

OPEN ACCESS



African Journal of
Microbiology Research

July 2021
ISSN 1996-0808
DOI: 10.5897/AJMR
www.academicjournals.org



**ACADEMIC
JOURNALS**
expand your knowledge

About AJMR

The African Journal of Microbiology Research (AJMR) is a peer reviewed open access journal. The journal commenced publication in May 2007. The journal covers all areas of microbiology such as environmental microbiology, clinical microbiology, immunology, virology, bacteriology, phycology, molecular and cellular biology, molecular microbiology, food microbiology, mycology and parasitology, microbial ecology, probiotics and prebiotics and industrial microbiology.

Indexing

[CAB Abstracts](#), [CABI's Global Health Database](#), [Chemical Abstracts \(CAS Source Index\)](#), [Dimensions Database](#), [Google Scholar](#), [Matrix of Information for The Analysis of Journals \(MIAR\)](#), [Microsoft Academic](#), [Research Gate](#)

Open Access Policy

Open Access is a publication model that enables the dissemination of research articles to the global community without restriction through the internet. All articles published under open access can be accessed by anyone with internet connection.

The African Journal of Microbiology Research is an Open Access journal. Abstracts and full texts of all articles published in this journal are freely accessible to everyone immediately after publication without any form of restriction.

Article License

All articles published by African Journal of Microbiology Research are licensed under the [Creative Commons Attribution 4.0 International License](#). This permits anyone to copy, redistribute, remix, transmit and adapt the work provided the original work and source is appropriately cited. Citation should include the article DOI. The article license is displayed on the abstract page the following statement:

This article is published under the terms of the [Creative Commons Attribution License 4.0](#). Please refer to <https://creativecommons.org/licenses/by/4.0/legalcode> for details about [Creative Commons Attribution License 4.0](#).

Article Copyright

When an article is published by in the African Journal of Microbiology Research, the author(s) of the article retain the copyright of article. Author(s) may republish the article as part of a book or other materials. When reusing a published article, author(s) should; Cite the original source of the publication when reusing the article. i.e. cite that the article was originally published in the African Journal of Microbiology Research. Include the article DOI, Accept that the article remains published by the African Journal of Microbiology Research (except in occasion of a retraction of the article).

The article is licensed under the Creative Commons Attribution 4.0 International License.

A copyright statement is stated in the abstract page of each article. The following statement is an example of a copyright statement on an abstract page.

Copyright ©2016 Author(s) retains the copyright of this article.

Self-Archiving Policy

The African Journal of Microbiology Research is a RoMEO green journal. This permits authors to archive any version of their article they find most suitable, including the published version on their institutional repository and any other suitable website.

Digital Archiving Policy

The African Journal of Microbiology Research is committed to the long-term preservation of its content. All articles published by the journal are preserved by [Portico](#). In addition, the journal encourages authors to archive the published version of their articles on their institutional repositories and as well as other appropriate websites.

<https://www.portico.org/publishers/ajournals/>

Metadata Harvesting

The African Journal of Microbiology Research encourages metadata harvesting of all its content. The journal fully supports and implement the OAI version 2.0, which comes in a standard XML format. [See Harvesting Parameter](#)

Memberships and Standards



Academic Journals strongly supports the Open Access initiative. Abstracts and full texts of all articles published by Academic Journals are freely accessible to everyone immediately after publication.



All articles published by Academic Journals are licensed under the [Creative Commons Attribution 4.0 International License \(CC BY 4.0\)](#). This permits anyone to copy, redistribute, remix, transmit and adapt the work provided the original work and source is appropriately cited.



[Crossref](#) is an association of scholarly publishers that developed Digital Object Identification (DOI) system for the unique identification published materials. Academic Journals is a member of Crossref and uses the DOI system. All articles published by Academic Journals are issued DOI.

[Similarity Check](#) powered by iThenticate is an initiative started by CrossRef to help its members actively engage in efforts to prevent scholarly and professional plagiarism. Academic Journals is a member of Similarity Check.

[CrossRef Cited-by](#) Linking (formerly Forward Linking) is a service that allows you to discover how your publications are being cited and to incorporate that information into your online publication platform. Academic Journals is a member of [CrossRef Cited-by](#).



Academic Journals is a member of the [International Digital Publishing Forum \(IDPF\)](#). The IDPF is the global trade and standards organization dedicated to the development and promotion of electronic publishing and content consumption.

Contact

Editorial Office: ajmr@academicjournals.org

Help Desk: helpdesk@academicjournals.org

Website: <http://www.academicjournals.org/journal/AJMR>

Submit manuscript online <http://ms.academicjournals.org>

Academic Journals
73023 Victoria Island, Lagos, Nigeria
ICEA Building, 17th Floor,
Kenyatta Avenue, Nairobi, Kenya.

Editors

Prof. Adriano Gomes da Cruz
University of Campinas (UNICAMP),
Brazil.

Prof. Ashok Kumar
School of Biotechnology
Banaras Hindu University Uttar Pradesh,
India.

Dr. Mohd Fuat Abd Razak
Infectious Disease Research Centre,
Institute for Medical Research, Jalan
Pahang, Malaysia.

Dr. Adibe Maxwell Ogochukwu
Department of Clinical Pharmacy and
Pharmacy Management,
University of Nigeria
Nsukka, Nigeria.

Dr. Nadezhda Fursova
Molecular Microbiology,
State Research Center for Applied
Microbiology and Biotechnology,
Russia.

Dr. Mehdi Azami
Parasitology & Mycology Department
Baghaeei Lab.
Isfahan, Iran.

Dr. Franco Mutinelli
Istituto Zooprofilattico Sperimentale delle
Venezie Italy.

Prof. Ebiamadon Andi Brisibe
University of Calabar,
Calabar,
Nigeria.

Prof. Nazime Mercan Dogan
Department of Biology
Faculty of Science and Arts
University Denizli Turkey.

Prof. Long-Liu Lin
Department of Applied Chemistry
National Chiayi University
Chiayi County Taiwan.

Prof. Natasha Potgieter
University of Venda
South Africa.

Dr. Tamer Edirne
Department of Family Medicine
University of Pamukkale
Turkey.

Dr. Kwabena Ofori-Kwakye
Department of Pharmaceutics
Kwame Nkrumah University of Science &
Technology
Kumasi, Ghana.

Dr. Tülin Askun
Department of Biology
Faculty of Sciences & Arts
Balikesir University Turkey.

Dr. James Stefan Rokem
Department of Microbiology & Molecular
Genetics
Institute of Medical Research Israel – Canada
The Hebrew University – Hadassah Medical
School Jerusalem, Israel.

Editors

Dr. Afework Kassu
University of Gondar
Ethiopia.

Dr. Wael Elnaggar
Faculty of Pharmacy
Northern Border University
Rafha Saudi Arabia.

Dr. Maulin Shah
Industrial Waste Water Research
Laboratory
Division of Applied & Environmental
Microbiology, Enviro Technology Limited
Gujarat, India.

Dr. Ahmed Mohammed
Pathological Analysis Department
Thi-Qar University College of Science
Iraq.

Prof. Naziha Hassanein
Department of Microbiology
Faculty of Science
Ain Shams University
Egypt.

Dr. Shikha Thakur
Department of Microbiology
Sai Institute of Paramedical and Allied
Sciences India.

Prof. Pongsak Rattanachaikunsopon
Department of Biological Science,
Ubon Ratchathani University,
Thailand.

Dr. Rafael Lopes e Oliveira
Chemical Engineering,
Amazon State University - Uea,
Brazil.

Dr. Annalisa Serio
Faculty of Bioscience and Technology for
Food, Agriculture and Environment,
University of Teramo.
Italy

Dr. Samuel K Ameyaw
Civista Medical Center
USA.

Dr. Mahmoud A. M. Mohammed
Department of Food Hygiene and Control
Faculty of Veterinary Medicine
Mansoura University Egypt.

Dr. Anubrata Ghosal
Department of Biology
MIT - Massachusetts Institute of Technology
USA.

Dr. Bellamkonda Ramesh
Department of Food Technology
Vikrama Simhapuri University
India.

Dr. Sabiha Yusuf Essack
Department of Pharmaceutical Sciences
University of KwaZulu-Natal
South Africa.

Dr. Navneet Rai
Genome Center
University of California Davis USA.

Dr. Iheanyi Omezuruike Okonko
Department of Virology
Faculty of Basic Medical Sciences
University of Ibadan
Ibadan, Nigeria.

Dr. Mike Agenbag
Municipal Health Services,
Joe Gqabi,
South Africa.

Dr. Abdel-Hady El-Gilany
Department of Public Health & Community
Medicine, Faculty of Medicine
Mansoura University
Egypt.

Dr. Bachir Raho Ghalem
Biology Department,
Faculty of natural sciences and life,
Mascara university,
Algeria.

Table of Content

Antifungal and antibacterial activity of the different parts of mature <i>Benincasa hispida</i> against various microbial infectious agents	349
Md. Khasrul Alam, Mohammad Abu Hena Mostofa Jamal, Morsed Ajam and Md. Rezuatul Islam	
Molecular diagnosis of multidrug-resistant tuberculosis from culture-positive isolates using line probe assay in North Karnataka	360
Reshma Bellad and Mahantesh Nagamoti	
Antibiotic susceptibility and production of extended-spectrum beta-lactamase (ESBL) of <i>E. coli</i> strains isolated from meat	370
Omer A. S., Salwa E., Sanaa O. Y. and Elzubeir I. E. M.	
Assessment of the physicochemical qualities and microbiological profile of Idah River, Kogi State, Nigeria	377
Deborah Ebunoluwa Adedire, Adijat Ozohu Jimoh, Zainab Kashim-Bello, Bilyaminu Adam Wase Shuaibu, Oluwabukola Atinuke Popoola, Kabiru Isa Pate, Onyinyechi Stainless Uzor, Eno Etingwa, Jumoke Florence Joda, Oyetola Olanrewaju Opaleye, Kehinde Rukayyat Adeniran, Ene Elizabeth Francis, Gbadebo Barnabas Olukotun and Oyekanmi Nashiru	
Partial chemical characterization of the yeast extracts <i>Lachancea thermotolerans</i> CCMA 0763	388
Moacir Torres, Marcos A. S. Ribeiro, João Arthur S. Oliveira, Eduardo C. Meurer and Kátia R. F. Schwan-Estrada	
Long-term effectiveness of commonly used hospital disinfectants against <i>Pseudomonas aeruginosa</i> and <i>Staphylococcus aureus</i>	396
Stephen T. Odonkor, Tahiru Mahami and Anthony M. Sallar	
Isolation, identification and antibiogram of verotoxin producing <i>Escherichia coli</i> from raw salad vegetables at Jashore, Bangladesh	401
Nigar Sultana Meghla, Debashish Mridha, Md. Sohel Rana, Md. Ahsanul Haque Shahid and Md. Muket Mahmud	

Full Length Research Paper

Antifungal and antibacterial activity of the different parts of mature *Benincasa hispida* against various microbial infectious agents

Md. Khasrul Alam, Mohammad Abu Hena Mostofa Jamal, Morsed Ajam and
Md. Rezuhanul Islam *

Department of Biotechnology and Genetic Engineering, Islamic University, Kushtia, Bangladesh.

Received 1 March, 2021; Accepted 8 June, 2021

In this study we assayed on antimicrobial activity of peel, pulp, waxy coating of *Benincasa hispida*. Various parts of the plant are reported as antibacterial agents worldwide. Leaves, flower, root, seed are the most studied elements as antibacterial agent. Some fruits are also proved as antibacterial agent and used as herbal medicine as well as nutritional supplements during disease. Different parts of mature and immature winter melon fruits were extracted with different organic solvents viz., methanol, ethyl acetate and chloroform. These extracts were subjected to test against selected pathogenic bacterial (*Bacillus subtilis* IFO 3026, *Sarcina lutea* IFO 3232, *Xanthomonas campestris* IAM 1671, *Escherichia coli* iw 3310 IFO 3007, *Pseudomonas denitrificans* KACC 32026, and fungal (*Fusarium oxysporum*, *Aspergillus niger* KTCC 1700, *Collectotrichum melongenae*) strains using the well diffusion method. The waxy coating and peel of mature fruit of *B. hispida* extracts has strong antibacterial activities than immature fruit. In these cases, the minimum inhibitory concentration was 128 µg/ml. Ethyl acetate and chloroform extracts of waxy coating of *B. hispida* showed inhibition rate against fungal infection 43% and 33%, respectively against *F. oxysporum* but there is no inhibition against *A. niger*, *C. melongenae*. These results suggested that. So, the *B. hispida* would be a potential source that may help to develop natural antimicrobial agents.

Key words: Antibacterial, a fruit, winter melon, infectious, extracts, minimum inhibitory concentration (MIC).

INTRODUCTION

The common agricultural products are rice, wheat, jute, tea, cotton, sugarcane, flower, vegetables, fishes and seed development, livestock, horticulture are main agricultural sectors. Microbial contamination is one of the most alarming causes of agricultural production.

According to the Food and Agriculture Organization (FAO), pests and diseases are responsible for about 25% of crop loss (Sheahan and Barrett, 2017). To solve this issue, new methods are needed to detect diseases and pests early such as novel sensors that detect plant

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

odours, and spectroscopy and biophotonics that are able to diagnostic plant health and metabolism (Martinelli et al., 2015). Globally, plant extracts are employed for their antibacterial, antifungal and antiviral activities. It has been reported that more than 400,000 species of tropical flowering plant showed medicinal properties and the usages of medicinal flowering plant make the folk medicine cheaper the other medicine (Yuan et al., 2016). *Benincasa hispida* (Thunb.) which commonly called as winter melon belongs to the *cucurbitaceae* family. It is a popular vegetable crop, especially among Asian communities both for nutritional and medicinal purposes (Al-Snafi, 2013; Arora and Kaushik, 2016). Phytochemical screening of various extracts of fruits indicate the major constituents of the fruit are triterpenoids, flavonoids, glycosides, saccharides, carotenes, vitamins, β -sitosterin, and uronic acid (Mandal et al., 2012).

Extracts of winter melon may be a natural drug with anti-ulcer activity and as well as antioxidant property (Lee et al., 2005). It contains terpenes, flavonoids, glycosides and sterols which have antioxidant effects (Shetty et al., 2008). Seed extract of winter melon howed anti-angiogenic property through inhibition of endothelial cell proliferation (Wen et al., 2008). The methanolic extracts of *B. hispida* showed excellent protection against histamine-induced bronchospasm probably through an antihistamine activity that is H1 receptor-antagonism (Simons and Simons, 2008). The ethanol extract of *B. hispida* have antinociceptive and antipyretic activity and used as herbal medicine against fever and pain conditions (Rahmatullah et al., 2012; Sharma et al., 2014). The antioxidant property of winter melon may be beneficial in the management of colchicine-induced rat model of Alzheimer's disease (Yagnik et al., 2009). The hypoglycemic effects of *B. hispida* waxy coating may be used to prevent diabetes mellitus (Gu et al., 2013). The methanol extract of winter melon fruit showed significant inhibition against fungus namely *Candida albicans* (Xing et al., 2012) also methanolic and petroleum ether extracts showed significant inhibition of carrageenan-induced paw edema, histamine induced paw edema and cotton pellet-induced granuloma in a rat model (Ashok et al., 2010; Elhassan et al., 2020; Patil and Patil, 2017). The ethanol extract of seeds showed an anti-urolithiatic effect with reduction in stone forming constituents in the urine and decreased kidney retention that reduced the solubility product of crystallizing salts (Juliana et al., 2018; Meshram et al., 2016; Tatiya et al., 2017). The seeds of *B. hispida* having bioactive peptide, Hispidalin showed inhibitory effects against human bacterial and fungal pathogens (Salas et al., 2015; Sharma et al., 2014). The aqueous extract of stem of winter melon for hypoglycemic effect in alloxan-induced diabetic rabbits showed significant dose-dependent reduction in blood glucose levels (Jhonatas et al., 2019; Khan et al., 2012).

In this study the authors tried to analyse antimicrobial activity of the different parts of mature and immature winter melon fruits. The different parts were extracted with

different organic solvents viz, methanol, ethyl acetate and chloroform. These extracts were subjected to test against selected pathogenic bacterial and fungal strains using the well diffusion method. The potentially of the different organic extract of *B. hispida* will be assayed to find strong antimicrobial activities. If they found *B. hispida* would be a potential source that may help to develop natural antimicrobial agents for the control of plant pathogens.

MATERIALS AND METHODS

Materials

The phytochemical analysis showed that different solvents are responsible for extracting different components from the plants. So, several solvents of different polarity are used to extract different plant components. Chloroform, Ethyl Acetate and Methanol were selected according to their degree of polarity. The different types of media were used during experiment Mueller-Hinton Agar (MHA), Lactose Broth, Potato Dextrose Agar (PDA) (Mahboob et al., 2019).

Microorganisms

Five different pathogenic bacteria and three fungi were collected from the microbiology laboratory of the department of Biotechnology and Genetic Engineering, Islamic University, Kushtia. The following strains were used as test strain *Bacillus subtilis* IFO 3026, *Sarcina lutea* IFO 3232, *Xanthomonas campestris* IAM 1671, *Escherichia coli* iw 3310 IFO 3007, *Pseudomonas denitrificans* KACC 32026, *Fusarium oxysporum*, *Aspergillus niger* KTCC 1700 and *Collectotrichum melongenae*.

Sample collection

The healthy, disease free, fresh immature and mature fruits of *B. hispida* were safely collected from farmers land. Various parts of *B. hispida* waxy coating, peel of mature and immature fruits were used.

Plant extract preparation

The fleshy surface, wax gourd of *B. hispida* dried under the shade and then powdered with mechanical grinder. It was later stored in air tight containers protected from direct sunlight for further experiments. The fine grinded powder was mixed (10 gm/100 ml) separately with chloroform, ethyl acetate and methanol. The samples with solvent were placed in rotatory shaker for 24 h at (30-36°C) (Elnaggar et al., 2019; Ikeda et al., 2007; Sasidharan et al., 2011). The extracts of plant material were filtered more than three times and performed by passing the extracts through filter paper. The extracts with different solvents then allowed to air dry after filtration to concentrate.

Antimicrobial Assay

The crude organic extracts of selected solvents were used *In vitro* screening of antibacterial and antifungal activity. The screening of antibacterial and antifungal activity is a typical microbiological assay which is performed with culture of microorganisms. One of the most commonly used methods of bacterial assay is the well diffusion method (Mohsenipour and Hassanshahian, 2016; Valgas et al.,

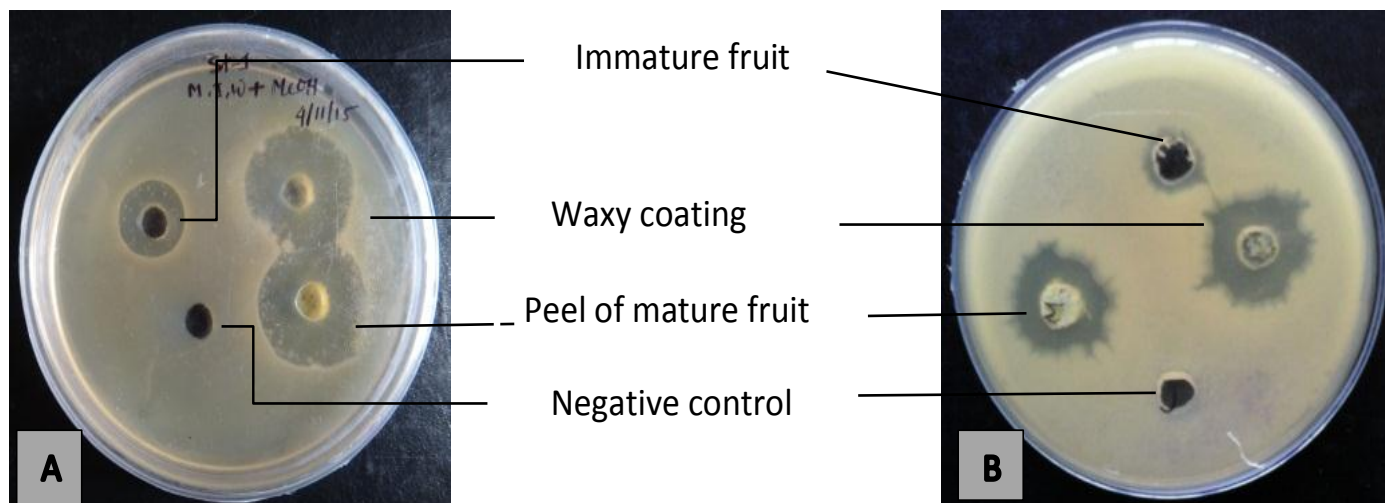


Figure 1. Antibacterial activity of methanol extract of *B. hispida* different parts against *B. subtilis* IFO 3026 (A), *E. coli coli iw 3310* IFO 3007 (B). **N.B:** Negative control-Methanol (only solvent).

2007). The antimicrobial activity was evaluated by measuring the zone of inhibition expressed as mm against test organisms.

The antibacterial activity of plant extract was carried out by well diffusion assay using 100 μ l of inoculum suspension of bacteria. Then each well was loaded with 100 μ l of the different concentrations of extracts. Negative controls were used to screen the activity of solvents which may have effect on antimicrobial activity, for this the same solvents which were used to dissolve the plant extracts employed as negative control. Positive control was used to measure the antibacterial sensitivity of the test strain as well as it can confirm the effectiveness of the methods. Streptomycin (10 μ g/disc from Banex Ltd., USA) was used as positive reference standard to determine the sensitivity of the tested strain in each bacterial species.

The crude sample which showed the best potentiality against the selected organisms used *In vitro* screening of minimum inhibitory concentration (MIC). In microbiological research, minimum inhibitory concentration (MIC) is the lowest concentration in which the extract showed inhibition of the visible growth of a microorganism after incubation. In this study, it was determined by the serial dilution technique (Meenakshi and Anoop, 2019). The process of serial dilution was continued up to nine tubes. It contained 16384 μ g ml⁻¹ in the first tube and 64 μ g ml⁻¹ in the last test tube.

The antifungal activity of plant extract was carried out by well diffusion assay using the mycelia from a 24-h culture. Each of them was well loaded with 100 μ l of the different concentrations of extracts. Negative controls were used for the same solvents as was discussed earlier. The plates were incubated at 27 \pm 2 $^{\circ}$ C for 48-72 h. The antifungal activity was evaluated by calculating Growth Inhibition Rate (%) against test organisms (Bhalodia and Shukla, 2011). Each test in this experiment was replicated four times.

Data analysis

Analysis of variance of antimicrobial activities of extracts from winter melon was assayed using SPSS-16 Program. Total antimicrobial activities were expressed as the mean \pm S.D. (n=4). Significance of difference was calculated by Duncan's new multiple range test and results with P<0.05 were considered statistically significant.

RESULTS

Agar well diffusion method was used to test antibacterial efficacy of crude aqueous extracts of waxy coating, peel (mature fruit) and immature fruit of winter melon. In this case 100 μ l aqueous methanol extract of different sample of *B. hispida* were applied in each well. The wells of 9 mm diameter were prepared in pre-inoculated Mueller-Hinton Agar (MHA) plates with the test organism.

Methanol extract

At the concentration of 4.096 μ g/ μ l (applied 100 μ l), the methanol extract of waxy coating showed zone of inhibition against *B. subtilis* IFO 3026, *S. lutea* IFO 3232, *X. campestris* IAM 1671, *E. coli iw 3310* IFO 3007, *P. denitrificans* KACC 32026 were 15.6 mm, 16.4 mm, 15.6 mm, 16.8 mm, 15.7 mm, respectively. Peel extract of mature fruit produced zone of inhibition against these strains 15.3 mm, 14.9 mm, 13.2 mm, 16.2 mm and 13.5 mm, respectively whereas immature fruit extract showed inhibition in the diameter 11.5 mm, 11.2 mm and 12.1 mm against *S. lutea* IFO 3232, *X. campestris* IAM 1671, *E. coli iw 3310* IFO 3007 but *B. subtilis*, *P. denitrificans* were not inhibited by methanol extract of immature fruit (Figures 1 and 2).

Ethyl acetate extract

At the concentration 4.096 μ g/ μ l (applied 100 μ l), ethyl acetate extract of waxy coating of winter melon, the zone of inhibition against *B. subtilis* IFO 3026, *S. lutea* IFO 3232, *E. coli iw 3310* IFO 3007, and *P. denitrificans* KACC

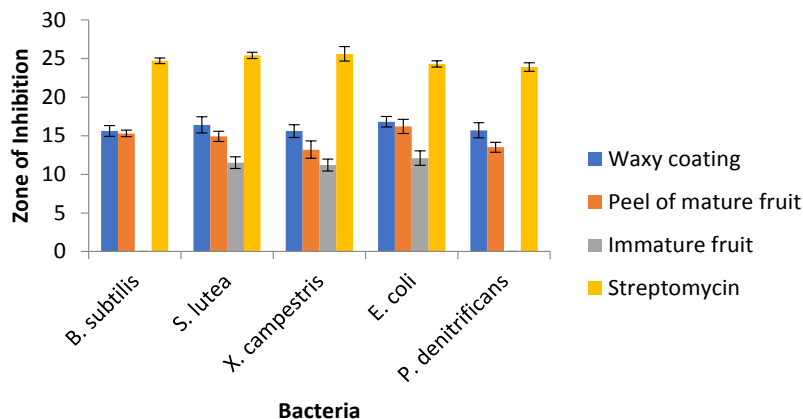


Figure 2. Comparative antibacterial activity of commercial antibiotic and methanol extract of *B. hispida* against selected pathogenic bacteria.

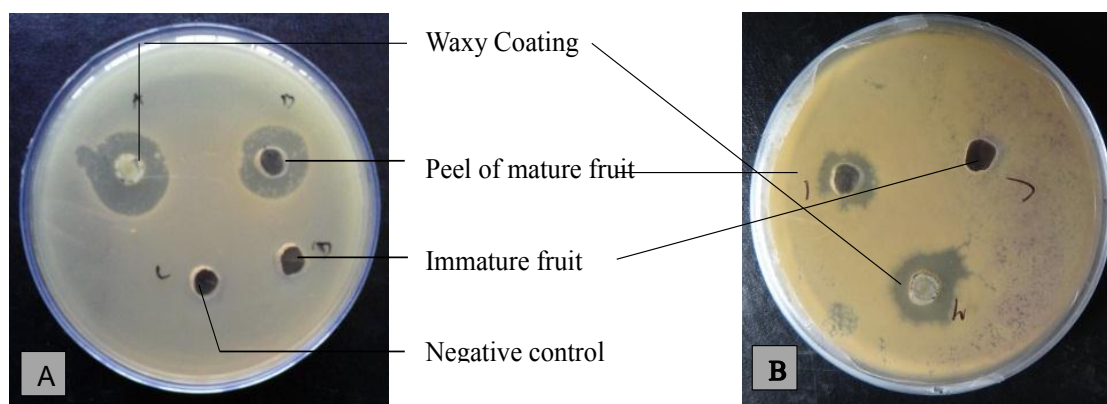


Figure 3. Antibacterial activity of ethyl acetate extract of *B. hispida* different parts against *B. subtilis* IFO 3026 (A) and *P. denitrificans* KACC 32026 (B). Negative control- Ethyl acetate (only solvent).

32026 produced 15.7 mm, 14.4 mm, 16.5 mm, and 16.9 mm, respectively. In case of mature fruit extract, the zone of inhibition showed 14.8mm, 13.2 mm, 15.6 mm, and 15.2 mm against tested bacterial strains *B. subtilis* IFO 3026, *S. lutea* IFO 3232, *E. coli* iw 3310 IFO 3007, and *P. denitrificans* KACC 32026, respectively. *X. campestris* IAM 1671 did not show inhibition zone when the waxy coating and peel extract were applied in the well. Immature fruit extract exhibited inhibition zone in the diameter of 11.5 mm and 12.1 mm against *S. lutea* IFO 3232, and *E. coli* iw 3310 IFO 3007, respectively. In this study, the most sensitive strain was *P. denitrificans* KACC 32026 with the inhibition zone of 16.9 mm produced by waxy coating of *B. hispida* (Figures 3 and 4).

Chloroform extract

Chloroform extract of waxy coating of *B. hispida* produced inhibition against *B. subtilis* IFO 3026, *S. lutea*

IFO 3232, and *E. coli* iw 3310 IFO 3007. At the concentration 4.096 $\mu\text{g}/\mu\text{l}$ (applied 100 μl), the diameter of zone of inhibitions were found 14.6 mm, 14.4 mm, and 16.4 mm, respectively. Peel extract of chloroform also produced inhibition zones against these strains in the diameter of 12.7 mm, 12.5 mm, and 13.4 mm, respectively. *X. campestris* IAM 1671 and *P. denitrificans* KACC 32026 did not show any sensitivity with these extracts. Immature fruit extracts showed small zone of inhibition in the diameter of 10.2 mm, and 10.1 mm against *S. lutea* IFO 3232, and *E. coli* iw 3310 IFO 3007, respectively but did not produce any inhibitions against other bacterial strains (Figures 5 and 6).

Comparative study of different crude extracts of waxy coating of *B. hispida* against tested pathogenic bacteria

Different concentration of crude extracts of waxy coating

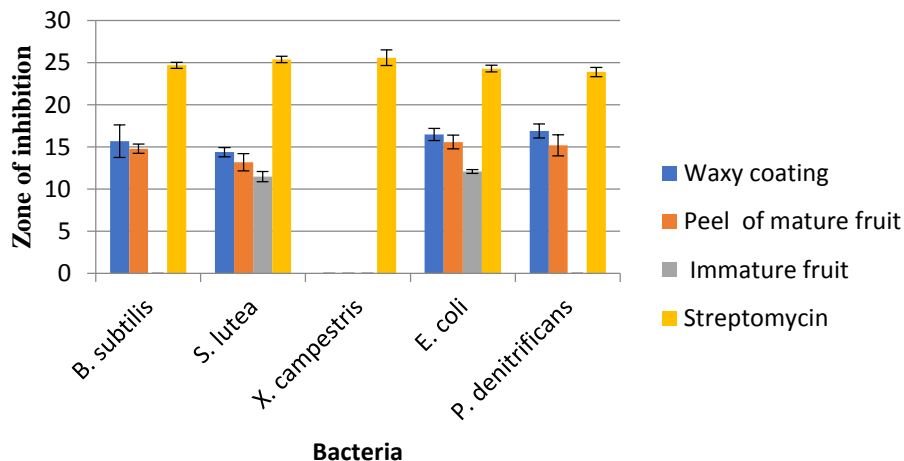


Figure 4. Comparative antibacterial activity of commercial antibiotic and ethyl acetate extract of *B. hispida* against selected pathogenic bacteria.

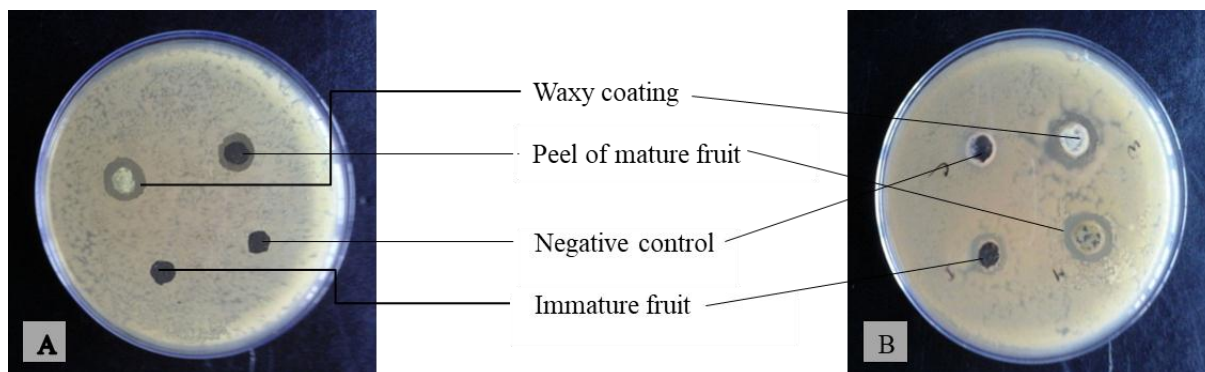


Figure 5. Antibacterial activity of chloroform extract of *B. hispida* different parts against *B. subtilis* IFO 3026 (A) and *S. lutea* IFO 3232 (B). Negative control- Chloroform (only solvent).

of *B. hispida* was examined. It is found that methanol and ethyl acetate extract of waxy coating showed better antibacterial activity than chloroform extract. It was found that *E. coli* iw 3310 IFO 3007 showed minimum inhibitory concentration (MIC) of Methanol and ethyl acetate extract at lower extent than other bacteria (Figures 7 and 8).

Comparative study of different crude extracts of mature fruit (peel) of *B. hispida* against tested pathogenic bacteria

The comparative study of peel extracts of *B. hispida* mature fruit were evaluated against different test strains. It is indicated that methanol and ethyl acetate extract of mature fruit (peel) showed better antibacterial activity against *E. coli* iw 3310 IFO 3007 than chloroform extract. Ethyl acetate extract produced better inhibition zone against *P. denitrificans* KACC 32026 than methanol

extract, whereas chloroform extract did not show inhibition zone against this bacteria and it was found that *E. coli* iw 3310 IFO 3007 showed minimum inhibitory concentration (MIC) of different extract at lower extent than other bacteria. Methanol extracts showed good results in case of MIC (Figures 9 and 10).

Comparative study of different crude extracts of immature fruit of *B. hispida* against tested pathogenic bacteria

The comparative study of immature fruit extracts of *B. hispida* along with standard antibiotic streptomycin. It is indicated that methanol, ethyl acetate and chloroform extract of immature fruit didn't show better antibacterial activity against *B. subtilis* IFO 3026, *X. campestris* IAM 1671 and *P. denitrificans* KACC 32026, whereas showed small inhibition zone against *S. lutea* IFO 3232, *E. coli* iw

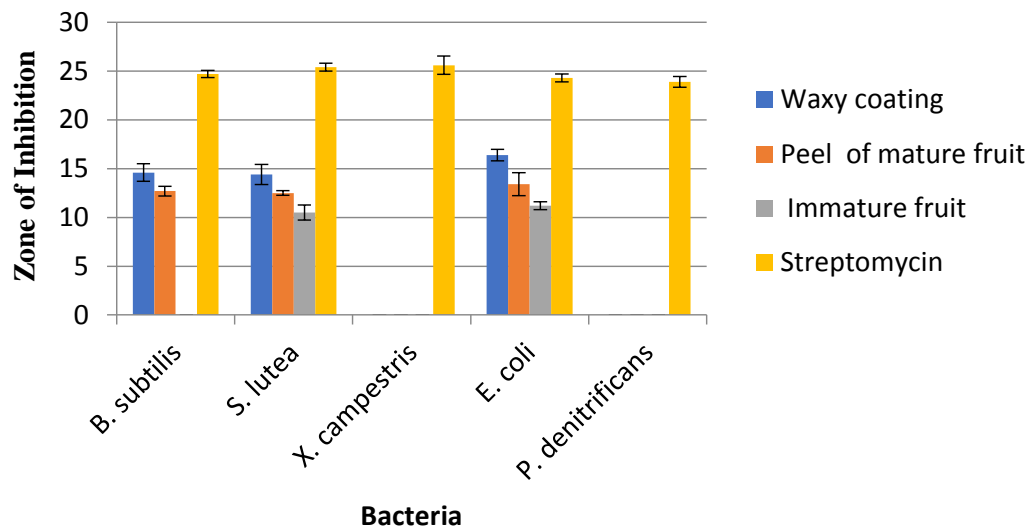


Figure 6. Comparative antibacterial activity of commercial antibiotic and chloroform extract of *B. hispida* against selected pathogenic bacteria.

