.00	2.25^{d}±0.01	3.31^{c}±0.00
SBLP	2.15^{a}±0.01	2.37^{a}±0.01	3.35^{a}±0.02	0.86^{a}±0.00	1.94^{a}±0.01	0.05^{a}±0.01	2.83^{a}±0.01	3.94^{a}±0.01

Values are mean ± standard deviation of triplicate readings. Values having the same superscript within the column are not significantly different ($p < 0.05$). Key: RS = Reducing sugar; Sap = Saponin; Alk = Alkaloid; Sol CH = Soluble carbohydrate; HCN = Hydrogen cyanide; Gly = Glycoside; Flav = Flavonoid; SBLP = Seedless breadfruit leaves powder; Phyt = Phytochemicals; Eth = Ethanol; n-H= n-hexane.

was 1.94±0.005 mg/100 g and this value was higher than the values recorded in the other samples. Water extract had the highest steroid content with a value of 1.92±0.00 mg/100 g of all the extracts. Ethanol extract had a steroid value of 1.72±0.00 mg/100 g while n-hexane had a value of 1.89±0.01 mg/100 g.

The seedless breadfruit leaves had the highest glycolside content (2.83±0.005 mg/100 g). The water extract had a glycoside content of 2.37±0.002 mg/100 g, hence contained the highest amount of glycosides as compared to the other extracts. This was followed by ethanol extract which had a value of 2.32±0.01 mg/100 g and the n-hexane extract which had a glycoside content value of 2.25±0.01 mg/100 g.

The seedless breadfruit leaves had the highest amount of soluble carbohydrate with a value of 0.86±0.02 mg/100 g while the water extract had the highest amount of soluble carbohydrate out of the extracts with a value of 0.79±0.005 mg/100 g. This was followed by the n-hexane extract which had a value of 0.71±0.002 mg/100 g and the ethanol extract whose value was 0.69±0.05 mg/100 g.

The seedless breadfruit leaves had the highest content of flavonoids (3.94±0.01 mg/100 g) among all the samples. This was followed by the value of 3.79±0.01 mg/100g recorded for ethanol extract. The n-hexane extract had a value of 3.31±0.00 mg/100g and the water extract had the least value of 3.11±0.01 mg/100 g.

Antimicrobial activity of seedless breadfruit

Antimicrobial activity of seedless breadfruit leaves extracts using the agar diffusion technique

Table 3 shows the antimicrobial activity of seedless breadfruit of *A. altilis* leaves extracts (ethanol, n-hexane, and water). From Table 3, it was observed that the ethanol extract was able to inhibit the growth of *B. subtilis* at concentrations of 100, 50, 25 and 12.5 mg/ml. However, the organism showed resistance at concentration levels of 6.25 mg/ml and at all the concentration levels used in n-hexane and water extracts. The other test microorganisms used for the antimicrobial testing showed resistance at the various concentrations and in all the extracts used for the analysis. The minimum inhibition concentration of the ethanol extract against *B. subtilis* is shown in Table 4 and it was deduced to be 3.98 mg/ml (Figure 2).

Antimicrobial activity of seedless breadfruit leaves extracts using the agar dilution technique (ADT)

From the Tables 5 - 7, it is observed that the ethanol extract has the highest inhibitory activity against the microorganisms as compared to the other solvents used (n-hexane and water). At concentrations of 10, 9, 8, 7 and 6 (mg/ml), it showed total inhibitory activity against all the test microorganisms used. However, the minimal inhibitory concentration (MIC) for *Pseudomonas auroginosa* was observed at 6 mg/ml, followed by *Candia albicans Aspergillus niger, Salmonella typhi, Shigella dysentariae and Klebsiella pneumonia* at concentration of 5 mg/ml and then *B. subtilis, P. mirabellis, and E. coli* at concentration level of 3 mg/ml. Finally, all the test organisms showed activity at the concentration level of 1 mg/ml. The n-hexane fraction had a minimal inhibitory concentration of 8 mg/ml for *P. aeruginosa* and *A. niger*. At the concentration level of 3 mg/ml, it was observed that all the test organisms became resistant. The aqueous fraction had no inhibitory activity against all the test organisms at all the concentration levels.

Anti-nutrient content of seedless breadfruit leaves (SBLP) powder and its extracts

Test for anti-nutrient was carried out on the seedless breadfruit leaves and the extracts in order to determine the anti-nutrient present and also to quantify the level at which they are present. The analysis confirmed the presence of phytate, oxalate, cyanide and tannin. Table 8 shows the anti-nutrient content of the seedless breadfruit leaves powder and the water, n-hexane and ethanol extracts.

The phytate content of the seedless breadfruit leaves was the highest (1.55±0.00 mg/100 g). The water extract had the highest level of phytate out of all the extracts with

Table 3. Antimicrobial activity of seedless breadfruit leaf extracts (ethanol, n-hexane and water extracts) using the agar well diffusion technique

Extract conc. (mg/ml)	*B. sub*	*Shig*	*Kleb.*	*P. mir*	*S. aureus*	*E. coli*	*Sal*	*C. alb.*	*A. niger*	*Ps.*
Ethanol	100	16.90^{a}±0.14	NI	NI	NI	NI	NI	NI	NI	NI
	50	14.15^{b}±0.21	NI	NI	NI	NI	NI	NI	NI	NI
	25	11.20^{c}±0.28	NI	NI	NI	NI	NI	NI	NI	NI
	12.5	9.00^{d}± 0.00	NI	NI	NI	NI	NI	NI	NI	NI
	6.25	NI	NI	NI	NI	NI	NI	NI	NI	NI
n-hexane	100	NI	NI	NI	NI	NI	NI	NI	NI	NI
	50	NI	NI	NI	NI	NI	NI	NI	NI	NI
	25	NI	NI	NI	NI	NI	NI	NI	NI	NI
	12.5	NI	NI	NI	NI	NI	NI	NI	NI	NI
	6.25	NI	NI	NI	NI	NI	NI	NI	NI	NI
Aqueous	100	NI	NI	NI	NI	NI	NI	NI	NI	NI
	50	NI	NI	NI	NI	NI	NI	NI	NI	NI
	25	NI	NI	NI	NI	NI	NI	NI	NI	NI
	12.5	NI	NI	NI	NI	NI	NI	NI	NI	NI
	6.25	NI	NI	NI	NI	NI	NI	NI	NI	NI

Values are means ± standard deviation of duplicate readings. Samples having the same superscript within the column are not significantly different ($p<0.05$). *B. sub= Bacillus subtilis; Kleb= Klebsiella pneumonia; Shig = Shigella dysenteriae; P. mir = Proteous mirabillis; S. aureus = Staphylococcus aureus; E. coli= Echerichia coli; Sal = Salmonella typhi; C.alb. = Candida albicans; A. niger = Aspergillus niger; Ps = Pseudomonas aeruginosa*; NI = No Inhibition; Conc =Concentration.

Table 4. Determination of the minimum inhibition concentration (MIC) of the ethanol extract against *Bacillus subtilis.*

Concentrations (mg/ml)	IZD (mm)	Log of Conc (mg/ml)
100	17	2.0
50	14	1.7
25	11	1.4
12.5	9	1.1
6.25	-	0.8

Key: IZD = Inhibition zone diameter; Conc = Concentration.

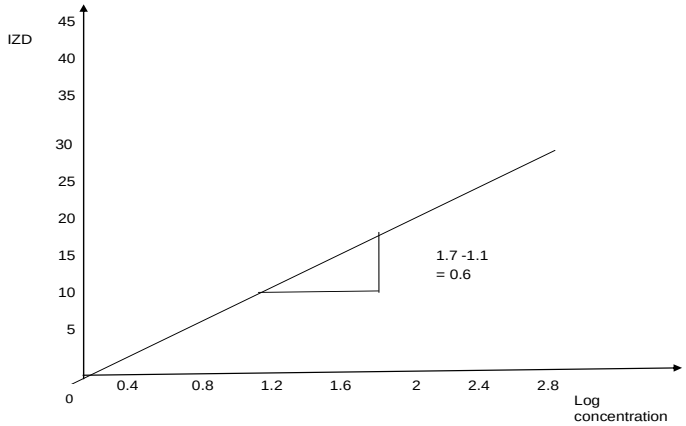

Figure 2. The minimum inhibition concentration of the ethanol extract against *B. subtilis*. Taking the antilog of 0.6; Antilog of 0.6 = 3.98. Therefore the minimum inhibition concentration of the ethanol extract against *B. subtilis* is 3.98 mg/ml.

Table 5. Antimicrobial activity of ethanol extract from seedless breadfruit leaves using agar dilution technique.

Conc (mg/ml)	Microorganism								
	B. sub Sal	***Shig***	***Kleb***	***P. mir***	***S. aureus***	***E. coli***	***C. alb***	***A. niger***	***Ps***
10	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	+
4	-	+	+	-	-	-	+	+	+
3	-	+	+	-	-	-	+	+	+
2	+	+	+	+	+	+	+	+	+
1	+	+	+	+	+	+	+	+	+

Key; + = Growth; - = No growth *B. sub = Bacillus subtilis; Kleb = Klebsiella pneumonia; Shig = Shigella dysenteriae; P. mir = Proteous mirabillis; S. aureus = Staphylococcus aureus; E. coli = Echerichia coli; Sal = Salmonella typhi; C. alb = Candida albicans; A. niger = Aspergillus niger; Ps = Pseudomonas aeruginosa*; Conc = Concentration

Table 6. Antimicrobial activity of n-hexane extract of seedless breadfruit leaves using agar dilution technique.

Conc (mg/ml)	Microorganism									
	B. sub	***Shig***	***Kleb***	***P. mir***	***S. aureus***	***E. coli***	***Sal***	***C. alb***	***A. niger***	***Ps***
10	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	+	+
6	-	-	+	-	-	-	-	+	+	+
5	-	+	+	+	-	-	+	+	+	+
4	+	+	+	+	-	-	+	+	+	+
3	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+	+
1	+	+	+	+	+	+	+	+	+	+

Keys; + = Growth; - = No growth
B. sub = Bacillus subtilis; Kleb = Klebsiella pneumonia; Shig = Shigella dysenteriae; P. mir = Proteous mirabellis; S. aureus = Staphylococcus aureus; E. coli = Echerichia coli; Sal = Salmonella typhi; C. alb = Candida albicans; A. niger = Aspergillus niger; Ps = Pseudomonas aeruginosa; Conc = concentration.

Table 7. Antimicrobial activity of aqueous (water) extract of seedless breadfruit leaves using agar dilution technique.

Conc (mg/ml)	Microorganism									
	B. sub	***Shig***	***Kleb***	***P. mir***	***S. aureus***	***E. coli***	***Sal***	***C. alb***	***A. niger***	***Ps***
10	+	+	+	+	+	+	+	+	+	+
9	+	+	+	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	+	+	+
7	+	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+	+
1	+	+	+	+	+	+	+	+	+	+

Key; + = Growth; - = No growth *B. sub = Bacillus subtilis; Shig = Shigella; Kleb = Klebsella pneumonia; P. mir = Proteus mirabillis; S. aureus = Staphylococcus aureus; E. coli = Escherichia coli; Sal = Salmonella typhi; C. alb= Candida albicans; A. niger = Aspergillus niger; Ps = Pseudomonas aeroginosa*; Conc = concentration.

Table 8. Anti-nutrient content of seedless breadfruit leaves powder (SBLP) and its extracts.