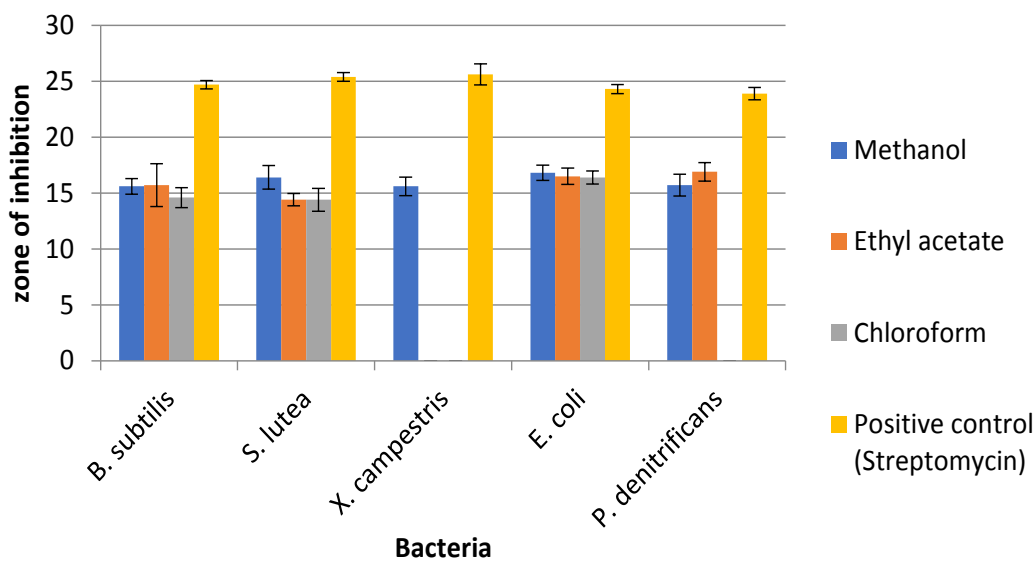


Figure 7. Comparative antibacterial activity study of different waxy coating extracts of *B. hispida* mature fruit.

3310 IFO 3007. It was found that methanol extract showed good results in case of MIC than ethyl acetate and chloroform extracts (Figures 11 and 12).

Antifungal activity study

Agar well diffusion method was used to test antifungal efficacy of crude aqueous extracts of waxy coating, peel (mature fruit) and immature fruit of winter melon. Ethyl acetate extract of waxy coating and peel of mature fruit

showed more positive results against *Fusarium oxysporum* than methanol or chloroform extract. Immature fruit extract did not show any considerable inhibition against this fungus (Figures 13 and 14).

DISCUSSION

The importance on the investigations of natural antimicrobials from plant extracts which can substitute synthetic chemicals may reduce harmful side effects due

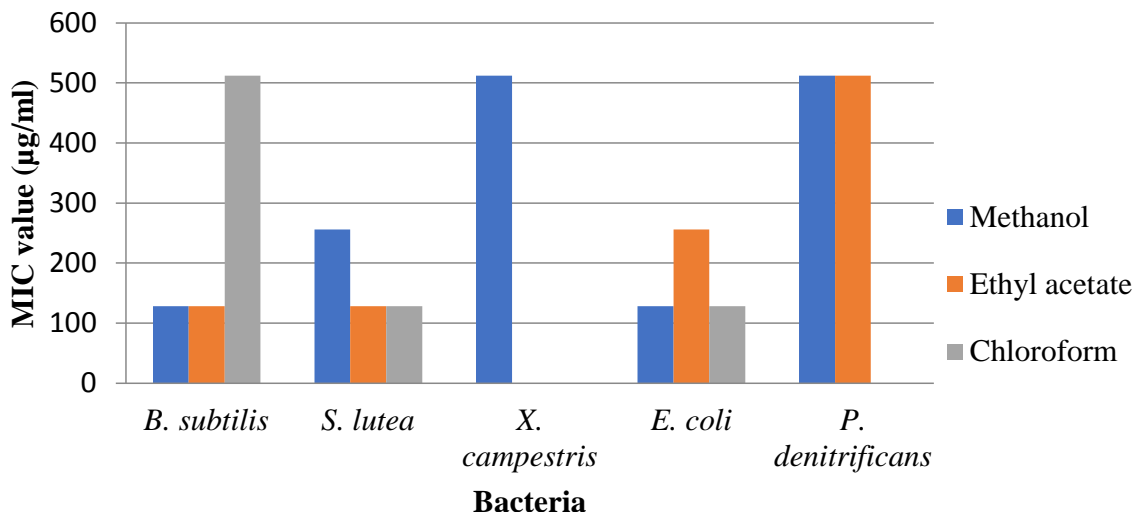


Figure 8. Comparative study of minimum inhibitory concentration (MIC) of different waxy coating extracts of *B. hispida* mature fruit against pathogenic bacteria.

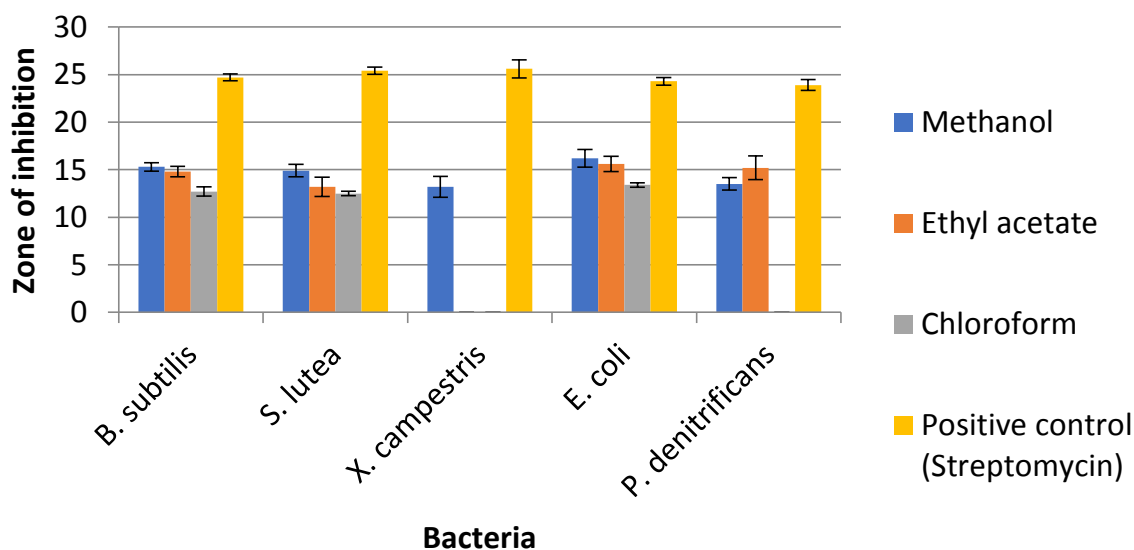


Figure 9. Comparative antibacterial activity study of different peel extracts of *B. hispida* mature fruit.

to potential toxicity of these commercially available drugs.

Now days multiple drug resistance become great threat of public health due to indiscriminate use of commercially available antimicrobial drugs. There is a significant role of phytochemical to mitigate societal health issues and to avoid drug resistance (Zhang et al., 2015; Karuppiyah and Rajaram, 2012). Humans from the pre-historical times have used the herbs, spices, and plants as a natural sources of antimicrobial agents, although the levels and range of activity were not fully defined. Studies have pointed out that many drugs that are used in commerce have come from folk-use and use of plants by indigenous cultures

(Cowan, 1999). The antimicrobial activity of the leaves of some wild Cucurbitaceae species against some human pathogenic microorganisms has investigated recently (Anyanwu and Okoye, 2017). The present study was conducted to find out the antimicrobial effects of different extracts of *B. hispida* belongs to *Cucurbitaceae* family.

In this study, different samples that are the waxy coating, peel of mature fruit, and immature fruit of *B. hispida* were extracted with methanol, ethyl acetate and chloroform. These extracts were evaluated against two species of Gram-positive bacteria *B. subtilis* IFO 3026, *S. lutea* IFO 3232 and three species of Gram-negative

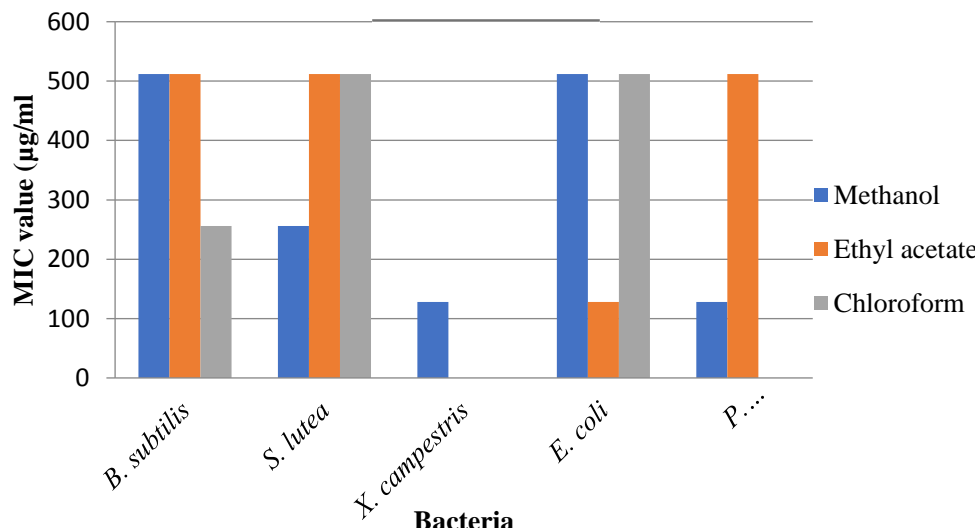


Figure 10. Comparative study of minimum inhibitory concentration (MIC) of different peel extracts of *B. hispida* mature fruit against pathogenic bacteria.

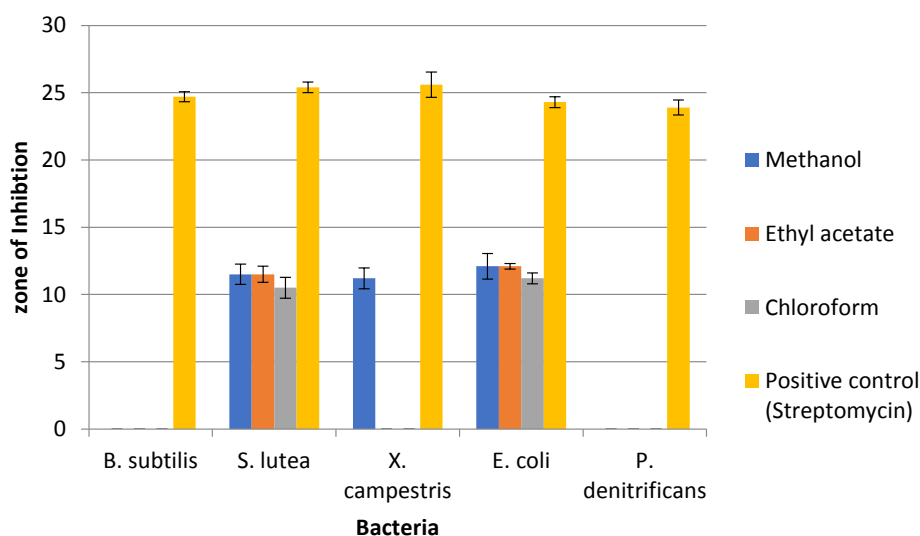


Figure 11. Comparative study of antibacterial activity of different immature fruit extracts of *B. hispida*.

bacteria, *X. campestris* IAM 1671, *Escherichia coli* iw 3310 IFO 3007, *P. denitrificans* KACC 32026. These organic extracts were also investigated against three pathogenic fungal species such as *F. oxysporum*, *A. niger* KTCC 1700, *C. melongenae*. The inhibition zone was the highest in case of standard antibiotic but methanol extract inhibited all bacterial strain efficiently than ethyl acetate or chloroform extract. Methanol extract has been reported effectiveness in many studies (Atef et al., 2019). This study explored a query about the reason of the effectiveness of methanol extract comparing other solvent extracts. Further study is required to find out the

chemical composition of methanol extract and other extracts to search the responsible components of antimicrobial activity. *B. hispida* known as water melon is a common and popular fruits all over the world. These findings explore the health benefit of *B. hispida* which the authors take very frequently. So, *B. hispida* can be recommended antimicrobial agent.

Conclusion

Winter melon is used for the management of various

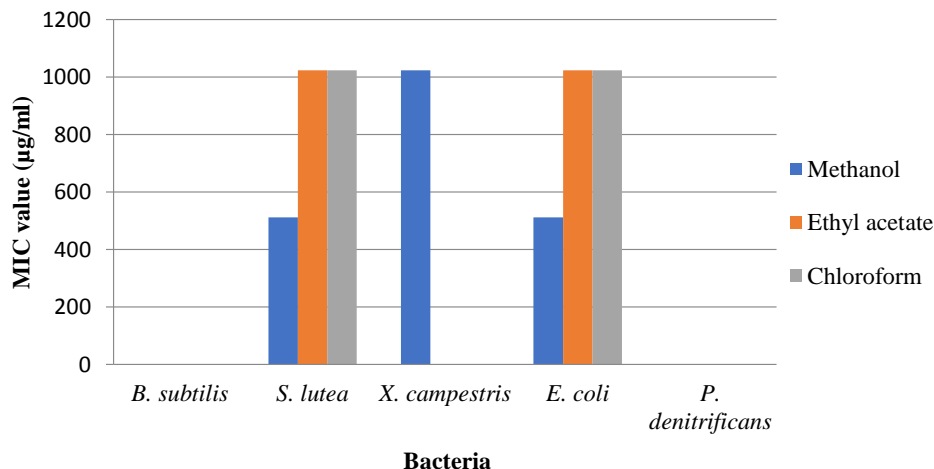


Figure 12. Comparative study of minimum inhibitory concentration (MIC) of different immature fruit extracts of *B. hispida* against pathogenic bacteria.

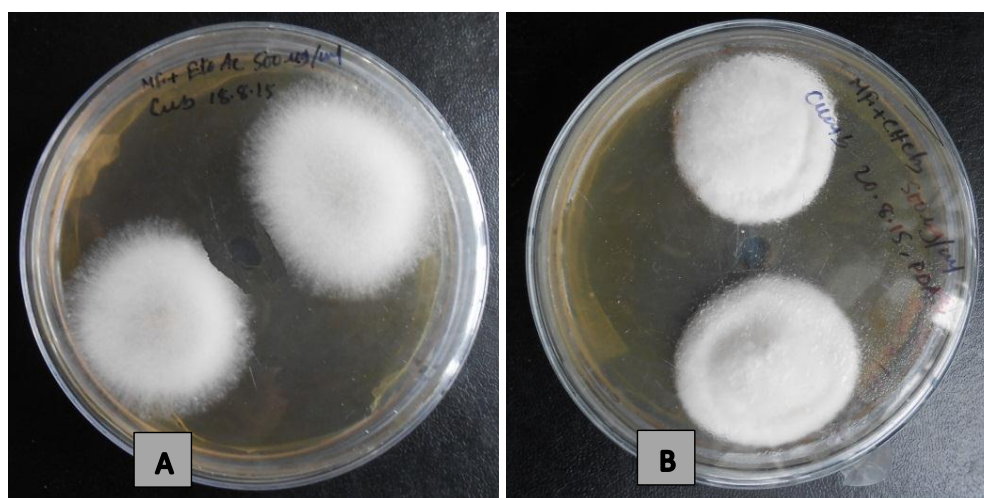


Figure 13. Antifungal activity of ethyl acetate (A) and chloroform (B) extracts of waxy coating of *B. hispida* mature fruit against pathogenic fungus *F. oxysporum*.

diseases and also used as antioxidant, styptic, anti-inflammatory, astringent, anthelmintic, aphrodisiac, demulcent, diuretic, febrifuge, and tonic agents. The results of the study revealed that the different extracts of waxy coating and peel of mature of *B. hispida* possess many active ingredients that inhibited the growth of selected pathogenic bacteria (*B. subtilis* IFO 3026, *S. lutea* IFO 3232, *X. campestris* IAM 1671, *E. coli* iw 3310 IFO 3007, *P. denitrificans* KACC 32026). On the other hand, ethyl acetate extracts of waxy coating and peel of mature winter melon fruit showed maximum antifungal activity against *F. oxysporum*. So, it is concluded that waxy coating and peel of mature fruit has better antibacterial and antifungal activities. If it is possible to find out the reason of self-protective mechanism of

mature fruits of *B. hispida*, there is enormous possibility to prevent immature fruit-rot of winter melon from various infections and also will help to develop antifungal and antibacterial agents.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

The entire work was funded by Islamic University research fund Ref.-141/Edu./IU-2019/385 Date: 16-11-

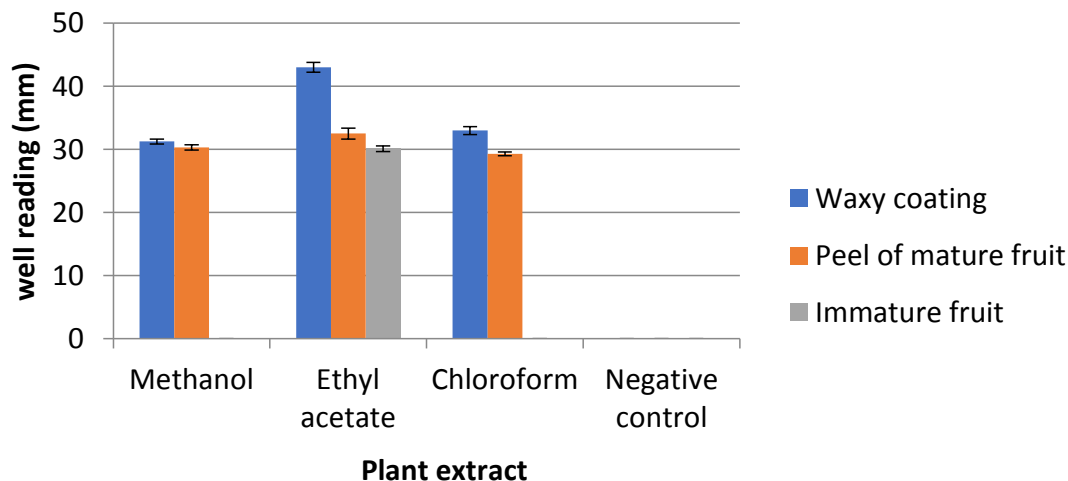


Figure 14. Growth Inhibition Rate (%) of different extracts of *B. hispida* against pathogenic fungi *F. oxysporum*.

2019.

REFERENCES

- Al-Snafi A (2013). The Pharmacological Importance of *Benincasa hispida*. A review. International Journal of Pharmaceutical Sciences and Research 4(12):165-170.
- Anyanwu MU, Okoye RC (2017). Antimicrobial activity of Nigerian medicinal plants. Journal of Intercultural Ethnopharmacology 6(2):240-259.
- Arora P, Kaushik D (2016). Therapeutic potential of Benincasa cerifera: A review. Chinese Journal of Integrative Medicine, pp. 1-14. doi.org/10.1007/s11655-016-2589-8
- Ashok P, Koti BC, Thippeswamy AHM, Tikare VP, Dabadi P, Viswanathaswamy AHM (2010). Evaluation of Antiinflammatory Activity of *Centratherum anthelminticum* (L) Kuntze Seed. Indian Journal of Pharmaceutical Sciences 72(6):697.
- Atef NM, Shanab SM, Negm SI (2019). Evaluation of antimicrobial activity of some plant extracts against antibiotic susceptible and resistant bacterial strains causing wound infection. Bulletin of the National Research Centre 43(1):1-11.
- Bhalodia NR, Shukla VJ (2011). Antibacterial and antifungal activities from leaf extracts of *Cassia fistula* L. An ethnomedicinal plant. Journal of Advanced Pharmaceutical Technology and Research 2:104-109.
- Cowan MM (1999). Plant products as antimicrobial agents. Clinical microbiology reviews 12(4):564-582.
- Elhassan B, Nouria N, Amal D (2020). Antimicrobial Effect of *Quercus robur* L Leaves Selective Extracts from the Mezi Mountain of Djeniene Bourezg (West of Algeria). Current Bioactive Compounds 16(8):1181-1190.
- Elnaggar M, Abdulla G, El-Shourbagy G, El-Badawi A, El-Sohaimy S (2019). Antimicrobial and antioxidant activities of some plant extracts. Polymers 44:1061-1071.
- Gu M, Fan S, Liu G, Guo L, Ding X, Lu Y, Zhang Y, Ji G, Huang C (2013). Extract of Wax Gourd Peel Prevents High-Fat Diet-Induced Hyperlipidemia in C57BL/6 Mice via the Inhibition of the PPAR γ Pathway. Evidence-Based Complementary and Alternative Medicine P 342561.
- Ikeda UP, Silva M, Di Stasi L, Barbosa L, Junior A (2007). Antibacterial activity of medicinal plant extracts. Brazilian Journal of Microbiology 38:717-719.
- Jhonatas ERC, Joyce FCG, Marcos SG, Guilherme ROF, Enyara RM (2019). Phytochemical Analysis and Evaluation of Antimicrobial Activity of *Peumus boldus*, *Psidium guajava*, *Vernonia polysphaera*, *Persea Americana*, *Eucalyptus citriodora* Leaf Extracts and *Jatropha multifida* Raw Sap. Current Pharmaceutical Biotechnology 20(5):433-444.
- Juliana MP, Priscilla CV, Grazielle NN, Meireles MAA (2018). Extraction Methods for Obtaining Natural Blue Colorants. Current Analytical Chemistry 16(5):504-532.
- Karuppiyah P, Rajaram S (2012). Antibacterial effect of *Allium sativum* cloves and *Zingiber officinale* rhizomes against multiple-drug resistant clinical pathogens. Asian Pacific journal of tropical biomedicine 2(8):597-601.
- Khan V, Najmi AK, Akhtar M, Aqil M, Mujeeb M, Pillai KK (2012). A pharmacological appraisal of medicinal plants with antidiabetic potential. Journal Of Pharmacy and Bioallied Sciences 4(1):27.
- Lee K, Choi HR, Kim CH (2005). Anti-angiogenic effect of the seed extract of *Benincasa hispida* Cogniaux. Journal of Ethnopharmacology 97(3):509-513.
- Mahboob N, Iqbal H, Ahmed M, Magnet MMH, Mamun K (2019). Disk diffusion method in enriched mueller hinton agar for determining susceptibility of candida isolates from various clinical specimens. Journal of Dhaka Medical College 28(1):28-33.
- Mandal U, De D, Ali K, Biswas A, Ghosh D (2012). Effect of different solvent extracts of *Benincasa hispida* T on experimental hypochlorhydria in rat. Journal of Advanced Pharmaceutical Technology and Research 3(1):41-46.
- Martinelli F, Scalenghe R, Davino S, Panno S, Scuderi G, Ruisi P, Villa P, Stroppiana D, Boschetti M, Goulart LR (2015). Advanced methods of plant disease detection. A review. Agronomy for Sustainable Development 35(1):1-25.
- Meenakshi G, Anoop K (2019). Comparison of Minimum Inhibitory Concentration (MIC) value of statin drugs: A Systematic Review. Anti-Infective Agents 17(1):4-19.
- Meshram GG, Kumar A, Rizvi W, Tripathi CD, Khan RA (2016). Evaluation of the anti-inflammatory activity of the aqueous and ethanolic extracts of the leaves of *Albizia lebeck* in rats. Journal of Traditional and Complementary Medicine 6(2):172-175.
- Mohsenipour Z, Hassanshahian M (2016). Antibacterial Activity of *Euphorbia hebecarpa* Alcoholic Extracts Against Six Human Pathogenic Bacteria in Planktonic and Biofilm Forms. Jundishapur Journal of Microbiology 9(6):e34701.
- Patil KR, Patil CR (2017). Anti-inflammatory activity of bartogenic acid containing fraction of fruits of *Barringtonia racemosa* Roxb. in acute and chronic animal models of inflammation. Journal of Traditional and Complementary Medicine 7(1):86-93.
- Rahmatullah M, Hasan A, Parvin W, Moniruzzaman M, Khatun A, Khatun Z, Jahan FI, Jahan R (2012). Medicinal plants and formulations used by the Soren clan of the Santal tribe in Rajshahi

- district, Bangladesh for treatment of various ailments. *African Journal of Traditional, Complementary and Alternative Medicines* 9(3):350-359.
- Salas CE, Badillo-Corona JA, Ramírez-Sotelo G, Oliver-Salvador C (2015). Biologically active and antimicrobial peptides from plants. *BioMed Research International* pp.102-129.
- Sasidharan S, Chen Y, Saravanan D, Sundram KM, Yoga Latha L (2011). Extraction, isolation and characterization of bioactive compounds from plants' extracts. *African Journal of Traditional, Complementary and Alternative Medicines* 8(1).
- Sharma S, Verma HN, Sharma NK (2014). Cationic Bioactive Peptide from the Seeds of *Benincasa hispida*. *International Journal of Peptides* Article ID 156060, 12 p, <https://doi.org/10.1155/2014/156060>
- Sheahan M, Barrett CB (2017). Food loss and waste in Sub-Saharan Africa. *Food Policy* 70:1-12.
- Shetty RP, Endy D, Knight TF (2008). Engineering BioBrick vectors from BioBrick parts. *Journal of Biological Engineering* 2:5.
- Simons FER, Simons KJ (2008). H1 antihistamines: current status and future directions. *World Allergy Organization Journal* 1(9):145-155.
- Tatiya AU, Saluja AK, Kalaskar MG, Surana SJ, Patil PH (2017). Evaluation of analgesic and anti-inflammatory activity of *Bridelia retusa* (Spreng) bark. *Journal of Traditional and Complementary Medicine* 7(4):441-451.
- Valgas C, Souza SMD, Smania EFA, Smania Jr A (2007). Screening methods to determine antibacterial activity of natural products. *Brazilian Journal of Microbiology* 38(2):369-380.
- Wen W, Lu J, Zhang K, Chen S (2008). Grape seed extract inhibits angiogenesis via suppression of the vascular endothelial growth factor receptor signaling pathway. *Cancer Prevention Research* 1(7):554-561.
- Xing Y, Xu Q, Li X, Che Z, Yun J (2012). Antifungal activities of clove oil against *Rhizopus nigricans*, *Aspergillus flavus* and *Penicillium citrinum* *in vitro* and in wounded fruit test. *Journal of Food Safety* 32(1):84-93.
- Yagnik B, Vaghasiya J, Nurudin J, Kanzariya N, Rameshwar P, Natavarlal P (2009). Antioxidant activity of *Benincasa hispida* on renal ischemia/reperfusion injury. *Pharmacologyonline* 1:44-49.
- Yuan H, Ma Q, Ye L, Piao G (2016). The Traditional Medicine and Modern Medicine from Natural Products. *Molecules* 21:559.
- Zhang YJ, Gan RY, Li S, Zhou Y, Li AN, Xu DP, Li HB (2015). Antioxidant Phytochemicals for the Prevention and Treatment of Chronic Diseases. *Molecules* 20(12):21138-56.

Full Length Research Paper

Molecular diagnosis of multidrug-resistant tuberculosis from culture-positive isolates using line probe assay in North Karnataka

Reshma Bellad and Mahantesh Nagamoti*

Department of Microbiology, JNMC, KAHER Nehru Nagar, Belgavai-590010, Karnataka, India.

Received 23 July, 2019; Accepted 13 July, 2020

The control of tuberculosis (TB) has become a global health challenge due to the emergence of multidrug-resistant tuberculosis (MDR-TB) in *Mycobacterium tuberculosis* (MTB). This highlights the need for faster and more accurate detection of tuberculosis cases. The study aims to detect MDR-TB strains of pulmonary tuberculosis using resistance ratio method and to compare the diagnostic value of drug susceptibility testing (DST) with line probe assay (LPA) using Genotype MTBDRplus. All the sputum samples were tested for Acid Fast Bacilli (AFB) by Ziehl-Neelsen's staining method and were cultured on Lowenstein-Jensen (L-J) media. The identification and confirmation of *M. tuberculosis* were done using various biochemical tests. DST was carried against the first line of anti-TB drugs. MTB positive samples were subjected to LPA. A total of 57 samples were subjected to DST and LPA for the detection of drug resistance of MTB to RIF and INH after conventional detection methods were applied to all the samples. Among these 57 MTB samples, 11 (19.29%) were resistant to INH, 4 (7.14%) were resistant to RIF; eleven (19.29%) isolates were identified as MDR-TB. LPA revealed 54 MTB positive among 57 MTB culture-positive samples and 3 showed invalid results. In LPA, MDR-TB was found in 10 samples (17.54%) in which one was RIF-resistant. The study concludes risk factors that resulted in the development of TB are biomedical, socio-cultural, and behavioral interactions. LPA can be used as a rapid diagnostic technique for the detection of MDR-TB.

Key words: Tuberculosis (TB), multidrug resistance (MDR-TB), drug susceptibility testing (DST), line probe assay (LPA).

INTRODUCTION

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (MTB) is one of the leading causes of deaths due to infectious diseases in developing nations, including India. According to the World Health Organization (WHO), 1.8 million people died due to TB, and 10.4 million people

were infected with TB in 2015. Overall, 95% of the total TB deaths occurred in low- and middle-income countries. Six countries had accounted for about 60% of the global total, with India leading the burden of tuberculosis in the world (WHO, 2016). Globally India has become a home

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

for one-fourth of TB burden patients. In 2015, around 280, 00, 00 TB cases occurred in India, and 48, 0000 people had died due to this disease. The global emergence of Multidrug-resistant tuberculosis (MDR-TB) is a major public health problem as it causes a major challenge to control the disease and the mortality rates associated with it. India has a high burden of patients with MDR-TB. Annually, according to India reports, 130,000MDR-TB cases among which 79,000 MDR-TB cases are pulmonary TB (TB Statistics for India; WHO, 2008).

Early diagnosis of MDR-TB is necessary for the effective treatment and control of MDR-TB strains. It is known that resistance to isoniazid (INH) and rifampicin (RIF) is a key factor to determine the efficacy of the currently recommended standard treatment regimens. (Telenti et al., 1997; Cavusoglu et al., 2002; de Viedma et al., 2002; Morcillo et al., 2002; Saribas et al., 2003). Conventional techniques for MDR-TB detection are time-consuming and require sophisticated laboratory infrastructure, which causes delay in reporting the results. This in turn delays the proper treatment which increases the risk of transmission of the disease (Chauhan and Arora, 2004). Despite various measures available for the detection of MDR-TB, the prevalence of MDR-TB rate has remained unchanged in recent years (Havumaki et al., 2017). There is an urgent need for standardizing rapid molecular tests such as Line Probe assay (LPA) (Hillemann et al., 2005).

Identification of infectious cases is an important step for TB control programs worldwide. Detection of AFB in sputum by smear microscopy continues to be the mainstay diagnostic technique since its introduction in the late nineteenth century (Chakravorty and Tyagi, 2005). Drug susceptibility testing in Lowenstein-Jensen (L-J) culture media remains the cornerstone and gold standard for the diagnosis of resistance patterns in TB. There are different conventional methods (proportion, resistance ratio, and absolute concentration methods), the radiometric method, and other newer methods used for determining antimicrobial susceptibility patterns. The most extensively used is the proportion (PR) and the resistance ratio (RR) methods. The RR method compares the resistance of unknown strain of tubercle bacilli (test organism) with that of a standard laboratory strain of *M. tuberculosis* (H37Rv) (Acharya et al., 2010).

The drug resistance of Indian isolates varies from 52.2 to 2% (Iqbal et al., 2003). As there are variations in the resistance pattern to address this, the hospital-based study was undertaken to perform microscopy, biochemical analysis, and drug susceptibility testing using the RR method for the first line of anti-TB drugs. This method gives faster results than other DST methods. The revolution of TB diagnosis started in 2008 when WHO and Foundation of Innovative Diagnostics (FIND) endorsed the use of Line Probe Assay (LPA) which was developed by Genotype MTBDRplus (Hain Life Science,

Nehren, Germany) for the detection of MDR-TB (Nathavitharana et al., 2017). The assay is based on the multiplex polymerase chain reaction (PCR) method and uses reverse hybridization to identify MDR-TB from MTB culture and smear-positive sputum specimens (Barnard et al., 2012). LPA detects the wild-type sequencing or specific mutations associated with the *rpoB* gene for RIF resistance, the *katG* gene for high-level INH resistance, and *inhA* regulatory region gene for low-level INH resistance (Chauhan and Arora, 2004). It targets a mutation in the 81 base pair “core region” of the *rpoB* gene which detects almost 95% of RIF resistant strains and *ahpC*-oxy R intergenic region which detects 5-10% mutation in INH resistant (Nathavitharana et al., 2017). The study aimed to compare the conventional drug susceptibility testing (DST) and LPA for the detection of RIF and INH resistance in MTB.

MATERIALS AND METHODS

Sample size and patient recruitment

The study was approved by the Institutional Ethical Board, KLE Academy of Higher Education and Research, Belagavi (Karnataka, India). Patients attending Out Patient Department (OPD) and wards of the Karnataka Lingayat Education Society (KLES) Dr.Prabhakar Kore Hospital and Medical Research Centre with symptoms such as fever, night sweats, and cough for more than 2 weeks with sputum, chest pain from January 2013 to December 2014 were included in the study. Non-TB patients, extra-pulmonary TB, and culture-negative samples excluded. The sampling method for this study was inverse sampling. The estimated sample size according to this method should be 125 positive cultures. However, in this study 232 smear, positive samples were obtained out of which 75 samples were culture-positive. Hence to establish the validity of results, bootstrapping analysis was carried out (Haldane, 1945).

Collection of samples

Samples were collected after the Ethical clearance was obtained from the Institutional Ethical Board of KLE University Belagavi (Karnataka, India).The study was carried out at the Department of Microbiology, J.N Medical College, KLE University, and Belagavi, India between January 2013 and December 2014. This is a Hospital-based study. Sputum samples of all the 3453 patients (1 sputum sample per patient) were collected from Hi-Tech Laboratory, KLES Dr.Prabhakar Kore Hospital, Belagavi from January 2013 to December 2014. Patients attending Out Patient Department (OPD) and wards of the KLES Dr. Prabhakar Kore Hospital and Medical Research Centre, who have fever, night sweats, cough for more than 3 weeks with sputum and chest pain were included sputum sample was collected in a clean, dry, sterile wide-neck, leak-proof screw cap container (Figure 1).