Anti-nutrients	Phytate (mg/100 g)	Oxalate (mg/100 g)	Tannin (mg/100 g)	Cyanide (mg/100 g)
Water extract	$1.46^{b} \pm 0.00$	$2.12^{b} \pm 0.00$	$4.95^{b} \pm 0.00$	$0.05^{ab} \pm 0.00$
n-hexane	$0.72^{c} \pm 0.00$	$0.59^{c} \pm 0.00$	$4.22^{d} \pm 0.00$	$0.04^{c} \pm 0.01$
Ethanol	$0.69^{d} \pm 0.00$	$0.57^{d} \pm 0.00$	$4.63^{c} \pm 0.00$	$0.04^{b} \pm 0.01$
SPLB	$1.55^{a} \pm 0.00$	$2.20^{a} \pm 0.01$	$5.75^{a} \pm 0.00$	$0.06^{a} \pm 0.00$

Values are mean ± standard mean deviation of triplicate readings. Values having the same superscript on the same column are not significantly different ($p<0.05$)
SBLP = Seedless breadfruit leaves powder

a value of 1.46±0.00 mg/100 g. This was followed by the n-hexane extract with a value of 0.72±0.00 mg/100 g and finally the ethanol having a value of 0.69±0.00 mg/100 g. The oxalate content of the seedless breadfruit leaves was highest with a value of 2.20±0.01 mg/100 g. Water extract was observed to have the highest level of oxalate with a value of 2.12±0.00 mg/100 g out of all the extracts. This was followed by the n-hexane extract having a value of 0.69±0.00 mg/100 g and the ethanol extract having the least oxalate content value of 0.57±0.00 mg/100 g.

The level of tannin observed in the seedless breadfruit leaves was highest with a value of 5.75±0.00 mg/100 g. The n-hexane had the least concentration value of 4.22±0.00 mg/100 g. Ethanol extract had a value of 4.63±0.00 mg/100 g while water extract had a tannin content value of 4.22±0.00 mg/100 g.

The cyanide content of the seedless breadfruit leaves and its extracts were observed to be low. The cyanide content of the seedless breadfruit leaves was observed to be 0.06±0.00 mg/100 g. This was higher than the values observed for all the extracts. The water extract had the highest cyanide content out of all the extracts with a value of 0.05±0.00 mg/100 g. The ethanol and n-hexane extract both had a cyanide content value of 0.04±0.01 mg/100 g.

DISCUSSION

Phytochemical composition of seedless breadfruit leaves powder (SBLP) using ethanol, n-hexane and water extracts

Earlier reports showed the presence of these bioactive components in the Moraceae family (Usman et al., 2000). Qualitative analysis indicated the presence of the phytochemicals and the level at which they are present in the various samples while quantitative analysis quantified the phytochemicals already identified in the qualitative analysis.

The amount of reducing sugar found in seedless breadfruit leaves powder is lower than the values reported by Salam et al. (2011) for some leafy plants such as *Chenopodium album* and *Stellaria media* (8.15 mg/100 g and 22.65 mg/100 g). It is also lower than the range of values obtained for reducing sugar (5.90±0.92 mg/100 g, 4.94±0.73 mg/100 g, 8.11±1.10 mg/100 g, 6.62±0.97 mg/100 g) reported by Mohammed et al. (2010) for *Morus alba, Morus nigrus, Morus laevigata* (white) and *Morus laevigata* (black), respectively.

The saponin content obtained in seedless breadfruit leaves agreed with the saponin value of 2.2±0.00 mg/100 g of *Vernonia amygdalina* (bitter leaf) reported by Nwaoguikpe (2010). The results obtained however contrasted with the values reported for various leafy vegetables consumed in Nigeria namely *V. amygdalina* (bitterleaf) (0.12± 0.02mg/100 g), *Corchorous olitorius* (ewedu) (0.13±0.01 mg/100 g), *Talirium triangulare* (water leaf) (0.12±0.01 mg/100 g), *Solanum monocarrnum* (garden egg) (0.28±0.01 mg/100 g), *Telfaririn occidentalis* (pumpkin leaf) (0.14±0.02 mg/100 g), *Abeimorchus esculentus* (okro) (0.24±0.13 mg/100 g), *Manihot esculenta* (cassava leaves) (0.25±0.11 mg/100 g) and *Amaranthus hybridus* (green leaves) (0.20±0.01 mg/100 g as reported by Agbaire (2012).

The alkaloid values observed in all the samples were higher than the range of values of 0.82±0.46 mg/100 g, 0.88±0.46 mg/100 g, 0.99±0.55 mg/100 g, 0.81±0.47 mg/100 g, 1.81±0.34 mg/100 g, 1.19±0.89 mg/100 g, 1.68±1.28 mg/100 g and 1.28±0.84 mg/100 g for “Utazi”, “Nturukpa”, “Nchanwu”, “Ugu”, “Onogbo”, “Oha”, “Ahihara” and “Okazi”, respectively reported by Onyeka and Nwambekwe (2007).

The steroid content obtained for all the extracts were higher than the range of values (0.07±0.01 mg/100 g to 0.27±0.01 mg/100 g) for common leafy vegetables consumed in the Eastern part of Nigeria as reported by Onyeka and Nwambekwe (2007).

The flavonoid values obtained for all the samples was higher than the range of values (0.03±0.01 mg/100 g to 0.22±0.06 mg/100 g) reported by Onyeka and Nwamkewe (2007) for common leaves such as ‘‘utazi”, nturukpa”, “nchanwu”, “ugu”, “onugbu”, “oha”, “ahihara” and “okazi” consumed in the Eastern part of Nigeria.

The exhibited antimicrobial properties of the *A. altilis* could be attributed to the presence of bioactive components such as saponins, steroids, alkaloids, tannins and flavonoids in the plant. Tomas-Barberan et al. (1990) reported that the presence of these compounds might have potentiated or complimented the saponins in the

antimicrobial activities in the plant where both alkaloids and alkaloids were present. These bioactive components have also been reported to have antimicrobial and medicinal effect medicinal effect (Iniaghe et al., 2009).

Antimicrobial activity of seedless breadfruit

The antimicrobial activity of seedless breadfruit using agar diffusion technique corresponds with the zone of inhibition range of 16.5 to 9 mm reported by Fakruddin et al. (2012) against Gram positive *B. subtilis* using the same solvent (ethanol). Antimicrobial activity of seedless breadfruit leaves extracts using agar dilution technique showed that the ethanol extract had a higher inhibitory activity against the test organisms used for the analysis than the n-hexane and water extracts as compared to the two methods used for the antimicrobial analysis, agar dilution technique proved to be a better method as compared to the agar diffusion technique in the present study. It was observed to produce inhibitory activity against all the test organisms used at concentration levels as low as 6 and 8 mg/ml for the ethanol and n-hexane fractions respectively. Whereas in the agar diffusion technique, inhibitory activity was very low with only the ethanol extract showing a degree of inhibition against *B. subtilis* at concentration level of 100, 50, 25 and 12.5 mg/ml. The low inhibitory activity of the extracts used in the agar diffusion technique could probably be as a result of the very low and diffusibility of the bioactive components present in the leaf.

Also, it could be observed that the ethanol extract had a higher inhibitory activity against the test organisms used for the analysis than the n-hexane and water extracts and this was in agreement with the report by Dash et al. (2011) that ethanol extracts of *Centella asiatica* had higher antimicrobial activities than n-hexane and water extracts. The results obtained also agrees with that of Vasugi et al. (2012) who reported that methanol and ethanol extracts of *Artocarpus altilis* showed high significant antibacterial and antifungal activities than aqueous extract. In the present study, with reference to the agar dilution technique, the ethanol and n-hexane extracts of the *A. altilis* leaves exhibited high inhibitory effect on all the test microorganisms used in the study and thus justifies the reports of its use by local communities for managing microbial infections in wounds, tooth ache and skin infections, ear infections and some intestinal infections (Ragone, 1997).

The antimicrobial study showed that the bacterial strains tested were more susceptible to the ethanol and n-hexane extract than the fungal strains. *P. aeruginosa* and *A. niger* which are both moulds were the first microorganisms to shows signs of resistance in the ethanol and n-hexane extract. The result was in agreement with the report of Lopolito et al. (2007) that moulds are more resistant than yeast and considerably more resistant than vegetative bacteria. It was also observed that the Gram-positive bacteria (*B. subtilis* and *S. aureus*) were the most inhibited of the test microorganisms in the ethanol and n-hexane extract. This result agreed with the report of Staden and Rube (1997) that Gram-positive bacteria are better inhibited than Gram-positive bacteria. The antimicrobial activities of the *A. altilis* could be attributed to the presence of bioactive compounds.

Anti-nutrient content of seedless breadfruit leaves (SBLP) powder and its extracts

The phytate content of all the samples were observed to be lower than those reported for some leafy edible vegetables of Southern Nigeria. The values include 13.00±0.40 mg/100 g, 6.11±0.22 mg/100 g, 5.39±0.22 mg/100 g, 4.12±0.45 mg/100 g for scent leaf, lemon grass, cassava leaf and green leaf, respectively (Agbaire, 2012).

In contrast to the test raw material, the oxalate range of values (0.09±0.01 mg/100 g to 1.00±0.22 mg/100 g) was reported by Agbaire (2012) for common leafy vegetables consumed in Southern Nigeria and observed to be lower than the value obtained for the seedless breadfruit leaves.

The tannin content obtained for all the samples were observed to be higher than those reported by Agbaire (2012) for some selected leafy vegetable. These include 0.26±0.03 mg/100 g (scent leaf), 0.12±0.02 mg/100 g (lemon grass), 0.20±0.01 mg/100 g (cassava leaf), and 0.19±0.01 mg/100 g (green leaf). Also, the values were found to contrast with the values reported by Onyeka and Nwambekwe (2007) as they are also found to be higher than the range of values (0.08±0.05 mg/100g to 1.68±1.28 mg/100 g) for some commonly consumed leafy vegetables in the Eastern part of Nigeria.

In contrast to the obtained cyanide content, Okoli et al. (2003) reported that the amount of hydrogen cyanide found in bitter leaf was 6.40 mg/100 g and this is higher than the values reported for both the seedless breadfruit powder and its extracts. The values were however in line with the values reported by Agbaire (2012) for selected leaves in Southern Nigeria, these include 0.13±0.03 mg/100 g (scent leaf), 0.23±0.01 mg/100 g (cassava leaf), 0.10±0.01 mg/100 g (green leaf) but was found to be lower than the 6.09±0.01 mg/100 g value reported for lemon grass.

From the results obtained on anti-nutrient composition, it is observed that the seedless breadfruit leaves contained the highest amount of anti-nutrients identified in the samples, while the ethanol extract contained the least anti-nutrients of all the samples. This could probably be due to the fact that the identified anti-nutrients in the leaves had the least solubility in ethanol leading to a lower yield as compared to the seedless breadfruit leaves in its intact state.

Earlier reports indicated that tannins act on the membrane of the microorganisms and shows the antibacterial

activity by precipitating the microbial protein (Rajesh et al., 2010; Qudsia et al. 2009; Hisonari et al., 2001) and flavonoids exhibit antibacterial activity by inhibition of the nucleic acid synthesis and cytoplasmic membrane function of the pathogen. Saponins also show antifungal, antibacterial and antiprotozoan effects (Morisaki et al., 1995). Studies have also shown that phytochemicals are present in all plant parts and that those present differ according to the type of extracting solvent used (Tijjani et al., 2009) hence this account for the differences observed in the content of content of phytochemicals in the ethanol, n-hexane and water extracts. The results from Table 8 confirmed the presence of anti-nutrients such as tannins, phytate, cyanide and oxalate. Phytates and oxalates are reported to bind to minerals such as calcium, magnesium, copper, iron and zinc hence making these minerals unavailable for absorption in the intestine. Tannins chelate metals such as iron and zinc and reduce the absorption of these nutrients. They also inhibit digestive enzymes and precipitate proteins (Bruno, 2010). However, processing such as cooking can destroy or reduce the effects of these anti-nutrients permitting the body to absorb the nutrient from them (Decker, 2011).

Conclusion

This research confirmed that the seedless breadfruit leaves extracts possess antimicrobial activity. The antimicrobial property of the seedless breadfruit leaves extracts can be attributed to the phytochemicals identified in the samples and the levels at which they were found to occur. Out of the two methods of antimicrobial analyses used (agar diffusion technique and agar dilution technique) which were compared in order to test for the antimicrobial properties of the samples, it was observed that the agar dilution technique produced a more effective antimicrobial activity as compared to the agar diffusion method. For the two methods compared, the ethanol extract was observed to have the highest inhibition against the microorganisms tested. This was followed by the n-hexane extract and finally the water extract had the least inhibition against the test organisms. With the low cost of the plant and its abundance especially in the rural parts, the leaves can be readily exploited for its potential in the production of medicinal herbs and food preservatives especially for the poor people in the rural areas where modern facilities are not readily available.

With the continuous development of resistant microorganisms to already developed antibiotics overtime, it is recommended that the use of natural extracts of the seedless breadfruit should be highly advocated since they contain chemicals which are similar to anti-bacterial agent and are also considered safer than the use of synthetic antibiotic drugs which sometimes may lead to one or more complications in the body.

It is also recommended that since the ethanol extract had the highest inhibition against the test organisms used, research should be carried out using other solvents in the same family with ethanol such as methanol, pro-panol, butanol and acetone in order to ascertain the level of their potency against pathogenic and spoilage organisms. Finally, it is recommended that more research work should be carried out on the seedless breadfruit leaves extracts as the inert potentials of the plant are not exhaustive in this research.

Conflict of Interests

The authors have not declared any conflict of interests.

REFERENCES

Agbaire PO (2012). Level of antinutritional factors in some common leafy vegetables of Southern Nigeria. 3(4):99-101.

Anowi CF, Nnabuife CC, Mbah, CJ, Onyekaba TC (2012). Antimicrobial evaluation of the methanolic extract of the leaves of *Nauclea latifolia*. Intl. J. Drug Res. Technol. 2(1):45-55.

AOAC (2010). Official Methods of Analysis. Association of Analytical Analysts, 18th edition, Washington D.C., USA.

Bruno J (2010). Phytates and Diet, www.livestrong.com/phytate/. Accessed 3rd August, 2013.

Dash BK, Faruquee HM, Biwas SK, Alam MK, Sisir SM Prodhan UK (2011). Antibacterial and antifungal activities of several extracts of *Centella asiatica L.* against some human pathogenic microbes. Life Sci. Med. Res. 35:1-4.

Decker F (2011). The effect of antinutrients in foods. www.livestrong.com>Home >Food&Drinks> Nutrition Information for Food> Nutrients in Foods. Accessed 3rdAugust, 2013.

Deivanaz S Subhash JB (2010). Breadfruit (*Artocarpus altilis* Fosb.)- An underutilized and neglected fruit plant species. Middle East J. Sci. Res. 6(5):418-428.

Fakruddin MD, Mannan KSB, Mazumdar RM, Afroz H (2012). Antibacterial, antifungal and antioxidant activities of the ethanol extracts of the stem bark of *Clausena heptaphylla*. BMC Complement. Altern. Med. 12:232.

Harborne JB (1973). Phytochemical method of analysis. Chapman and Hall Publications, New York.

Hisonari A, Kazuyasu F, Osamu Y, Takashi O, Keiji I (2001). Antibacterial action of several tannins against *Staphylococcus aureus.* J. Antimicrob. Chemother. 48 (4):487-491.

Iniaghe OM, Malomo SO, Adebayo JO (2009). Proximate composition and phytochemical constituents of leaves of some acalpha species. Pak. J. Nutr. 8(3):256-258.

Krishnaraju AV, Rao-Tayi VN, Sundararaju D, Vanisree M, Tsay HS, Subbaraju GV (2005). Assessment of bioactivity of Indian medicinal plant using brine shrimp (*Artemia salina*) Lethality Assay. Int. J. Appl. Sci. Eng. (2):125-134.

Lopolito P, Cabernett C, Polarine J (2007). Control Strategies for Fungal Contamination in Clean Rooms. www.ncbi.nlm.nih.gov/pubmed/3089567. Accessed on 4th of August 2013.

McCoy M (2010). Introduction to Breadfruit (*Artocarpus altilis)* to the Hawaaiian Island. Econ. Bot. 64(4):374-381.

Mclintoch C, Manchew P (1993). The breadfruit nutrition and health. Tropical fruits Newsletter 6:5-6.

Mohammed I, Hamayun K, Mohibullah SI, Rasool K, Faridullah K (2010). Chemical composition and antioxidant activity of certain morus species. J. Zhejiang Univ. 11(12):973-980.

Morisaki NS, Watanab M, Tezeka M, Zenikaya R, Shina N, Koyema T, Kanzaki, Saitto Y (1995). Mechanism of Angiogenic effects of Saponin from ginseng dixruberin human umbilical vein. Endothelial cells. Brit. J. Pharmacol. 11(7):1188-1193.

Natural Standard (2013). Breadfruit. www.naturalstandard.ccom>Home>Database>food,herbs and supplements>all topics. Accessed on 15th of January 2013.

Nisbet LJ, Moore M (1997). Will natural products remain an important source of drug research for the future? Curr. Opin. Biotechnol. 8: 706-712.

Nwaoguikpe RN (2010). The effect of extract of bitter leaf (*Vernonia amygdalina*) on blood of glucose level of diabetic rats. Int. J. Biol. Chem. Sci. 4(3)721-729.

Okoli IC, Anunobi MO, Obua BE, Enemuo V (2003). Studies on selected browses of South-Eastern Nigeria with particular reference to their proximate and some endogenous antinutritional constituent. Livest. Res. Rural Dev. 15:9.

Oladunjoye IO, Ologhobo AD, Olaniyi CO (2010). Nutrient composition, energy value and residual anti-nutritional factors in differently processed breadfruit (*Artocarpus altilis)* meal. Afr. J. Biotechnol. 9:4259-4263.

Oloyed OI (2005). Chemical Profile of Carical papaga. Pak. J. Nutr. 4: 379-381.

Onyeka EU, Nwambekwe IO (2007). Phytochemical profile of some green leafy vegetables in some South-East Nigeria. Nig. Food J. 25 (1):67-76.

Oxoid (1982). Oxoid manual: culture media, ingredients and other laboratory services, 5th ed. Oxoid Limited, Basingstoke. pp. 73-91.

Pearson DA (1976). Chemical analysis of foods, 7th Ed; Church Hill Livingstone, Edinburgh.

Qudsia K, Ishtiaq H, Hamid LS, Arshad J (2009). Flavonoids from mango leaves with antibacterial activity. J. Serbian Chem. Soc. 74 (12):1389-1399.

Ragone D (1991). Collection, establishment and evaluation of a germplasm collection of pacific island breadfruit. Ph.D dissertation, University of Hawaaii. Honolulu, Hawaaii, USA.

Ragone D (1997). Breadfruit, *Artocarpus altilis* (Parkinson) Fosberg. In: Promoting the conservation and use of underutilized and neglected crops Vol.10. Institute of Plant Genetics and Crop Plant Research, Gatersleben/International Plant Genetic Resources Institute, Rome, Italy. p. 37.

Ragone D (2001). Chromosome numbers and pollen stability of three species of Pacific Island breadfruit (Artocarpus Moraceae). Amer. J. Bot. 88:693-698.

Ragone D (2006). *Artorcarpus altilis* (breadfruit)–Guamsustainableag. Org. www.guam sustainable ag-org/fruittrees/altilis-breadfruit%20(8).pdf. Accessed on 5th of March 2013.

Rajesh K, Harsha R., Mohammed GA, Hareesh AR, Thammanna GSS, Dinesha R, Satiskumar BP, Irfan AM (2010). Antimicrobial activity of ethanol extract of leaf and flower of *Spathodea campanulata* P. Beauv. Res. J. Pharm. Biol. Chem. Sci. 1(3):692-698.

Rincon AM (2004). Physicochemical properties of breadfruit (*Artocarpus altilis*) starch from Margarita island, Venezuela. Archivos Latinoamericanos de Nutrición 54:4

Russell AD (2002). Antibiotic and biocide resistance in bacteria: Introduction. J. Appl. Microbiol. Symp. Suppl. 2:176-181.

Stuartxchange (2012). Phillipine medicinal plants. www.stuartxchange.com/ Rimas.html. Accessed on 7th March, 2013.

Sagita D (2009). Scientists on Indonesia's plant science frontier reporting fruitful results, Jakarta globe. www.thejakartaglobe.com/Indonesia/scientists-on-Indonesia's-plant-science-frontier-reporting-fruitful-results/18325.

Salam, JS, Joylani SD, Rebika ND, Priyadarshini S (2011). Secondary metabolites, antioxidant status and nutritive composition of two non-conventional leafy vegetables-*Stellaria media.* L and *Chenopodium album.* L. Indian J. Agric. Biochem. 24(2):136-140.

Shahia M, Alireza F, Teimour G, Ahmad GN (2001). Antibacterial Activity of the Crude Extracts and Fractionated Constituents of *Myrtus communis*. Pharm. Biol. 39 (5):399-401.

Staden VJ, Rube T (1997). Antibacterial Activity of South African Plants used for Medicinal Purposes. J. Ethnopharmacol. 56:61-67.

Tijjani M, Bello I, Aliyu A, Olurishe T, Maidawa S, Habila J, Balogun B (2009). Phytochemical and antimicrobial studies of root extract of *Cochlospermum tintorium.* Afr. Res. J. Med. Plants 3:16-22.

Tomas-Barberan FE, Iniesta SF, Tomas-lorente, Rumbero A (1990). Antimicrobial phenolic compounds from three Spanish *Hellichrysum* species. Phytochemistry 29:1093-1366.

Usman H, Abdulrahman FI, Usman A (2000). Qualitative phytochemical screening and in-vitro antimicrobial effect of methanol stem bark extract of *Ficus thonningi* (Moraceae). Afr. J. Tradit. Complement. Altern. Med. 6 (3):289-295.

Vasugi R, Sudhahar D, Anandajogopal K (2012). Preliminary phytochemical investigation and screening of antimicrobial activity of leaf extracts of *Artocarpus altilis.* Asian J. Biol. Life Sci. 4: 105-106.

Wan J, Wilcock A, Conventry MJ (1998). Comparative evaluation of methods commonly used to determine antimicrobial susceptibility to plant extracts and phenolic compounds. J. Appl. Microbiol. 84: 152-158.

Wiegand I, Hilpert K, Hancock RE (2008). Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat. Protoc. 3(2):163-175.

academicJournals

Vol. 8(37), pp. 3422-3434, 10 September, 2014
DOI: 10.5897/AJMR2014.6802
Article Number: B5A96EE47927
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Morphological, physiological and pathogenic variability of small-spore *Alternaria* sp. causing leaf blight of Solanaceous plants in Algeria

BESSADAT Nabahat[1*], SIMONEAU Philippe[2], BENICHOU Soumaya[1], SETTI Benali[3], Kihal Mabrouk[1] and HENNI Djamel Eddine[1]

[1]Laboratoire de Microbiologie Appliquée, Université d'Oran Es-Senia, BP1524 El M'naouer 31000 Oran, Algeria.
[2]SFR QUASAV 4207, UMR 1345 IRHS, Université d'Angers, 2 boulevard Lavoisier, France.
[3]Institut des Sciences Agronomiques, Université de Chlef, Algeria.

Received 25 February, 2014; Accepted 9 June, 2014

Due to premature defoliation, early blight epidemics can cause major yield losses. Large-spore *Alternaria* species such as *A. solani* and *A. tomatophila* have long been recognized as important pathogens responsible for such blight disease in the family *Solanaceae* and thus represent a serious risk for crop production. Small-spore *Alternaria* species have also been frequently isolated from plant samples with typical blight symptoms but their incidence as primary pathogens is often controversial. In order to study the diversity of small-spore *Alternaria* species, 32 isolates were selected from a larger collection of 130 isolates from infected leaves, fruits and stems of tomato from various growing regions of North-West Algeria. Morphological characterization under standard conditions and polymerase chain reaction (PCR) analyses using specific primers to amplify a part of the ITS regions and the 5.8S gene were conducted to confirm their identification as members of the *alternata* section. They were then examined according to morphological characteristics of conidia and sporulation patterns on potato carrot agar (PCA) and were segregated into three morphological species: *A. alternata*, *A. tenuissima* and *A. arborescens.* Colony type, substrate colour, margin, zonation, pigmentation, colony diameter and conidia production were studied on potato sucrose agar (PSA). Physiological parameters and nutritional requirements of the isolates were also assessed and a data matrix based on cluster analysis and Euclidean distance was constructed. Results of pathogenicity test on tomato showed obvious diversity among the isolates and they could be separated into two groups based on their virulence. The dendrogram based on the influence of cultural, nutritional and physiological characters suggests moderate heterogeneity within the populations of *A. alternata* and *A. tenuissima*. The small-spore species formed five clusters that fundamentally paralleled the morphological groupings. However, the results provided no evidence for geographical and pathogenicity clustering of isolates.