Microscopic technique

From purulent part of the specimen, and subjected to smear microscopy by Ziehl-Neelsen (ZN) staining for acid-fast bacilli in laboratory level 3 biosafety level 2. The grading of smears was done according to the guidelines provided by the Revised National Tuberculosis Control Program (RNTCP) of India (WHO, 2009). The

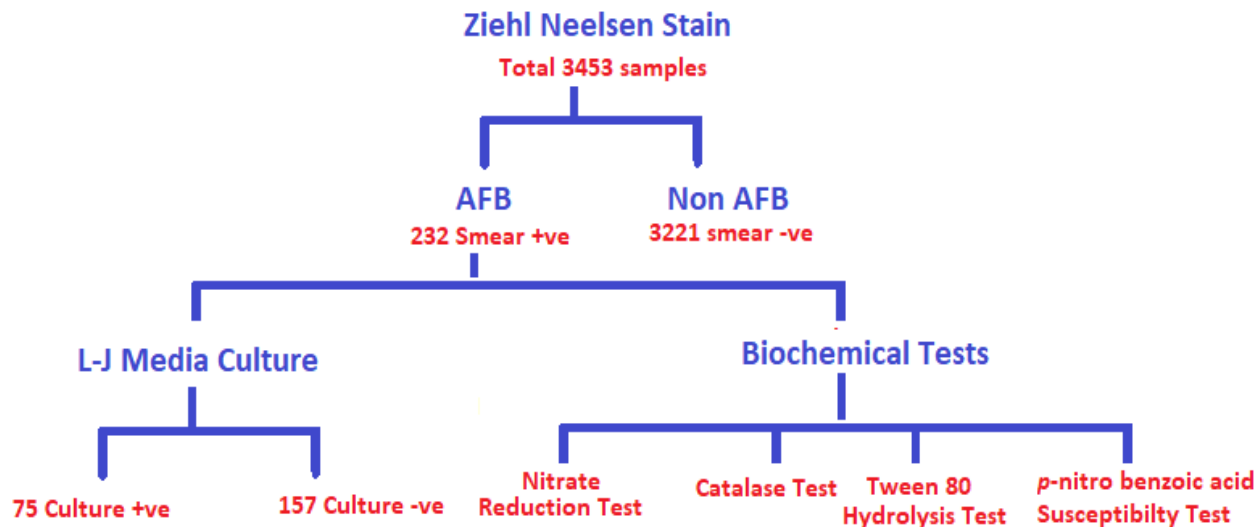


Figure 1. Schematic flow of procedure followed for confirmation of the species of *Mycobacterium tuberculosis*.

results of the microscopic examination were reported according to the Revised National Tuberculosis Control Programme (RNTCP). The processing of samples was carried out in a biosafety cabinet with standard procedures (Revised TB Programme, 2005; Central TB Division, 2005).

Culture technique

All sputum specimens were handled at the bio-safety level (BSL-3). Only smear-positive samples were cultured by Petroff's method (N-acetyl-L-cysteine and sodium hydroxide (NALC-NaOH) the sediments were suspended in distilled water. The supernatant was discarded after centrifugation and two slopes of Löwenstein–Jensen (LJ) media was taken for inoculation of each sample. The inoculated LJ media was incubated at 37°C. Reading and interpretation was done for the colony formation every week, preferably twice within the first week. The contaminated cultures and rapidly growing mycobacteria (colonies apparent in less than 7 days) were removed. *M. tuberculosis* colonies were developed within 3-4 weeks. Cultures were kept for up to 8 weeks before being reported as negative (Figure 2a) (Protocol for Processing, 1998).

Biochemical tests

The identification and confirmation of *M. tuberculosis* were done using various biochemical tests. **Catalase Test**- Into two screw-cap test-tubes 0.5 ml of phosphate buffer was dispensed (pH7.0). One tube was placed in the water-bath at 68°C for 20 min. Another tube was left at room temperature (Figure 2c).

Nitrate reduction test

Two loopful of bacterial growth was emulsified in 0.2 ml distilled water; then 2 ml of the substrate medium was added. Incubation was done at 37°C for 2 h. To each tube in sequence one drop of reagent 1 (HC1), two drops of reagent 2 (Sulfanilamide), and two drops of reagent 3 (n-naphthyl ethylenediamine dihydrochloride) was added. The development of a red colour indicated a positive

reaction (Figure 2b).

Tween-80 hydrolysis

A volume of 0.5 ml freshly prepared Tween 80–peroxide substrate was added to each tube. The formation of bubbles was observed. The release of the oleic acid from Tween 80 results in the change of colour from the neutral indicator yellow to red within 5-10 days.

p-nitro benzoic acid (PNB) susceptibility

Single slope of LJ medium was inoculated containing Para-Nitro Benzoic acid (PNB Sodium Salt) 500 mcg/ml with bacterial suspension. It was incubated at 37°C and growth was recorded on the 28th day (Manual on isolation, 1998).

Drug susceptibility testing (DST)

Minimal Inhibitory Concentration (MIC) on solid LJ medium was used for DST which is expressed as a resistance ratio method according to the standard operating procedure of RNTCP. All the culture-positive MTB isolates (showing colonies more than 20) were further analyzed by DST resistant ratio method. The media containing two-fold dilutions of the primary anti-TB drugs were prepared as follows: INH, 0.5 and 1.0 µg/ml; RIF, 32.0 and 64.0 µg/ml; streptomycin (SM), 16.0 and 32.0 µg/ml; and ethambutol (EMB), 4.0 and 8.0 µg/ml. One drop (100 µl) of 1 mg/ml bacillary suspension (McFarland No.1) from a Pasteur pipette was spread on the surface of each drug-containing slope of media of different concentrations. The same procedure was done for the H₃₇RV strain, which was used as a positive control for the test. All the tubes were incubated at 37°C for 4 weeks and were observed every week. The growth was examined after 28 days and this was defined by the presence of 20 or more colonies in the drug-containing media (Figure 2a and b). The isolates were considered resistant when the growth appeared on the drug-containing media (Revised TB Programme 2014). MTB in all isolates were identified by the slow

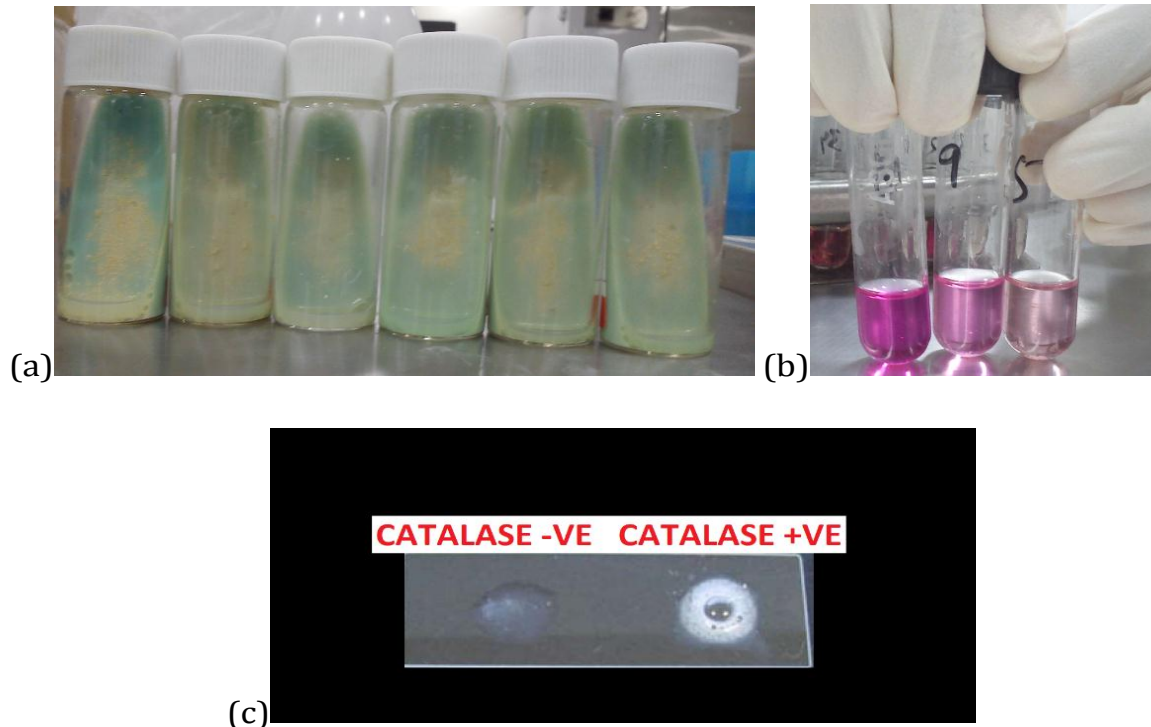


Figure 2. *M. tuberculosis* a) colonies on L-J Media, Biochemical tests b) Nitrate Reduction Test c) Catalase Test.

Table 1. Cycling reaction for PCR.

Temperature (°C)	Time	Cycle
95	15 min	1
95	30 s	10
58	120 s	
95	25 s	20
53	40 s	
70	40 s	
70	8 min	1

growth rate, colony morphology and biochemical tests such as the incapability to grow on PNB acid, niacin positive and catalase-negative test, nitrate negative test (Chauhan et al., 1998).

Line probe assay (LPA)

The bacterial colonies from cultures were used for DNA extraction (Bhawan, 2009). The bacterial DNA was extracted from the colonies of solid media according to the manufacturer's instructions. Molecular grade water of 300 μ l was added to and the DNA was by vortexing. This bacterial suspension was centrifuged for 15 min at 10,000 rpm. The supernatant was aspirated and the pellet was resuspended in 100 μ l distilled water. The specimens were heat-killed at 95°C for 20 min, sonicated for 15 min and centrifuged at 13000 rpm for 5min. The supernatant containing DNA was transferred into a fresh tube. The extracted DNA was kept in 4°C and was used within 1-7 days. The procedure of LPA was performed according to the manufacturer's instructions (Hain Lifescience, Nehren, Germany, 2012). It consists of three steps: 1)

multiplex PCR, 2) amplification and 3) reverse hybridization (Khanna et al., 2010). To avoid contamination, these steps were carried out in three separate rooms with restricted access and unidirectional flow.

A total of 5 μ l DNA was added to 45 μ l of the master mix for each PCR reaction and the amplification procedure for cultured isolates were followed as per the directions given by the manufacturer (QIAGEN, Hilden, Germany). The cycling reaction is given in Table 1. Hybridization and detection is the final step in the assay, which was performed by using all the materials and reagents provided by the kit manufacturer (GmbH, Hain Life Science). A twelve well plastic tray was used for all three steps: denaturation, hybridization, and detection. In this tray, a denaturation buffer of 20 μ l was dispensed to this 20 μ l of DNA amplicons and mixed thoroughly. The solution was incubated at room temperature for 5 min. After denaturation, a 30 min hybridization step was done by adding 1ml of pre-warmed green hybridization buffer (HYB). After the aspiration of HYB 1ml of preheated red stringent wash buffer was added. To remove the excess STR buffer 1 ml of rinse (RIN) solution was added which was followed by the addition of previously prepared

Table 2. Age-gender distribution of pulmonary tuberculosis.

S/ N	Age group	Total no. of patients = 57					
		Male		Female		Total	
		Total count	%	Total count	%	Total count	%
1	>18	3	5.26	1	1.75	4	7.01
2	20-30	6	10.54	5	8.77	15	26.31
3	30-40	11	19.3	4	7.01	2	3.51
4	40-50	1	1.75	1	1.75	15	26.31
5	50-60	12	21.05	3	5.26	8	14.04
6	61-70	7	12.28	1	1.75	2	3.51
7	71-80	1	1.75	1	1.75	57	100
	Total	40	71.18%	16	28.07%		

Table 3. The general characters distribution of pulmonary tuberculosis.

Character	Number of patients = 57	
Habits	Smoking	38 (67.85%)
	Alcoholism	32 (49.23%)
Health status	Diabetes Mellitus	07 (10.76%)
	HIV	-
Socioeconomic status	Labour	25 (38.46%)
	Farmer	22 (33.84%)
	Business	09 (13.84%)

conjugate solution. Once again the strips were washed by 1ml of RIN solution and 1 ml of distilled water. In the final step, 1 ml of diluted substrate solution was added to each well and the tray was washed with 1 ml of distilled water. The bands were air-dried and the staining reaction was observed, showing whether the probes have been hybridized or not (GmbH, Hain Life Science).

The LPA strip has 27 reaction zones or bands for verification of the test procedures. The strips of LPA consist of two internal controls; conjugate control (CC) and amplification control (AC). The presence of positive MTB control tuberculosis-complex DNA band (TUB) reaction indicates the given specimen is positive for MTB complex. The test is considered valid only when wild type (WT) and mutation (MUT) bands are present, if these two bands are absent the test is considered invalid (GmbH, Hain Life Science).

RESULTS

All the 3453 sputum samples were subjected to Z-N staining. The smear examination yielded the following bacillary load: +1 AFB in 92 (39.65%), 2+ in 69 (29.75%) and 3+ in 71 (30.6%). Of these 232, 167 samples (71.18%) were males and 65 samples (28.07%) females (Table 2). Smoking (67.85%) and alcoholism (49.23%) were the commonest predisposing conditions and 72.3%

were labour and farmer by profession (Table 3).

Out of 232 smear-positive samples, 75 were culture-positive, 70 were culture-negative, 34 were contaminated and 53 had less than 20 colonies. Biochemical tests were performed for 65 samples (10 were excluded due to issues associated with culture handling). The samples were further tested by biochemical analysis using catalase, nitrate reduction test, tween-80 hydrolysis, and *p*-nitrobenzoic acid (PNB) susceptibility for the confirmation of MTB. Out of these, 60 (25.86%) isolates were MTB and 5 isolates (2.1%) were non-tuberculosis Mycobacteria (NTM).

Among 60, 3 yielded less than 20 colonies in a culture which were excluded from further tests. The remaining 57 samples were subjected to DST analysis (Figure 4). Among these 57 MTB samples, 11 (19.29%) were resistant to INH, 4 (7.14%) were resistant to RIF, and 11 (19.29%) isolates were identified as MDR-TB through the resistance ratio method of DST (Table 4). LPA revealed 54 MTB positive among 57 MTB culture-positive samples and 3 showed invalid results (Figure 3). The TUB band was absent in all five NTM specimens. In LPA 10 samples (17.54%) were MDR-TB, in which one was RIF-resistant (Table 5).

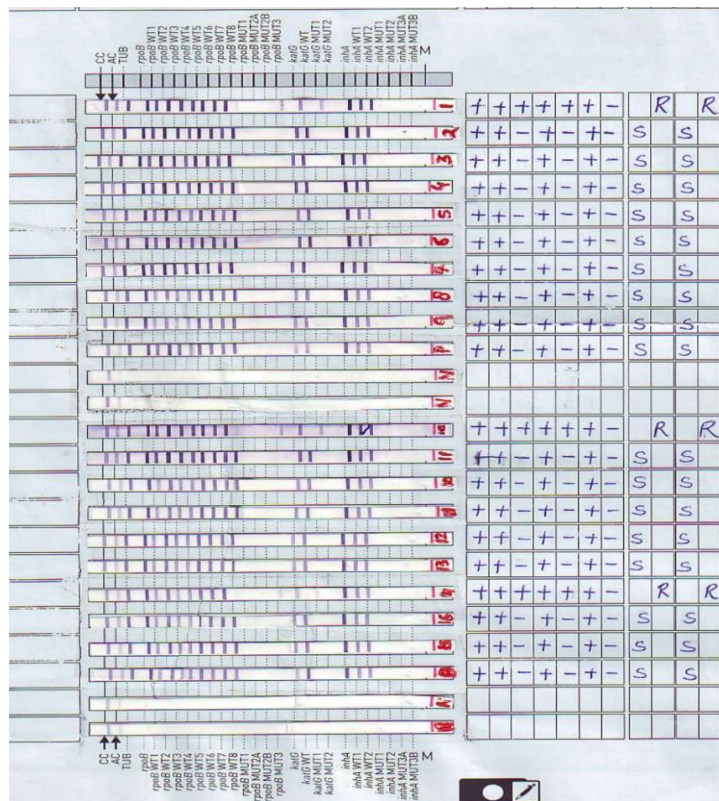
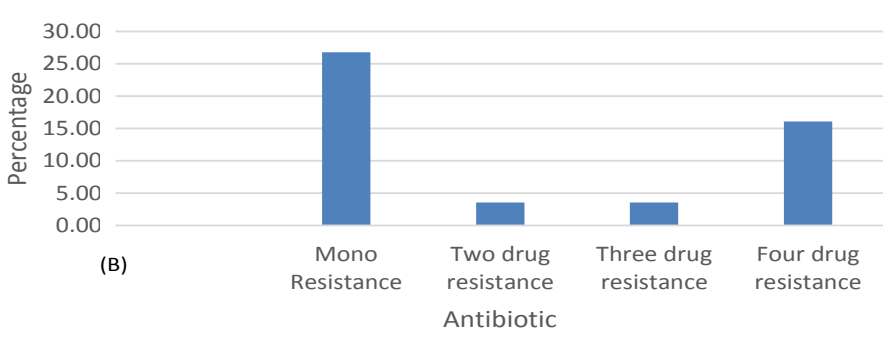
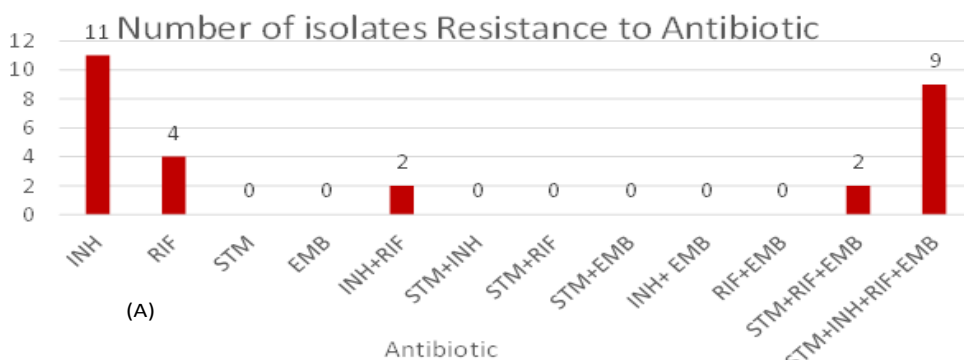


Figure 3. LPA results.



Percentage of strains resistance to antibiotics

Figure 4. Graphical representation of (a) Drug resistance pattern obtained by DST (b) Drug resistance percentage obtained by DST.

Table 4. Drug resistance pattern obtained by DST.

Resistance	Number of isolates (N=57)	Percentage	Total Percentage
Mono-resistance	11		
INH	4	19.64	26.78
RIF	0	7.14	
STM	0	-	
EMB		-	
Resistance to two drugs			
INH+RIF	2	3.57	3.57
STM+INH	0	-	
STM+RIF	0	-	
STM+EMB	0	-	
INH+ EMB	0	-	
RIF+EMB	0	-	
Resistance to three drugs			
STM+RIF+EMB	2	3.57	3.57
Resistance to four drugs			
STM+INH+RIF+EMB	9	16.07	16.07

INH: Isoniazid, RIF: Rifampicin, STM: Streptomycin, EMB: Ethambutol .

DISCUSSION

TB has existed for millennia and remains a major global health problem. In developing countries laboratory plays a crucial role in diagnosing TB. It is one of the top 10 causes of death worldwide, ranking above HIV/AIDS as one of the leading causes of death from an infectious disease (WHO, 2016). Thus, the present study was undertaken to study the drug resistance pattern, the risk factor associated with TB, and recommending the use of the resistance ratio method for DST. The risk factors which resulted in the development of TB were biomedical, socio-cultural, and behavioral interactions (Balaji et al., 2010).

One of the predisposing factors for the cause of Pulmonary TB was low economic status as these classes of people were more illiterate and have fewer health facilities which resulted in more mortality (Gupta et al., 2011). TB was more common in males (71.18%) when compared to females (28.07%). The accompanying risk factors (Smoking, Alcoholism, and DM), may have triggered the disease more in males as they are prone to such lifestyle (Acharya et al., 2010; Deodhar et al., 1999). Alcoholism is one of the most important morbid factors for TB infection (Fleming et al., 2006). This study predicts that Ziehl-Neelsen staining is rapid and inexpensive, but lacks sensitivity and specificity. It cannot be used to distinguish between the various members of the Mycobacterium and also requires a high amount of organisms in the specimen. Due to its low sensitivity, there are high chances of false negatives. Resistance testing is too expensive with modern techniques. In such cases as conventional L-J based approach, the DST

method may be a suitable alternative. There are studies carried out in Bijapur and Pakistan where the isolation rate was 34.74 and 25.84% respectively, which can be compared to these results. There can be a variety of reasons for culture-negative; the organism may have lost their ability to grow on culture media or patients on treatment with regimens have negative results (Gaude et al., 2014).

Drug susceptibility testing was carried on all first-line anti-tubercular drugs-INH, RIF, EMB, and STM. It was observed that 11 specimens were sensitive to all 4 drugs. INH mono-resistance was comparable, INH+RIF resistance is nearly 50% reduced in our case, resistance to STM+RIF+EMB combination is reduced to one third, and resistance to all four drugs is double in this study. The MDR-TB rate is highly variable between countries and in between the states of India i.e. Delhi (33.7%), Bihar (15%), Mumbai (51%), Gujarat (17.4%) and Tamil Nadu (20.3%) (Tripathy et al., 2015). The resistance of MTB in this study was (19.64%) which can be compared to Tamil Nadu and Gujarat. Since drug-resistant TB has increased in incidence and interfered with TB control programs, monitoring of drug resistance patterns is very much important to prevent MDR-TB outbreaks. So, all isolates of *M. tuberculosis* should be tested for their susceptibilities to the primary anti-tubercular drugs.

Of the conventional culture-based techniques for antimicrobial susceptibility testing, the Resistance Ratio (RR) and the Proportion (PR) methods are commonly used. The resistance ratio method is still in use in many countries especially the United Kingdom (Kent and Kubica, 1985). However, WHO has recommended the use of the proportion method to be used for determining

Table 5. The pattern of genetic mutation in drug-resistant *Mycobacterium tuberculosis* isolates using the Genotype MTBDRplus assay.

Gene	Band	Gene region of the mutation	RIF mono	INH mono	MDR ^{*,^}
			resistance ^{*,^} N=1	resistance ^{*,^} N=0	N=10
katG	WT	315	1 (100%)	0 (0%)	9 (90%)
	MUT1	S315T1	0 (0%)	0 (0%)	1 (10%)
	MUT2	S315T2	0 (0%)	0 (0%)	8 (80%)
inhA	WT1	0.9375	1 (100%)	0 (0%)	10 (100%)
	WT2	-8	1 (100%)	0 (0%)	10 (100%)
	MUT1	C15T	0 (0%)	0 (0%)	10 (100%)
	MUT2	A16G	0 (0%)	0 (0%)	0 (0%)
	MUT3A	T8C	0 (0%)	0 (0%)	0 (0%)
	MUT3B	T8A			0 (0%)
rpoB	WT1	506-509	1 (100%)	0 (0%)	10 (100%)
	WT2	510-513	1 (100%)	0 (0%)	10 (100%)
	WT3	513-517	1 (100%)	0 (0%)	10 (100%)
	WT4	516-519	1 (100%)	0 (0%)	10 (100%)
	WT5	518-522	1 (100%)	0 (0%)	10 (100%)
	WT6	521-525	1 (100%)	0 (0%)	10 (100%)
	WT7	526-529	1 (100%)	0 (0%)	10 (100%)
	WT8	530-533	0 (0%)	0 (0%)	10 (100%)
	MUT1	D516V	0 (0%)	0 (0%)	6 (60%)
	MUT2A	H526Y	0 (0%)	0 (0%)	0 (0%)
	MUT2B	H526B	0 (0%)	0 (0%)	0 (0%)
	MUT3	S531L			9 (90%)

*Definitions of abbreviations: INH = isoniazid; RIF = rifampicin; MDR = multidrug-resistant. ^Values are numbers, with percentages in parentheses.

drug susceptibility of *M. tuberculosis*. The RR method has been used in this study this compares the resistance of the unknown strain with that of the control strain on the same batch of the medium. Resistance can be expressed as the ratio of the MIC (Minimum Inhibitory Concentration) of the test strain to the MIC of the control strain in the same test. The RR method was convenient for inoculum preparation and it required a shorter time to perform. Interpretation of the result was rather simple when it can compare to other methods.

In MDR-TB detection, the conventional culture and DST on the solid LJ media is a time-consuming process. However, an early diagnosis of RIF and INH drug-resistant MTB is essential for effective treatment and control of MDR-TB. With the advent of molecular techniques and the development of commercial or in-house DNA hybridization or amplification methods, the results of MDR-TB can be obtained fast (Ahmed et al., 2017). With the introduction of LPA for the rapid diagnosis of drug-resistant TB, there has been a significant reduction in time to start the treatment in MDR suspected cases.

The present study was conducted to determine the

pattern of mutations in MTB using LPA and to compare with DST for the detection of RIF and INH resistance in culture-positive isolates. The findings of this study showed that many mutations, which occurred in the *rpoB* and *katG* genes, are comparable to those reported in other countries (Taniguchi et al., 1996; Siddiqi et al., 2002). The existence of common mutations in the *rpoB* gene, at codons 526 and 531 isolates from India and other countries, supports the assumption that these mutations are common for many drug-resistant strains around the globe (Telenti et al., 1993; Sun et al., 2008). In this study, resistance to RIF was higher than that of INH in LPA. This similarity was also observed in Ethiopia (Meaza et al., 2017). The common mutation associated with RIF was similar to the present study.

The LPA test failed to detect mono- INH resistant strains in 11 specimens, which were detected by conventional DST. One MDR-TB sample detected by DST showed the false result in LPA as MTB which could be due to the presence of PCR inhibitors during the process of DNA extraction. Similar results were also observed in another study conducted in central India (Desikan et al., 2017). Among 57 isolates, 10 were RIF

resistant strains and one was RIF mono-resistant of MDR-TB which had a mutation on *rpoB* S531L diagnosed by the presence of MUT3 band (Table 3). The most frequent mutation found in INH was a *katG* mutation, which occurred more commonly in MDR-TB strains than in INH mono-resistant strains. RIF resistance is associated with the mutations in 81 base pairs region (codon 527 to 533) of the *rpoB* gene (Yue et al., 2003). The finding of dominant mutations for RIF resistance in *rpoB* S531L in the present study is similar to a previously published report (Miotto et al., 2006).

In this study, no mutations were observed in the *inhA* gene region. Similarly, a low frequency of *inhA* gene mutations was reported in Ethiopia (Omer et al., 2016). A study from north India also has reported a low frequency of INH resistance mutation in the *inhA* gene. This could suggest that there are possibilities of mutation in other codons of the *katG* and *inhA* gene (Omer et al., 2016). The high prevalence of mutations within the *rpoB* core region in the MDR-TB strains isolated from India indicates the potential of a rapid diagnostic test for the detection of drug-resistant MTB. As stated, the detection of mutations in the *rpoB* gene is very effective for the diagnosis of drug resistance to RIF in MTB complex since the mutations in the hot spot region are prevailing. The invalid results observed in the present study were due to the lower bacillary load in sputum specimens or culture-negative samples, which emphasizes not using the LPA test, directly for smear-negative clinical specimens (Chauhan and Arora, 2004).

The limitation of LPA by Genotype MTBDR plus assay is that it requires sophisticated infrastructure, well-trained and skilled laboratory personnel. The test does not provide convenient results with sputum specimens, which have a lower bacillary load (Yadav et al., 2013). However, the use of LPA can lead to the earlier initiation of appropriate drug therapy which will thereby prevent further transmission of the drug-resistant strains. Applying the LPA method to detect drug resistance in the MTB isolates in clinical laboratories, require further research and method validation, aiming to enable the patients receive appropriate standardized MDR-TB treatment regime at an early stage of the illness.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGMENTS

The authors appreciate the Head of Department of Microbiology and Dean, J.N. Medical College, KAHER, Belagavi, and National JALMA Institute for Leprosy and other Mycobacterial Diseases, Agra (UP) for their help in providing the conventional and molecular technical platform for the study.

REFERENCES

- Acharya S, Ghimire P, Khadka D, Nepali S (2010). Comparison of Proportion and Resistance Ratio Methods for Drug Susceptibility Testing of *Mycobacterium tuberculosis* isolated from Patients Attending National Tuberculosis Centre, Nepal. SAARC Journal of Tuberculosis, Lung Diseases and HIV/AIDS 5(1):13-20.
- Ahmed S, Shukla I, Fatima N, Varshney SK, Shameem M (2017). Evaluation of genotype MTBDRplus line probe assay in detection of rifampicin and isoniazid resistance in comparison to solid culture drug susceptibility testing in a tertiary care centre of western Uttar Pradesh. Indian Journal of Medical Microbiology 35:568-574.
- Balaji V, Daley P, Anand AA, Sudarsanam T, Michael JS, Sahni DR, Chordia P, George IA, Thomas K, Ganesh A, John KR (2010). Risk Factors for MDR and XDR-TB in a Tertiary Referral Hospital in India. PLoS ONE 53:e9527.
- Barnard M, Parsons L, Miotto P, Cirillo D, Feldmann K, Gutierrez C, Somoskovi A (2012). Molecular detection of drug resistant tuberculosis by line probe assay-Laboratory manual for resource-limited settings. Geneva: FIND.
- Bhawan N (2009). Training Manual for *Mycobacterium tuberculosis* Culture and Drug susceptibility testing. National Tuberculosis Institute, Bangalore.
- Cavusoglu C, Hilmioğlu S, Guneri S, Bilgic A (2002). Characterization of *rpoB* mutations in rifampin-resistant clinical isolates of *Mycobacterium tuberculosis* from Turkey by DNA sequencing and line probe assay. Journal of Clinical Microbiology 40(12):4435-4438.
- Central TB Division (2005). Directorate General of Health Services, Ministry of Health & family Welfare, Government of India.
- Chakravorty S, Tyagi J (2005). Novel Multipurpose Methodology for Detection of Mycobacteria in Pulmonary and Extrapulmonary Specimens by Smear Microscopy, Culture, and PCR. Journal of Clinical Microbiology 43:2697-2702.
- Chauhan LS, Arora VK (2004). Central TB Division, Directorate General of Health Services, Ministry of Health and Family Welfare; Indian Academy of Paediatrics, Management of Paediatric Tuberculosis under the Revised National Tuberculosis Control Program (RNTCP). Indian Paediatrics 41:901-905.
- Chauhan MM, Mahadev B, Balasangameshwara, VH (1998). Manual on isolation, identification and sensitivity testing of *Mycobacterium tuberculosis*. National Tuberculosis Institute, Bangalore, India.
- de Viedma DG, Infantes MDSD, Lasala F, Chaves F, AlcaláL, Bouza E (2002). New real-time PCR able to detect in a single tube multiple rifampin resistance mutations and high-level isoniazid resistance mutations in *Mycobacterium tuberculosis*. Journal of Clinical Microbiology 40(3): 988-995.
- Deodhar LL, Miskeen P, Chomal S (1999). Drug resistance in tuberculosis. Bombay Hospital Journal 41:253.
- Desikan P, Panwalkar N, Mirza SB, Chaturvedi A, Ansari K, Varathe R, Chourey M, Kumar P, Pandey M (2017). Line probe assay for detection of *Mycobacterium tuberculosis* complex: An experience from Central India. The Indian Journal of Medical Research 145(1):70.
- Fleming MF, Krupitsky E, Tsoy M, Zvartau E, Brazhenko N, Jakubowiak, W, McCaul ME (2006). Alcohol and drug use disorders, HIV status and drug resistance in a sample of Russian TB patients. International Journal of Tuberculosis Lung Diseases 10:565-570.
- Gaude GS, Hattiholli J, Kumar P (2014). Risk factors and drug-resistance patterns among pulmonary tuberculosis patients in the northern Karnataka region, India. Nigerian Medical Journal 55:327-333.
- HainLifesciences GmbH Germany (2012). GenoType MTBDR Plus VER 2.0. Instructions for Use IFU-304A-02 https://www.ghdonline.org/uploads/MTBDRplusV2_0212_304A-02-02.pdf.
- Gupta S, Shenoy VP, Mukhopadhyay C, Bairy I, Muralidharan S (2011). Role of risk factors and socioeconomic status in pulmonary tuberculosis: A search for the root cause in patients in a tertiary care hospital, South India. Tropical Medicine and International Health 16:74-78.
- Haldane JBS (1945). On a method of estimating frequencies. Biometrika 33(3):222-225.