Key words: Epidemiology, *Solanaceae*, small-spore *Alternaria* species, pathogenic variability, cultural characters, physiological diversity.

INTRODUCTION

Tomato *(Lycopersicon esculentum L)* often called as "queen of kitchen" is one of the oldest known and important vegetable crops grown in the world. It is commonly used for culinary purposes and consumed in

diverse ways, including raw, as an ingredient in many dishes, sauces, salads, and drinks. In Algeria, it represents a surface of 12173 Ha which covers 57.36% of the global cultivated area. Its production is ca 10 MQx of which 3.8 MQx is for industrial use (that is equivalent to 95% of the total industrial culture production) (Snoussi, 2009). Due to its high adaptability, early blight has the potential to become a serious threat for tomato crops in northwestern Algeria, because of the favorable environment; it affects foliage as well as tomato fruit reducing yield both qualitatively and quantitatively. Epidemics occur when the weather is warm and dry with short periods of high moisture and moderate temperatures (18 to 30°C). The fungal pathogens responsible for the disease belong to the genus *Alternaria* and infect the plant through conidia which are either wind-blown or splashed onto plant surfaces (Andersen and Frisvad, 2004; Leiminger et al., 2010). Both large-spore forming species (*A. solani* and *A. tomatophila*) and small-spore *Alternaria* spp. have been isolated from diseased tomato (Simmons, 2000).

The *Alternaria* genus includes nearly 275 species (Simmons, 2007) with saprophytic and plant-pathogenic lifestyles that may affect crops in the field or cause harvest and post-harvest decay of plant products (Logrieco et al., 2009). *Alternaria* taxonomy has long been mainly based on conidia morphology and sporulation pattern. Nishimura and Kohmoto (1983), using a statistical analysis of the size of conidia concluded that *Alternaria* isolates producing small spores belong to what they called a "collective species" *alternata*. From then, several authors (Kusaba and Tsuge, 1995; Johnson et al., 2000; Tsuge, 2003) have accepted these findings. To facilitate segregation and identification, Simmons and Roberts (1993) introduced the 3-dimensional sporulation pattern as a means of scoring small-spore isolates from Asian pears into groups. They described six major groups with characteristic sporulation patterns of which three will be treated in this paper. More recently based on phylogenetic studies, the *Alternaria* genus was separated into 24 sections and small-spore forming *Alternaria* were grouped into the *alternata* section that comprises almost 60 *Alternaria* species (Woudenberg et al., 2013; Lawrence et al., 2013). The molecular variation within the *alternata* section is low and these species were thus mainly differentiated based on phenotypic variation.

Phenotypic variations within fungal populations can generally be detected using morphological, cultural and pathogenic criteria. Understanding pathogen population structure and mechanisms by which such variations arise within a population is of paramount importance for devising a successful disease management strategy.

Most important environmental factors controlling the growth and sporulation of fungi are temperature, hydrogenion concentration and both carbon and nitrogen sources. A small variation in these factors may induce clear differences in their morphological characters, growth and sporulation (Bilgrami and Verma, 1978). Concerning pathogenic variability, it has been well established that *A. solani* and *A. tomatophila* could be considered as primary pathogens responsible for early blight of tomato (Simmons, 2000; Lourenço et al., 2009), while tomato isolates belonging to the *alternata* section have often been considered as successful invaders of preformed necrotic lesions with poor capability to infect green foliage in the field (Spits et al., 2005). Despite this, it is also well recognized that pathogenic populations (pathotypes) with narrow host range exist within the *alternata* section. *A. arborescens* responsible for the tomato stem canker constitutes a typical example (Grogan et al., 1975, Mesbah et al., 2000).

The objective of this study was to evaluate the importance of small–spore *Alternaria* species and to analyze the phenotypic variability within these populations isolated from tomato with early blight symptoms. We have therefore collected several isolates from diseased tomato in Algeria, analyzed their morphological characteristics based on growth and sporulation patterns and cultural behaviour on different media. We have also studied their virulence on tomato and showed that they can be divided into two groups based on their relative aggressiveness.

To the best of our knowledge, this constitutes the first report of leaf blight in solanaceous crops caused by small-spore *Alternaria* that is belonging to the *alternata* section, in the northwestern Algeria.

MATERIALS AND METHODS

Isolation

Samples of fresh infected leaf, stem and fruit of tomato were collected from 65 fields of commercial farmers located in the main vegetable producing cities in northwestern of Algeria including: Oran, Mascara, Mostaganem, Ain témouchent Relizane, Tlemecen and Sidi Bel-Abbès.

Identification of isolates

Isolation of *Alternaria* strains was done from small pieces of infected tissues after surface disinfection using 3% sodium hypochlorite (NaOCl) for 5 min. Tissue samples were then rinsed with sterile water and placed onto potato sucrose agar (PSA) medium and incubated at 25°C for 7 days. Cultures that were contaminated with bacteria were transferred on PSA amended with streptomycin (50 µg ml^{-1}). Pure cultures were obtained for each of the isolates using the single spore technique according to Hansen (1926).

Single spore colony was transferred to the same PSA medium. The

*Corresponding author. E-mail: bessadatnabahat@hotmail.com.

Table 1. Scale for evaluation of the mean mycelia growth and conidial production of *Alternaria* isolates.

Mean mycelial growth (mm/day)	Conidial production (×10^5 spores.mm^{-2})	Rating
0 to 3.0	0 to 10	0
3.1 to 6.0	10.1 to 20.0	1
6.1 to ≤	20.1 to ≤	2

130 pure isolates were stored at 4°C on PSA slants for further study. Identification of small spore *Alternaria* was performed according to a previously established protocol (Simmons, 2007).

DNA isolation and molecular characterization

DNA isolation from the studied *Alternaria* isolates was carried out according to the microwave miniprep procedure described by Goodwin and Lee (1993). The PCR amplification was carried out in a Biorad thermocycler using the following scheme: initial denaturation at 95°C for 3 min, then 30 cycles consisting of 95°C for 30 s, 55°C for 50 sand 72°C for 1 min, and a final elongation at 72°C for 10 min. Amplifications were carried out using primers AAF2 (5'-TGCAATCAGCGTCAGTAACAAAT-3') and AAR3 (5'-ATGGATGCTAGACCTTTGCTGAT-3') able to amplify a part of the ITS regions and the 5.8S gene specific to *A. alternata*-related species (Konstantinova et al., 2002). Amplification products were resolved on 1.2% agarose gels in 0.5×TAE buffer (20 mMTris-acetate pH 8, 0.5 mM EDTA) followed by ethidium bromide staining.

Cultural, nutritional and physiological characters of fungal strains

Thirty-two isolates were studied for their cultural and morphological variations on six culture media, potato carrot agar (PCA) (Simmons, 2007), malt extract agar (Messiaen et al., 1991), potato sucrose agar (PSA) (Samson et al., 2002), Czapek dox agar (Salam et al., 2006), Mathur (Mathur et al., 1950) and Sabouraud (Dongyou, 2010) selected for their ability to support *A. alternata* growth. Each figure was inoculated by transferring 5-mm mycelia disc from seven days old culture of each isolate and was incubated at 25±1°C for growth.The different colony characters like pigmentation, sporulation, and zonation were recorded in PSA medium by visual observation after 7 days. For morphological conidia chain examinations, the unsealed inoculated PCA figures were incubated under a daily natural light/dark cycle and maintained at a moderate temperature (22°C); cultures were observed after 5 days at 50× magnification with a stereomicroscope. Further examination was done at ×400 magnification using a compound microscope. Each spore suspension was mounted in lactophenol on a microscope slide and measured using a micrometer (septation and size). Thirty records per isolate were made for the purpose.

Effect of carbon and nitrogen sources

Czapekdox was used as a basal medium to study the effect of nutrients such as carbon and nitrogen compounds. Ten carbon sources were tested at 3% (v/v): sucrose, glucose, fructose, lactose, maltose, starch, cellulose, glycerol, mannitol and citric acid. Eight nitrogen sources were also tested at 0.2% (v/v): potassium nitrate, sodium nitrate, ammonium sulfate, asparagine, valine, leucine, arginine and peptone as described by Attrassi et al. (2007).

Effect of pH

To test the influence of pH on fungal growth, isolates were cultured in PSA initially adjusted to different pH ranging from 4.0 to 10.0 using dilute acid or alkali (Vijayalakshmi et al., 2012).

Effect of temperature

Petri dishes containing PSA medium were inoculated with 5-mm mycelial discs from ten-day-old culture of different isolates. The inoculated figures were incubated at different temperature: 5, 10, 15, 20, 25, 30 and 35°C (Hubballi et al., 2010).

Fungal growth and sporulation

Measurements on radial colony diameter were recorded 2^{th}, 4^{th}, 6^{th}, 8^{th}, 10^{th} and 12^{th} day after inoculation. Growth rate per day was calculated by the formula (Sofi et al., 2012):

χ = *Growth at day N (mm) - Growth at day N-2 (mm)/2*

Sporulation was determined by harvesting the conidia from the surface of ten-day-old colonies by flooding each figure with 10 ml of sterilized distilled water and scratching the agar surface with the help of rubber spatula. The resulting suspension was filtered through muslin cloth and concentration of the spores was measured with the help of haemacytometer. Total number of spores on the colony and number of spores per unit area were calculated. In this study, all assays were replicated three times.

All the 32 isolates were categorized into groups depending on rates attributed on the mean mycelia growth and conidial production using the scale described by Attrassi et al. (2007) and modified as follow in Table 1.

Bioassays for pathogenicity of fungal isolates

In order to confirm the identification of the disease and its causal agent, the pathogenicity test was performed using a detached leaf technique as described by Reni et al. (2007). Detached healthy leaflets of tomato cultivar Saint Pierre from leaf N^o 5 were cleaned with sterile distilled water, dried and transferred to Petri dishes with moisten filter papers. Leaflets were inoculated by two different methods. For the first method, conidial suspensions of 7 days old culture grown on PSA medium were prepared as previously described. Spore density was adjusted using a heamacytometer technique to 10^5 cfu/ml (Brame and Flood, 1983). A 20µl drop of the suspension was then applied to the surface of the leaf. A control experiment was carried out simultaneously using sterile distilled water. For the second method, leaflets were inoculated with 5-mm plugs of 7-day-old cultures of the isolate. The inoculum was placed in the middle of each leaf. Leaves for control were mock inoculated with a plug of sterile PDA (Loladze et al., 2005; Park et al., 2008).

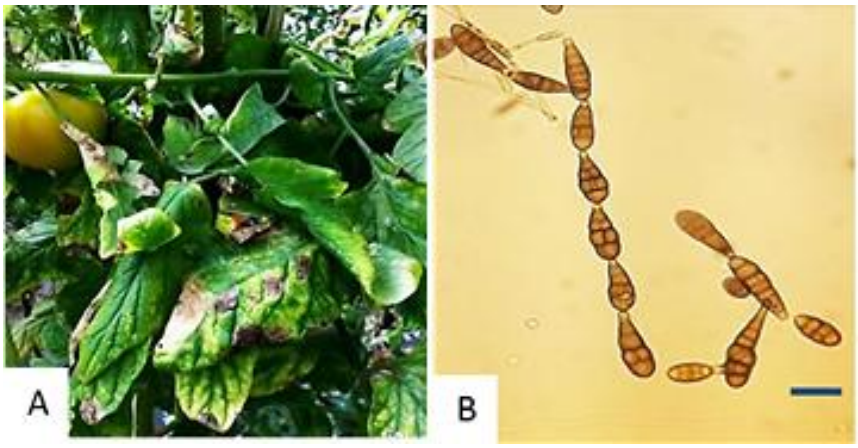

Figure 1. **A)** Leaves of tomato plants showing typical symptoms of early blight disease. **B)** Light micrographs of conidia of strains isolated from diseased tomato organs. Bars= 15 µm.