- Havumaki J, Hillemann D, Ismail N, Omar SV, Georgiou SB, Schumacher SG, Boehme C, Denkinger CM (2017). Comparative accuracy of the REBA MTB MDR and HainMTBDRplus line probe assays for the detection of multidrug-resistant tuberculosis: A multicenter, non-inferiority study. *PLoS ONE* 12(3):0173804.
- Hillemann D, Weizenegger M, Kubica T, Richter E, Niemann S (2005). Use of the genotype MTBDR assay for rapid detection of rifampin and isoniazid resistance in *Mycobacterium tuberculosis* complex isolates. *Journal of Clinical Microbiology* 43(8):3699-3703.
- Iqbal S, Iqbal R, Khan MM, Hussain I, Akhtar A, Shabbir I (2003). Comparison of Two Conventional Techniques used for the Diagnosis of Tuberculosis. *International Journal of Agriculture and Biology* 5:545-547.
- Kent PT, Kubica GP (1985). *Public health Mycobacteriology: A guide for the level III laboratory*. US Department of Health and Human Services, Public health service, CDC, Georgia.
- Khanna A, Raj VS, Tarai B, Sood R, Pareek PK, Upadhyay DJ, Sharma P, Rattan A, Saini, KS, Singh H (2010). Emergence and molecular characterization of extensively drug-resistant *Mycobacterium tuberculosis* clinical isolates from the Delhi Region in India. *Antimicrobial agents and chemotherapy* 54(11):4789-4793.
- Manual on Isolation, Identification and Sensitivity (1998). Testing of *Mycobacterium tuberculosis* in india. www.kar.nic.in/cdc district level.
- Meaza A, Kebede A, Yaregal Z, Dagne Z, Moga S, Yenew B, Diriba G, Molalign H, Tadesse M, Adisse D, Getahun M (2017). Evaluation of genotype MTBDR plus VER 2.0 line probe assay for the detection of MDR-TB in smear-positive and negative sputum samples. *BMC Infectious Diseases* 17(1):280.
- Miotto P, Piana F, Penati V, Canducci F, Migliori GB, Cirillo DM (2006). Use of genotype MTBDR assay for molecular detection of rifampin and isoniazid resistance in *Mycobacterium tuberculosis* clinical strains isolated in Italy. *Journal of Clinical Microbiology* 44(7):2485-2491.
- Morcillo N, Zumarraga M, Alito A, Dolmann A, Schouls L, Cataldi A, Kremer K, Van Soolingen D (2002). A low-cost, home-made, reverse-line blot hybridization assay for rapid detection of rifampin resistance in *Mycobacterium tuberculosis*. *International Journal of Tuberculosis and Lung Disease* 6:959-965.
- Nathavitharana RR, Cudahy PGT, Schumacher SG, Steingart KR, Pai M, Denkinger CM (2017). Accuracy of line probe assays for the diagnosis of pulmonary and multidrug-resistant tuberculosis: A systematic review and meta-analysis. *European Respiratory Journal* 18:49-51.
- Omer ZB, Mekonnen Y, Worku A, Zewde A, Medhin G, Mohammed T, Pieper R, Ameni G (2016). Evaluation of the GenoTypeMTBDRplus assay for detection of rifampin and isoniazid-resistant *Mycobacterium tuberculosis* isolates in central Ethiopia. *International Journal of Mycobacteriology* 5(4):475-481.
- Protocol for Processing Specimens for Culture (1998). www.tbcare1.org/publications/toolbox/tools/dst/module_6_2_Annex_6.1.doc.
- Revised National TB Control Programme (2014). *Training Manual for Mycobacterium tuberculosis Culture & Drug susceptibility testing*. <https://tbcindia.gov.in>.
- Revised National Tuberculosis Control Programme Laboratory Network (2005) Guidelines for quality assurance of smear microscopy for diagnosing tuberculosis. TBC India. <http://www.tbcindia.nic.in/pdfs/RNTCP%20Lab%20Network%20Guidelines.pdf>.
- Saribas Z, Kocagoz T, Alp A, Gunalp A (2003). Rapid Detection of Rifampin Resistance in *Mycobacterium tuberculosis* Isolates by Heteroduplex Analysis and Determination of Rifampin Cross-Resistance in Rifampin-Resistant Isolates. *Journal of Clinical Microbiology* 41(2):816-818.
- Siddiqi N, Shamim M, Hussain S, Choudhary RK, Ahmed N, Prachee, Banerjee S, Savithri GR, Alam M, Pathak N, Amin A, Hanief M, Katoch VM, Sharma SK, Hasnain SE (2002). Molecular Characterization of Multidrug-Resistant Isolates of *Mycobacterium tuberculosis* from patients in North India. *Antimicrobial Agents and Chemotherapy* 46 (2):443-450.
- Sun Z, Chao Y, Zhang X, Zhang J, Li Y, Qiu Y, Liu Y, Nie L, Guo A, Li C (2008). Characterization of Extensively Drug-Resistant *Mycobacterium tuberculosis* Clinical Isolates in China. *Journal of Clinical Microbiology* 46(12):4075-4077.
- Taniguchi H, Aramaki H, Nikaido Y, Mizuguchi Y, Nakamura M, Koga T, Yoshida S (1996). Rifampicin resistance and mutations of the rpoB gene in *Mycobacterium tuberculosis*. *FEMS Microbiology Letters* 144:103-108.
- TB Statistics for India (National and State Statistics) www.tbfacts.org/tb-statistics-india.
- Telenti A, Honoré N, Bernasconi C, March J, Ortega A, Heym B, Takiff HE, Cole ST (1997). Genotypic assessment of isoniazid and rifampin resistance in *Mycobacterium tuberculosis*: A blind study at reference laboratory level. *Journal of Clinical Microbiology* 35(3):719-723.
- Telenti A, Imboden P, Marchesi F, Lowrie D, Cole S, Colston MJ, Matter L, Schopfer K, Bodmer T (1993). Detection of rifampicin-resistance mutations in *Mycobacterium tuberculosis*. *Lancet* 341:647-650.
- Tripathy S, Kumar R, Singh SD (2015). Prevalence of Multidrug Resistant Pulmonary Tuberculosis in North Bihar. *Journal of Clinical and Diagnostic Research* 9:09-12.
- World Health Organization (2008). Molecular line probe assays for rapid screening of patients at risk of multi-drug resistant tuberculosis (MDR-TB). Policy statement WHO, Geneva, Switzerland TBC. http://www.who.int/tb/features_archive/policy_statement.pdf.
- World Health Organization (2009). Guidelines for surveillance of drug resistance in tuberculosis (No. WHO/HTM/TB/2009.422). Geneva: World Health Organization.
- World Health Organization (2016). *Global Tuberculosis Report*, WHO, Geneva, Switzerland. <http://www.who.int/mediacentre/documents/s23098en/s23098en.pdf>
- Yadav RN, Singh BK, Sharma SK, Sharma R, Soneja M, Sreenivas V, Myneedu VP, Hanif M, Kumar A, Sachdeva KS, Paramasivan CN, Vollepore B, Thakur R, Raizada N, Arora SK, Sinha S (2013). Comparative Evaluation of GenoTypeMTBDRplus Line Probe Assay with Solid Culture Method in Early Diagnosis of Multidrug-Resistant Tuberculosis (MDR-TB) at a Tertiary Care Centre in India. *PLoS ONE* 8(9):72036.
- Yue J, Shi W, Xie J, Li Y, Zeng E, Wang H (2003). Mutations in the rpoB Gene of Multidrug-Resistant *Mycobacterium tuberculosis* isolates from China. *Journal of Clinical Microbiology* 41(5):2209-2212.

Full Length Research Paper

Antibiotic susceptibility and production of extended-spectrum beta-lactamase (ESBL) of *E. coli* strains isolated from meat

Omer A. S.¹, Salwa E.², Sanaa O. Y.^{1*} and Elzubeir I. E. M.³

¹Department of Microbiology and Molecular biology, Faculty of Science and Technology, El Neelain University, Sudan.

²Department of Biomedical Science, Faculty of Pharmacy, Omer AlMukhtar University, Libya.

³Department of Dairy production, Faculty of Animal production, Khartoum University, Sudan.

Received 13 March, 2020; Accepted 13 May, 2021

The zoonotic potential of *Escherichia coli* from chicken and beef food products is well documented. The uses of antibiotics on agriculture encourage the development of resistance bacteria capable of causing human disease and passing resistance to human pathogens. This study aimed to detect the antibiotic susceptibility and production of extended-spectrum beta-lactamase (ESBL) of *E. coli* strains isolated from meat. *E. coli* was isolated and identified according to standard techniques using traditional and chromogenic media and confirmed by biochemical reaction. Kirby-Bauer disk diffusion method was used to determine antimicrobial susceptibility towards twelve commonly used antibiotics. The resistance of the isolated *E. coli* towards the third generation of cephalosporins was detected using cefotaxime (30µg), ceftriaxone (30 µg) and ceftazidime (30 µg). ESBL producer *E. coli* was investigated using combination test. The results showed that 135 (75%) of the 180 meat samples revealed positive isolation of *E. coli*. 77.33% of the chicken meat samples showed positive isolation of *E. coli*, while 63.33% (19/30) of minced beef meat samples showed positive growth of *E. coli*. From these isolates, it was clear that most of them were highly resistant to tetracycline (10 µg), amoxiclav (30 µg) and cefalexin (30 µg). The lowest resistance was observed with ceftriaxone (30 µg) and ceftazidime (30 µg). The resistance of the isolated *E. coli* towards the third generation of cephalosporins was ranged between 5 to 33%. This study revealed that the isolated *E. coli* was ESBL producer as 85.71, 83.33, 70.83, 68.18 and 66.66% were detected in chickens leg, skin, wing, abdomen and chest respectively; while minced meat showed isolation of 15.78% of the ESBL producer *E. coli*. The study concluded that chicken and beef minced meat sold in Khartoum state have high hazardous risk for transmission of ESBLs producing *E. coli*; thus quality control application is highly needed. Policy actions should be implemented in order to prevent cross transmission of ESBLs producer *E. coli* to human.

Key words: *E. coli*, ESBL producer *E. coli*, susceptibility pattern, meat quality.

INTRODUCTION

Microbes in meat have a matter of great public health concern especially those causing food borne diseases

(Pepin et al., 1997), particularly poultry, can be a source of ExPEC strain transmission to humans (Jakobsen et al.,

*Corresponding author. E-mail: soyagoub08@hotmail.com.

Author(s) agree that this article remain permanently open access under the terms of the [Creative Commons Attribution License 4.0 International License](https://creativecommons.org/licenses/by/4.0/)

2010; Overdevest et al., 2011; Manges and Johnson, 2012). Meats are especially common source of *Escherichia coli* contamination, which may be acquired during slaughter through fecal contact (Cohen et al., 2007).

Extraintestinal pathogenic *E. coli* (ExPEC) is an important group of pathogenic *E. coli* causing a diversity of infection in both animals and humans including septicemia, meningitis and urinary tract infections (UTIs). Also they are a major cause of economic loss to the poultry industry (Mellata, 2013; Köhler and Dobrindt, 2011; Russo and Johnson, 2003). Antibiotic resistance is a worldwide health problem in many fields, such as environment, livestock, human, veterinary medicine, and agriculture (Marshall and Levy, 2011). Livestock animals are considered important reservoirs of antibiotic-resistant Gram-negative bacteria (Aiello and Larson, 2003; Seiffert et al., 2013); these bacteria automatically bring antibiotic-resistant from animals to humans via consumption of meat. Major bacterial infections in humans can be traced back to livestock (Jakobsen et al., 2010; Overdevest et al., 2011). The overuse of antibiotics in food animal production contributes to increasing rates of antibiotic resistance (Ventola, 2015).

ESBL producing *E. coli* strains have emerged as a potential health hazard from food producing animals (Costa et al., 2009; Smet et al., 2008), they confer resistance to penicillins, cephalosporins and aminopenicillins including the third-generation cephalosporins cefovecin and ceftiofur and the fourth-generation cephalosporin cefquinome, which are approved veterinary drugs (Ewers et al., 2012; Madec et al., 2017). ESBL-producing *E. coli* has also been detected in wild animals, emphasizing the wide distribution of these resistance determinants (Guenther et al., 2011). Many studies have demonstrated the presence of ESBL-producing *E. coli* in animals and meat, most likely caused by the use of the third-generation cephalosporin ceftiofur in food animals. Since the late 1990s, ESBL-producing *E. coli* have been detected in retail meat and production animals in Europe, Asia, Africa, and the United States (Jouini et al., 2007; Blanc et al., 2006). This study aimed to detect presence of *E. coli* in frozen packed raw chicken meat and red minced meats, determine the antibiotic susceptibility of the isolated *E. coli* and to detect the presence of the extended spectrum beta lactamase enzyme in the isolated *E. coli*.

MATERIALS AND METHODS

Collection of samples

A total of 180 samples were collected as 150 samples of poultry included thirty samples from each part (wing, leg, abdomen, skin and chest). The chickens were purchased randomly from different factories in the Khartoum state from July 2015 to March 2018. The chicken samples were divided with sterile knives and assessors and kept separately in sterile collection bags at 4°C. 30 minced beef

meat from different company were purchased from super markets. Each package of minced meat was opened with sterile knife and forceps, and then collected in sterile bags.

Isolation and identification of *Escherichia coli*

Five grams of each food parts sample were blended by stomacher blinder. The samples were enriched in 45 ml Brain Heart Infusion Broth (Micromaster, Maharashtra-India) and incubated aerobically at 35°C for 3 h, then pre-enrichment by transferred to 45 ml of tryptone phosphate (TP) broth and incubated at 44°C for 20 h. Each broth samples were inoculated on MacConkey agar medium (Himedia, Mumbai-India) and Eosin methylene blue (Levine) (Oxoid, Hampshire-England), then incubated aerobically for 18 h at 37°C; then confirmed by cultured on chromogenic agar (brilliance green *E. coli* coliform agar (Himedia, Mumbai-India) at 37°C for 18 h. A positive *E. coli* bacteria were observed as purple colonies, then confirmed using indole test, vogues proskauer test, methyl red test, citrate test, motility test, Oxidase test and sugar fermentation test according to Cowan and Steel (1999).

Antibiotic susceptibility test

The standard Kirby-Bauer disk diffusion method was used to determine antimicrobial susceptibility of *E. coli* isolates according to Clinical and Laboratory Standards Institute guidelines (Clinical Laboratory Standards Institute Manual, 2013). The following antibiotics discs (Himedia, India) were tested: ciprofloxacin (5µg), cefixime (5µg), ceftazidime (30µg), ceftriaxone (30µg), cefotaxime (30 µg), amoxiclav (10µg), cefalexin (30µg), tetracycline (10µg), gentamicin (10µg), chloramphenicol (30µg), amikacin (30µg), and co-trimexazole (30µg). 18 h broth cultures were prepared and equivalent to the 0.5 McFarland turbidity standards. The antibiotic discs were impressed on inoculated plates and incubated at 37°C for 24 h. Diameter of inhibition zones of *E. coli* isolates around each antimicrobial disc was measured in mm, then the results were reported as sensitive (S), intermediate (I), and resistant (R).

Screening of extended spectrum beta lactamase enzyme production

The discs of antibiotics containing cephalosporin alone (cefotaxime 30 ug, ceftazidime 30 ug, ceftriaxone 30 ug) and in combination with clavulanic acid were applied onto isolated *E. coli* inoculated plates, and then sufficient space between individual discs was ensured to allow proper measurement of inhibition zones. The plates were incubated at 37°C for 18 h. The inhibition zone around the cephalosporin discs combined with clavulanic acid was compared with the zone around the disc with the cephalosporin alone. The test is positive (ESBL producer) if the inhibition zone diameter is ≥ 5 mm larger with combined cephalosporin and clavulanic acid than cephalosporin alone.

RESULTS

Number and percentage of the isolation of *E. coli*

E. coli was identified according to the morphological culture characteristic and biochemical reactions as shown in Table 1 and Figure 1. *E. coli* was isolated from 135 (75.00%) out of 180 samples, while the negative isolation

Table 1. Identification of *E. coli*.

Test	Isolated <i>E. coli</i> reaction	Test	Isolated <i>E. coli</i> reaction
Gram stain	Gram negative	MR	Positive
Motility	Motile	VP	Negative
Oxidase production	Negative	Citrate	Negative
Catalase production	Positive	Growth on MacConkey agar	Pink colonies
Indole production	Positive	Growth on chromogenic media	Purple colonies

**Figure 1.** Purple colonies of *E.coli* chromogenic agar (brilliance green *E. coli*/ coliform agar).**Table 2.** Number and percentage of *Escherichia coli* isolated from meats.

Samples	Number of samples	No. (%) of positive isolation of <i>E. coli</i>	No. (%) of negative isolation of <i>E. coli</i>
Chicken Inside wing	30	24 (80.00)	6(20.00)
Chicken Chest	30	18 (60.00)	12(40)
Chicken Abdomen	30	22 (73.33)	8(26.66)
Chicken Leg	30	28 (93.33)	2(6.66)
Chicken Skin	30	24 (80.00)	6(20.00)
Red minced meat	30	19 (63.33)	11(36.66)
Total number	180	135 (75.00)	45 (25%)

of *E. coli* was observed in 45 (25%). The highest number of isolation of *Escherichia coli* was recorded generally in chicken meats part samples as 116 (64.44%), while red minced meats showed percentage of 63.33%. There were variations in the number of isolation of *E coli* among chicken meats. The highest isolates was observed in leg part samples as 28 (93.33%) out of 30 samples, followed by both inside wing and skin part which showed isolation of 24 (80.00%) out of 30 samples of each. The abdomen revealed 22 (73.33%) out of 30 samples, while chest showed 18 (60.00%) out of 30 samples. A positive isolates of *E. coli* from red minced meat were 19 (63.33%) out of 30 samples (Table 2).

Antimicrobial susceptibility of the isolated *E. coli*

The antibiotic resistance pattern of the isolated *E. coli*

from wing parts of frozen chicken meats was recorded as 79.2% resistance against tetracycline, 66.7% towards both of gentamicin and amoxiclav, while cefalexin and co-trimexazole were resistant with percentage of 45.8%. *E. coli* isolated from skin of the chicken showed highest percentage of resistance towards tetracycline and amoxiclav as 70.8% and Cefalexin as 54.2%. On the other hand, all isolated *E. coli* showed clear sensitivity towards ceftriaxone. Similarly *E. coli* isolated from chest part of the chickens showed the highest percentage of resistance for tetracycline (72.2%) followed by amoxiclav (55.6%), cefalexin (50%) while all isolates (100%) showed clear sensitivity towards both ceftriaxone and ceftazidime. The *E. coli* isolated from the abdomen of the chicken showed resistance towards cephalixin and tetracycline as 63.6% and cefixime as 54.5%. The isolated *E. coli* showed resistance to amoxiclav and gentamicin with percentage of 50% (Table 3). On the

Table 3. Percentage of the resistance of the isolated *E. coli* towards antibiotics.

Antibiotics (μ g)	Percentage of the resistance of isolated <i>E. coli</i>					
	Chicken					Cattle red minced Meat
	Wing	Skin	Leg	Chest	Abdomen	
Cefalexin (30)	45.8	54.2	50	50	63.6	31.6
Ceftriaxone (30)	4.2	0	7.1	0	4.5	10.5
Cotrimexazole (25)	45.8	29.2	35.7	38.9	36.4	15.8
Gentamicin (10)	25	45.8	46.4	16.3	50	10.5
Cloramphenicol (30)	41.7	37.5	39.3	38.9	36.4	10.5
Cetazidime (30)	8.3	8.3	3.6	0	4.5	5.3
Tetracycline (10)	79.2	70.8	57.1	72.2	63.6	26.3
Amikacin (30)	16.7	41.7	39.3	22.2	18.2	15.8
Ciprofloxacin (5)	29	29.2	32.1	22.2	31.8	0
Cefixime (5)	37.5	50	32.1	44.4	54.5	57.9
Cefotaxime (30)	33.3	12.5	17.9	22.2	18.2	5.3
Amoxiclav (30)	66.7	70.8	17.9	55.6	50	31.6

Table 4. Percentage of resistance of isolated *E. coli* towards third generation of cephalosporins.

Antibiotic (30 μ g)	Percentage of resistance of isolated <i>E. coli</i>					
	Chest	Abdomen	Skin	Leg	Wing	Cattle red minced meat
Ceftriaxone	0	5	0	7	4	11
Ceftazidime	0	5	8	4	8	5
Cefotaxime	22	18	13	18	33	5

other hand *E. coli* isolated from minced meat showed lower resistance towards tested antibiotics. The highest resistance was observed with cefixime as 57.9%, followed by cefalexin and amoxiclav as 31.6% for both. All isolated *E. coli* from minced meat were sensitive to ciprofloxacin.

Resistance of the isolated *E. coli* towards third generation of cephalosporins

The study revealed that the highest percentage of resistance to third generation cephalosporins were detected in chicken parts, especially wing parts as 33% to cefotaxime followed by ceftazidime (8%) and ceftriaxone (4%) compared with *E. coli* isolated from red minced meats which showed 11% resistance for ceftriaxone, 5% to both cefotaxime and ceftazidime. The highest percentages of resistance were detected in *E. coli* isolated from chicken parts towards cefotaxime which were distributed as follows: wing parts, 33%, chest parts, 22%; both abdomen and leg 18%; and skin 13%. All isolated *E. coli* from chest parts showed clear sensitivity towards ceftriaxone and ceftazidime. In skin parts isolates, no resistance was observed towards ceftriaxone.

Among all isolates of *E. coli* from red minced meats, the highest rate of resistance towards ceftriaxone was 11% (Table 4).

Detection of extended spectrum beta lactamase enzyme

The results revealed that out of 116 isolates of *E. coli* from chicken samples, 75.86% were positive as ESBL *E. coli* producer, while out of 19 isolates of *E. coli* from red minced meat samples only three of them showed production of ESBL enzyme (15.78%). Among the isolated *E. coli* from chicken meats parts, the leg part represented the highest percentage of isolation of ESBL *E. coli* producer (24%), followed by skin samples (20%), wing samples (17%); whereas abdomen samples represented 15% (Table 5).

DISCUSSION

In this study, 75% of the collected samples revealed positive growth of *E. coli*. The isolation of *E. coli* indicates low quality of food, fecal contamination and the presence

Table 5. Percentage of ESBL producer *E. coli* from chicken meats and cattle red minced meat.

Test	Percentage of positive isolation of ESBL <i>E. coli</i>					
	Cattle red minced meat	skin	abdomen	wing	leg	chest
Positive for combination test	3(15.78%)	20(83.33%)	15(68.18%)	17(70.83%)	24(85.71%)	12(66.66%)
Number of the positive <i>E. coli</i>	19	24	22	24	28	18

of high risk of transmission of enteric dangerous pathogens; otherwise some strain of *E. coli* is considered as pathogens that causes very serious disease. In fact, during and after slaughtering, the bacteria from the animal microbiota, the slaughter house environment, hands and equipment might contaminate carcasses. Some of these bacteria may grow and survive during storage, other pathogenic bacteria such as *Salmonella*, *Listeria*, *Campylobacter*, *Aeromonas*, *Staphylococcus* and toxin producing aerobic and anaerobic gram positive bacteria might be present. These confirm the findings of Authority (2016), Praveen et al. (2016), Höll et al. (2016), Line et al. (2013), and Veluz et al. (2012) who reported isolation of *E. coli* and pathogenic bacteria from poultry meat.

In this study, the isolated *E. coli* showed high resistance towards many commonly used antibiotics, these strongly pointed to the misuse of the antibiotic in animal production sector. Antimicrobials are used extensively in food animal production for disease prevention, treatment and growth promotion. Sarma et al. (1981) discovered that approximately 80% of isolated *E. coli* from healthy and diseased poultry was resistant to chlortetracycline, tetracycline, oxytetracycline and triple sulfas. Similarly, Paula Signolfi et al. (2019) found that more than 67% of isolated *Escherichia coli* were resistant to tetracycline, nalidixic acid and ampicillin. The inappropriate use of antibiotics, not only in human medicine but also in animal husbandry has been considered a main driver leading to the increase of multi-drug resistant bacteria (Chantizaras et al., 2014). The higher rates of antimicrobial resistance and multi-drugs resistance of the isolated *E. coli* in this study could be due to poor monitoring by regulatory bodies as the use of antibiotics in animal farms that used production of meat for human consumption, which have been prohibited in several countries. Furthermore, transmission via consumption of meat products has been suggested as a potential source of multidrug resistant bacteria in Africa (Alonoso et al., 2017; Eibach et al., 2018).

The increasing incidence of infections caused by extended-spectrum beta-lactamase (ESBL) *Escherichia coli* is of serious concern, as many studies from countries with a highly industrialized poultry industry suggested that meat products of poultry farms might be an important source for transmission of (ESBL) *Escherichia coli* to human (Linda et al., 2019; Poirel et al., 2018). Hawkey (2008), Kumarasamy et al. (2010) and Mathai et al.

(2002) found that 70-90% of *Enterobacteriaceae* are ESBL producers in India. Kar et al. (2015) conducted systematic study on multiple drugs resistant ESBL producing *E. coli* in food producing animals. Paula Signolfi et al. (2019) found that more than 31% of the isolated *Escherichia coli* were ESBL producer. Furthermore, ESBL producer *E. coli* was found to be more resistant to a higher number of antimicrobial substances compared to non ESBL producing *E. coli*. Alonso et al. (2017) reviewed lower prevalence of (ESBL) *Escherichia coli* among poultry meat products in African countries compared with European countries. However previous studies did not use any (ESBL) screening plates for the detection of *Escherichia coli* which might under estimate the ESBL production. Studies from the Netherland, Sweden and Vietnam detected (ESBL) *Escherichia coli* not only in chickens but also in high numbers among humans (Borjesson et al., 2016). These studies concluded that poultry farms or meat products might be an important source of (ESBL) *Escherichia coli*. Furthermore, ESBL strains of *Escherichia coli* were found to be 1.40 times more likely to contain more virulence genes than non ESBL – producing strain and it could be transmitted to human via food chain.

The percentage of the isolation of third generation cephalosporin resistant *E. coli* varied in this study according to meat source and parts in chicken, wing, chest, abdomen, legs and skin showed resistance of 33, 22, 18, 18 and 13% respectively towards cefotaxime (30 µg). These results indicate the highest use of cefotaxime in poultry farms and confirm the fact that the broiler farms are beginning to shift to more recently developed drugs, such as third generation cephalosporin as mentioned by Zekar et al. (2017). Third generation cephalosporin are used to treat urinary tract infections caused by Gram negative bacteria and have recently received research attention because of the rapid spread of multidrug-resistance. This resistance related to a novel gene called *fos A3*, which has been reported in *Escherichia coli* and *Klebsiella pneumonia* and often detected in *bla*_{CTX-M} producing and multi-resistant *Escherichia coli* both in animals and in clinical isolates (Ho et al., 2013). These findings raised the possibility that *Escherichia coli* present in the intestinal tract of healthy individuals could acquire those genes from *Escherichia coli* derived from chicken meat, which could act as reservoir for bacteria harboring resistance genes (Manges and Johnson, 2012). This study concluded that there is a high need for application

of the quality control measurements to ensure serving good and safe food as well as prevent transmission of food borne pathogen and control the rise of antimicrobial resistance microorganisms.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interest.

REFERENCES

- Aiello AE, Larson EI (2003). Antibacterial cleaning and hygiene products as emerging risk factor for antibiotic resistance in the community. *The Lancet Infectious Diseases* 3(8):501-506.
- Alonso CA, Zarazaga M, Ben Sallem R, Jouini A, Ben Sallem K, Torres C (2017). Antibiotic resistance in *Escherichia coli* in husbandry animals, the African perspective. *Letters in Applied Microbiology* 64(5):318-334.
- Authority EFS. (2016). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2015. *EFSA Journal* 14(12):e04634.
- Blanc V, Mesa R, Saco M, Lavilla S, Prats G, Miró E, Navarro F, Cortés P, Llagostera M (2006). ESBL- and plasmidic class C β -lactamase-producing *E. coli* strains isolated from poultry, pig and rabbit farms. *Veterinary Microbiology* 118(3-4):299-304.
- Borjesson S, Jernberg C, Brolund A, Edquist P, Finn M, Landen A (2016). Limited dissemination of extended-spectrum beta-lactamase and plasmid-encoded AmpC-producing *Escherichia coli* from food and farm animals Sweden. *Emerging Infectious Diseases* 22(4):634-640.
- Chantziaras I, Boyen F, Callens B, Dewulf J (2014). Correlation between veterinary antimicrobial use and antimicrobial resistance in food-producing animals: a report on seven countries. *Journal of Antimicrobial Chemotherapy* 69(3):827-834.
- Clinical and Laboratory Standards Institute (2013). Performance standards for antimicrobial susceptibility testing. 23rd informational supplement. CLSI Document M100-S23, CLSI, Wayne, Pa, USA.
- Cohen N, Ennaji H, Bouchrif B, Hassar M, Karib H (2007). Comparative study of microbiological quality of raw poultry meat at various seasons and different slaughtering process in Casablanca (Morocco). *Journal of Applied Poultry Research* 16(4):502-508.
- Costa D, Vinue L, Poeta P, Coelho AC, Matos M, Saenz Y, Somalo S, Zarazaga M, Rodrigues J, Torres C (2009). Prevalence of extended-spectrum beta-lactamase-producing *Escherichia coli* isolates in faecal samples of broilers. *Veterinary Microbiology* 38(3-4):339-344.
- Cowan I, Steel KJ (1999). *Manual for identification of medical bacteria*. Third edition. Cambridge University Press. United Kingdom.
- Eibach D, Dekker D, Gyau Boahen K, Wiafe akenten C, Sarpong N, Belmar campos C (2018). Extended-spectrum beta-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* in local and imported poultry meat in Ghana. *Veterinary Microbiology* 217:7-12.
- Ewers C, Bethe A, Semmler T, Guenther S, Wieler LH (2012). Extended-spectrum lactamase-producing and AmpC-producing *Escherichia coli* from livestock and companion animals, and their putative impact on public health: a global perspective. *Clinical Microbiology and Infection* 18(7):646-655.
- Guenther S, Ewers C, Wieler LH (2011). Extended-spectrum β -lactamases producing *E. coli* in wildlife, yet another form of environmental pollution? *Frontiers in Microbiology* 2:246-259
- Hawkey PM (2008). Prevalence and clonality of extended-spectrum beta-lactamases in Asia. *Clinical Microbiology and Infection* 14(1):159-165.
- Ho PL, Chan J, Lo WU, Law PY, Li Z, Lai EL (2013). Dissemination of plasmid-mediated fosfomycin resistance *fos A3* among multidrug-resistant *Escherichia coli* from livestock and other animals. *Journal of Applied Microbiology* 114(3):695-702.
- Höll L, Behr J, Vogel RF (2016). Identification and growth dynamics of meat spoilage microorganisms in modified atmosphere packaged poultry meat by MALDI-TOF MS. *Food Microbiology* 60:84-91.
- Jakobsen L, Spangholm DJ, Pedersen K, Jensen LB, Emborg HD, Agersø Y (2010). Broiler chickens, broiler chicken meat, pigs and pork as sources of ExPEC related virulence genes and resistance in *Escherichia coli* isolates from community-dwelling humans and UTI patients. *International Journal of Food Microbiology* 142(1-2):264-272.
- Jouini A, Vinue L, Slama KB, Saenz Y, Klibi N, Hammami S, Boudabous A, Torres C (2007). Characterization of CTX-M and SHV extended-spectrum β -lactamases and associated resistance genes in *Escherichia coli* strains of food samples in Tunisia. *Journal of Antimicrobial Chemotherapy* 60(5):1137-1141.
- Kar D, Bandyopadhyay S, Bhattacharyya D, Samanta I, Mahanti A, Nanda PK (2015). Molecular and phylogenetic characterization of multidrug resistant extended spectrum beta-lactamase producing *Escherichia coli* isolated from poultry and cattle in Odisha, India. *Infection, Genetics and Evolution* 29:82-90.
- Köhler CD, Dobrindt U (2011). What defines extraintestinal pathogenic *Escherichia coli*? *Journal of Medical Microbiology* 301(8):642-647.
- Kumarasamy KK, Toleman MA, Walsh TR, Bagaria J, Butt F, Balakrishnan R (2010). Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. *The Lancet Infectious Diseases* 10(9):597-602.
- Linda F, Can I, Kwabena O, Charity WA, Benedikt H, Ralf K, Sven P, Vinzent L, Oliver S, Nimako S, Ellis Owusu D, Jurgen M, Daniel E (2019). Detection and characterization of ESBL-producing *Escherichia coli* from human and poultry in Ghana. *Frontiers in Microbiology* 9:3358.
- Line JE, Oakley BB, Stern NJ (2013). Comparison of cumulative drip sampling with whole carcass rinses for estimation of *Campylobacter* species and quality indicator organisms associated with processed broiler chickens. *Poultry Science* 92(1):218-224.
- Madec JY, Haenni M, Nordmann P, Poirel L (2017). Extended spectrum β -lactamase/AmpC- and carbapenemase-producing *Enterobacteriaceae* in animals: a threat for humans? *Clinical Microbiology and Infection* 23(11):826-833.
- Manges AR, Johnson JR (2012). Food-borne origins of *Escherichia coli* causing extraintestinal infections. *Clinical Infectious Diseases* 55(5):712-719.
- Marshall BM, Levy SB (2011). Food animals and antimicrobials: impacts on human health. *Clinical Microbiology Reviews* 24(4):718-733.
- Mathai D, Rhomberg PR, Biedenbach DJ, Jones RN (2002). India Antimicrobial Resistance Study Group, 2002. Evaluation of the in vitro activity of six broad-spectrum β -lactam antimicrobial agents tested against recent clinical isolates from India: a survey of ten medical center laboratories. *Diagnostic microbiology And Infectious Disease* 44(4):367-377.
- Mellata M (2013). Human and avian extraintestinal pathogenic *Escherichia coli*: infections, zoonotic risks, and antibiotic resistance trends. *Foodborne Pathogens and Disease* 10(11):916-932.
- Overdeest I, Willemsen I, Rijnsburger M, Eustace A, Xu L, Hawkey P, Heck M, Savelkoul P, Vandenbroucke- Grauls C, Van der Zwaluw K, Huijsdens X, Kluytmans J (2011). Extended-spectrum β -lactamase genes of *Escherichia coli* in chicken meat and humans, the Netherlands. *Emerging Infectious Diseases* 17(7):1216-1222.
- Paula Signolfi C, Vanessa LK, Erick KN, Sebastien H, Charles MD, Kelly CT, Benito G, Gerson N, Renata Katsuko TK (2019). Distribution of ExPEC virulence factors, bla_{CTX-M}, fos A3 and mcr-1 in *Escherichia coli* isolated from commercialized chicken carcasses. *Frontiers in Microbiology* 9:3254. <https://doi.org/10.3389/fmicb.2018.03254>.
- Pepin M, Russo P, Pardon P (1997). Public health hazards from small ruminant meat products in Europe. *Revue scientifique et technique (International Office of Epizootics)* 16(2):415-425.
- Poirel L, Madec JY, Lupo A Schink AK, Kieffer N, Nordmann P (2018). Antimicrobial resistance in *Escherichia coli* Microbia spectrum 6:ARBA-0026-2017. doi: 10.1128/microbiolspec.ARBA-0026-2017.
- Praveen PK, Debnath C, Shekhar S, Dalai, Ganguly S (2016). Incidence of *Aeromonas spp.* infection in fish and chicken meat and its related public health hazards: A review. *Veterinary World* 9(1):6-11.
- Russo TA, Johnson JR (2003). Medical and economic impact of

- extraintestinal infections due to *Escherichia coli*: focus on an increasingly important endemic problem. *Microbes and infection* 5(5):449-456.
- Sarma DK, Sambyal DS and Baxi KK (1981). Drug susceptibility of *Escherichia coli* from domestic fowl. *Zentralbl Veterinarmed B* 28(4):333-335.
- Seiffert S, Hilty M, Perreten V, Endimiani A (2013). Extended-spectrum cephalosporin-resistant gram-negative organisms in livestock: An emerging problem for human health? *Drug Resistance Updates* 16(1-2):22-45.
- Smet A, Martel A, Persoons D, Dewulf J, Heyndrickx M, Catry B, Herman L, Haesebrouck F and Butaye P (2008). Diversity of extended-spectrum beta-lactamases and class C beta-lactamases among cloacal *Escherichia coli* isolates in Belgian broiler farms. *Antimicrobial Agents and Chemotherapy* 52(4):1238-1243.
- Veluz GA, Pitchiah S, Alvarado CZ (2012). Attachment of *Salmonella* serovars and *Listeria monocytogenes* to stainless steel and plastic conveyor belts. *Poultry science* 91(8):2004-2010.
- Ventola C (2015). The antibiotic resistance crisis. *US National Library of medicine National Institutes of Health* 40(4):277-228.
- Zekar FM, Granier SA, Marault M, Yaici L, Gassilloud B, Manceau C, Touati A, Millemann Y (2017). From Farms to Markets: Gram-Negative Bacteria Resistant to Third-Generation Cephalosporins in Fruits and Vegetables in a Region of North Africa. *Frontiers in Microbiology* 8:1569.