Table 2. Collected samples from northwestern Algeria regions from different fields of tomato production.

Zone	Season	Isolate	Sample
Oran	Spring and Summer	A26, A21	48
Mascara	Spring and Summer	A13, A15, A17, A18, A19, A20, A24	11
Mostaganem	Spring and Summer	A4, A6, A7, A8, A9, A10, A11, A16, A22, A23, A25, A27, A30	31
Ain témouchent	Summer	A1, A3, A12, A14, A28	10
Relizane	Summer	A31, A32	3
Telemcen	Summer	A2	11
SidiBel-Abbès	Summer	A5, A29	16

(20−23°C). All isolates were evaluated and each assay was replicated three times.

Disease assessment

The impact of the pathogens was determined by visual rating of disease symptoms 1 week after inoculation; the assessment was carried out by measuring the diseased area of the leaflets and compared to the control using a scale as follows: 0 = no symptoms, 1 = 0 to 30% of leaf area necrotic and chlorotic, 2 = 31 to 60% of leaf area necrotic and chlorotic, and 3 = 61 to 100% of leaf area necrotic and chlorotic (Van der Waals et al., 2004; Kheder et al., 2012).

Statistical analysis

Averages of colony diameter and number of conidia/ml for each treatment combination were evaluated for subsequent data analysis. Analysis of variance and separation of means to determine differences in growth rates and sporulation under different growth conditions was carried out. The data were statistically analyzed and the treatment means were compared. Agglomerative hierarchical clustering (Ward, 1963) of cultural, nutritional and physiological parameters were combined to construct a dendrogram using the STATISTICA 5.0 software. Euclidean distances were transformed according to the formula: 100 × distance between two points / maximal distance. This enabled the interpretation of results as 0–250%.

On the other hand, the analysis of the results from the virulence assays was performed on the basis of the means and standard deviation using nonhierarchical classification (Digby and Kempton, 1987). It is often found that nonhierarchical methods provide a more acceptable classification into fewer major groups. The groupings obtained are also more robust to any aberrant similarities between individual pairs of units (Digby and Kempton, 1987).

RESULTS AND DISCUSSION

Isolation of *Alternaria*

All isolates were collected from plants displaying similar symptoms characteristic of the early blight disease. They affected leaves, stems and fruits (Figure 1A).

Mycological analysis indicated that tomatoes in the seven main producer cities of vegetables in northwestern of Algeria were strongly invaded by small-spore *Alternaria* spp (section *alternata*). The cultural and morphological characters of 130 isolates were closely similar to those described by Simmons (2007) (Table 2). Conidiophores arising singly or in small groups produced spores in chains and conidiospores were large with longitudinal and transverse septa and a short beak typical for *A. alternata* and related species (Figure 1B). Their identification was further confirmed by molecular analysis using specific primers. As shown in Figure 2, the amplification of DNA from these small-spores isolates using the AAF2

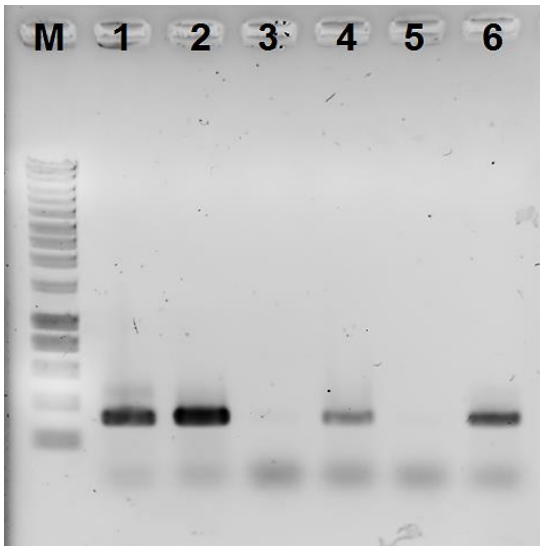

Figure 2. Sample gel showing amplification products from *Alternaria* genomic DNA using the AAR2/AAF3 primer pair. Template DNA was isolated from: *A. alternata* (lane 1), *A. solani* (lane 3), *A. tomatophila* (lane 5), *Alternaria* isolates from diseased tomato (lanes 2,4, and 6). Lane M, 100 bp molecular weight marker.

/AAR3 primer pair resulted in a specific 341-bp PCR product. At the same time, no amplification product was observed using the same primer pair and DNA from *A. tomatophila* and *A. solani*, two species often isolated from symptomatic tomato leaves, confirming the identity of the chosen isolates as members of the *alternata* section.

Cultural characteristics of the isolates on PSA medium

Thirty-two fungal isolates were randomly selected for further characterization of their colony morphology on potato sucrose agar (PSA). The results revealed a considerable variation in macroscopic characters of these isolates collected from different locations this morphological species were. Colony color varied from light to dark olivaceous with greenish or brownish tinge. Mostly, the colonies had velvety or cottony mycelia growth with slight variations and regular to irregular margin and without concentric zonation. All the isolates impregnated the media with a color mostly grey to brown with some variations that were clearly visible from the underside of figures. These observations are in agreement with Pusz (2009) who reported that the colonies of *A. alternata* isolated from *Amaranthus retroflexus* varied from light grey to dark grey. Similarly, Rai and Kumari (2009) observed loose, cottony, compact and dense colonies with light to dark black color for *A. alternata* infecting Periwinkle. The findings of Hubballi et al. (2010) showed variation in the pigmentation of 15 *A. alternata* isolates producing black, brownish black, greenish black, brown and yellow pigmentation.

Variation with respect to topography of the colony type was also observed. Two isolates (A21 and A26) had cottony sub-aerial mycelia growth while, 13 isolates (A4, A6, A7, A8, A9, A10, A11, A16, A22, A23, A25, A27 and A30) had medium cottony growth. Five isolates (A1, A3, A12, A14 and A28) had cottony central growth. Seven isolates (A13, A15, A17, A18, A19, A20 and A24) produced velvety, oppressed mycelium whereas, two isolates (A5 and A29) grown with velvety mycelium and furrowed margin. One isolate (A2) had velvety mycelium with oppressed margin.

All the isolates produced spores on PSA (Figure 5B). High sporulation was observed for one isolate (A12), while for most of the isolates moderate sporulation was recorded. Poor sporulation was observed only for one isolate (A7). Variation in growth rates on PSA was also observed (Figure 3). The fastest mean growth rate was obtained for isolate A31 (mean 14.90 ± 0.614 mm/day) and the slowest in the (A2) isolate (mean 8 ± 0.487 mm/day). Isolates in the present study depicted periodic changes in their growth rates. Four isolates grew very fast in the initial 2–4 days of observation (A12, A13, A16 and A17) but decreased afterwards while most of the isolates had the highest growth rate at 6 days and one isolate (A10) grew faster at the end of the experiment. Almost similar observations were performed by Pusz (2009) and Hubballi et al. (2010) when measuring growth rates of *A. alternata* and *A. mali* isolates, respectively.

Sporulation patterns of the isolates on PCA medium

All the 130 *Alternaria* isolates collected from plants in the family *Solanaceae* in northwestern Algeria were characterized for their sporulation patterns and conidial characteristics on potato carrot agar (PCA) at 7 days. All isolates could be grouped into three types that corresponded to type 3, 4 and 5 sporulation patterns as defined by Simmons and Roberts (1993). Figure 4 shows the morphological characteristics of conidia and sporulation patterns of representative isolates of each group. Type 4 sporulation pattern (Figure 4A) appeared as low bushy clumps of well-branched chains. Conidia varied from obpyriform to ovate to obclavate, yellowish-brown to brown, with 1-8 transverse and 0-3 longitudinal or oblique septa. Spore body size was 6.5-59.8 × 4.2-16.5µm. This typical *A. alternata* accounted for 33. 85% and the selected isolates from this morphological species were A1, A2, A10, A11, A12, A13, A15, A16, A19, A21, A24, A26, A28, A30 and A32. Type 5 sporulation pattern (Figure 4B) corresponded to moderately long to long chains of more than 9 conidia, branching of chains usually was minor (1 to 2 conidia) or lacking. Conidia produced were obclavate or ellipsoidal, brown to golden brown, some conidia with minutely verrucose walls. Mature conidia with 4-9 transverse septa and 0-4 longitudinal or oblique septa were

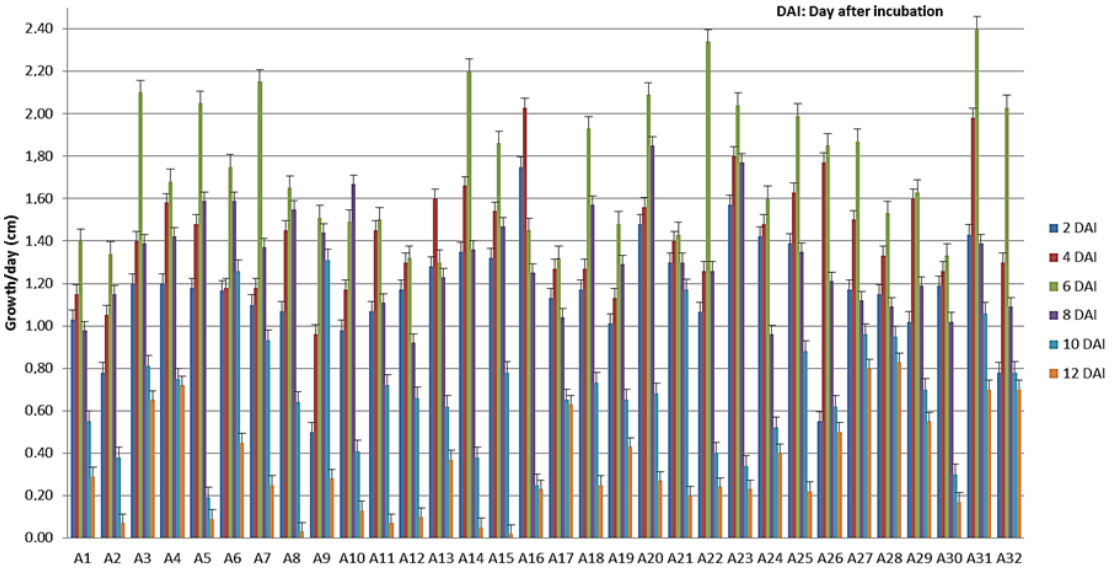

Figure 3. Variability in the growth rates of *Alternaria* isolates on PSA medium.

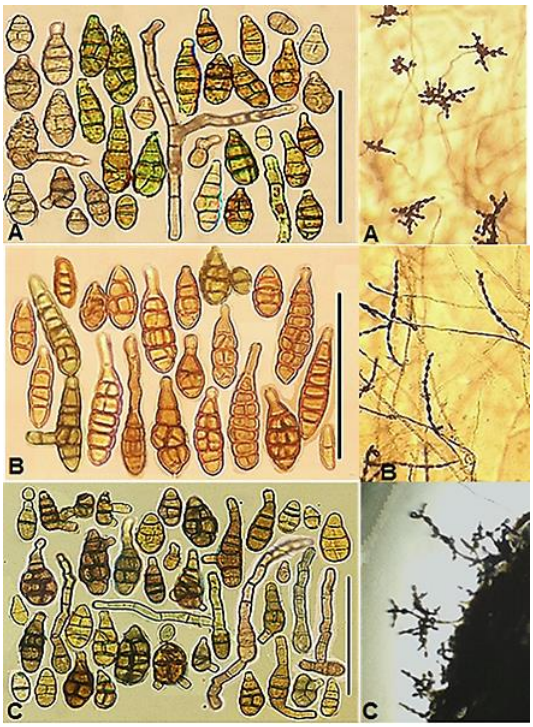

Figure 4. (A) *Alternaria alternata* conidia and sporulation pattern (PCA 7d) from isolate A15. **(B)** *Alternaria tenuissima* conidia and sporulation pattern (PCA 7d) from isolate A8. **(C)** *Alternaria arborescens* conidia and sporulation pattern (PCA 7d) from isolate A23. Bars=50 µm.

observed. Conidium body size was 9.8-60.20 × 8.6-15.5 µm. This typical *A. tenuissima* accounted for 64.61% and the chosen isolates were A3, A4, A5, A6, A7, A8, A9, A14, A17, A18, A20, A22, A25, A27, A29 and A31. Type 3 sporulation pattern (Figure 4C) appeared as conidial chains of 2-6 units long and typically produce branches

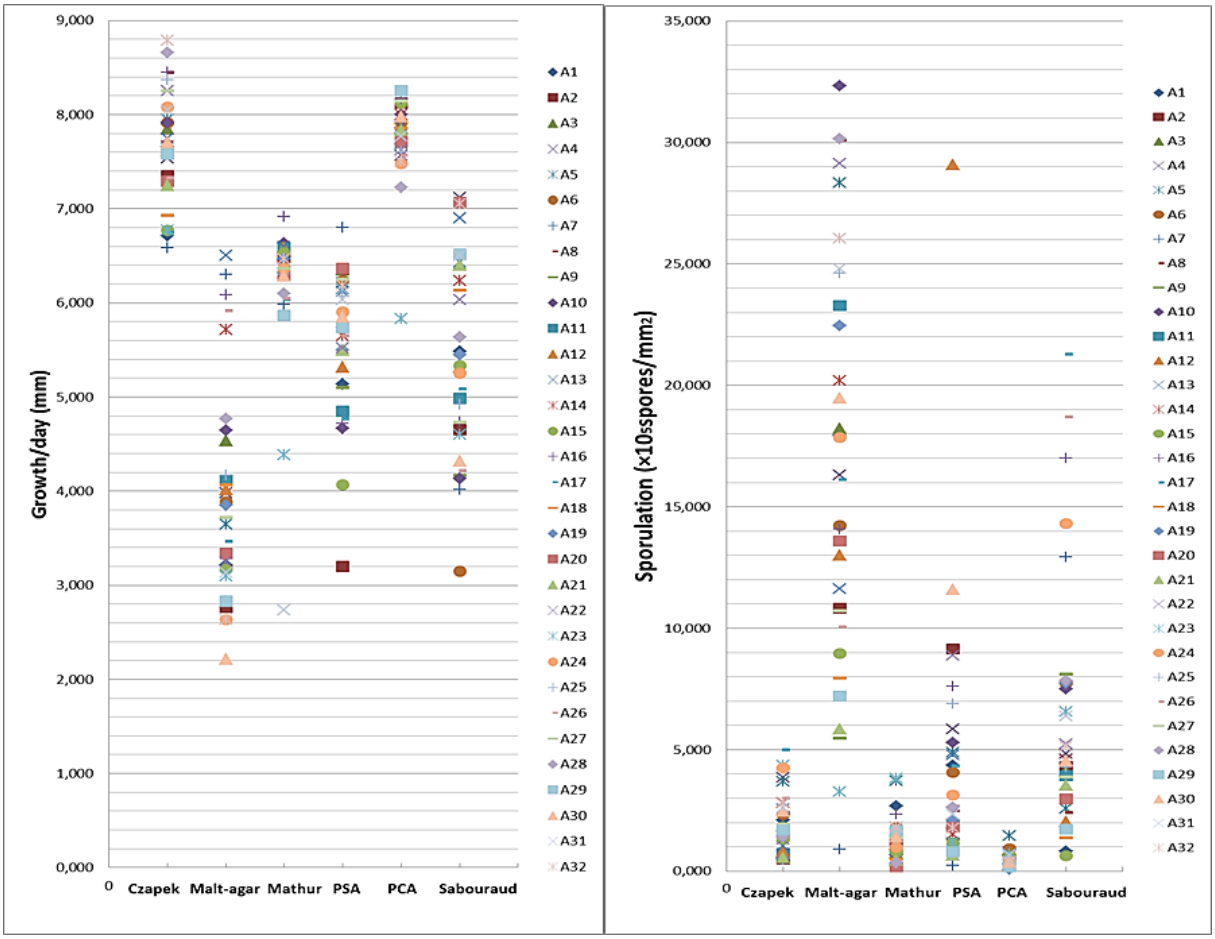

Figure 5. Variability in the growth (A) and sporulation (B) rates of small- spore *Alternaria* isolates on different medium.

(1 to 5 conidia) having a long defined primary conidiophore with few terminal and sub terminal branches. Conidia were ovate, obpyriform to ellipsoidal, mature conidia had 1-6 transverse septa and 0-3 longitudinal or oblique septa; spore body measured 9.6-38.4 × 3.2-12.8 µm. This group accounted for only 1.53% of the isolates and A23 was chosen for further studies. To compare, Xia and Tian-Yu (2008) reported that the conidia of 27 *A. alternata* isolates varied from 16.5-56.5 × 6.5-14.5 µm and conidia size variability among 53 isolates of *A. tenuissima* was 22.5-42 × 8.5-12.5 µm. Although morphological characteristics of conidia and conidiophores have often provided the major taxonomic criteria for delimitation of fungal species, these characters may be strongly affected by environmental factors. Thus in quantifying size dimensions, number of transverse or longitudinal septa, isolates often fit a number of different species and accurate assignment of small-spore *Alternaria* to one of the described species is often difficult based on this sole criteria. By contrast, 3D-sporulation patterns provide a much robust morphological criteria for the delineation of *Alternaria* species within the *alternata* section.

Response of the isolates to different culture conditions

Base medium

Growth and sporulation of the 32 selected isolates was first tested on different culture media. Significant vegetative growth was observed on all tested media (Figure 5). The relatively high growth rates recorded irrespective of the medium indicate that small-spore *Alternaria* isolates have the ability to utilize a wide range of carbon sources and other nutrients. For all the 32 isolates, Czapek Dox agar supported the maximum growth (mean: 7.667±0.591 mm/day) but poor sporulation (2.166 ± 0.98210^5 spores /mm^2) maybe due to the presence of chloride ion. Growth rates on PCA medium were almost similar (mean: 7.763±0.425 mm/day) with the minimum fungal sporulation (mean: 0.450± 0.207 10^5 spores/mm^2). This medium, which is rich in nutrients, may probably favor mycelia growth with ultimate loss of sporulation (Nasraoui, 2006). By contrast, the slower mycelia growth rate was measured on malt extract agar (mean: 3.902±1.141 mm/day) which supported the maximum sporulation

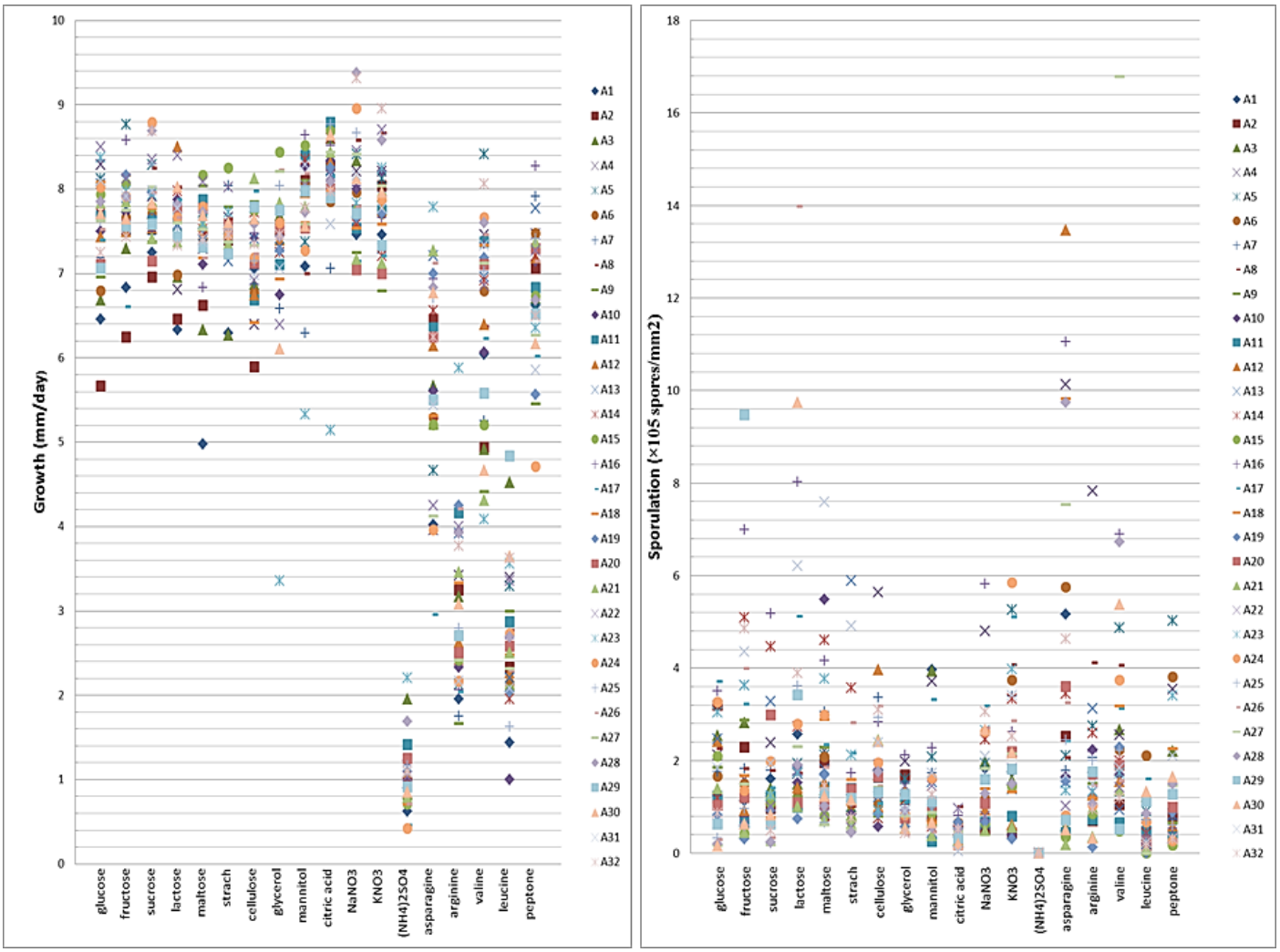

Figure 6. Variability in the growth (A) and sporulation (B) rates of small –spore *Alternaria* isolates on different carbon and nitrogen sources.

(mean: 16.721±6.915 10^5 spores/mm^2), albeit with large variation between isolates. Poor sporulation (mean: 1.334 ±0.581 10^5 spores/mm^2) and moderate mycelia growth (mean: 6.212±1.072 mm/day) was observed on Mathur medium. Sabouraud medium and PSA both supported moderate mycelia growth (mean: 5.501±1.072 mm/day and 5.639±0.766 mm/day, respectively) and sporulation (6.365±3.618 10^5 spores/mm2 and 4.348 ± 3.06410^5 spores/mm^2, respectively), and were the preferred media for culturing small-spore *Alternaria*. The influence of media base revealed a negative correlation between growth and sporulation of the isolates. The composition of the culture media thus constitutes an important physiological parameter that significantly affects the mycelia growth rate and conidial production of the isolates.