Full Length Research Paper

Assessment of the physicochemical qualities and microbiological profile of Idah River, Kogi State, Nigeria

Deborah Egunoluwa Adedire¹, Adijat Ozohu Jimoh¹, Zainab Kashim-Bello¹, Bilyaminu Adam Wase Shuaibu³, Oluwabukola Atinuke Popoola¹, Kabiru Isa Pate⁴, Onyinyechi Stainless Uzor¹, Eno Etingwa¹, Jumoke Florence Joda¹, Oyetola Olanrewaju Opaleye¹, Kehinde Rukayyat Adeniran², Ene Elizabeth Francis², Gbadebo Barnabas Olukotun⁵ and Oyekanmi Nashiru^{1,2*}

¹Genetics, Genomics and Bioinformatics Department, National Biotechnology Development Agency, Abuja, Nigeria.

²Center for Genomics Research and Innovation, National Biotechnology Development Agency, Abuja, Nigeria.

³Aquatic Bioresources Training Center, National Biotechnology Development Agency, Idah, Kogi, Nigeria.

⁴Aquatic Bioresources Training Center, National Biotechnology Development Agency, Tunari, Taraba, Nigeria.

⁵Bioentrepreneurship and Extension Services Department, National Biotechnology Development Agency, Abuja, Nigeria.

Received 11 May, 2021; Accepted 30 June, 2021

Analysis of water bodies, such as rivers and lakes can provide an insight into their intrinsic composition and potential impact on the environment. Water samples collected from four designated sites in Idah River, were assessed for their physicochemical parameters and microbial diversity using standard procedures. The temperature from all sites was 26.00°C and the pH ranged from 6.93 to 7.08. Turbidity values ranged between 32.00 and 38.00 NTU, while dissolved oxygen ranged between 6.28 and 9.28 ppm. Heavy metals, such as Selenium and Arsenic (with peak values of 0.10 and 0.09 ppm, respectively) were detected in the river. However, dissolved oxygen, arsenic and turbidity values across all sites exceeded the maximum limit set by World Health Organisation and the Standard Organisation of Nigeria. The total heterotrophic bacterial counts showed excessive bacterial load from all sample sites, while pathogenic bacteria, including *Escherichia coli*, *Klebsiella* and *Shigella* species, were isolated from regions with intense anthropogenic activities along the river, indicative of microbial pollution. Fungal studies identified the presence of *Fusarium*, *Aspergillus* and *Trichoderma* species as the most abundant in the river. Obtained results showed that Idah River is exposed to heavy metal seepage and subject to microbial contamination. Therefore, continuous monitoring and better management of the river body is recommended to prevent disease outbreak.

Key words: Aquatic ecosystems, Idah River, microbial diversity, physicochemical analysis.

INTRODUCTION

Water can be sourced from water bodies such as rivers, boreholes, lakes, springs and other large water bodies.

However, the quality of water bodies can be adversely affected by man-made activities. For example, pollution

*Corresponding authors. E-mail: oyekan.nash@gmail.com, ebun.fasesan@gmail.com.

Author(s) agree that this article remain permanently open access under the terms of the [Creative Commons Attribution License 4.0 International License](https://creativecommons.org/licenses/by/4.0/)

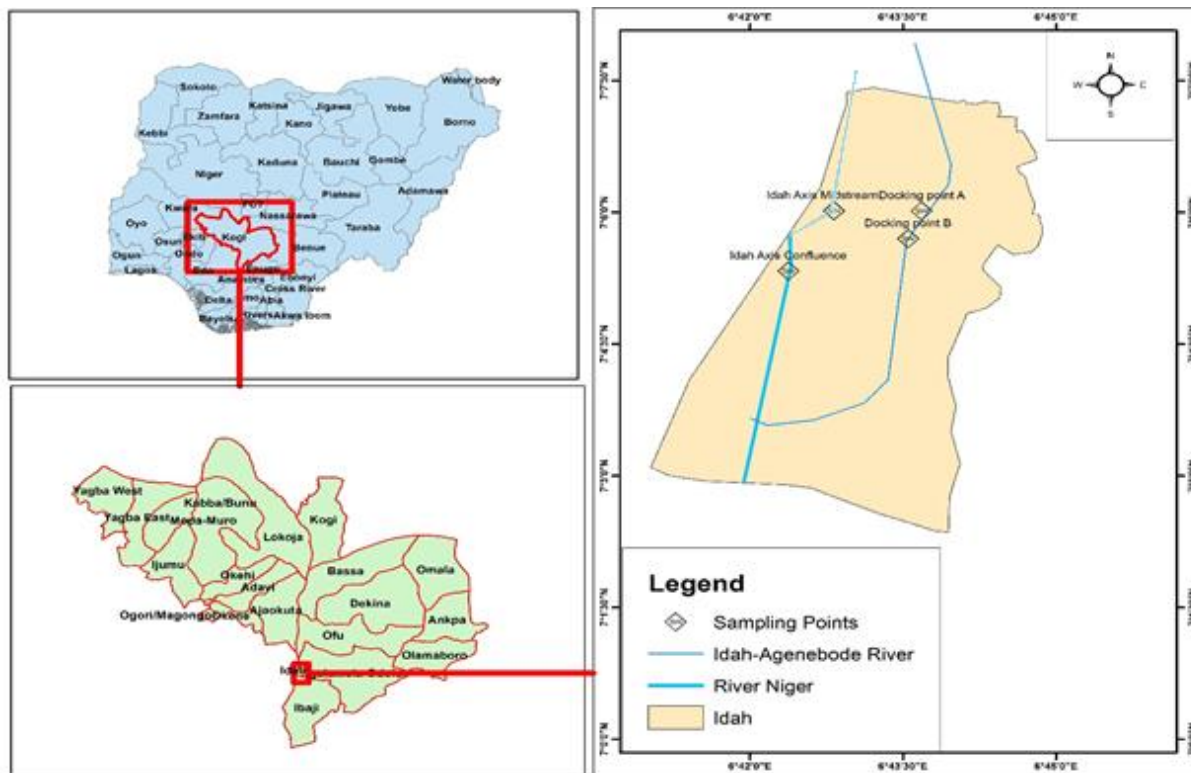


Figure 1. Map of the study area within Nigeria and its sampling points.

of freshwater bodies such as rivers (e.g. Idah River, Kogi State), streams, lakes and ponds are mostly experienced due to industrial discharge, municipal waste disposal and surface run-off (Akaniwor et al., 2007). Anthropogenic activities, such as discharges of domestic waste, untreated waste from sewage treatment plants, plastic materials, disposal of personal care products and household chemicals, improper disposal of car batteries, construction activities, mining activities, and pilgrim activities constantly deteriorate the water quality of rivers (Environmental Pollution Centers, 2018). Such deterioration in water bodies include an alteration in pH, increased turbidity, higher content of total dissolved solids and metals, as well as a higher risk of such water body hosting water-borne pathogens (Shaji et al., 2009; Ananthkrishnan et al., 2012). Water-borne pathogens pose a great health risk to humans, animals and plants, most especially, infants, young children under the age of five and immunocompromised individuals (EPA, 2016; WHO, 2014).

River Niger, at Idah, is an extension of the two major Nigerian rivers; River Niger- after which the country Nigeria is named, and River Benue, after their confluence in Lokoja. The Idah River is located between latitude $7^{\circ}6'1''N$ and longitude $6^{\circ}42'23''E$ (Figure 1) and it serves as a boundary between Kogi and Edo states. The temperature of the water ranges between 22 and $31^{\circ}C$

(which greatly depends on the season). It is relatively turbid and has a slightly alkaline pH between 7.5 and 7.8 (depending on the sampling location). The highest water levels are usually recorded between August and September, while the lowest water level is usually recorded between March and April (Adeyemi, 2010).

Although, microbial communities represent a fundamental part of aquatic ecosystems, and are of great importance for matter and energy flux (Kavka et al., 1996), little is known about the microbial biodiversity of River Niger (Idah Axis), despite its popularity in Nigeria and beyond. Local communities at the different axis of River Niger bank exploit the water for the fishery, aquatic medicinal plants, and domestic purposes, such as cooking, drinking, bathing, washing utensils and clothes, while the water is exposed to both human and animal waste, discharge of untreated industrial and domestic wastewater, runoff and dead organic plants and animals. Studying the unique diversity and functions of microbes in their ecological niche, as well as establishing the factors that affect them would aid in unraveling the role they play in animal, human, plant health, and in their environment (Marchesi, 2017).

This study is therefore aimed at evaluating the total microbial content of water at different points of River Niger at Idah axis, using a conventional approach. In addition, the study seeks to link the community present to

the physicochemical factors that characterize the river, in a bid to proffer necessary recommendations to avert infectious disease outbreaks.

MATERIALS AND METHODS

Study site

Idah is a town in Kogi State, in the middle belt region of Nigeria and situated on the east bank of Niger River. It has a land mass of 36 km² and located on latitude 7°06'48.42"N and longitude 6°44'19.18" E. Its human population was around 79,815 as at 2006 (National Population Commission, 2006). It has a tropical savannah climate with a wet season between late March and early November, and dry season between early November and late March. Idah River is exposed to water pollutants, such as chemical waste from farming activities, as well as discharge from gullies and streams, including the Inachalo River. The locals consume the water for domestic and dry farming activities. During rainy season, run-off washes domestic and municipal sewage, abattoir effluents and remnants of open defecation of both human and animals into the river.

Sample collection

Towards the end of the dry season in February 2020, a total of 20 water samples were aseptically collected in replicates from 4 selected sites in the river (designated Docking Point A, Docking Point B and the Idah Axis - Midstream and Confluence Area); all characterized by different levels of anthropogenic activities (Figure 1). Samples for bacteriological, mycological and physicochemical analyses were collected, at a depth of 20 cm below the water surface and against the water current into 250 mL sterile clean bottles (Ademorati, 1996). The temperature and water pH were monitored *in situ* using a mercury glass thermometer and a pH meter (ROHS Model), respectively before sampling (APHA, 2005). Collected samples were preserved in an icebox and transported immediately to the laboratory for microbiological and physicochemical analyses.

Physicochemical examination of water samples

Water samples placed on icepacks were transported to Soil Microbiology Laboratory at the International Institute of Tropical Agriculture, Ibadan, Oyo State, Nigeria within 24 to 48 h, for physicochemical analysis. In addition to pH and temperature (recorded *in situ*), a total of eighteen physicochemical parameters were evaluated. These included dissolved oxygen, specific ions (calcium, magnesium, potassium, sodium, iron, copper, nickel, arsenic, and selenium), turbidity, nitrates, sulphates, total dissolved solids, total nitrogen and electrical conductivity using titrimetric, colorimetric or spectrophotometric assays (Feng et al., 2009; Ologbosere et al., 2016; Adedire et al., 2021).

Isolation and identification of bacteria

Using aseptic techniques, four-fold serial dilution of each water sample was made, and isolation of bacteria from a serially diluted sample (1 mL) was done in Petri dish through the pour plate method on Nutrient Agar, prepared according to manufacturer's specification. The plates were incubated at 37°C for 24 h. The mean total viable count of the isolated bacterial colonies was enumerated and recorded. Colonies with distinct morphological differences were randomly picked from the nutrient agar plates and

repeatedly streaked on fresh, sterile agar plates (Nutrient Agar (Lifesave Biotech, USA), MacConkey Agar (Lifesave Biotech, USA), Eosin Methylene Blue Agar (Lifesave Biotech, USA), Salmonella-Shigella Agar (Lifesave Biotech, USA), and Centrimide Agar (Oxoid, UK) and subsequently incubated at 37°C for 24 h. Pure cultures were stored in the refrigerator at 4°C for further characterization and analysis. Pure cultures of isolates were characterized and identified using their macroscopic and microscopic characteristics, as well as biochemical tests (Catalase, Oxidase, Centrimide, Citrate Utilization, Methyl Red, Voges Proskauer, Urease, Gelatin Hydrolysis, Motility, Sugar Fermentation: Glucose, Lactose, Maltose, Sorbitol) as described by Cheesebrough (2000) and following the Bergey's Manual of Determinative Bacteriology (Genhardt et al., 1994).

Isolation and identification of fungi

Fungi were isolated through serial dilution (10⁻² and 10⁻⁴) method using sterile distilled water. One milliliter of each dilution was aseptically transferred into Potato Dextrose Agar (Lifesave Biotech, USA) media plates supplemented with Streptomycin (0.03 g/L) to inhibit bacterial growth (Hageskal et al., 2006). The plates were incubated at room temperature (25 ± 3.00°C) for 5 to 7 days. Using a flamed inoculating needle, the edge of each growing colony was picked and slides of the different colonies were made. A drop of Lactophenol cotton blue stain was added to the prepared slides, covered with a coverslip before microscopic examination using 10x and 40x magnifications to observe the microscopic features (hyphal characteristics, shape of sporangia, conidia and spores) of each isolate. Fungal colonies were also identified using cultural (macroscopic) characteristics (colony texture, elevation, chromogenesis/pigmentation, opacity and size) (Watanabe, 2002).

Statistical analysis

Data from physicochemical determinations and bacteriological plate counts were analyzed using Duncan's Multiple Range Test using SPSS (version 25.0). Mean occurrences of bacterial and fungal isolates were tested using Tukey Pairwise comparison of grouping at a 5% level of probability using SPSS (version 25.0).

RESULTS

Physicochemical characteristics of water samples from different sites of Idah River

The results of the physicochemical analyses of water samples collected from four different sites in Idah River are shown in Table 1. The observed water quality of the river was compared to acceptable standards reported by the World Health Organization (WHO, 2011) and Standard Organization of Nigeria (SON, 2007). The pH of water samples taken from different sampling points within Idah River was significantly different. However, the pH of Docking point A (DPA) and Idah Axis Confluence (IAC) water samples, as well as Docking point B (DPB) and Idah Axis Midstream (IAM) water samples, respectively were not significantly different. The pH ranges obtained also showed that IAM had the highest mean pH value of 7.08, which was quite similar to that obtained at Docking Point B (7.07). The lowest pH recorded was at the IAC

Table 1. Physicochemical quality of Idah River samples.

Parameter	Sample sites				Significance	WHO Limits (2011)	SON limits (2007)
	DPA	DPB	IAM	IAC			
pH	6.95 ^b	7.07 ^a	7.08 ^a	6.93 ^b	*	6.50 - 8.50	6.50 - 8.50
Temperature (°C)	26.00	26.00	26.00	26.00	NS	-	22.00 - 32.00
Ca (ppm)	5.98 ^c	6.93 ^a	7.01 ^a	6.37 ^b	*	200.00	-
Mg (ppm)	1.83 ^c	2.02 ^{bc}	2.30 ^a	2.12 ^{ab}	*	200.00	0.20
K (ppm)	2.96 ^{ba}	3.08 ^a	3.05 ^{ab}	2.89 ^c	*	30.00	-
Na (ppm)	3.98 ^b	4.24 ^a	4.12 ^{ab}	4.23 ^{ab}	*	200.00	200.00
Fe (ppm)	0.49 ^b	0.60 ^{ab}	0.56 ^b	0.78 ^a	*	0.50 - 50.00	0.30
Cu (ppm)	0.34 ^b	0.34 ^b	0.36 ^a	0.34 ^b	*	2.00	1.00
Ni (ppm)	0.01	0.01	0.01	0.01	NS	0.07	0.02
As (ppm)	0.04 ^b	0.04 ^b	0.09 ^a	0.04 ^b	*	0.01	0.01
Se (ppm)	0.05 ^b	0.06 ^b	0.10 ^a	0.05 ^b	*	0.04	-
Turbidity (NTU)	33.10	33.10	32.43	37.67	NS	5.00	5.00
NO ₃ (ppm)	3.73 ^a	3.20 ^b	1.22 ^c	1.19 ^c	*	<50.00	50.00
DO (ppm)	9.28 ^a	9.28 ^a	6.67 ^b	6.28 ^b	*	<5.00	3.00 - 5.00
SO ₄ (ppm)	1.89 ^a	1.84 ^a	0.60 ^c	0.84 ^b	*	250.00	100.00
TDS (ppm)	0.01 ^a	0.00 ^b	0.00 ^b	0.00 ^b	*	600.00	500.00
Total N (%)	0.00	0.00	0.00	0.00	NS	-	-
Electrical conductivity (S/m)	70.67 ^c	78.00 ^b	80.00 ^a	80.00 ^a	*	<1000.00	1000.00

DPA - Docking Point A, DPB – Docking Point B, IAM – Idah Axis Midstream, IAC – Idah Axis Confluence, Ca – Calcium, Mg – Magnesium, K – Potassium, Na – Sodium, Fe – Iron, Cu – Copper, Ni – Nickel, As – Arsenic, Se – Selenium, NO₃ – Nitrates, DO – Dissolved Oxygen, SO₄ – Sulphates, TDS – Total Dissolved Solids. Mean values with similar letter(s) across rows are not significantly different at 5% level of significance by Duncan's Multiple Range Test (DMRT). NS: Not significant; *: Significant.

site (6.93) ($p < 0.05$).

The recorded temperature was uniform across all sampled sites at 26.00°C. The mean values of calcium (7.01 ppm), magnesium (2.30 ppm), copper (0.36 ppm), arsenic (0.09 ppm) and selenium (0.10 ppm) were all found to be the highest in IAM. DPA had the lowest mean values for calcium (5.98 ppm), magnesium (1.83 ppm), sodium (3.98 ppm) and iron (0.49 ppm). The mean values of nickel, at 0.01 ppm, were uniform across all sampled sites. The highest potassium (3.08 ppm) and sodium (4.24 ppm) were recorded at DPB, and they were significantly higher than values recorded from other locations. Copper had a uniform value (0.34 ppm) at three sites: DPA, DPB and IAC areas. Turbidity mean values ranged from 33.10 NTU (at DPA and DPB) to 37.67 NTU (at IAC) and the values were not significantly different from one another. Nitrates, dissolved oxygen and sulphates were all found to be the highest in DPA with mean values of 3.73, 9.28 and 1.89 ppm, respectively.

Bacteria distribution and frequency

The total heterotrophic bacteria counts (THBC) of bacteria are seen in the four sites shown in Table 1. The highest microbial load was recorded at both DPA and

DPB with mean values of 1.55×10^4 and 2.52×10^4 CFU/mL, respectively. The lowest microbial load was recorded at IAM with a mean population of 1.27×10^2 CFU/mL. Ten genera of bacteria were identified from a total of 97 isolates. These genera included *Acinetobacter* species, *Staphylococcus* species, *Bacillus* species, *Pseudomonas* species, *Escherichia coli*, *Klebsiella* species, *Bacillus subtilis*, *Aeromonas hydrophilia*, *Shigella* species and *Streptococci* species. Out of these ten species, *Acinetobacter* spp. had the highest percentage frequency of occurrence (50.52%), followed by *Staphylococcus* (18.56%) and *Bacillus* spp. (10.31%) (Figure 2). Across all sampled sites, *Acinetobacter*, *Staphylococcus* and *Bacillus* spp. were the most predominant. The least dominant microbes from each sample site were *B. subtilis* (in DPA and IAM), *A. hydrophilia* (in DPB and IAM), *Shigella* spp. (DPB and IAC) and lastly, *Streptococcus* spp. (in IAM only). Cumulatively, *Acinetobacter* spp. had a mean microbial prevalence of 12.25 and was the most predominant bacteria ($P < 0.05$), while *Streptococcus* spp. was the least with a cumulative mean value of 0.25. Also, the two docking points A and B had the highest number of bacterial isolates with 30.00 and 39.00, respectively, while the least number of bacterial isolate (10) was observed at the Idah midstream area (Table 2). Concerning the total viable THBC observed in water

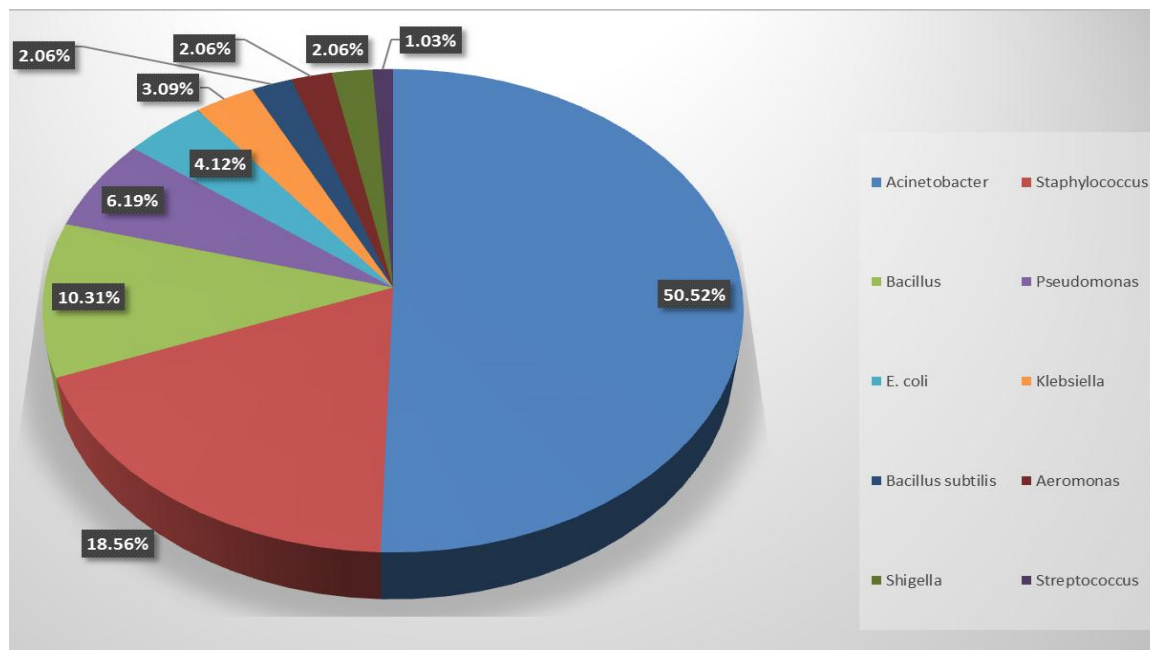


Figure 2. Percentage frequency of total bacteria isolates found in sites of study at Idah River.

Table 2. Number and types of bacteria species isolated from different sites in Idah River.

Genus or species	Docking point A	Docking point B	Idah Axis Midstream	Idah axis confluence	Mean values (Tukey's test)
<i>Acinetobacter</i> spp.	12.00	24.00	5.00	8.00	12.25 ^a
<i>Staphylococcus aureus</i>	5.00	9.00	2.00	2.00	4.50 ^{ab}
<i>Bacillus</i> spp.	4.00	2.00	0.00	4.00	2.50 ^b
<i>Pseudomonas aeruginosa</i>	5.00	0.00	0.00	1.00	1.50 ^b
<i>Escherichia coli</i>	2.00	1.00	0.00	1.00	1.00 ^b
<i>Klebsiella</i> sp.	1.00	1.00	0.00	1.00	0.75 ^b
<i>Bacillus subtilis</i>	1.00	0.00	1.00	0.00	0.50 ^b
<i>Aeromonas hydrophilia</i>	0.00	1.00	1.00	0.00	0.50 ^b
<i>Shigella</i> spp.	0.00	1.00	0.00	1.00	0.50 ^b
<i>Streptococcus</i> spp.	0.00	0.00	1.00	0.00	0.25 ^b
THBC (CFU/mL)	1.55 × 10 ⁴	2.52 × 10 ⁴	1.27 × 10 ²	4.50 × 10 ³	11.33 × 10 ^{4ab}
Total	30.00	39.00	10.00	18.00	-

Means values (across the row) with same letters are not significantly different ($P < 0.05$) using Tukey's test at 95% confidence interval. THBC: Total Heterotrophic Bacteria Count.

samples collected from the four sample sites, bacteria colonies ranged from 1.27×10^2 CFU/mL (IAM) to 2.52×10^4 CFU/mL (DPB).

Fungi distribution and frequency

Seven fungal genera were identified from a total of 23 fungal isolates. These included *Aspergillus flavus*, *Aspergillus niger*, *Penicillium* species, *Fusarium* species,

Cladosporium species, *Trichoderma* species and *Curvularia* species. Table 3 shows their distinct morphological differences and microscopic characteristics with 2 of the fungi species belonging to *Aspergillus* genus. *Fusarium* spp. had the highest percentage occurrence (39.13%), followed by *Trichoderma* spp., *A. flavus* and *Penicillium* spp. (all at 13.04%) (Figure 3). *Fusarium* spp. had the highest microbial incidence across all sites with a mean value of 2.25 ($P < 0.05$), and the least occurring isolate being *Curvularia* spp. with a cumulative

Table 3. Fungi isolates and their probable identification.

Isolate	Morphological observations						Microscopic characteristics	Probable fungal identity
	Form	Elevation	Surface	Opacity	Chromogenesis	Size		
1	Irregular	Flat	Glistening	Opaque	White edge, yellowish green in the center	Large	Septate hyphae with long and erect conidiophores	<i>Aspergillus flavus</i>
2	Irregular	Raised	Rough	Opaque	Initially white, then changes to dark brown/black	Covers entire surface	Long smooth conidiophores with very rough conidia	<i>Aspergillus niger</i>
3	Irregular	Flat	Rough	Opaque	Green and black edge with a velvety look	Large	Broom-like conidiophores with septate hyphae	<i>Penicillium</i> spp.
4	Circular	Flat	Smooth	Opaque	White cottony look	Medium	Hyaline and abundant microconidia with one or two septa, short conidiophores	<i>Fusarium</i> spp.
5	Irregular	Umbrate	Smooth	Opaque	Suede-like and black	Medium	Erect conidiophores, conidia in branched chains	<i>Cladosporium</i> spp.
6	Circular	Raised	Rough	Translucent	Yellowish green and white in the center	Medium	Repeatedly branched conidiophores with clusters of bent phialides	<i>Trichoderma</i> spp.
7	Irregular	Flat	Rough	Opaque	Black, spiky and fluffy	Medium	Curved conidia	<i>Curvularia</i> spp.

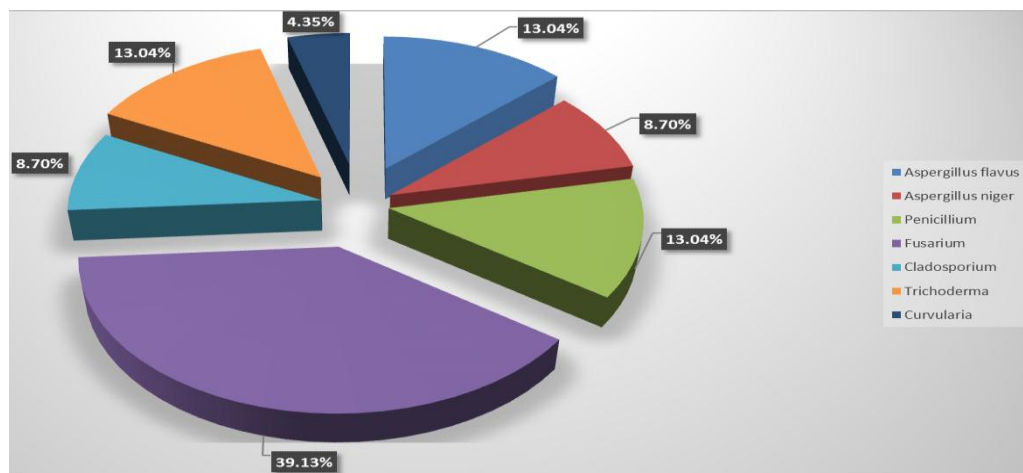


Figure 3. Percentage frequency of total fungi isolates found in sites of study at Idah River.

mean of 0.25. Also, DPA had the highest number of fungi isolates (9.00), followed by DPB (7.00) while the IAM area had the lowest number of fungi isolates 2 (Table 4).

DISCUSSION

From the analysis of the physicochemical features of water samples, recorded pH values were within

a narrow range of 6.93 to 7.08. This fell within the acceptable range according to the standards set by WHO (2011) and SON (2007), thus making it suitable for aquatic life. This observation was in

Table 4. Number and types of fungi species isolated from different sites in Idah River.

Genus or Species	Docking point A	Docking point B	Idah Axis Midstream	Idah Axis Confluence	Mean values (Tukey's test)
<i>Aspergillus flavus</i>	2.00	0.00	1.00	0.00	0.75 ^{ab}
<i>Aspergillus niger</i>	1.00	0.00	0.00	1.00	0.50 ^{ab}
<i>Penicillium</i> spp.	1.00	2.00	0.00	0.00	0.75 ^{ab}
<i>Fusarium</i> spp.	2.00	2.00	1.00	4.00	2.25 ^a
<i>Cladosporium</i> spp.	1.00	1.00	0.00	0.00	0.50 ^{ab}
<i>Trichoderma</i> spp.	2.00	1.00	0.00	0.00	0.75 ^{ab}
<i>Curvularia</i> spp.	0.00	1.00	0.00	0.00	0.25 ^b
Total	9.00	7.00	2.00	5.00	-

Mean values (across the row) with same letters are not significantly different ($P < 0.05$) using Tukey's test at 95% confidence interval

agreement with the findings of Adesakin et al. (2020) who reported a similar pH range for domestic water sources in Zaria, Nigeria. The pH values observed in this study could be attributed to the major soil type in the area or the buildup of organic materials from runoff, as described by Taiwo et al. (2020).

The pH in water bodies is among the major physiological factors that play a critical role in shaping microbial structures and other biological activities in water (Adesakin et al., 2020). There appears to be a correlation between the microbial population observed across all sample sites and the pH of Idah River. All isolated microbes were neutrophilic in nature and this may be related to the near-neutral pH of the river body, encouraging the proliferation and prevalence of neutrophils.

The temperature of any water body determines the proliferation and survival rate of microorganisms (Bouزيد, 2016). In this study, the temperature value of 26.00°C in water samples could be regarded as optimal for the growth of heterotrophic mesophilic bacteria and fungi. However, this was within the standard permissible temperature limit of SON (2007) set for aquatic water life. Igbinsosa et al. (2012) also observed a similar temperature range of 26 to 27.2°C in Shanomi creek, in the Niger Delta region. In addition, Nwoko et al. (2015) in their report on the assessment of seasonal physicochemical parameters of Oguta Lake in Nigeria, observed a similar temperature range.

The calcium (Ca) levels in water samples collected from different points along the river, was significantly different from each other and ranged from 5.98 ppm (DPA) to 7.01 ppm (IAM); however, these levels were below the permissible limit (200.00 mg/L) recommended by WHO (2011). As such, there might be no detrimental effect on aquatic life and on humans who ingest foods harvested from the river. Magnesium is generally associated with calcium in water bodies and with a concentration usually lower than that of calcium (Venkatasubramani and Meenambal, 2007). Magnesium values observed in this study support this statement as

they ranged from 1.83 ppm (DPA) to 2.30 ppm (IAM).

The potassium and sodium values ranged from 2.89 ppm (IAC) to 3.08 ppm (DPB) and 3.96 ppm (DPA) to 4.23 ppm (IAC), respectively. Many water bodies have sodium concentrations well below 50.00 mg/L (Ikhuorhah et al., 2016). Copper levels ranged narrowly from 0.34 to 0.36 ppm and they were notably lower than what Kuz'mina and Ushakova (2007) reported in their metal assessment of fishponds (10.6 ppm). The heavy metal, Nickel is known to be associated with gastrointestinal irritation without inherent toxicity (Hammer and Hammer, 2003). In this study, Nickel value was recorded as 0.01 ppm across the four different samples, which was within the acceptable limit in WHO (2011) recommendations, hence, the river appeared devoid of the heavy metal. These values were contrary to the much higher values reported by Okereke (2014), who assessed the physicochemical properties of the Ihuku River (0.07 - 0.082 mg/L).

Arsenic is a metalloid widely distributed in the earth and usually found in natural groundwater (Thi et al., 2009) but its concentrations vary based on the geological formations, weathering processes of rocks, microbial activities, leaching or other anthropogenic activities such as mining and application of pesticides (Katsoyiannis et al., 2004; Oremland and Stolz, 2003). The metal has no known biological function and is extremely toxic in high concentrations. The arsenic mean values in this study were slightly above the WHO permissible limit, with the highest being 0.09 ppm in the IAM area. This can pose certain risks to consumers of water from the river, as this metal has been characterized as a carcinogen. Regular consumers of contaminated water and contaminated aquatic foods may be at risk of arsenic buildup in the body. Selenium is an essential micronutrient found in groundwater as well but toxic at elevated levels. Its concentration levels usually rise when there is an occurrence of industrial emissions or mining activities (Fernandez-Martinez and Charlet, 2009). Selenium recorded for the different water samples were relatively close to recommended WHO limit (0.05 mg/L) except for

the IAM sample, which showed a high value of 0.10 ppm.

Turbidity is the degree of clarity or cloudiness of water and it is evaluated by the presence of suspended solids in the water (Health Canada, 2012). The mean turbidity values obtained from all water samples ranged from 32.43 to 37.67 NTU. This raises another concern, as the values obtained exceeded recommended limits by WHO and SON, thereby rendering it unfit for drinking. Suspended solids in water promote the growth of microorganisms and as such, high turbidity is often correlated with a high presence of disease-causing microorganisms (Shittu et al., 2008). The high turbidity values obtained are clear indicators of Idah River pollution. The concentrations of the nutrients (nitrates and sulphates) were within the acceptable limits for both WHO and SON standards. Therefore, they did not pose a serious water quality issue.

Dissolved oxygen (DO) is a measure of oxygen levels dissolved in an aqueous solution, which plays a major role in the biological activities of cultured organisms (Murphy, 2005). It is an important criterion for assessing quality, as it provides details on pollution levels, metabolic activities of microorganisms and nutrient availability (Premlata, 2009). The DO concentration range for the four sites: DPA (9.28 ppm), DPB (9.28 ppm), IAM (6.67 ppm) and IAC (6.27 ppm) were all higher than the statutory permissible limit (5 mg/L) necessary for aquatic life. Although, slightly above the permitted threshold, there have been higher concentrations of DO reportedly used for agricultural purposes in brackish aquaculture (Ayedun et al., 2011). Also, microorganisms use DO for the decomposition of organic materials at the bottom of water bodies. Low DO may lead to an anaerobic environment, resulting in a bad odour of water (Adekunle et al., 2007).

Electrical conductivity is defined as a measure of the degree of ions in an aqueous solution or the capacity of water to pass electrical flow. The source of these conductive ions could be from dissolved salts and inorganic materials such as chlorides, carbonate compounds or sulfides (Miller et al., 1988). The range of electrical conductivity values recorded from all sample sites was within the acceptable limit reported by WHO (2011). This may imply that the river receives low amounts of dissolved salts and inorganic substances (Kidu et al., 2015).

Results also showed that lesser concentrations of calcium, magnesium, potassium, nitrates and sulphates were recorded in IAM and IAC sample sites. This might be connected to the continuous movement of water, thereby diluting the concentrations of organic and inorganic wastes that might have been introduced at the docking sites of the river.

The total heterotrophic bacteria counts of bacteria exceeded the WHO's stipulated standards for water bodies. Notably, the high population of bacteria seen at the two docking points could be due to the fishing and other observed anthropogenic activities occurring around

the Idah riverbank. The high abundance of bacteria isolated from these two areas could also be linked to the bathing activities, washing and sewage run-offs, which was noticed at the riverbank, and is most likely to transmit an array of infectious diseases (Anyanwu and Okoli, 2012).