Carbon and nitrogen sources

Effects of ten carbon sources and eight nitrogen sources (Figure 6) were then tested using Czapek dox as basal medium. All the ten carbon sources were found suitable to sustain mycelia growth. Although the type of carbon source had low effect on the recorded growth rates, sporulation was much more affected by this parameter and varied from 0.431±0.215 10^5 spores/mm^2 on citric acid to 2.89±2.6910^5 spores/mm^2 on lactose. Contrarily to what was recorded for carbon sources, a stronger effect of the nitrogen source on growth parameters was observed. The mycelia growth of the isolates on different nitrogen sources was found to be highest on two inorganic nitrogen sources, sodium nitrate (7.977±0.584 mm/day) and potassium nitrate (7.827±0.503 mm/day) with moderate (1.778±1.242 spores/mm^2 and 2.297 ± 1.462 10^5 spores/mm^2, respectively) sporulation. The lowest fugal growth was recorded on ammonium sulfate containing medium (1.054±0.386 mm/day) with no sporulation. These observations are in agreement with those of Attrassi et al. (2007) studying *A. alternata*

Figure 7. Variability in the growth (A) and sporulation (B) rates of small-spore *Alternaria isolates* on different pH and temperatures.

isolated from pears and Snapbeans, respectively, while Ramjegathesh and Ebenezar (2012) found that ammonium sulfate was the optimum nitrogen source of *A. alternata* isolated from leaf onion. While many fungi assimilate well the inorganic nitrogen sources like nitrates, they can also use a wide range of amino-acids which evoke different fungal responses and usually fungi grow well on asparagine unlike on leucine which supports low fungi growth (Nasraoui, 2006; Brzonkalik et al., 2011). In line with this, our results show that growth and sporulation were very poor on leucine and arginine as organic nitrogen sources while valine and asparagine yielded moderate growth (6.453±1.181 and 5.741±1.184 mm/day, respectively) and sporulation (2.862±3.019 and 3.552 ± 3.64810^5 spores/mm^2, respectively).

pH and temperature

The impact of the medium pH on mycelial growth and sporulation (Figure 7) of the 32 isolates was studied in the range of pH 4 - 10. In accordance with previous observations showing that generally fungi grew best at neutral or slightly acidic pH (Madan and Thind, 1998), the optimum pH for growth of our isolates was between 6 and 8 (ca. 6.6 mm/day). The pH values below 6 and above 9 led to a decreased mycelia growth and minimum growth rate was recorded at pH 10 (5.678±0.883 mm/day). Similar trend has already been reported by Ramjegathesh and Ebenezar (2012) and alkaline media are generally not recognized as favorable for fungal growth and sporulation. By contrast, pH 4 allowed the maximum mean of sporulation (4.058±3.309 10^5 spores/mm^2) followed by pH 8 (3.918±3.190 10^5 spores/mm^2). To compare, optimal pH values for sporu-lation varying between 4.8 and 6.3 have been reported for different *Alternaria* species (Mathur and Sarboy, 1977; Gemawat and Ghosh, 1980). In the present study, a single peak was recorded for optimum pH value for mycelia growth, which agrees with the behavior of fungi studied by Mehrotra (1964). However, two peaks were observed according to the mean sporulation of the isolates as already reported by Mathur et al. (1950) for *C. lindemuthianum*.

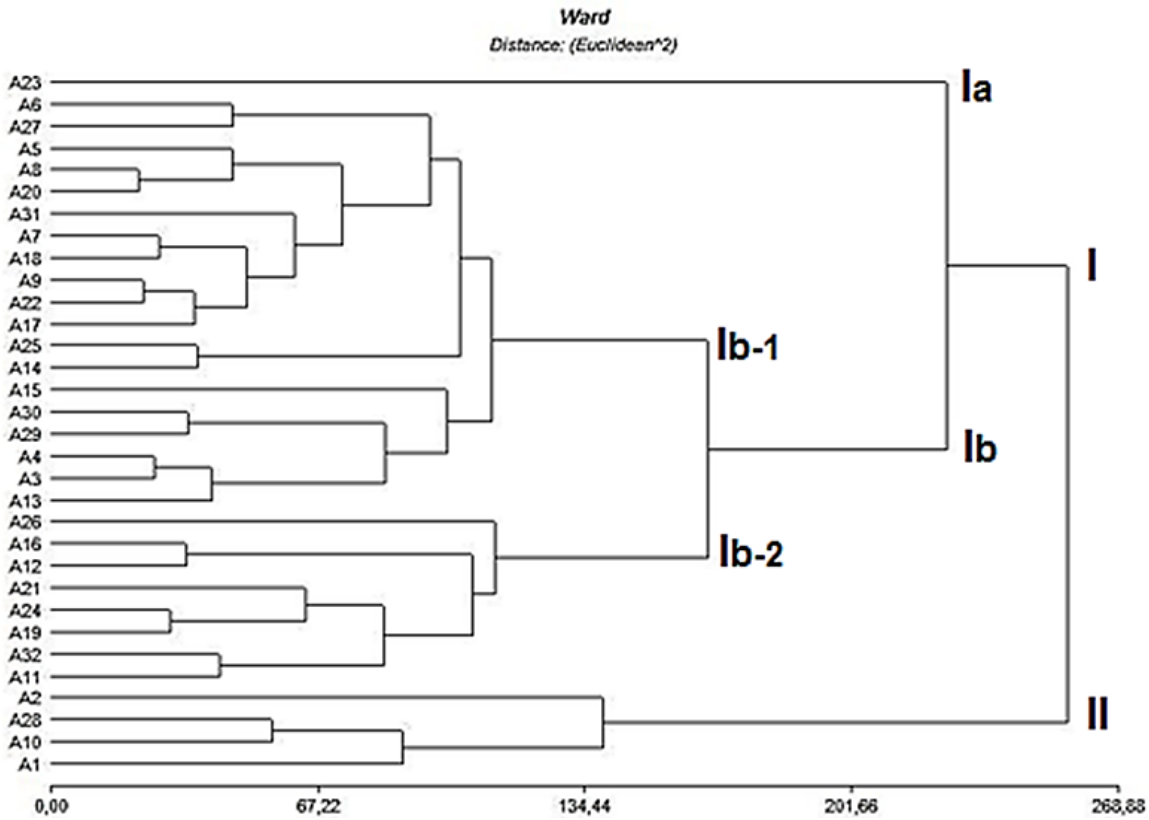

Figure 8. Dendrogram presenting the cluster analysis of morphological, cultural and physiological characteristics of 32 small-spored*Alternaria* isolates.

Temperature is often considered as the most important physical environmental factor for regulating the growth and reproduction of fungi. All the 32 isolates grew well at a temperature of 30°C (7.434±0.612 mm/day) followed by 25°C (6.751±0.457 mm/day). The lowest growth was observed at 35°C (1.108±0.678 mm/day). From this study, it is clear that temperatures ranging from 25 - 30°C are optimal for the growth of small-spore *Alternaria*. These results are in agreement with those of Hubballi et al. (2010), reporting that 27°C was the optimum temperature for the growth of *A. alternata*. The most suitable temperature for sporulation was 20°C (7.648±6.358 10^5 spores/mm^2) followed by 30 and 25°C. Poor sporulation was observed at 5°C (1.381±1.54610^5 spores/mm^2).

Global analysis of the cultural variability of small-spore *Alternaria* isolates from tomato

Combined results of growth rates at different temperatures, colony morphology, and metabolite profiles were found to be useful in characterization and differentiation of small-spore *Alternaria* spp. when standardized conditions were applied and representative isolates employed for comparison (Andersen et al., 2005). We therefore performed a global analysis of the above-described phenotypical traits from our selected isolates collection. The matrix shown on Figure 8 consisted of 32 isolates and 65 characters. All the isolates were categorized into two main clusters (I and II). Cluster I contained one isolate of *A. arborescens* and 27 isolates of *A. tenuissima* and *A. alternata* species group respectively, cluster II accommo-dated only four isolates of *A. alternata* group. Cluster I was further subdivided into two groups accommo-dating the *A. arborescens* isolate A23 (Ia) and 16 A. tenuissima isolates as well as 11 isolates of *A. alternata species* (Ib), respectively. Similarly, cluster Ib was further subdivided into two subgroups. The Ib-1 subgroup accommodated the 19 isolates from our collection that had a typical A. *tenuissima* sporulation pattern and three isolates thathad the typical *A. alternata sporulation* pattern. The Ib-2 subgroup consisted of eight isolates that had the typical *A. alternata* sporulation pattern. Several workers have already reported cultural, morphological and pathogenic variability among isolates of small spore *Alternaria* spp. (Slavov et al., 2004; Tetarwal et al., 2008; Sofi et al., 2012) and Rotem (1994) claimed that the variation on the cultural characteristics (color, growth, and sporulation) allowed to identify almost as many races as the number of isolates tested. The observed diversity for the phenotypic characters within the isolates from tomato and assigned to the *A. alternata* and *A. tenuissima* morphological species might reflect various environmental conditions in regions

Figure 9. Tomato leaf blight disease development on detached leaves pathogenicity tests on *Lycopersicon esculentum L*; leaf inoculated with sterile growth medium and sterile distilled water (far left) remained free of symptoms 1 week after inoculation. Leaflets inoculated with mycelia plug (A) leaflets inoculated with conidial suspension (B).

where these isolates have been collected. However, no geographical clustering was obtained; all groups and sub groups contained isolates from different districts having almost similar agro-climatic conditions.

Pathogenicity variability among small-spore *Alternaria* isolates from tomato

In order to test whether the cultural characteristics of the 32 selected isolates could reflect differences in virulence, pathogenicity tests were performed by inoculating detached leaves either with conidial suspensions or with mycelial plugs. Similar results were obtained for all isolates with both methods. The small-spore *Alternaria* isolates differed in their ability to produce lesions from the point of inoculation on leaflets. Fifteen isolates were weakly pathogenic comprising eight isolates of the *A. alternata* species and seven for the *A. tenuissima species*. For these isolates, we observed that spores germinated profusely on the surface of tomato leaflets inoculated with conidial suspensions, but failed to cause disease. However, 17 of the isolates tested exhibited relatively strong ability to produce infection on leaflets of which seven were of the *A. alternata* species, nine from the *A. tenuissima* species and the *A. arborescens* isolate A23. Nonhierarchical grouping of results from virulence assays on detached leaves showed that isolates could be divided into two main groups (Table 3). Isolates in group 1 had average virulence ratings of either 0 or 1, while those in group 2 had average ratings of either 2 or 3. The highest incidence of disease on leaflets inoculated with conidial suspensions was observed in A22 isolate (86.51±5.60%) followed by A5 (76.90±3.94 %) while isolate A23 recorded 56.73 ±12.20%. The lowest disease incidence was noted with the A16 isolate (11.81±3.86 %). Disease incidence in leaflets inoculated with mycelia plug shows almost same rates with high ability to produce lesions for A22 (90.18±6.44 %), A5 (84.23±6.00 %) and A23 isolate (75.59±15.67%) and the lowest incidence (13.33±4.30%) for A16 isolate as it is represented in Figure 9.

The pathogenicity assays in this study showed a high degree of variation in virulence of the different isolates. Pathogen entry into the tissues may be an active process but opportunistic infections may also occur as a result of sunburns, or cracking of leaves by the wind. Given the abundance of aerial spores, both active and opportunistic infections could result in lesions being composed of genetic mixtures. Two types of small-spore *Alternaria* isolates have previously been isolated from diseased tomato: the saprophytic form, for which all *Lycopersicon esculentum* cultivars are resistant, but develops symptoms only on ripe tomato fruits (blackmold) (Cassol and St. Clair, 1994) and the pathotype *A. alternata* f. sp. *lycopersici*, also reported as *A. arborescens* (Simmons, 1999), which induces lethal dark brown cankers on stems, leaf necrosis and wilting in susceptible tomato cultivars by the action of its host-specific AAL-toxins (Grogan et al., 1975; Mesbah et al., 2000). Several *A.*

Table 3. Nonhierarchical grouping of *Alternaria* isolates from northwestern Algeria into two groups based on virulence on detached leaves of tomato.

Zone	Isolate	
	Group 1	**Group 2**
AinTemouchent	A1, A12	A3, A14, A28
Mascara	A13, A17, A18, A19	A15, A20, A24
Mostaganem	A4, A6, A8, A9, A16, A30	A7, A10, A11, A22, A23, A25, A27
Oran	A26	A21
Relizane	A31	A32
SidiBelabbes		A29, A5
Tlemcen	A2	

Group 1 is least virulent (average ratings of either 0 or 1) and group 2 is most virulent (average ratings of either 2 or 3).

alternata pathotypes are known as host specific-toxins (HST) producers, which are essential virulence factors and determine their host range (Kohmoto et al., 1995). Although we have not investigated the production of HST in this study, AAL-toxin might be synthesized by isolate A23. However, further studies are needed to investigate the other isolates from our collection of small spore *Alternaria* based on their secondary metabolites patterns to check whether they might also be considered as potential HST-producers.