In the IAM area, the highest arsenic and selenium values were recorded, which were above the WHO limit. This might be indicative of toxicity, and could be the reason for the low heterotrophic bacterial plate count and the low number of bacteria and fungi isolated (Leon et al., 2018).

The bacteriological analysis showed that Gram-negative bacteria were predominant amongst the bacterial isolates from Idah River. The microorganisms isolated in order of prevalence were *Acinetobacter* spp., *Staphylococcus aureus*, *Bacillus* spp., *Pseudomonas aeruginosa*, *E. coli*, *Klebsiella* spp., *B. subtilis*, *Aeromonas hydrophilia*, *Shigella* spp. and *Streptococcus* spp. The bacterial diversity reported in this study had some similarities to previous reports by Anyanwu and Okoli (2012) who reported the presence of *Enterobacter* species, *Alcaligenes* species, *E. coli*, *Proteus* species, *Klebsiella* spp., *P. aeruginosa*, *Acinetobacter* spp., *S. aureus* and *Bacillus* spp. in different water supplies at Nsukka, Nigeria. Also, Umeh et al. (2020) reported the prevalence of *Klebsiella pneumoniae*, *Acinetobacter calcoaceticus*, *E. coli*, *S. aureus*, *Vibrio* species, *Pseudomonas* spp., *B. subtilis*, *Shigella flexneri*, *Salmonella* Typhi in selected fish ponds in Anambra State, Nigeria.

Based on the frequency rate of isolated bacteria, *Acinetobacter* spp. was found to be the most predominant across the four sites, constituting 50.52% of the total bacterial community. The high prevalence of this species is most likely linked to the observed discharge of abattoir effluents into the Idah River, which was also reported in the study of Tsai et al. (2018). This is also in agreement with the report of Okechi et al. (2020) who found that *Acinetobacter* was the most predominant bacteria in the sediment area of Otamiri River, constituting about 42.10%. In Northeastern China, Zhao et al. (2014) found that the genus *Acinetobacter* was also the most prominent group of isolates in zinc and arsenic polluted rivers. Species of the genera *Acinetobacter* are ubiquitous in a wide range of ecological areas like water, soil, sludge, and wastewater (Hamouda et al., 2011). However, some scientific reports argued that the *Acinetobacter* genus is a nosocomial pathogen and the possibility of it thriving in natural environments is low (Peleg et al., 2008). It would also seem that they are resilient, as documented by few reports, which showed that they can survive in various environments containing low amounts of nutritious components, as well as their resistance to adverse environmental conditions (Gospodarek and Ziolkowski, 2000). Strains of this organism are commonly known to harbor antibiotic resistance genes and they are regarded as emerging opportunistic pathogens of fish farmed in Poland

(Kozłńska et al., 2014). There might be a possibility of this occurring in Idah River, if precautionary measures are not taken.

The isolated Gram-positive bacteria, *S. aureus*, were also widespread across the river with a total percentage of 18.56%. The daily routine of washing, sand packing and fishing activities observed at the riverbank might account for its abundance at the docking sites. At the midstream and confluence areas of the river, its prevalence was relatively low. *S. aureus* is known to be commensal in the mucosa of mammals and birds. However, they can also be opportunistic pathogens (Quinn et al., 2004). The risk of *S. aureus* infections is premised on the possibility of its resistance to beta-lactamase antibiotics, including penicillin and thus, its presence in water calls for public health concern. *Streptococcus* spp. was found only in the midstream area of the river. This organism has been associated with illnesses such as pneumonia and upper respiratory tract infections. *Bacillus* spp. were identified across all sample sites. *Bacillus* spp. can occupy a wide range of ecological niches and its spores are quite ubiquitous (Nicholson, 2004). In particular, *B. subtilis* found in the docking and midstream area could have the potential to be used as probiotic additives in aquaculture, as reported by Guo et al. (2016).

The main indicator of faecal pollution, *E. coli*, was found in all sites, except for the midstream area of the water. These bacterial isolates were discovered from both docking areas, which is suggestive of sewage pollution and abattoir effluents observed at the Idah riverbank. Contamination of the river by other members of the Enterobacteriaceae family seen in this study – *Shigella* spp., *A. hydrophilia*, *P. aeruginosa* and *Klebsiella* spp. could be related to a combination of direct faecal contamination and agricultural run-off at the docking sites. These Gram-negative bacteria are typically responsible for some waterborne diseases, including Shigellosis, typhoid, dysentery, diarrhea, as well as urinary tract infections, and they have been implicated in high mortality rate across the world (WHO, 2011). *Pseudomonas* spp. has been isolated from fishes in contaminated rivers. When consumed raw or insufficiently processed, such fishes could serve as a vector for the transmission of pathogens to humans (Jeyasekaran et al., 2006). Generally, the prevalence of Gram-negative bacteria in this river could be as a result of faecal contamination and other human interference, which could result in the proliferation of pathogenic organisms in fish, affecting human consumers (Kay et al., 2008). Their presence is most likely linked to the washing activities in the area, as well as the agricultural/seepage runoffs entering the river (Abulreesh, 2012). This implies a significant health risk for humans consuming this water (Franciska et al., 2005).

The mycological analysis in this study showed the presence of *A. niger*, *A. flavus*, *Penicillium* spp., *Fusarium*

spp., *Cladosporium* spp., *Trichoderma* spp. and *Curvularia* spp. in Idah River. This agrees with the observations of Ili et al. (2019), who reported *Candida* species, *Fusarium* spp., *A. flavus*, *Penicillium* spp., *A. niger* and *Mucor* species in Okokpon River, Edo State. Furthermore, Agbabiaka and Oyeyiola (2012) documented the presence of *Curvularia*, *Aspergillus*, *Penicillium*, *Saccharomyces*, *Cladosporium*, *Geotrichum*, *Trichoderma*, *Mucor*, *Rhizopus*, *Fusarium* and *Mortierella* (fungi) as sediment contaminants of Foma River, Ita-Nmo, Ilorin.

The abundance of fungi, in the docking areas of Idah River, is likely linked to its high sulphate concentration, thus constituting an indicator of water pollution from anthropogenic and agricultural origins. Pietryczuk et al. (2018) reported a similar observation in their study of fungi diversity in selected rivers. Fungal population increases with pollution and this might be connected to the high number of fungal contaminants observed at the docking areas of the river.

Amongst the various fungi species isolated in this river samples, *Fusarium* spp. was the most predominant, constituting about 39.13%. *Fusarium* spp. are plant pathogens or rhizosphere fungi, and their presence in Idah River could be attributed to agricultural activities occurring around this river. This is quite similar to the observation of Sharma and Tiwari (2015) who reported *Fusarium* spp. as one of the most abundant soil fungi isolated from the Shivnath River and it is known to cause several superficial infections. *A. niger* and *A. flavus* found in this river are known to be among the main agents of food spoilage but it is co-related to a range of infections such as Aspergillosis, leading to respiratory infections. Refai et al. (2010) also confirmed *Aspergillus* as pathogenic fungi of freshwater fishes. *Trichoderma* spp. was found only in DPA and DPB, which had the most observed anthropogenic activities. It is one of the beneficial fungi in the environment that can serve as a bio-fungicide against various fungal pathogens (Schuster and Schmoll, 2010). It was suggested that it might have the potential to also control infectious diseases in aquaculture (Citarasu et al., 2012). *Penicillium* spp. was prevalent only in the two docking points, and the genus was reported to produce mycotoxins (Pohland and Wood, 1997). Contamination of fish with these mycotoxins could accumulate in their tissues, which could invariably affect humans when consumed. The processing of fish does not necessarily eliminate the presence of mycotoxins in their tissues. *Cladosporium* and *Curvularia* spp. isolated from the docking points in the river are predominantly saprophytic in nature, and they are known to colonize and parasitize aquatic plants.

Conclusion

In this study, physicochemical and microbial

(bacteriological and mycological) profiling of Idah River were assessed from four designated sampling points. Although, thirteen physicochemical parameters were within the safe limits as indicated by the WHO (2011) and SON (2007) standards, parameters indicative of pollution were obtained. They included high total heterotrophic bacterial counts, presence of pathogenic microbes and toxic levels of certain physicochemical factors, such as arsenic, selenium, turbidity and nickel, when compared with standards. Such extreme values derived from physicochemical analysis might be connected to anthropogenic activities of locals of the community, resulting in inorganic pollution. In addition, the various fungi and bacterial species found in the river raises health concerns, with regards to direct consumption of water and ingestion of food (such as fishes and aquatic plants) sourced from the water body. It is therefore recommended that programs and policies, such as continuous monitoring and public health awareness programs are set in place to enlighten locals of the community, about the dangers of unsuitable discharge of animal, human and inorganic wastes into the river. This could in turn, prevent the river from being an environmental reservoir of antibiotic-resistant pathogens, hence preventing public disease outbreaks.

This study provides the first report about physicochemical properties and microbial diversity (using culture-based techniques) inherent in the Idah River. However, additional microbial analysis, such as metagenomic studies, could be used to reveal a deeper microbial structure inherent in the river, which culture-dependent methodology might not have captured.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

We would like to thank Mrs. Roseline Ideh of Bio entrepreneurship and Extension Services Department, National Biotechnology Development Agency (NABDA), Abuja, for her technical support. We also appreciate Ms. Halima Idris of the National Space Research and Development Agency (NASRDA), Abuja, for assisting with the GIS map of the sample sites in Idah River.

REFERENCES

- Abulreesh HH (2012). Salmonellae in the environment. In: Annous, B, Gurtler, JB (Eds.), *Salmonella-Distribution, Adaptation, Control Measures and Molecular Technologies*. InTech. pp. 19-50.
- Adedire OM, Atere A, Ogundipe WF, Farinu AO (2021). Effects of Direct and Indirect Sunlight on Polythene Packs, Sensory, Microbial and Chemical Properties of Sachet Water. *Journal of Advances in Biology and Biotechnology* pp. 25-34.
- Adekunle IM, Adetunji MT, Gbadebo AM, Banjoko OB (2007). Assessment of groundwater quality in a typical rural settlement in Southwest Nigeria. *International Journal of Environmental Research and Public Health* 4(4):307-318.
- Ademorati MA (1996). *Standard Methods for water and effluent analysis*. Foludex Press Ltd, Ibadan. 1st ed. pp. 80-83.
- Adesakin TA, Oyewale AT, Bayero U, Mohammed AN, Aduwo IA, Ahmed PZ, Abubakar ND, Barje IB (2020). Assessment of bacteriological quality and physico-chemical parameters of domestic water sources in Samaru community, Zaria, Northwest Nigeria. *Heliyon* 1-14:e04773.
- Adeyemi SO (2010). Food and feeding habits of *Synodontis resupinatus* (Boulenger, 1904) at Idah area of River Niger, Kogi state, Nigeria. *Animal Research International* 7(3):1281-1286.
- Agbabiaka TO, Oyeyiola GP (2012). Microbial and physicochemical assessment of Foma River, Ita-Nmo, Ilorin, Nigeria: an important source of domestic water in Ilorin Metropolis. *International Journal of Plant, Animal and Environmental Sciences*, 2(1):209-216.
- Akaniwor JO, Anoksike E, Akaniuk JO (2007). Indomine industrial effluent discharge on microbial properties of new Calabar River. *Scientific Research and Essays* 2(1):001-005.
- Ananthkrishnan S, Loganathan K, Ahamed AJ (2012). Study on ground water quality and its suitability or drinking purpose in alathur block—Perambalur district. *Archives of Applied Science Research* 4(3):1332-1338.
- Anyanwu CU, Okoli EN (2012). Evaluation of the bacteriological and physicochemical quality of water supplies in Nsukka, Southeast, Nigeria. *African Journal of Biotechnology* 11(48):10868-10873.
- American Public Health Association (APHA) (2005). *Standard methods for examination of water and wastewater* (20th edn.) Washington P 1220.
- Ayedun H, Taiwo AM, Umar BF, Oseni OA, Oderinde AA (2011). Potential groundwater contamination by toxic metals in Ifo, Southwest Nigeria. *Indian Journal of Science and Technology* 4(7):820-823.
- Bouزيد M (2016). Waterborne diseases and climate change: Impact and Implications. Examining the Role of Environmental Change on Emerging Infectious Diseases and Pandemics IGI Global. pp. 89-108.
- Cheesebrough M (2000). *Medical Laboratory Manual for Tropical Countries*. Butter worth and Co. Ltd., UK pp. 21-32.
- Citarasu T (2012). Natural antimicrobial compounds for use in aquaculture. In: Austin B., editor. *Infectious Disease in Aquaculture*. Woodhead Publishing; Cambridge, UK pp. 419-456.
- Environmental Pollution Centers (2018). *Water Pollution Causes*. Available online: <https://www.environmentalpollutioncenters.org/water/causes/> (accessed on 19 April 2018).
- Feng BW, Li XR, Wang JH, Hu ZY, Meng H, Xiang LY, Quan ZX (2009). Bacterial diversity of water and sediment in the Changjiang estuary and coastal area of the East China Sea. *FEMS Microbiology Ecology* 70(2):236-248.
- Fernandez-Martinez A, Charlet L (2009). Selenium environmental cycling and bioavailability. A structural chemist point of view. *Reviews in Environmental Science and Biotechnology* 8(1):81-110.
- Franciska MS, Marcel D, Rinald LH (2005). *Escherichia coli* 0157: H7 in drinking water from private supplies. *Journal of Netherlands Water Resource* 39:4485-4493.
- Gerhardt P, Murray EGR, Wood AW, Krieg RN (1994). *Methods for General and Molecular Bacteriology*. ASM Press, Washington DC. pp. 340-791.
- Gospodarek E, Ziolkowski G (2000). Antibiotic-resistant strains of *Acinetobacter baumannii* occurring in Poland. *Epidemiologic Reviews* 54:88-96.
- Guo X, Chen DD, Peng KS (2016). Identification and characterization of *Bacillus subtilis* from grass carp (*Ctenopharyngodon idellus*) for use as probiotic additives in aquatic feed. *Fish and Shellfish Immunology* 52:74-84.
- Hageskal G, Knutsen AK, Gaustad P, de Hoog GS, Skaar I (2006). Diversity and significance of mold species in Norwegian drinking water. *Applied Environmental Microbiology* 72(12):7586-7593.
- Hammer JM, Hammer JMJr (2002). *Water and wastewater technology* (New Delhi, Prentice-Hall).
- Hamouda A, Findlay J, Al Hassan L, Amyes SG (2011). Epidemiology of *Acinetobacter baumannii* of animal origin. *Journal of Antimicrobial Agents* 38(4):314-318.
- Health Canada (2012). *Guidelines for Canadian drinking water quality:*

- Guideline technical document—Turbidity. Water and Air Quality Bureau, Healthy Environments and Consumer Safety Branch; Health Canada: Ottawa, ON, Canada.
- Ifi F, Enerijiofi K, Ekhaie FO (2019). Investigation of the physicochemical and bacteriological qualities of Okokpon River, Edo State, Nigeria for its portability status. *FUDMA Journal of Sciences* 3(2):185-194.
- Igbinsola EO, Uyi OO, Odjajare EE, Ajuzie CU, Orhue PO, Adewole EM (2012). Assessment of physicochemical qualities, heavy metal concentrations and bacterial pathogens in Shanomi Creek in the Niger Delta, Nigeria. *African Journal of Environmental Science and Technology* 6:419-424.
- Ikhuorihah SO, Oronsaye CG, Adebajo IA (2016). Assessment of some physical characteristics and some heavy metals of River Ossiomo, Ologbo, a tributary of the Benin River, southern Nigeria. *Journal of Applied Science and Environmental Management* 20(2):472-481.
- Jeyasekaran G, Ganesan P, Anandaraj R, Jeya Shakila R, Sukumar, D (2006). Quantitative and qualitative studies on the bacteriological quality of Indian white shrimp (*Penaeus indicus*) stored in dry ice. *Journal of Food Microbiology* 23(6):526-533.
- Katsoyiannis IA, Zouboulis AI, Jekel M (2004). Kinetics of bacterial As (III) oxidation and subsequent As (V) removal by sorption onto biogenic manganese oxide during groundwater treatment. *Industrial and Engineering Chemistry Research* 43(2):486-493.
- Kay D, Crowther J, Stapleton CM, Wyer MD, Ewtrell LF, Edwards A, Francis CA, McDonald AT, Watkinson J (2008). Fecal indicator organism concentrations in sewage and treated effluents. *Water Resources* 42:442-445.
- Kavka GG, Berger B, Hoch BM, Herndl, GJ (1996). Assessment of microbiological water quality in the Austrian section of the river Danube. *Archiv fur Hydrobiologie, Supplementband. Larger Rivers*. 113(10): 79-86.
- Kidu M, Abraha G, Hadera A, Yirgaalem W (2015). Assessment of physico-chemical parameters of Tsaeda-Agam River in Mekelle City, tigray, Ethiopia. *Bulletin of the Chemical Society of Ethiopia* 29(3):377.
- Kozínska A, Pażdziej E, Pękala A, Niemczuk W (2014). *Acinetobacter johnsonii* and *Acinetobacter iwoffii* the emerging fish pathogens. *Bulletin of the Veterinary Institute in Pulawy* 58(2):193-199.
- Kuz'mina VU, Ushakova NY (2007). Metal assessment of fish ponds. *Journal of Biomedical Life Sciences* 47(6):837-846.
- Leon CG, Moraga R, Valenzuela C, Gugliandolo C, Lo Giudice A, Papale M (2018). Effect of the natural arsenic gradient on the diversity and arsenic resistance of bacterial communities of the sediments of Camarones River (Atacama Desert, Chile). *PLoS ONE* 13(5):e0195080.
- Marchesi J (2017). *Unlocking the Microbiome*. Microbiology Society, Charles Darwin House, London. PDF file. 11 July, 2019.
- Miller RL, Bradford WL, Peters NE (1988). Specific Conductance: Theoretical Considerations and Application to Analytical Quality Control. In U.S. Geological Survey Water-Supply Paper. Retrieved from <http://pubs.usgs.gov/wsp/2311/report.pdf>
- Murphy S (2005). General information on Dissolved Oxygen Basin Project, city of Boulder.
- National Population Census (NPC) (2006). Federal Republic of Nigeria Official Gazette No.2 Abuja. 96 B. 25 Federal Government Printer.
- Nicholson WL (2004). Ubiquity, longevity, and ecological roles of *Bacillus* spores. In *Bacterial spore formers: Probiotics and emerging applications*, In: E. Ricca, A.O. Henriques, and S.M. Cutting, eds. (Norfolk, Horison Bioscience) pp. 1-15.
- Nwoko CIA, Ukiwe LN, Egereonu UU, Ukachukwu SN (2015). Assessment of seasonal physicochemical parameters of Oguta Lake, Nigeria. *Journal of Advances in Chemistry* 11(7):3759-3764.
- Okechi RN, Chukwura EI (2020). Physicochemical and Bacteriological Qualities of Otamiri River Water and Sediment in South Eastern Nigeria. *Frontiers in Environmental Microbiology* 6(2):18-26.
- Okereke IJ (2014). An assessment of the physicochemical parameters of Ihuku River. *IOSR Journal of Environmental Science Toxicology and Food Technology* 8(1):27-30.
- Ologbosere OA, Aluyi HSA, Ogofure AG, Beshiru A, Omeje FI (2016). Physico-chemical and microbiological profile of bacterial and fungal isolates of Ikpoba River in Benin City: Public health implications. *African Journal of Environmental Science and Technology* 10(3):67-76.
- Oremland RS, Stolz JF (2003). The ecology of arsenic. *Science* 300(5621):93-94.
- Peleg AY, Seifert H, Paterson DL (2008). *Acinetobacter baumannii*: emergence of a successful pathogen. *Clinical Microbiology Reviews* 21(3):538-582.
- Pietryczuk A, Cudowski A, Hauschild T (2018). Abundance and Species Diversity of Fungi in Rivers with Various Contaminations. *Current Microbiology* 75(5):630-638.
- Premalata V (2009). Multivariate analysis of drinking water quality parameters of Lake Pichhola in Udaipur, India. *Biological Forum International Journal* 1(2):97-102.
- Pohland AE, Wood GE (1997). Occurrence of Mycotoxins in Food. In: *Mycotoxins in Food*, Krogh, P(Ed.). Academic Press, London, UK. pp. 35-64.
- Refai MK, Mohammed LA, Kenawy AM, Shima El-SMA (2010). The Assessment of Mycotic Settlement of Freshwater Fishes in Egypt. *Journal of American Science* 6(11):823-831.
- Quinn PJ, Carter ME, Markey B, Garter GR (2004). *Clinical Veterinary Microbiology, USA*. Mosby an imprint of Elsevier Limited. pp. 43-49.
- Schuster A, Schmoll M (2010). *Biology and Biotechnology of Trichoderma*. *Applied Microbiology and Biotechnology* 87(3):787-799.
- Shaji C, Nimi H, Bindu L (2009). Water quality assessment of open wells in and around Chavara industrial area, Quilon, Kerala. *Journal of Environmental Biology* 30(5):701-704.
- Sharma MS, Tiwari KL (2015). Isolation and identification of soil fungi from mahamera aniket, shivnath river durg. *IOSR Journal of Environmental Science, Toxicology and Food Technology* 1(5):24-26.
- Shittu OB, Olaitan JO, Amusa TS (2008). Physico-chemical and bacteriological analyses of water used for drinking and swimming purposes in Abeokuta, Nigeria. *African Journal of Biomedical Research* 11(3):285-290.
- Standard Organization of Nigeria (SON) (2007). *Nigerian Standard for Drinking Water Quality*, SON, Abuja, Nigeria.
- Taiwo AA, Abayomi TO, Umar B, Abubakar NM, Iduwo AA, Ahmed PZ, Abubakar ND, Ibrahim BB (2020). Assessment of bacteriological quality and physico-chemical parameters of domestic water sources in Samaru community, Zaria, Northwest Nigeria. *Heliyon* 6(8) 04773-8440.
- Thi TGL, Suthipong S, Kyoung-Woong, K (2009). Arsenic and other trace elements contamination in groundwater and a risk assessment study for the residents in the Kandal Province of Cambodia. *Environment International* 35(3):455-460.
- Tsai HC, Chou MY, Shih YJ, Huang TY, Yang PY, Chiu YC, Chen JS, Hsu BM (2018). Distribution and genotyping of aquatic *Acinetobacter baumannii* strains isolated from the Puzi River and its tributaries near areas of livestock farming. *Water* 10:1374.
- Umeh OR, Chukwura EI, Ibo EM (2020). Physicochemical, bacteriological and parasitological examination of selected fish pond water samples in Awka and its environment, Anambra State, Nigeria. *Journal of Advances in Microbiology* pp. 27-48.
- U.S. Environmental Protection Agency (EPA) (2016). *Six-Year Review 3 Technical Support Document for Microbial Contaminant Regulations*. Available online at: <https://www.epa.gov/sites/production/files/2016-12/documents/810r16010.pdf>
- Venkatasubramani R, Meenambal T (2007). Study on subsurface water quality in Mettupalayam taluk of Coimbatore district, Tamil Nadu. *Nature Environment and Pollution Technology* 6(2):307-310.
- World Health Organization (WHO) (2011). *Guidelines for Drinking-Water Quality*, Fourth 565 edition. WHO Library Cataloguing-in-Publication, Switzerland.
- World Health Organization (WHO) (2014). *The United Nations International Children's Emergency Fund (UNICEF). Joint Monitoring Programme for Water Supply and Sanitation. In Progress on Drinking Water and Sanitation: 2014 Update*; UNICEF: New York, NY, USA.
- Zhao J, Zhao X, Chao L, Zhang W, You T, Zhang J (2014). Diversity change of microbial communities responding to zinc and arsenic pollution in a river of northeastern China. *Journal of Zhejiang University-Science B Biomedicine and Biotechnology* 15(7):670-680.

Full Length Research Paper

Partial chemical characterization of the yeast extracts *Lachancea thermotolerans* CCMA 0763

Moacir Torres^{1*}, Marcos A. S. Ribeiro², João Arthur S. Oliveira², Eduardo C. Meurer³ and Kátia R. F. Schwan-Estrada¹

¹Agronomy Department, Universidade Estadual de Maringá, Maringá/87020-900, Brazil.

²Department of Biotechnology, Genetics, and Cell Biology, Universidade Estadual de Maringá, Maringá/87020-900, Brazil.

³FENN Mass Spectrometry Laboratory, Universidade Federal do Paraná, Jandaia do Sul 9/86900-000, Brazil.

Received 25 November, 2020; Accepted 2 March, 2021

The fungi are living organisms of great importance around agriculture, food industry, and the pharmaceutical industry. The usage of these microorganisms in agriculture is for the biocontrol of diseases in plants of commercial interest. Some yeasts can fight phytopathogens through secondary metabolites produced, inhibiting their development. Some species show positive results in the control of pathologies in different cultures. Yeasts have been used as biocontrol agents, and among them, a *Lachancea (Kluyveromyces) thermotolerans*, which is present in plants (such as grapes), soil and insects, can be adapted to different environments and, possibly perform biological control, although it is not known its mode of action. This work aimed to analyze and identify secondary metabolites used by the yeast *L. thermotolerans* CCMA 0763 isolated from commercial grapes in the Agricultural Microbiology Laboratory (Federal University of Lavras-MG/BR), using the analytical method of Ultra High-Efficiency Liquid Chromatography (UHPLC) (HRMS) and Tandem Mass Spectrometry (MS/MS). Four heterocyclic compounds of the Alkaloids class were identified, three (03) natural: 4-Hydroxyquinoline, Xanthine and Calistegine A3, and one (01) synthetic: Clausehainanine C. Therefore, these compounds can be tested against phytopathogenic microorganisms.

Key words: Yeast, biological control, secondary metabolite, ultra high-efficiency liquid chromatography (UHPLC), mass spectrometry.

INTRODUCTION

Non-*Saccharomyces* yeasts are sources of complex aromatics, glycerol content and responsible in ethanol reduction and polysaccharide concentrations in industrial processes. Its main functionality is associated with the production of alcoholic beverages from fermentation

processes (Contreras et al., 2014; Belda et al., 2015, 2017; Domizio et al., 2017). This fungus is mainly in the production of wine, on substrates containing hexoses, playing an important role in the characteristics of the drink not producing spores or allergenic toxins; and are

*Corresponding author. E-mail: mtorres@uem.br.

not nutritionally demanding. They present rapid growth, they do not present risks to the consumer and they act as antagonists, either through competition for nutrients, antibiosis or hyperparasitism (Domizio et al., 2017; Mehlomakulu et al., 2014, 2015). However, its use is not limited to wines, beers, breads and cheeses productions, but on other products for example control of phytopathogens. The good performance of yeasts as biocontrol agents is due to the different modes of action of these microorganisms against phytopathogens. Among these, we can highlight the competition with phytopathogens for space and nutrients, mycoparasitism, antibiosis, resistance induction in plants and the production of antimicrobial toxins and alcohol by yeasts. Understanding these biocontrol mechanisms is essential to improve the action of these microorganisms in combating plant diseases (Hatoum et al., 2012; Muccilli and Restuccia, 2015). Biological control is an important management strategy for fungal diseases, with the basic principle of using antagonistic microorganisms to reduce and/or keep the population of a phytopathogen below levels that cause economic loss (Carmona-Hernandez et al., 2019). Organic control using biological agents against mycotoxins is considered a safer option until now, gaining popularity in the food industry (Tsitsigiannis et al., 2012). In fact, the application of yeasts (cells and their volatiles) and yeast derivatives has great potential to minimize the economic losses caused by mycotoxigenic fungi. Several of yeast and bacteria species have been identified as having effective fungi biological control activities at pre- and post-harvest, where they were tested against toxigenic fungi to inhibit their growth and mycotoxin synthesis (Catara, 2007; Haiissam, 2011). Within the microorganisms tested, the non-saccharomyces yeasts are promising antimicrobial and bio-remediation agents (Übeda et al., 2014). However, the antimicrobial properties associated with yeast are linked to the production of killer toxins. Killer toxins have been identified as glycosylated proteins that bind to specific receptors. Their strains were called killer yeast. Killer yeasts secrete lethal toxins to and their strains are insensitive to their own toxins (Alturki et al., 2019; Mannazzu et al., 2019).

In addition, the biological properties associated with this type of microorganism are not limited only to the production of proteins, but in the direct and/or indirect interactions between the different strains through the physical-transformed changes in the environment caused by the reaction of one strain to another. The effect of a specific yeast strain on its environments is unique suited to its metabolites and their respective (Roullier-Gall et al., 2020). Thus, it is evident that non-saccharomyces yeasts have a favorable metabolic engineering for the production of new and well-known compounds with wide biological activity (Kulkarni et al., 2015; Liu et al., 2020). Currently, thousands of secondary metabolites have been identified from various living organisms, mostly plants. However, it

is estimated that this number is higher, being, in part, limited to analytical techniques. With the instrumental advance, compounds with a high degree of difficulty in analysis, whether due to their abundance, structural diversity or the complexity of the matrix, have been constantly identified (Zhao et al., 2013). Among the technological tools used to identify these molecules, is Ultra High Efficiency Liquid Chromatography (UHPLC) (HRMS), to analyze the yeast metabolome, for its excellent resolution, mass accuracy, new possibilities for improving analytical methods for matrices complex samples (ingredients, food and biological samples) and a wide range of spectrometric data (Motilva et al., 2013). Thus, the present work aimed to partially characterize the secondary metabolites produced by the yeast *Lachancea thermotolerans* CCMA 0763, isolated from commercial raisin, using the analytical technique of (UHPLC)(HRMS) and Tandem Mass Spectrometry (MS/MS).

MATERIALS AND METHODS

Crude extract obtainment

The yeast *L. thermotolerans* CCMA 0763 (Sample) belonging to the Agricultural Microbiology Culture Collection at the Federal University of Lavras - Minas Gerais/Brazil was grown in a Petri dish containing Potato-Dextrose-Agar (PDA) (Culture Medium) for 2 days and inoculated in 500 ml Erlenmeyers containing Potato-Dextrose (PD) with pH 6.8 and incubated without shaking for 21 days at 28°C (Quality Control). The mycelium was separated from the fermented broth by filtration in cotton and hydrophobic gauze and the aqueous phase was centrifuged at 2750 rpm for 15 min. The liquid-liquid extraction was performed with Ethyl Acetate in the proportion of 1:3 (Ethyl acetate: fermented broth) in a separating funnel. This step was repeated three times. The solvent was collected and evaporated in a Rotary Evaporator (Tecnal TE-210) at 400°C and the Crude Extract (1.014 g) subjected to Ultra High Efficiency Liquid Chromatography Analysis (UHPLC) (HRMS) and Tandem Mass Spectrometry (MS/MS). The same procedure was used for the Quality Control sample.

Chemical analyses

The solvents used were: Acetonitrile grade UHPLC-MS (JT Baker-Phillipsburg, NJ, USA), Deionized Water from a Millipore Milli-Q water reagent system (Millipore, Bedford, MA, USA) and Analytical grade Formic Acid (85%). The extract of the yeast *L. thermotolerans* (1.0141 g) was solubilized in methanol and the concentration of the solution corrected to 1.0 mg. mL⁻¹. For the Mass Spectrometry Analysis, a volume of 1.0 ml of Yeast Extract was collected and 10.0 µl of 0.1% Formic Acid (v/v) (Analysis in positive mode) was added and stored in a vial for analysis.

Liquid chromatography

The extracts (Sample and Control) were analyzed by Ultra-High Performance Liquid Chromatography (Shimadzu, Nexera X2, Japan) coupled to a High Resolution Mass Spectrometer (Impact II, Bruker Daltonics Corporation, Germany) equipped with an ionization source by electrospray. The chromatographic separation was performed with an Acquity UPLC® CSHTM C18 column,

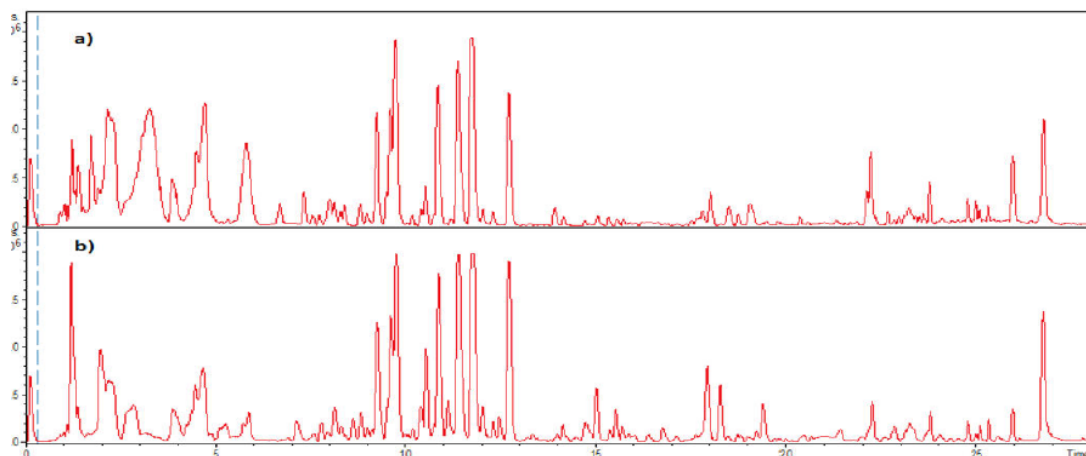


Figure 1. UPLC-Q-TOF/MS BPI chromatogram in mode positive from Quality Control (a), and yeast *Lachancea thermotolerans* (b).

particle size of 2.1 μm , with 2.1 \times 100 mm (Waters, Ireland) and flow rate of 0.200 ml min^{-1} . The gradient of the solvent mixture A (H_2O with 0.1% (v/v) formic acid) and B (acetonitrile with 0.1% (v/v) formic acid) as follows: 5% B 0-1 min, 50% B 1- 5 min, 95% B 5-10 min and maintained at 95% B 10-16 min, 5% B 16-18 min and maintained at 5% B 18-32 min at 40°C.

Mass spectrometry

The ionization source was operated in positive ionization modes and set to 4500V with a potential displacement of the final plate of 500V. The drying gas parameters were adjusted to 8 L min^{-1} at 180°C and the nebulizer gas pressure was set to 4 bar. The data were collected from m/z 50 to 1300 with an acquisition rate of 5 Hz and the 4 most intense ions were selected for automatic fragmentation (Auto MS/MS).

Data processing and statistical analyses

The software used for data processing for peak detection, multivariate analysis and identification was Data Analysis 4.0 (Bruker, Germany). The specialized databases were: MoNa (<http://mona.fiehnlab.ucdavis.edu/>), ChemSpider (<http://www.chemspider.com/>) and Metlin (<https://metlin.scripps.edu/>) for the identification of compounds. The search parameters in the online databases were: precursor mass with error \leq 10 ppm and fragment tolerance \leq 10 ppm. Principal component analysis (PCA) and projection discrimination in an orthogonal latent structure (OPLC-DA) were generated using the ProfileAnalysis 2.1 software.