Conclusion

Small-spore *Alternaria* have consistently been isolated from tomato organs showing symptoms of early blight in the northwestern region of Algeria. Based on their sporulation patterns, among the 32 studied isolates, only one corresponded to the tomato pathogenic *A. arborescens* species while all the other isolates were assigned to the *A. tenuissima and A. alternata* species. Members of the latter groups have often been considered as fungi with saprophytic or opportunistic lifestyles. Despite this, pathogenicity assays conducted on detached tomato leaves showed that some of the studied isolates, either from the *A. tenuissima* or the *A. alternata* species, were highly virulent resulting in almost complete browning of inoculated leaves in one week. Diversity within the *A. alternata* species, and to a lesser extent within the *A. tenuissima* species was also evidenced from their morphological characteristics and cultural behavior. Agglomerative hierarchical clustering of such parameters provided no correlation with virulence nor with geographic origin of the isolates, although the resulting dendrogram was coherent with the species grouping and confirmed that the identified morphotypes corresponded to different species.

Besides isolates belonging to the *alternata* section, *Alternaria* species belonging to the *porri* section (*A. solani* and *A. tomatophila*) are often considered as major cause for early blight on tomato. Future studies will therefore investigate the occurrence of this *Alternaria* lineage in northwestern Algeria and their possible interactions with isolates of the *alternata* section.

Conflict of Interests

The authors have not declared any conflict of interests.

REFERENCES

Andersen B, Frisvad JC (2004). Natural occurrence of fungi and fungal metabolites in moldy tomatoes. J. Agric. Food Chem. 52: 7507-7513.

Andersen B, Hansen ME, Smedsgaard J (2005). Automated and Unbiased Image Analyses as Tools in Phenotypic Classification of Small-Spored *Alternaria* spp. Phytopathology 95(9):1022-1029.

Attrassi K, Benkirane R, Attrassi B, Badoc A, Douira A (2007). Effect ofcarbon and nitrogen sourceson the growth and sporulation of fungal species responsible for apple rot in fruit conservation. Bull. Soc. Bordeaux 211-224.

Bilgrami KS, Verma RN (1978). Physiology of fungi. Vikas Publishing House. New Delhi 597.

Brame C, Flood B (1983). Antagonism of *Aureobasidium pullutans* towards *Alternaria solani.* Trans. Br. Mycol. Soc. 81:621-624.

Brzonkalik K, Herrling T, Syldatk C, Neumann A (2011). The influence of different nitrogen and carbon sources on mycotoxin production in *Alternaria alternata*. Int. J. Food Microbiol. 147:120-126.

Cassol T, St Clair DA (1994). Inheritance of resistance to Blackmold (Alternaria alternata (Fr. Keissler) in two interspecific crosses of tomato (Lycopersicon esculentum L. cheesmanii f. typicum). Theor. Appl. Genet. 88:581-88.

Digby PGN, Kempton RA (1987). Multivariate analysis of ecological communities, Chapman and Hall, London. pp. 131-147.

Dongyou L (2010). Molecular Detection of Foodborne Pathogens. CRC Press Taylor and Francis Group, USA. pp. 524-905.

Gemawat PD, Ghosh SK (1980). Studies on the physiology of growth of Alternaria solani. Indian J. Mycol. Plant Pathol. 9:138-139.

Goodwin DC, Lee SB (1993). Microwave miniprep of total genomic DNA from fungi, plants, protists and animals for PCR. Biotechniques 15:438-444.

Grogan RG, Kimble KA, Misaghi I (1975). A stem canker disease of tomato caused by *Alternaria alternata f. sp. lycopersici.* Phytopathology 65:880-886.

Hansen HN (1926). A simple method of obtaining single spore cultures. Science 64:384-389.

Hubballi M, Nakkeeran S, Raguchander T, Anand T, Samiyappan R (2010). Effect of Environmental Conditions on Growth of *Alternaria alternata* Causing Leaf Blight of Noni. World J. Agric. Sci. 6(2): 171-177.

Johnson RD, Johnson L, Itoh Y, Kodama M, Otani H, Kohmoto K (2000). Cloning and characterization of a cyclic peptide synthetase gene from *Alternaria alternata* apple pathotype whose product is involved in AM-toxin synthesis and pathogenicity. Mol. Plant Microbe Interact. 13(7): 742-753.

Kheder AA, Yanaga K, Tsuge T, Akagi Y, Maekawa N, Kodama M, Akamatsu H, Otani H (2012). Functional analysis of the melanin biosynthesis genes ALM1 and BRM2-1 in the tomato pathotype of *Alternaria alternata.* J. Gen. Plant Pathol. 78: 30-38.

Kohmoto K, Otani H, Tsuge T (1995). *Alternaria alternata* pathogens In Pathogenesis and Host Specificity in Plant Diseases: Histopathological Biochemical, Genetic and Molecular Bases, Eukaryotes. (eds) Oxford Pergamon. 2:51-63.

Konstantinova P, Bonants PJM, van Gent-Pelzer MPE van der Zouwen P, van den Bulk R (2002). Development of specific primers for detection and identification of *Alternaria spp.* in carrot material by PCR and comparation with blotter and plating assays. Mycol. Res. 106: 23-33.

Kusaba M, Tsuge T (1995). Phylogeny of *Alternaria* fungi known to produce host specific toxins on the basis of variation in internal transcribed spacers of ribosomal DNA. Curr. Genet. 28(5): 491-498.

Lawrence DP, Gannibal PB, Peever TL, Pryor BM (2013). The sections of *Alternaria*: Formalizing species-groups concepts. Mycologia 105: 530-546.

Leiminger J, Bahnweg G, Hausladen H (2010). Population genetics – consequences on early blight disease. Twelfth Euro Blight workshop Arras (France). PPO-Special Report 14: 171-178.

Logrieco A, Moretti A, Solfrizzo M (2009). *Alternaria* toxins and plant diseases: an overview of origin, occurrence and risks. World Mycotoxin J. 2(2):129-140.

Loladze A, Garland Campbell KA, Chen X (2005). Development of a detached leaf assay for stripe rust resistance screening. Agronomy Abstracts.http://crops.confex.com/crops/2005am/techprogram/P3298.HTM

Lourenço Jr V, Moya A, González-Candelas F, Carbone I, Maffia LA Mizubuti ESG (2009). Molecular diversity and evolutionary processes of *Alternaria solani* in Brazil inferred using genealogical and coalescent approaches. Phytopathology 99: 765-774.

Madan M, Thind KS (1998). Physiology of fungi. A.P.H. Publishing Corporation. New Delhi, India.

Mathur RS, Barnett HL, Lilly VG (1950). Sporulation of Coletotrichum lindcmuthianum in culture. Phytopathology 40:104.

Mathur SB, Sarboy AK (1977). Physiological studies on *Alternaria alternata* from sugar beet. Indian J. Phytopathol. 30:432-434.

Mehrotra MD (1964). Studies on *Choanephoraceae*. Effect of Hydrogen-ion Concentration. Sydowia. Ann. Mycolog. Ser. (2)17:223-229.

Mesbah LA, van der Weerden GM, Nijkamp HJJ, Hille J (2000). Sensitivity among species of Solanaceae to AAL toxins produced by *Alternaria alternata* f. sp*. lycopersici.* Plant Pathol. 49: 734-741.

Messiaen CM, Blancard D, Rouxel F, Lafon R (1991). Les maladies des plantes maraichères, INRA Paris. p. 552.

Nasraoui B (2006). Parasitic fungi of crops.Center of academic publication. Tunisia 456:41.

Nishimura S, Kohmoto K (1983). Host-specific toxins and chemical structures from *Alternaria* species. Annu. Rev. Phytopathol. 21: 87-116.

Park DS, Sayler RJ, Hong YG, Nam MH, Yang Y (2008). A method for inoculation and evaluation of rice sheath blight disease. Plant Dis. 92:1.

Pusz W (2009). Morpho-physiological and molecular analyses of *Alternaria alternata* isolated from seeds of *Amaranthus*. Phytopathologia 54: 5-14.

Rai PK, Kumari L (2009). Variability in *Alternaria alternata* infecting periwinkle (*Catharanthus roseus*). Prog. Agric. 9: 269-272.

Ramjegathesh R, Ebenezar EG (2012). Morphological and Physiological Characters of *Alternaria alternata* Causing Leaf Blight Disease of Onion. Int. J. Plant Pathol. 3: 34-44.

Reni CG, Remmelt S, Roeland EV (2007). Assessment of early blight (*Alternaria solani*) resistance in tomato using a droplet inoculation method. J. Gen. Plant Pathol. 2(73): 96-103.

Rotem J (1994). The genus *Alternaria*, biology and pathogenicity. APS Press, St. Paul, Minnesota 326.

Salam AA, Jarjees MM, Juer KS (2006). Enhancement of *Alternaria solani* isolates for sporulation on culture media and evaluation of their pathogenicity. State Board for Agricultural Research, College of Agricultural, University of Baghdad, Abo-Gharib, Baghdad, Iraq.

Samson RA, Hoekstra ES, Frisvad JC, Filtenborg O (2002). Introduction to food and air borne fungi. 6th ed. Utrecht: Central bureau voorSchimmel cultures 379.

Simmons EG (1993). Alternaria themes and variations (63-72).Mycotaxon 48: 91-107.

Simmons EG (1999). *Alternaria* themes and variations (236-243).Host-specific toxin producers. Mycotaxon 70: 325-69.

Simmons EG (2000). *Alternaria* themes and variations (244-286) species on *Solanaceae*. Mycotaxon75: 1-115.

Simmons EG (2007). *Alternaria*. An Identification Manual: CBS Biodiversity Series No. 6. CBS Fungal Biodiversity Centre, Utrecht.The Netherlands 775.

Simmons EG, Roberts RG (1993). *Alternaria* themes and variations (73). Mycotaxon 48: 109-140.

Slavov S, Mayama S, Atanassov A (2004). Some aspects of epidemiology of *Alternaria alternata* tobacco pathotype*.* Biotechnol. Biotechnol. Equip. 18: 85-89.

Snoussi SA (2009). Basic study on tomato in Algeria. Regional Programme for Integrated Pest Management for the Middle East.www.ipm-neareast.com

Sofi TA, Muzafer A, Beig GH, Hassan D, Mushtaq A, Aflaq H, Ahangar FA, Padder BA, Shah MD (2012). Cultural, morphological, pathogenic and molecular characterization of *Alternaria mali* associated with *Alternaria* leaf blotch of apple. Afr. J. Biotechnol. 370-381.

Spits HG, Raay van H, Flier WG, Schepers HTAM (2005). The fight against *Alternaria* in potatoes.Praktijkonderzoek Plant- en Omgeving (PPO)-Rapport 520356.

Tetarwal ML, Rai PK, Shekhawat KS (2008). Morphological and pathogenic variability of *Alternaria alternata* infecting senna (*Cassia angustifolia*). J. Mycol. Plant Pathol. 38:375-377.

Tsuge T (2003). Studies on pathogenicity genes of *Alternaria alternata*. J. Gen. Plant Pathol. 69: 418-420.

Van der Waals JE, Korsten L, Slippers B (2004). Genetic diversity among *Alternaria solani* isolates from potatoes in South Africa. Plant Dis. 88:959-964.

Vijayalakshmi M, Madhavi M, Kavitha A (2012). Studies on *Alternaria porri* (Ellis) Ciferri pathogenic to Onion (*Allium cepa L*.). Arch. Appl. Sci. Res. 4(1):1-9.

Ward JH (1963). Hierarchical grouping to optimize an objective function. J. Am. Stat. Assoc. 58: 236-244.

Woudenberg JHC, Groenewald JZ, Binder M, Crous PW (2013). *Alternaria* redefined. Mycology 75:171–212.

Xia S, Tian-Yu Z (2008). Morphological and molecular characterization of *Alternaria* isolates on fruits of *Pyrus bretschneideri Rehd. "YaLi".* Mycosystema 27(1):105-11.


African Journal of
Microbiology Research
Related Journals Published by Academic Journals
African Journal of Biotechnology
African Journal of Biochemistry Research
Journal of Bacteriology Research
Journal of Evolutionary Biology Research
Journal of Yeast and Fungal Research
Journal of Brewing and Distilling
academicJournals