RESULTS AND DISCUSSION

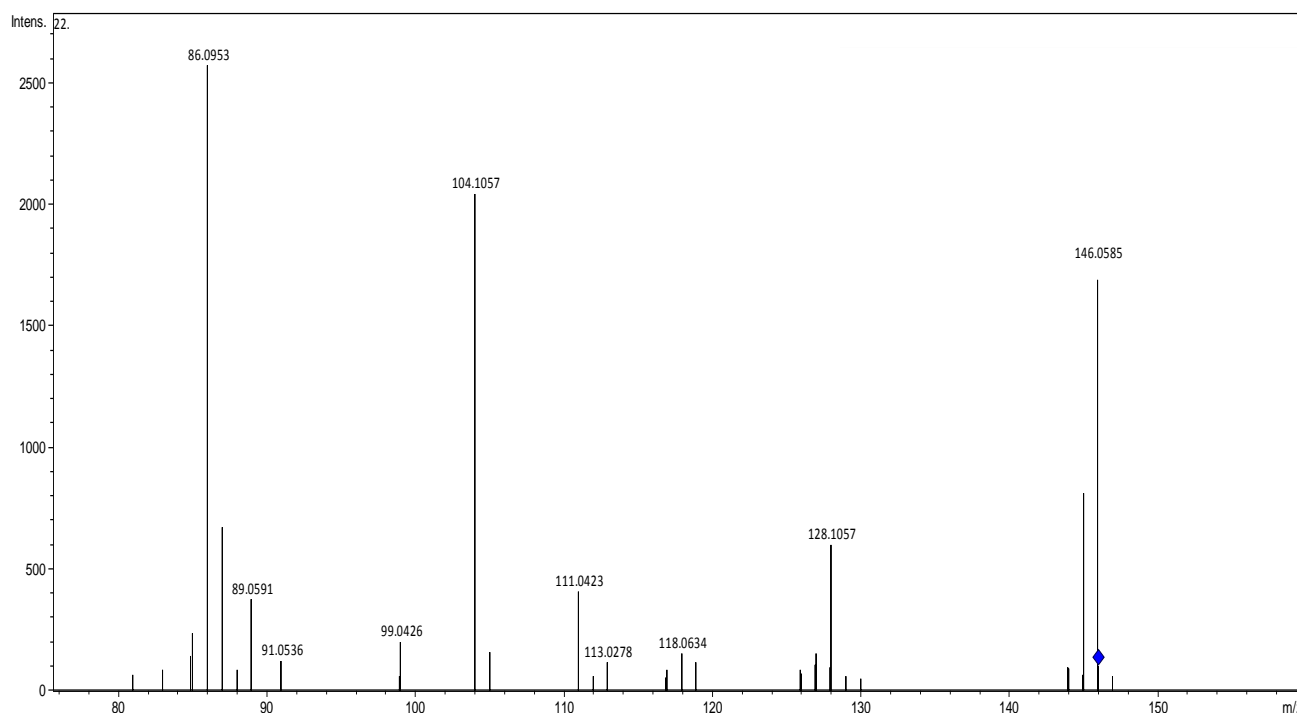
Figure 1 represents the diversity of the chemical profiles of *L. thermotolerans* and Quality Control extracts in the positive mode chromatogram generated from the UHPLC-HRMS. The chromatographic run had an injection time of 28.0 min with 4.0 min for column balance and cleanliness.

It was revealed in this first analysis that in the time between 1.0 to 6.0 min, the two chromatograms behave differently in relation to the intensity and areas of the peak. This is also seen in the time between 14.0 and 25.5 min. However, the chromatographic profile is very similar between the Control and the Sample, little difference between them. However, UHPLC-HRMS analyzes provide coverage of the chemical space based on MS and MS/MS data and are capable of presenting the best differentiation between Sample and Control. For this experiment, the molecular masses studied were 50 to 1300 Dalton (Da). This mass distribution of precursors was based on studies in the literature (Maciá-Vicente et al., 2018).

The global chemical space observed in the complete data set comprised eight thousand exclusive MS/MS spectra after the first performed for a sample and control. To minimize and discard potential noise or artifacts present in the UHPLC-HRMS analyzes, three boundary conditions were imposed: first, the removal of the spectra present only in the control or in the whites; second, consider the final spectra that occurred only in the sample; third, consider spectra with acquired MS/MS, an important identification of the compounds in the sample. All spectral data were collected and processed using centralization, isotopic pattern, filtering, retention time and peak recognition methods to generate a datamatrix including sample identity, ion identity and ion abundance. The result of this mathematical deconvolution procedure provided 126 exclusive ions from the sample. The software used for data processing for peak detection, multivariate analysis and identification was DataAnalysis 4.0 (Bruker, Germany) and Software ProfileAnalysis 2.1. For the identification of compounds, specialized Database sites were used, such as: MoNa, ChemSpider and Metlin. Other parameters, such as fragmentation

Table 1. Results of high resolution UHPLC-HRMS mass spectrum analysis.

Library	Compound	[M+H] ⁺ m/z	RT (min)	Exact mass	Formula	MS/MS
Mona	1	1.460.585	6.49	1.450.528	C ₉ H ₇ NO	128.1057; 118.0634; 111.0423; 104.1057;99.0426; 86.0953
Mona	2	1.530.395	2.70	1.520.334	C ₅ H ₄ N ₄ O ₂	136.0133; 110.0343 142.0853; 130.0854; 124.0748; 112.07
Goldmann et al. (1990)	3	1.600.958	1.03	1.590.895	C ₇ H ₁₃ NO ₃	52; 103.0385; 94.0648; 86.0600; 82.0648
Ma et al. (2018)	4	2.921.332	0,34	2.911.259	C ₁₉ H ₁₇ NO ₂	246.1301; 200.1253; 158.1159; 1121110

**Figure 2.** ESI-MS/MS spectrum for 4-Hydroxyquinoline.

score and bibliographic record on the occurrence of the molecules, were also considered for disambiguation. The main exclusion factors used for identification were: mass error less than or greater than 10 ppm to decrease the number of candidates, fragmentation profile to justify and prove the identification and the probability of ionization by ESI.

This attention with several exclusion factors is associated with the possibility of compounds within a mass range of 0.5 to 5 ppm, which generally have very different numbers of carbon atoms and the biosynthesis of units with a common elementary composition that provides information limited. Thus, mass accuracy and isotopic ratio, are almost orthogonal parameters that can be used to exclude hypothetical elementary compositions,

but do not establish a consistent identification (Nielsen et al., 2011). In these cases, MS/MS, or preferably by Sequential Mass Spectrometry (MS_n), with subsequent fragmentation patterns is more efficient, assuming that reference standards are available to model the fragmentation of the class of compounds (Sharma et al., 2007). Thus, all precursors were submitted to the parameters presented here to support their identification. Four compounds were identified (Table 1), with their ESI-MS/MS spectra, respectively (Figures 2 to 5).

All compounds identified are alkaloids (Figure 6). They have nitrogen atoms and a differentiated fragmentation profile in their chemical skeleton. The compound identified as 4-Hydroxyquinoline belongs to the group of nitrogenous heterocyclic compounds of the quinoline

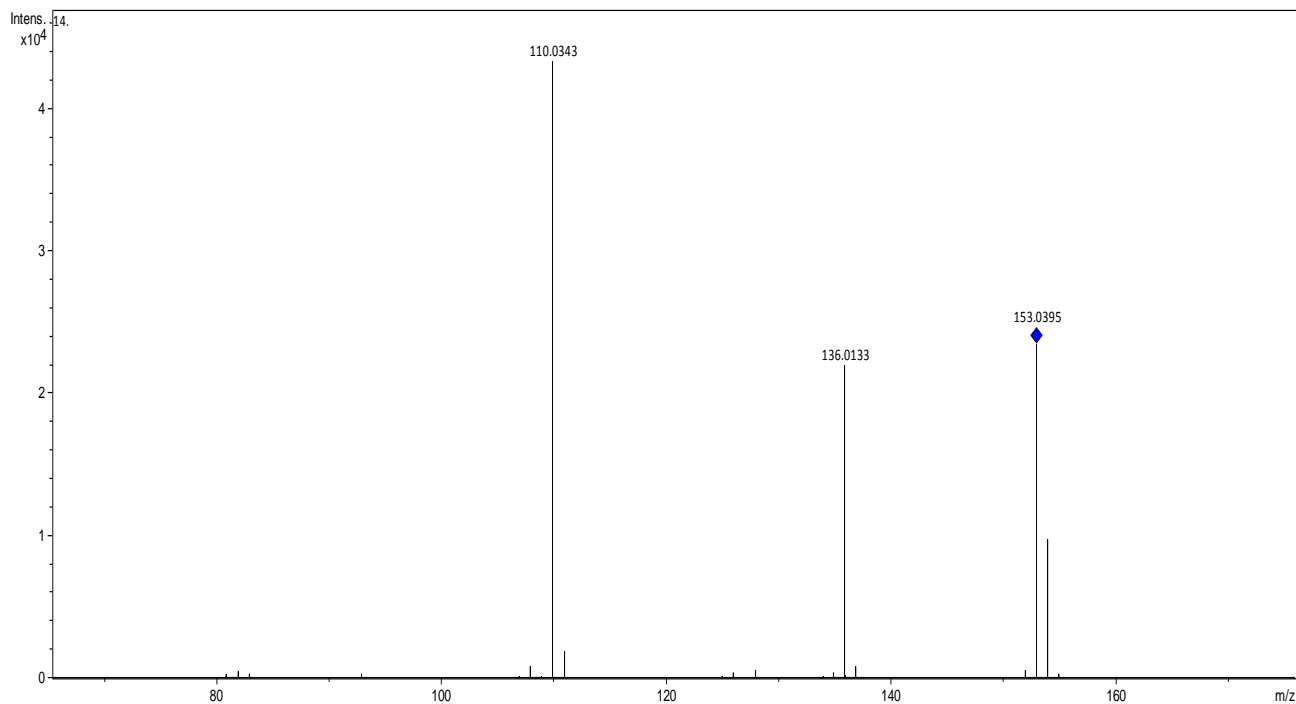


Figure 3. ESI-MS/MS spectrum for Xanthine.

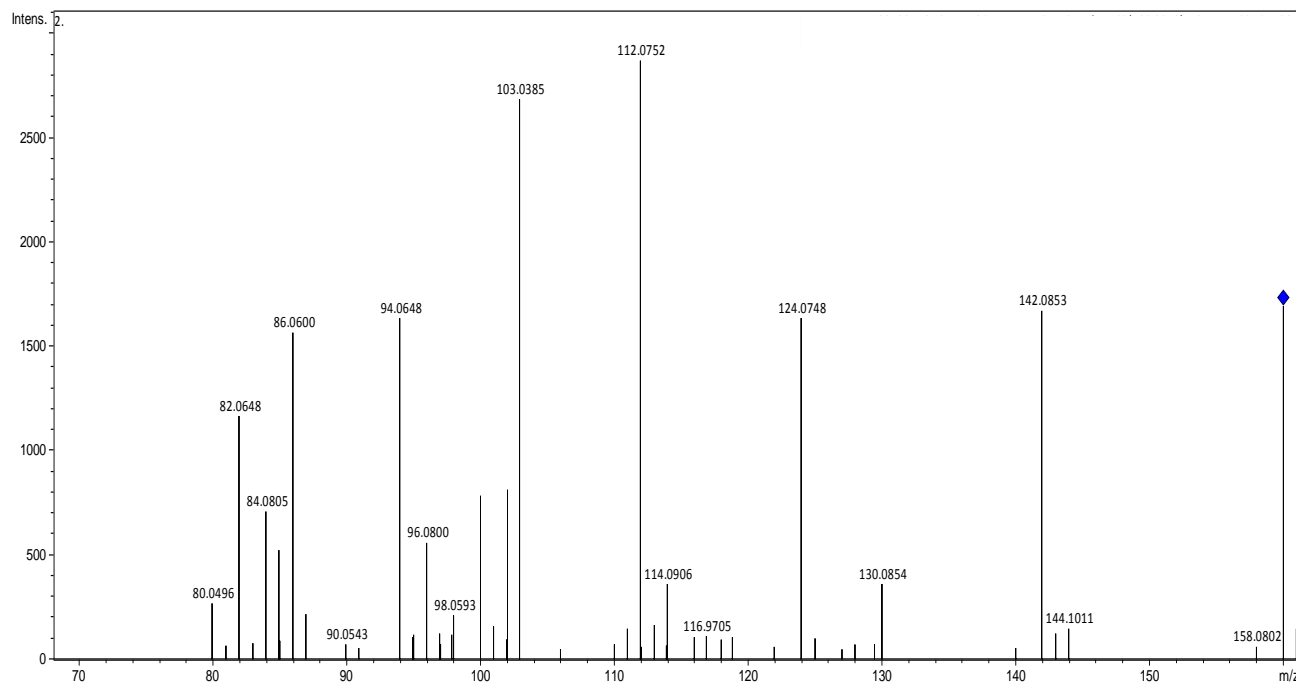


Figure 4. ESI-MS/MS spectrum for Calystegine A3.

alkaloids class. They play an important role in several areas of knowledge and have a wide range of natural substances. Quinolones are molecules structurally

derived from the heterocyclic Quinoline, being distributed in nature as a product of the secondary metabolism of several species of plants and fungi, mainly in species of

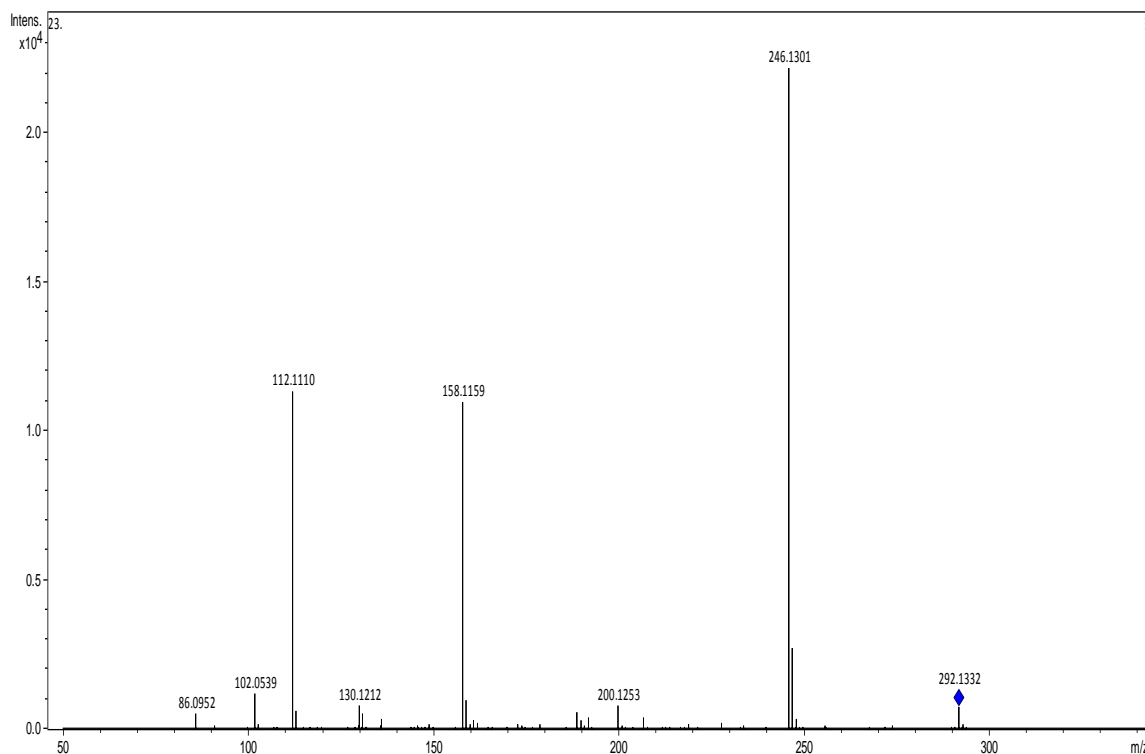


Figure 5. ESI-MS/MS spectrum for Clausehainanine C.

the family *Rutaceae* (Suárez et al., 2011). Several derivatives of Quinolone have been isolated from this family with analgesic, amebicidal, antiviral, herbicidal and fungicidal activities (Barrera and Suarez, 2007). The compound 8-Hydroxyquinoline and its derivatives are a subclass of Quinolines with a wide variety of biological activities. They have been used as a fungicide in agriculture and a preservative in the textile, wood and stationery industries (Oliveri and Vecchio, 2016).

Xanthines are a class of Purine Alkaloids found in many different plants, including yerba mate (1,3- and 3,7-Dimethylxanthine), coffee (1,3,7-Trimethylxanthine) and cocoa (Athayde et al., 2000). Another particularity associated with these compounds is their biological function in conjunction with the enzyme Xanthine A dehydrogenase (XDH), a Hydroxylase containing Molybdenum, being important for regulating the metabolism of Purines and Uric Acid responsible for the regulation of *Arabidopsis* aging and resistance to stress, peas, corn and grapes (Barabás et al., 2000; Zdunek-Zastocka and Lips, 2003; Brychkova et al., 2008; Werner and Witte, 2011; Shuanghong et al., 2017; Han et al., 2020).

Calistegines are a group of alkaloids (glycoalkaloids) initially discovered in the roots of cultures of *Calystegia sepium* and *Atropa belladonna*. The chemical structures contain a Nortropene ring system with 3-, 4-, or 5-Hydroxyl groups (Calistegines A, B or C, respectively), located in various positions with different stereochemistry

(Aminocetal functionality), and an Amino bridge group (Asano et al., 2000). These compounds were identified in several genera such as *Atropa*, *Datura*, *Duboisia*, *Hyoscyamus* and *Escopolia* belonging to the *Solanaceae*, *Convolvulaceae*, *Erythroxylaceae* and *Brassicaceae* families (Schimming et al., 2005; Brock et al., 2005, 2006; Torres-Romera et al., 2019). The biological activities associated with polyhydroxylated alkaloids are their levels of toxicity to bacteria, fungi, viruses, insects, animals and humans (Friedman, 2006).

Carbazolic Alkaloids are characterized by a basic aromatic tricyclic skeleton consisting of a central Pyrrole ring fused with two Benzene rings. Carbazol itself was originally isolated from the Anthracene fraction of coal tar (Greger, 2017). They have been found in bacteria, myxomycetes, fungi, sponges, tunicates and in the related plant families *Apocynaceae* and *Loganiaceae*. The vast majority of Carbazoles, comprising more than 330 derivatives, have been shown to be derived from 3-Methylcarbazole as a common precursor (Ryvolova et al., 2012). The vast majority of Carbazoles, comprising more than 330 derivatives, have been shown to be derived from 3-Methylcarbazole as a common precursor. This type of Carbazoles was called Fitocarbazoles (Chakraborty and Roy 2003). However, there is no scientific evidence from recent studies related to the application of this compound in the area of agriculture, so it can be the object of research to be investigated.

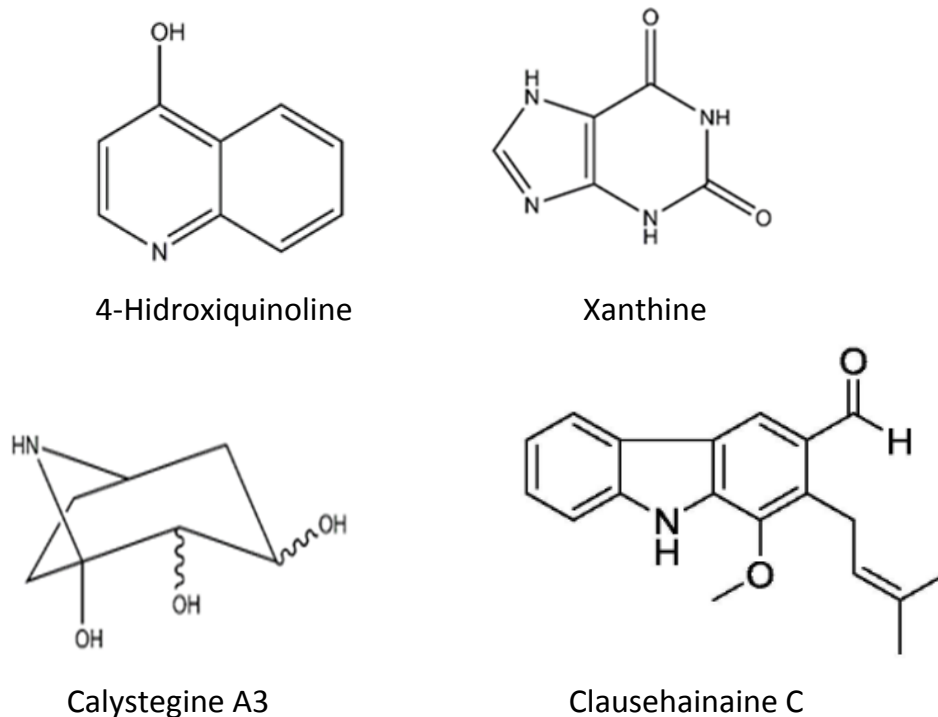


Figure 6. Compounds identified in the extract of *Lachancea thermotolerans*.

Conclusion

The crude extract of secondary metabolites from the yeast *L. thermotolerans* presents eight thousand exclusive MS/MS spectra through the UHPLC-HRMS analysis. These data were included in the mathematical deconvolution procedure, obtaining 126 exclusive ions from the sample. The analytical method of UHPLC-HRMS has been a high performance tool for the identification of compounds and biomolecules of great structural diversity for the analysis of secondary metabolites. Thus, four components of the class of heterocyclic alkaloids were identified, three (03) naturals: 4-Hydroxyquinoline, Xanthina and Calistegina A3; and one (01) synthetic: Clausehainanina C from the crude extract of *L. thermotolerans*.

These compounds present, through the literature: antimicrobial, antiproliferative, inhibitory and regulatory activities, applied in the most diverse areas of knowledge, being able to be tested as biocontrol agents in antagonistic tests of biological tests against phytopathogens that cause fungal and bacterial diseases in agricultural cultures

ACKNOWLEDGMENT

This study was partially funded by the Coordination for the Improvement of Higher Education Personnel - Brazil (CAPES) - Financial Code 001.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

- Alturki SN, Al-Saud NS, Alhenjin AM, Hassan SM (2019). Study of Yeast Killer Toxins and Their Interactions. *International Journal Advanced of Research* 7(5):314-320.
- Asano N, Nash RJ, Molyneux J, Fleet GWJ (2000). Sugar-mimic glycosidase inhibitors: natural occurrence, biological activity and prospects for therapeutic application. *Tetrahedron: Asymmetry* 11(8):1645-1680.
- Athayde ML, Coelho GC, Schenkel EP (2000). Caffeine and theobromine in epicuticular wax of *Ilex paraguariensis* A. St.-Hil.," *Phytochemistry* 55(7):853-857.
- Barabás NK, Omarov RT, Erdei L, Lips SH (2000). Distribution of the Mo-enzymes aldehyde oxidase, xanthine dehydrogenase and nitrate reductase in maize (*Zea mays* L.) nodal roots as affected by nitrogen and salinity. *Plant Science* 155(1):49-58.
- Barrera CAC, Suarez LEC (2007). Metabolitos secundarios aislados de los géneros *Raputia* y *Esenbeckia* (Rutaceae). *Scientia et Technica* 1(33):337-338.
- Belda I, Navascués, E, Marquina D, Santos A, Calderon F, Benito S (2015). Dynamic analysis of physiological properties of *Torulasporea delbrueckii* in wine fermentations and its incidence on wine quality. *Applied Microbiology and Biotechnology* 99(4):1911-1922.
- Belda I, Ruiza J, Beata Beisert B, Navascués E, Marquina D, Calderón F, Rauhut D, Benito S, Santos A (2017). Influence of *Torulasporea delbrueckii* in varietal thiol (3-SH and 4-MSP) release in wine sequential fermentations. *International Journal of Food Microbiology* 257:183-191.
- Brock A, Bieri S, Christen P, Dra B (2005). Calystegines in wild and cultivated *Erythroxylum* species. *Phytochemistry* 66(11):1231-1240.

- Brock A, Herzfeld T, Paschke R, Koch M, Dräger B (2006). *Brassicaceae* contain nortropane alkaloids. *Phytochemistry* 67(18):2050-2057.
- Brychkova G, Alikulov Z, Fluhr R, Sagi M (2008). A critical role for ureides in dark and senescence-induced purine remobilization is unmasked in the *Atxdh1* *Arabidopsis* mutant. *The Plant Journal* 54(3):496-509.
- Catara V (2007). *Pseudomonas corrugata*: plant pathogen and/or biological resource? *Molecular Plant Pathology* 8(3):233-244.
- Carmona-Hernandez S, Reyes-Péres JJ, Chiquito-Contreras RG, Rincon-Enriquez G, Cerdan Cabrera CR, Hernandez-Montiel LG (2019). Biocontrol of Postharvest Fruit Fungal Diseases by Bacterial Antagonists: A Review. *Agronomy* 9(3):121.
- Chakraborty DP, Roy S (2003). Carbazole Alkaloids IV. In: *Fortschritte der Chemie organischer Naturstoffe / Progress in the Chemistry of Organic Natural Products*. Fortschritte der Chemie organischer Naturstoffe/Progress in the Chemistry of Organic Natural Products, vol 85. Springer, Vienna. https://doi.org/10.1007/978-3-7091-6051-0_3
- Contreras A., Hidalgo C, Henschke PA, Chambers PJ, Curtin C, Varela C (2014). Evaluation of non-*Saccharomyces* yeasts for the reduction of alcohol content in wine. *Applied and Environmental Microbiology* 80(5):1670-1678.
- Domizio P, Liu Y, Bisson LF, Barile D (2017). Cell wall polysaccharides released during the alcoholic fermentation by *Schizosaccharomyces pombe* and *S. japonicus*: quantification and characterization. *Food Microbiology* 61:136-149.
- Friedman M (2006). Potato glycoalkaloids and metabolites: roles in the plant and in the diet. *Journal of Agricultural and Food Chemistry* 54(23):8655-8681.
- Greger H (2017). Phytocarbazoles: alkaloids with great structural diversity and pronounced biological activities. *Phytochemistry Reviews* 16:1095-1153.
- Haijssam JM (2011). *Pichia anomala* in biocontrol for apples: 20 years of fundamental research and practical applications *Antonie Van Leeuwenhoek* 99(1):93-105.
- Han R, He X, Pan X, Shi Q, Wu Z (2020). Enhancing xanthine dehydrogenase activity is an effective way to delay leaf senescence and increase rice yield. *Rice* 13(1):1-14.
- Hatoum R, Labrie S, Fliss I (2012). Antimicrobial and probiotic properties of yeasts: from fundamental to novel applications. *Frontiers in Microbiology* 3:421.
- Kulkarni P, Loira I, Morata A, Tesfaye W, González MC, Suárez-Lepe JA (2015). Use of Non-*Saccharomyces* yeast strains coupled with ultrasound treatment as a novel technique to accelerate ageing on lees of red wines and its repercussion in sensorial parameters. *Food Science and Technology* 64(2):1255-1262.
- Liu S, Laaksonen O, Marsol-Vall A, Zhu B, Yang B (2020). Comparison of Volatile Composition between Alcoholic Bilberry Beverages Fermented with Non-*Saccharomyces* Yeasts and Dynamic Changes in Volatile Compounds during Fermentation. *Journal of Agricultural and Food Chemistry* 68(11):3626-3637.
- Maciá-Vicente JG, Shi YN, Cheikh-Ali Z, Grün P, Glynou K, Hagi Kia S, Piepenbring M, Helge B, Bode HB (2018). Metabolomics-based chemotaxonomy of root endophytic fungi for natural products discovery. *Environmental Microbiology* 20(3):1253-1270.
- Mannazzu I, Domizio P, Carboni G, Zara S, Zara G, Comitini F, Budroni M, Ciani M (2019). Yeast killer toxins: from ecological significance to application. *Critical Reviews in Biotechnology* 39(5):603-617.
- Mehlomakulu NN, Setati ME, Divol B (2015). Non-saccharomyces killer toxins possible biocontrol agents against *Brettanomyces* in wine. *South African Journal of Enology and Viticulture* 36(1):94-104.
- Mehlomakulu NN, Setati ME, Divol B (2014). Characterization of novel killer toxins secreted by wine-related non-*Saccharomyces* yeasts and their action on *Brettanomyces* spp. *International Journal of Food Microbiology* 188:83-91.
- Motilva MJ, Serra A, Macià A (2013). Analysis of food polyphenols by ultra high-performance liquid chromatography coupled to mass spectrometry: An overview. *Journal of Chromatography A* 1292:66-82.
- Muccilli S, Restuccia C (2015) Bioprotective Role of Yeasts. *Microorganisms* 3(4):588-611. doi: 10.3390/microorganisms3040588.
- Nielsen KF, Ma M, Rank C, Frisvad JC, Larsen TO (2011). Dereplication of microbial natural products by LC-DAD-TOFMS. *Journal of Natural Products* 74(11):2338-2348.
- Oliveri V, Vecchio G (2016). 8-Hydroxyquinolines in medicinal chemistry: A structural perspective. *Journal of Medicinal Chemistry* 120:252-274.
- Roullier-Gall C, David V, Hemmler D, Schmitt-Kopplin P, Alexandre H (2020). Exploring yeast interactions through metabolic profiling. *Scientific Reports* 10(1):1-10.
- Ryvolova M, Adam V, Eckschlager T, Stiborova M, Kizek R (2012). Study of DNA-ellipticine interaction by capillary electrophoresis with laser-induced fluorescence detection. *Electrophoresis* 33(11):1545-1549.
- Shuanghong Y, Zhu Bo, Wang F, Han H, Sun M, Zhu H, Peng R, Yao Q (2017). A *Vitis vinifera* xanthine dehydrogenase gene, *VvXDH*, enhances salinity tolerance in transgenic *Arabidopsis*. *Plant Biotechnology Reports* 11(3):147-160.
- Schimming T, Jenett-Siems K, Mann P, Tofern-Reblin B, Milson J, Johnson RW, Deroin T, Daniel F, Austin DF, Eich E (2005). Calystegines as chemotaxonomic markers in the Convolvulaceae. *Phytochemistry* 66(4):469-480.
- Sharma A, Jansen R, Nimitz M, Johri BN, Wray V (2007). Rhamnolipids from the rhizosphere bacterium *Pseudomonas* sp. GRP3 that reduces damping-off disease in chilli and tomato nurseries. *Journal of Natural Products* 70(6):941-947.
- Suárez LEC, Barrera CAC, Barrera EDC, Moreno JML (2011). Antibacterial activity of terpenes and alkaloids isolated from three Colombian plants. *Cuban Magazine of Pharmacy* 45(2):275-282.
- Torres-Romera A, Arrebola-Liebanas J, Vidal JLM, Frenich AG (2019). Determination of Calystegines in Several Tomato Varieties Based on GC-Q-Orbitrap Analysis and their Classification by ANOVA. *Journal of Agricultural and Food Chemistry* 67(4):1284-1291.
- Tsitsigiannis DI, Dimakopoulos M, Antoniou PP, Tjamos EC (2012). Biological control strategies of mycotoxigenic fungi and associated mycotoxins in Mediterranean basin crops. *Phytopathologia Mediterranea* 51(1):158-174.
- Úbeda JF, Maldonado M, Briones AI, González FJ (2014). Bio-prospecting of Distillery Yeasts as Bio-control and Bio-remediation Agents. *Current Microbiology* 68(5):594-602.
- Zhao N, Wang G, Norris A, Chen X, Chen F (2013). Studying Plant Secondary Metabolism in The Age of Genomics. *Critical Reviews in Plant Sciences* 32(6):369-382.
- Zdunek-Zastocka E, Lips HS (2003). Is xanthine dehydrogenase involved in response of pea plants (*Pisum sativum* L.) to salinity or ammonium treatment? *Acta Physiologiae Plantarum* 25(4):395-401.
- Werner AK, Witte CP (2011). The biochemistry of nitrogen mobilization: Purine ring catabolism. *Trends in Plant Science* 16(7):381-387.

Full Length Research Paper

Long-term effectiveness of commonly used hospital disinfectants against *Pseudomonas aeruginosa* and *Staphylococcus aureus*

Stephen T. Odonkor^{1*}, Tahiru Mahami² and Anthony M. Sallar³

¹School of Public Services and Governance, Ghana Institute of Management and Public Administration Accra, Ghana.

²Biotechnology and Nuclear Agriculture Research Institute, Ghana Atomic Energy Commission Accra, Ghana.

³School of Liberal Arts and Social Sciences, Ghana Institute of Management and Public Administration Accra, Ghana.

Received 27 May, 2021; Accepted 16 July, 2021

Nosocomial infections are enhanced due to a flop in the infection control processes such as disinfection. The aim of this study was to assess long term effectiveness and the stability of disinfectants currently used within healthcare settings in Accra, Ghana against two indicator pathogens. Two locally produced and two imported disinfectants usually used in health care settings were obtained. The efficacy and long-term stability of the disinfectants was done using the in-use method, with identified microbial culture isolates of *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Counts of both test organisms (*Pseudomonas aeruginosa* and *Staphylococcus aureus*) were over and above the $5 \geq$ CFU recommended standard. All the disinfectants failed the test. However, contamination was observed to be higher in local disinfectants as compared to those imported. Furthermore, *Enterobacter spp* was isolated as contaminant from one imported disinfected and *Proteus spp* was also isolated from one local disinfectant. Interestingly, one of the imported disinfectants (Disinfectant I) showed more stability and was effective in the long term. All disinfectants did not pass the in-use test. However, disinfectants that are produced locally are more unstable and ineffective.

Key words: Infections, disinfectants, drug stability, hospital, pathogens, contamination.

INTRODUCTION

Disinfectants are widely used in hospitals and various health care facilities for diverse applications, including topical as well as hard surface. Generally, they are important part of infection control practices and help in the prevention of hospital acquired infections (HAI) or nosocomial infections (Cadnum et al., 2017; Liu and

Dickter, 2020; Rutala, 1951; Ling, 2020). HAI are one of the foremost infectious diseases that present an enormous economic effect globally (Barrasa-Villar et al., 2017; Kollef et al., 2021). HAI accounts for the hospitalization of about two (2) million people worldwide annually (Abbasi et al., 2010). Empirical evidence shows

*Corresponding author. E-mail: stodonkor@gimpa.edu.gh Tel: +233 243 709 702.

Table 1. Composition and pH of the concentrated product.

Disinfectant	Active ingredient	Concentration (%)	pH of concentrated product
I	Dichloro-meta-xyleneol	65	7
	Chloroxylenol	4.8	
II	Oleum pini Aromaticum Isopropyl Alcohol	8.3	11
	Sapo vegetails	9.4	
		5.6	
III	Chlorine	70	8
IV	Chlorine	33	8.3

that the occurrence of HAI is more than two folds higher in the developing world (Nasiri et al., 2021; Nejad et al., 2011). The hospital environment serves as an essential reservoir for various infectious microorganisms. Thus, the prevention and control of HAI is a matter of grave concern and a key challenge to contend with (Liu and Dickter, 2020; Ling, 2020). This is because within the health care environment, inanimate objects are potential conduits for the transmission of infectious microorganisms. However, disinfection provides the avenue in to help break the epidemiological sequence of infections (Liu and Dickter, 2020). Disinfection involves the application of chemical agents to remove microorganisms, except spores of bacteria (Liu and Dickter, 2020; Ling, 2020). The level of annihilation of microorganisms is largely dependent on their relative sensitivities to the chemical disinfection process. Usually high-level disinfection comprises the removal of all except huge quantities of bacteria spores, second, intermediate level disinfection provides for the annihilation of all microbial life excluding spores. Low-level disinfection will not dependably destroy mycobacteria or spores.

Multiple HAI outbreaks resulting from the use of a contaminated disinfectants are well documented (Kampf, 2018; Rosca et al., 2021). For example, in 1966, Mitchell and Hayward reported that seven different incidents of urinary tract infections observed in children following a cystoscopy procedure, were later traced to the contamination of chlorhexidine solution that was applied for the disinfection to bladder-irrigation reservoir. Similarly, Dulake and Kidd (Dulake and Kidd, 1966; Pitten et al., 2003) described the isolation of *Alcaligenes foecalis* from samples of urine obtained from thirty gynaecological patients. These patients underwent bladder drainage via indwelling catheter. The cause of the infection was subsequently traced to a jar that contained 0.1% chlorhexidine, which was used for spigots storage (Dulake and Kidd, 1966). Disinfectants could be contaminated due to extrinsic and intrinsic occurrences. Inappropriate manufacturing techniques or through transportation unusually accounts for intrinsic occurrences (intrinsic contamination). Extrinsic contamination on the other hand occurs during the use of the disinfectants within the healthcare environment Extrinsic contamination

is further reflected in contaminated in stock of disinfectants, that are contaminated not replacing disinfectants after long usage, failure to wash disinfectant jars prior to refilling as well as refilling containers that are contaminated (Rosca et al., 2021).

However, on the market today there are varieties of products available that presents with moderate or even inadequate antimicrobial action (Pitten et al., 2003). Ideally disinfectants must possess 'permanent' antimicrobial activity without compromising the danger of engendering resistant microorganisms. Thus, disinfectants must not only be easy to use but safe, and effective against an extensive range of microbial pathogens, without leaving any toxic residues (Tipton et al., 2018; Simoes et al., 2010; Fraise, 1999). However, many medical facilities in developing nations are still actively using phenolic disinfectants, contrary to the developed nations where their use is being discouraged. Similarly, the use of gluteraldehydes has been ceased, due to toxicity and related issues (BSG Guidelines, 2003). However, in developing nations, they are still in active use. Additionally, some local companies have gotten into the production of disinfectants locally with very little or no regulation to guarantee quality.

Regrettably, most healthcare facilities such as hospitals in developing world, lack the needed resources to evaluate and monitor the efficacy of these new disinfectants against set microbiological standards before usage. The aim of this paper was to assess long term effectiveness and the stability of disinfectants currently used in healthcare settings in Accra, Ghana.

MATERIALS AND METHODS

Disinfectants usually used in hospitals and other health care settings in Accra were randomly selected for this research work. Two locally produced and two imported disinfectants were obtained randomly from different places within Accra. The samples obtained were labelled and transported to the laboratory in their original packages. Contents of the various disinfectants were aseptically withdrawn from their respective containers for the antimicrobial study. They were then prepared according to manufacturer's instructions. The composition and pH of the disinfectants used in the study are presented in Table 1. Data analysis was performed using GenStat statistical analysis soft software.

Table 2. Inhibition of disinfectants against *Pseudomonas aeruginosa* microbial count (Log₁₀ cfu/ml) of survivors.

Disinfectant Identity	Working concentration	Bacteria Count		Interpretation	Standard
		Bacteria count at 37°C (3-day contact time)	Bacteria count at room temperature (7 days contact time)		
I	81 to 2919 ml Dist. H ₂ O	100 (2)	230(2.36)	Fail	5≥CFU on
II	13 to 2000 ml Dist. H ₂ O	307(2.49)	TNTC	Fail	either
III	50 ml to 4.5 l of water	TNTC	TNTC	Fail	plate=Fail, 5≤CFU on
IV	One part to three parts of water (1:3)	TNTC	TNTC	Fail	either plate=Pass

Log₁₀ counts are shown in brackets while raw counts are outside. TNTC implies too numerous to count

Table 3. Inhibition of disinfectants against *Staphylococcus aureus* (Microbial count (Log₁₀ cfu/ml) of survivors.

Disinfectant Identity	Working concentration	Bacteria Count		Interpretation	Standard
		Bacteria count at 37°C (3-day contact time)	Bacteria count at room temperature (7 days contact time)		
I	81 to 2919 ml Dist. H ₂ O	92 (1.96)	197(2.29)	Fail	5≥CFU on
II	13 to 2000 ml Dist. H ₂ O	288(2.46)	320(2.51)	Fail	either
III	50 ml to 4.5 L of water	TNTC	TNTC	Fail	plate=Fail, 5≤CFU on
IV	One part to three parts of water (1:3)	TNTC	TNTC	Fail	either plate=Pass

Log₁₀ counts are shown in brackets while raw counts are outside. TNTC implies Too Numerous to Count.

Microbial culture and inoculum preparation

Identified microbial culture isolates of *Pseudomonas aeruginosa* - ATCC 15442 and *Staphylococcus aureus* - ATCC 8043 were obtained for this study. A 24 h culture of the test isolates was prepared; *Staphylococcus aureus* isolate was prepared on a Baird Parker agar (Oxoid). *Pseudomonas aeruginosa* on the other hand was prepared on a Pseudomonas C-N agar (Oxoid). Each of the isolates was subsequently sub-cultured into 1% sterile peptone water and the turbidity concentration standardized to 0.5 McFarland standards corresponding to 10⁸ cfu ml⁻¹. Pure culture inoculum of both microbes was thoroughly mixed on a vortex mixer for 2 min after which 2.5 ml of each organism was pipetted aseptically into a sterile universal bottle. A mixed inoculum from the two microbes was also prepared and the turbidity standardized as above.

Disinfectant testing

The efficacy and long-term stability of the disinfectants obtained was done using the in-use method (Burdon and Whitby, 1967). The in-use test method was opted for because of its ease to use and requires no sophisticated or complicated equipment. Plastic screw-capped tubes, each containing 9 ml of disinfectants, were used. The manufacturers' instructions were followed to the letter during the preparation of the disinfectant for the test. Tubes were prepared in triplicates (3 sets) for each disinfectant as follows: 1 for *Pseudomonas aeruginosa*, 1 for *Staphylococcus aureus* and 1 for a mixture of both microbes. Dirty conditions were stimulated by the addition of 1ml of blood to each test tube. 1 ml of each standard inoculum was then added to each disinfectant. The preparations

were then kept at room temperature in the laboratory for 4 days. 1 ml of the prepared sample was aseptically withdrawn from each disinfectant after a proper shake to mix. It was then added to sterile peptone water (0.1%) that contained 3% between 80 (an activator). Ten drops from each diluted sample were subsequently placed on Nutrient Agar plates, and kept for seven days at 37°C in an incubator and also at room temperature. Contamination was indicated by the presence of 5 or more colonies on either plate.

RESULTS

The results in Table 2 present the sensitivity results of *Pseudomonas aeruginosa* to the four disinfectants that were tested. The results as shown in Table 2 indicate that at both room temperature and at 37°C incubation all the disinfectants (4) failed the test against *Pseudomonas aeruginosa*. A similar observation is seen in Table 3, where *Staphylococcus aureus* was used as the test organism. However, Local products (disinfectant III and disinfectant IV) had higher counts (Too Numerous to Count- TNTC) of *Staphylococcus aureus* than imported products (disinfectant I and disinfectant II) which ranged from 92CFU-320CFU per plate. Sensitivity of a mixed inoculum of *Staphylococcus aureus* and *Pseudomonas* concentrations tested. However, locally produced disinfectants (disinfectant III and disinfectant IV) had

Table 4. Inhibition of disinfectants against a mixed population of *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Microbial count (Log₁₀ cfu/ml) of survivors).

Disinfectant Identity	Working Concentration	Bacteria count		Interpretation	Standard
		Bacteria count at 37°C (3-day contact time)	Bacteria count at room temperature (7 days contact time)		
I	81 m to 2919 ml Dist. H ₂ O	120(2.08)	260(2.41)	Fail	5≥CFU on either
II	13 ml to 2000 ml Dist. H ₂ O	TNTC	TNTC	Fail	plate=Fail, 5≤CFU on either
III	50 ml to 4.5 L of water	TNTC	TNTC	Fail	plate=Pass
IV	One part to three parts of water (1:3)	TNTC	TNTC	Fail	plate=Pass

Log₁₀ counts are shown in brackets while raw counts are outside. TNTC implies Too Numerous to Count.

higher microbial counts were compared to imported products (disinfectant II and disinfectant I). Interestingly, *Enterobacter* spp was isolated as a contaminant in disinfectant II, while *Proteus* spp was also isolated from disinfectant III.

DISCUSSION

The results from this current study found all of disinfectants tested to be contaminated at the conditions and concentrations tested. Generally, locally produced disinfectants, both of which contain chlorine compounds were found to be more contaminated compared to disinfectants that were imported. The very high resistance of *Staphylococcus aureus* and *Pseudomonas aeruginosa* to the tested disinfectants (Table 4) is of great concern. *Staphylococcus aureus* and *Pseudomonas aeruginosa* are associated pneumonia, cystic fibrosis and chronic wound (Kawamura-Sato et al., 2010; Harrison, 2007). Originally, only a hostile relationship between both organisms was portrayed as the existence of one is associated with the absence of the other (Harrison, 2007). In wound infection for example, *Staphylococcus aureus* mostly inhabits the wound surface; whereas *Pseudomonas aeruginosa* is located in the deep layers (Kirketerp-Møller, 2008). However, in this study *Staphylococcus aureus* and *Pseudomonas aeruginosa* appeared to have acted in synergy presenting with high resistance and contamination (Table 4).

This study also found that disinfectant I and III were contaminated with *Enterobacter* spp and *Proteus* spp respectively. *Proteus* rods are well known opportunistic microbial pathogen. They cause urinary tract infection under favorable conditions (Hasan et al., 2021; Hamilton and Kamm, 2018). They have also caused rheumatoid arthritis, meningitis in infants, and wound infection (Hasan et al., 2021). *Enterobacter* species on the other hand are known to cause a wide range of hospital acquired infections including but not limited to those that affect the urinary tract, the lungs, intraabdominal cavity

(Toleti et al., 2015; Dautzenberg et al., 2018). *E. sakazakii* causes meningitis and neonatal sepsis (Elkhawaga et al., 2020). Thus, the contamination of disinfectants as observed in this study by the aforementioned pathogenic microorganisms is of huge significance. In the case of disinfectant II and disinfectant IV, no microorganisms aside the test bacteria were identified; which suggest these disinfectants were more active in the presence of organic matter than the other disinfectants.

Contamination of disinfectants has been linked to nosocomial infection and thus it is of a serious concern. Several studies have demonstrated this. For example, contaminated solution of chlorhexidine was reported by Mitchell and Hayward (1966) to have caused urinary tract infections in children after a cystoscopy procedure.

Contaminated disinfectants could arise due to a number of reasons including but not limited to contaminated disinfectant stock, prolonged usage of disinfectants without changing, improper washing and cleaning of jars before the next refilling and refill of containers that are contaminated (Kirketerp-Møller, 2008). In this present study, the contamination observed in the disinfectants could be as a result of overgrowth of test organisms and residual microorganisms as a result of the inactivation of some of the disinfectants tested. This may be accounted for when the inoculum was left until the fourth day in the disinfectant. Furthermore, the blood that was added to stimulate dirty condition may have also aided the subsequent inactivation of the active ingredients in the disinfectants. In an earlier study, Lewis and Arens (1995) indicated all manner of organic matter presented in the form of fecal, pus, blood or lubricant material may have an adverse interference with the antimicrobial activity and potency of disinfectants. This may occur in two ways first, through a chemical reaction between disinfectant and the organic material, thus less active ingredient in the disinfectant to attack the microbes. Second, the organic material may shield the microbes from attack by forming a physical barrier (Lewis and Arens, 1995; Sasaki and Imazato, 2020).

CONCLUSION AND RECOMMENDATIONS

In conclusion, all though, all disinfectants did not pass the in-use test, disinfectants that are produce locally are more unstable and ineffective. However, the key purpose of disinfectant usage is premised on their ability to efficiently and effectively curtail the spread of pathogenic microorganisms transmitting by direct or indirect contact within the healthcare environment. This has dire implication for infection control measures. This is because health workers and patients seeking health services in healthcare settings may acquire infection through contaminated and ineffective disinfectants. Efforts ought to be made towards the regular testing of disinfectants that are used in the hospital to monitor and ensure its efficacy.

Limitation

The efficacy and long-term stability of the disinfectants was done using only, two identified microbial culture isolates (*Pseudomonas aeruginosa* and *Staphylococcus aureus*). Thus, recommendations cannot be generalized to all bacteria. However, this effect is mitigated since *Pseudomonas aeruginosa* (gram negative) and *Staphylococcus aureus* (gram positive), represents the two main classes of bacteria that is, gram-negative and gram-positive bacteria.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

- Abbasi FM, Ahmad H, Perveen F, Inamullah, Sajid M, Brar DS (2010). Assesment of genomic relationship between *Oryza sativa* and *Oryza australiensis*. African Journal of Mathematics and Computer Science Research 9(12):1312-1316.
- Barrasa-Villar JI, Aibar-Remón C, Prieto-Andrés P, Mareca-Doñate R, Moliner-Lahoz J (2017). Impact on morbidity, mortality, and length of stay of hospital-acquired infections by resistant microorganisms. Clinical Infectious Diseases 65:644-52.
- BSG Guidelines: Guidelines for Decontamination of Equipment for Gastrointestinal endoscopy, 2003 at www.bsg.org.uk
- Burdon D, Whitby JL (1967). Contamination of hospital disinfectants with *Pseudomonas* species. British Medical Journal 2(5545):153.
- Cadnum JL, Shaikh AA, Piedrahita CT, Sankar T, Jencson AL, Larkin EL, Ghannoum MA, Donskey CJ (2017). Effectiveness of disinfectants against *Candida auris* and other *Candida* species. Infection Control and Hospital Epidemiology 38(10):1240-1243.
- Dautzenberg MJ, Bayjanov JR, Leverstein-van Hall MA, Muller AE, Gelinck LB, Jansen CL, Leyten EM, Ruys T, Scharringa J, van der Starre RE, Fluit AC (2018). Dynamics of colistin and tobramycin resistance among *Enterobacter cloacae* during prolonged use of selective decontamination of the digestive tract. Antimicrobial Resistance and Infection Control 7(1):1-9.
- Dulake C, Kidd E (1966). Contaminated irrigating fluid. The Lancet 287(7444):980.
- Elkhwaga AA, Hetta HF, Osman NS, Hosni A, El-Mokhtar MA (2020). Emergence of *Cronobacter sakazakii* in cases of neonatal sepsis in upper Egypt: first report in North Africa. Frontiers in Microbiology 11:215.
- Fraiese AP (1999). Choosing disinfectants. Journal of Hospital Infection 43(4):255-264.
- Hamilton AL, Kamm MA, Ng SC, Morrison M (2018). *Proteus* spp. asymptomatic gastrointestinal pathogens. Clinical Microbiology Reviews 31(3):e00085-17.
- Harrison F (2007). Microbial ecology of the cystic fibrosis lung. Microbiology 153(4):917-923.
- Hasan TH, Alasedi KK, Jaloob AA (2021). *Proteus Mirabilis* Virulence Factors. International Journal of Pharmaceutical Research 13(1).
- Kampf G (2018). Efficacy of ethanol against viruses in hand disinfection. Journal of Hospital Infection 98(4):331-338.
- Kawamura-Sato K, Wachino JI, Kondo T, Ito H, Arakawa Y (2010). Correlation between reduced susceptibility to disinfectants and multidrug resistance among clinical isolates of *Acinetobacter* species. Journal of antimicrobial chemotherapy 65(9):1975-1983.
- Kirketerp-Møller K, Jensen PØ, Fazli M, Madsen KG, Pedersen J, Moser C, Tolker-Nielsen T, Høiby N, Givskov M, Bjarnsholt T (2008). Distribution, organization, and ecology of bacteria in chronic wounds. Journal of clinical microbiology 46(8):2717-2722.
- Kollef MH, Torres A, Shorr AF, Martin-Loeches I, Micek ST (2021). Nosocomial Infection. Critical care medicine 49(2):169-187.
- Lewis DL, Arens M (1995). Resistance of microorganisms to disinfection in dental and medical devices. Nature Medicine 1(9):956-958.
- Ling HW (2020). What do We Need to Know to Prevent and Control Nosocomial Infections Completely? International Journal of Collaborative Research on Internal Medicine and Public Health 12(4):1021-1021.
- Liu JY, Dickter JK (2020). Nosocomial Infections: A History of Hospital-Acquired Infections. Gastrointestinal Endoscopy Clinics 30(4):637-652.
- Mitchell RG, Hayward AC (1966). Postoperative urinary-tract infections caused by contaminated irrigating fluid. The Lancet 287(7441):793-795.
- Nasiri N, Gholipour S, Akbari H, Koolivand A, Abtahi H, Didehdar M, Rezaei A, Mirzaei N (2021). Contamination of obstetrics and gynecology hospital air by bacterial and fungal aerosols associated with nosocomial infections. Journal of Environmental Health Science and Engineering 19(1):663-670.
- Nejad SB, Allegranzi B, Syed SB, Ellis B, Pittet D (2011). Health-care-associated infection in Africa: a systematic review. Bulletin of the World Health Organization 89:757-765.
- Pitten FA, Werner HP, Kramer A (2003). A standardized test to assess the impact of different organic challenges on the antimicrobial activity of antiseptics. Journal of Hospital Infection 55(2):108-115.
- Rosca I, Ursu EL, Fifere A (2021). A Microbiological Epilogue-Nosocomial Infections. New Trends in Macromolecular and Supramolecular Chemistry for Biological Applications, pp. 179-189. Springer, Cham.
- Rutala WA (1951). Guidelines Committee: Draft Apic guideline for selection and use of disinfectants. American journal of infection control 17(1):24A-38A.
- Sasaki JI, Imazato S (2020). Autoclave sterilization of dental handpieces: A literature review. Journal of Prosthodontic Research 64(3):239-242.
- Simoes M, Simoes LC, Vieira MJ (2010). A review of current and emergent biofilm control strategies. LWT-Food Science and Technology 43(4):573-583.
- Tipton KA, Chin CY, Farokhyfar M, Weiss DS, Rather PN (2018). Role of capsule in resistance to disinfectants, host antimicrobials and desiccation in *Acinetobacter baumannii*. Antimicrobial Agents and Chemotherapy 62(12):e01188-18.
- Toleti S, Manivasagan M, Prudhve S, Raavi T, Shaik N, Myneni RB (2015). *Candida* and *Enterobacter* co-infection in a case of periampullary carcinoma with obstructive jaundice. International Journal of Research in Medical Sciences 3(7).

Full Length Research Paper

Isolation, identification and antibiogram of verotoxin producing *Escherichia coli* from raw salad vegetables at Jashore, Bangladesh

Nigar Sultana Meghla^{1*}, Debashish Mridha², Md. Sohel Rana¹, Md. Ahsanul Haque Shahid² and Md. Muket Mahmud²

¹Department of Microbiology, Faculty of Biological Science and Technology, Jashore University of Science and Technology, Jashore, Bangladesh.

²Department of Microbiology and Hygiene, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh, Bangladesh.

Received 3 June, 2021; Accepted 23 July, 2021

Verotoxin-producing *E. coli* are getting an emergence for human health and day by day, it is also becoming more alarming in the fact that, they are acquiring multidrug-resistant profiles. This study aimed at isolation and identification of verotoxigenic *E. coli* followed by the antibiogram of the species from salad vegetables at Jashore district in Bangladesh. A total of 119 raw vegetable salad samples were collected from different areas of the Jashore district. The collected samples were initially enriched in nutrient broth and then used for streak plating on MacConkey and followed by Eosin Methylene Blue (EMB) agar media. *Escherichia coli* species were isolated and identified following observation of the cultural, microscopic, and biochemical characteristics and further analyzed to determine the presence of verotoxin (VT) producing genes through polymerase chain reaction (PCR) using specific primers (VT1, VT2, and Eae). A panel of antibiotics were tested following the disc diffusion method for determining the antibiotic profile. Total 55 (46.22%) *E. coli* was isolated phenotypically. 6 (10.9%) isolated *E. coli* showed positive by amplifying verotoxin-producing gene *vt1*, but none of the samples were positive *vt2* and *eaeA* gene. Antibiogram for 55 *E. coli* isolates showed the highest resistance to Erythromycin (83.64%), Streptomycin (78.18%), Ceftriaxone (70.91%). On the other hand, the most heightened susceptibility was observed for Nalidixic acid (76.36%). Moreover, six *vt1* positive isolates showed a multidrug-resistant profile. Thus, the presence of toxigenic genes in the isolates suggests the proper maintenance and regular monitoring of raw salad items should be increase to ensure healthy life in this region.

Key words: Vegetable salad, *Escherichia coli*, verotoxin, antimicrobial resistant, polymerase chain reaction (PCR).

INTRODUCTION

Escherichia coli is one of the most common bacteria on the planet. Theodor Escherich, a German bacteriologist,

was the first to discover it in 1885. As a result, this bacteria was named after him. *E. coli* is a Gram-negative

*Corresponding author. E-mail: nigar@just.edu.bd.

facultative anaerobe that does not generate spores (Parvej et al., 2018). The cells are generally rod-shaped, with a diameter ranging from 0.2 to 1 μm and a length of around two μm . Flagella-containing strains are motile, and the flagella are frequently arranged in a peritrichous pattern. It is typically found in animal excrement, mammalian lower intestines, and on the outskirts of hot springs. They thrive at 37°C. These bacteria live mainly in the intestines of warm-blooded mammals. *E. coli* contains a broad spectrum of strains with actions ranging from harmful to helpful (Law, 2000).

Verotoxin-producing *E. coli* (VTEC) is an unusual *E. coli* strain. O26:H11 was the most prevalent VTEC isolated from sporadic instances of hemolytic uremic syndrome (HUS) (Foxman, 2010). Verotoxins, also known as Shiga toxin, initially found in *Shigella* dysentery. However, the genes for their manufacture are easily spread across *E. coli* strains by toxin-encoding bacteriophages (Herold et al., 2004). The antigenically unique toxins VT1 and VT2 are encoded by two individual special lysogenic lambdaoid bacteriophages (933J and 933W) introduced into the chromosome of *E. coli* 933 (Nazmul et al., 2008). VT phages have also been found in strains belonging to serogroups O111, O119, O128, and O157 (Ranjbar et al., 2017). Although this virulence factor (VT) may be found in a variety of serotypes, only a few well-characterized bio-serotypes and clones, especially O157:H7 and O26:H11, are the most significant hosts for the verotoxin phage(s) and are distributed globally (Nazmul et al., 2007).

Infections produced by isolates of VTEC serotypes other than the O157 have received more attention in recent years. Non-O157 VTEC infections are becoming more common in several countries. Many non-O157:H7 isolates linked to outbreaks do not have *eae* or the pO157 plasmid (Gyles, 2007), suggesting more non-O157 VTEC strains are not yet non-pathogenic. As a result, enhancing our understanding of these organisms requires efficient detection, isolation, and characterization of non-O157 VTEC isolates. The universality of the Polymerase Chain Reaction (PCR) test for amplification of *vt1* and *vt2* gene sequences has been proven, with both toxin genes being identified and distinguished across a wide range of verotoxigenic *E. coli*. The PCR approach allows for the quick, reliable, and low-cost identification of these critical toxin genes in clinical samples and potentially contaminated foods (Hamed et al., 2017).

Recently all over the world, food safety issues are given much concern because of the increased number of foodborne illnesses (WHO, 2002; Peattie, 2006). According to World Health Organization (WHO), Foodborne illness is referred to as hazardous for human health when the pathogen-associated contaminated food enters the human body (WHO, 2002). VTEC isolates are commonly present in food. Predictions are made of the possible increase in problems associated with these

emerging pathogens (Younus et al., 2020; Parvej et al., 2018). As time passed, the food habits of ordinary people have changed rapidly in Bangladesh. Nowadays it is more popular in this country to take a meal in a nearby hotel or restaurant rather than eating homemade food. Here in Bangladesh, Restaurant and hotels are not only just food places but also assessed as refreshment sites. In restaurants, hotels, roadside food carts, along with different fast or Bengali foods, salad are common for all types of dishes. Mainly seasonal vegetables are used for making this salad, such as tomato, carrot, cucumber, coriander leaf, radish, and green chili are very common among them (Younus et al., 2020).

Jashore is the third-largest city in the Khulna Division and one of the most populous cities in Bangladesh's southwestern area. It is the second most developed city in the Khulna Division and Bangladesh's most industrious and progressive city. According to the Jashore municipality's records, it has a population of around 0.298 million people. There are various roadside food stores or restaurants that offer a range of foods with a vegetable salad throughout the city. A vast variety of microorganisms, particularly verotoxin-producing *E. coli*, can be found in vegetables. This organism has been associated with outbreaks connected to fresh vegetable intakes, such as lettuce, spinach, carrots, sprouts, and alfalfa (Younus et al., 2020). According to outbreak research, *E. coli* may live and thrive in a variety of minimally processed vegetables (Kabir et al., 2014). It can also withstand freezing temperatures and extreme environmental conditions. Still now there is no study on the microbiological aspect of this vegetable salad in this region. It is necessary to evaluate the food safety issue of this served vegetable salad from a microbial aspect. So, this study was conducted for isolation, identification, and antibiogram of verotoxin-producing *E. coli* from raw salad vegetables at Jashore, Bangladesh.

MATERIALS AND METHODS

Sample collection

In this study, raw vegetables were selected as they are often eaten raw without any heat treatment, sometimes without washing and peeling. Total 119 vegetable samples (25 carrots, 22 cucumbers, 27 tomatoes, 20 radishes, 15 coriander leaves, and 10 green chilies) were collected from different locations at Jeshore in Bangladesh. Sterile zip bags (wiped with 70% ethanol) were used to collect salad vegetables, and the samples were carried to the laboratory without any delay.

Isolation and identification

After processing the collected samples, the nutrient broth was used for preliminary enrichment of the bacteria where the incubation temperature was 37°C for overnight. Overnight incubated test tubes then streaked on MacConkey and Eosin Methylene Blue (EMB) (Both were HiMedia, India) agar media for isolation of the

Table 1. Oligonucleotide sequences used in this study to detect verotoxin producing *E. coli*.

Primer	Target gene	Primer sequence	Annealing Tm (°C)	Amplification size (KB)	Reference
VT1	<i>vt1</i>	5'-CAAGAGTCCGTGGGATTACG-3' 5'-AGCGATGCAGCTATTAATAA-3'	55	130	
VT2	<i>vt2</i>	5'-ACCGTTTTTCAGATTTACACATA-3' 5'-TACACAGGAGCAGTTTCAGACAGT-3'	56	298	Lindeberg et al. (2018)
Eae	<i>eaeA</i>	5'-CACACGAATAAACTGACTAAAATG-3' 5'-AAAAACGCTGACCCGCACCTAAAT-3'	58	376	

Table 2. Prevalence of *E. coli* in different vegetable samples.

Types of vegetables	No. of examined vegetables	No. of vegetable with culture-positive <i>E. coli</i>	% of vegetables
Carrot	25	13	52
Cucumber	22	7	31.81
Tomato	27	10	37.04
Radish	20	12	60
Coriander leaf	15	8	53.33
Green chili	10	5	50
Total	119	55	46.22

suspected organism. *E. coli* produced a pinkish colony on MacConkey agar and a green metallic sheen containing colony on EMB agar. To confirm the microscopic examination, a loop of bacteria from the suspected colony was taken for Grams stain and placed the slide under 100X microscopic view (Punom et al., 2020).

Biochemical tests

Oxidase test and citrate utilization test were performed with culture-positive isolates according to the methods described in the Microbiology Laboratory Manual (Cheesbrough, 2006). Different sugar fermentation tests such as Dextrose, Mannitol, Lactose, Maltose, and Sucrose were done to confirm the organism (Punom et al., 2020).

Molecular detection

Genomic DNA was extracted by the conventional crude boiling method (Mishra et al., 2020). In short, 2-3 fresh cultured colonies were taken in a sterile 1.5 ml microcentrifuge tube and added 200 µl sterile Milli-Q water. Vortexed thoroughly and heated the microcentrifuge tube at 99°C for 10 min, followed by rapidly frozen at -20°C and centrifuged at 13,000 rpm for 5 min. Finally, 100 µl supernatant was collected and used as the DNA template in a PCR. The oligonucleotide primers used to amplify the genes by PCR are mentioned in Table 1, and the PCR reaction mixture was prepared as the author described in their study. The PCR products were visualized on a gel documentation system (Biometra, Jena, Germany) after electrophoresis with 1.5% agarose gel (SeaKem® LE Agarose from Lonza) as described by other researcher (Shahid et al., 2021).

Antimicrobial susceptibility test

The antimicrobial susceptibility was carried out for all the phenotypically positive isolates using the standardized agar disc diffusion method recommended by the Clinical Laboratory and Standards Institute (CLSI, 2018). Commercially available disc and Mueller Hinton Agar (Both were HiMedia, India) were used for the antimicrobial assay. Total 10 antibiotics disc from a different group of antimicrobial agents (Amoxicillin, 10 µg; Azithromycin, 15 µg; Ceftriaxone, 30 µg; Chloramphenicol, 30 µg; Ciprofloxacin, 5 µg; Erythromycin, 15 µg; Gentamycin, 10 µg; Nalidixic acid, 30 µg; Streptomycin, 10 µg and Tetracycline, 30 µg) were used in this study. Interpretation of the test results was made according to the CLSI guidelines and recorded in an excel file.

RESULTS AND DISCUSSION

Out of 119 vegetable samples, 55 samples were positive for the presence of *E. coli*. These positive samples were phenotypically confirmed where cultural characteristics on different agar media, microscopic examination, and biochemical test results were the parameters. The present study showed that the overall prevalence of *E. coli* was 46.22% (n=55/119). The highest and lowest incidence of *E. coli* were 60% and 31.81% found in the radish and cucumber, respectively (Table 2). *E. coli* produced small, round, smooth, and greenish colonies onto EMB agar and pink-colored colonies on MacConkey agar. They were Gram-negative and small rod-

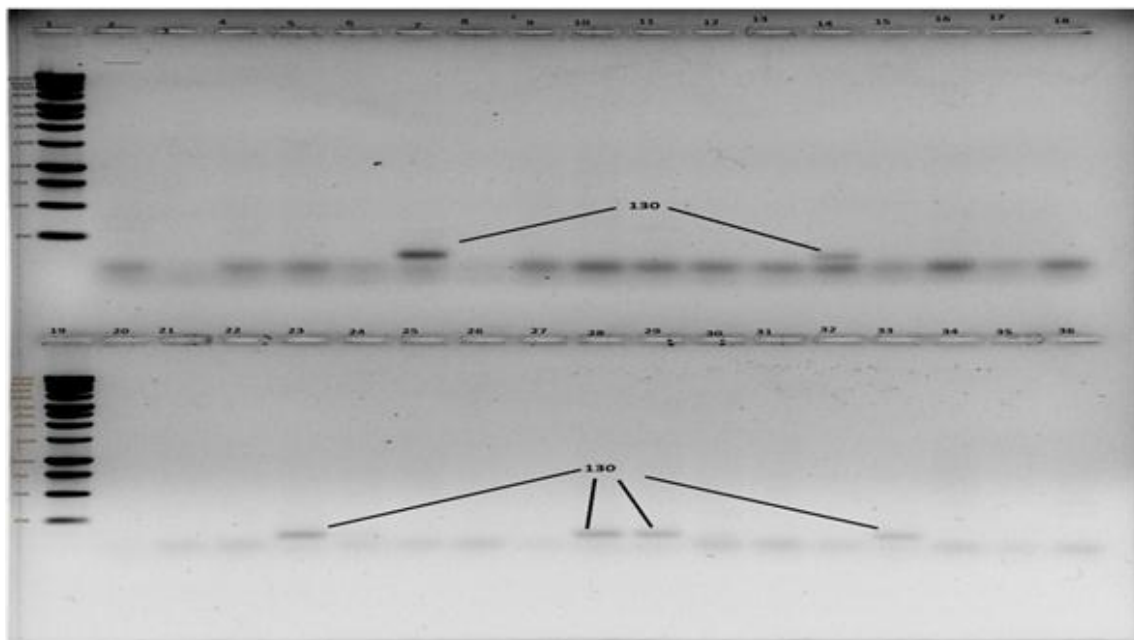


Figure 1. PCR amplification of *Vt1* gene of verotoxin-producing *E. coli*; Lane 1 and 19 are 1kb DNA ladder; Lane 7, 14, 23, 28, 29, and 33 are the positive *E. coli* isolates where they amplify verotoxin-producing *Vt1* gene at 130 bp.

Table 3. Antibiotic susceptibility profile of *E. coli* isolates.

Antibiotic name	Antibiotic sensitivity pattern (%) of <i>E. coli</i>		
	Susceptible % (n/N)	Intermediate % (n/N)	Resistant % (n/N)
Azithromycin	61.82 (34/55)	14.55 (8/55)	23.64 (13/55)
Ceftriaxone	12.73 (7/55)	16.64 (9/55)	70.91 (39/55)
Nalidixic acid	76.36 (42/55)	12.73 (7/55)	10.91 (6/55)
Chloramphenicol	58.18 (32/55)	25.45 (14/55)	16.36 (9/55)
Gentamycin	25.45 (14/55)	36.36 (20/55)	38.18 (21/55)
Erythromycin	0	16.36 (9/55)	83.64 (46/55)
Streptomycin	0	21.82 (12/55)	78.18 (43/55)
Ciprofloxacin	52.72 (29/55)	27.27 (15/55)	20 (11/55)
Amoxicillin	69.09 (38/55)	18.18 (10/55)	12.73 (7/55)
Tetracycline	63.64 (35/55)	20 (11/55)	16.36 (9/55)

shaped under microscopic view. Oxidase and Citrate tests were both negative for these isolates. All the suspected isolates fermented the sugars without producing any gas. The findings of microscopic exam and biochemical results were similar to the previously described in Punom et al. (2020), Younus et al., (2020) and Hasan et al. (2018).

A total of 6 isolates (cucumber-6a, cucumber-15c, carrot-11a, radish-4a, coriander leaf-13d, and coriander leaf-14a) were confirmed verotoxin producing *E. coli* by amplification of the *vt1* gene (Figure 1), but none of the samples were positive for *vt2* and *eaeA* verotoxin

producing genes. Antibioqram study of all *E. coli* isolates revealed that the highest was 83.64% (46), 78.18% (43), and 70.91% (39) isolates found resistant to Erythromycin, Streptomycin, and Ceftriaxone, respectively. 76.36% (42) and 69.09% (38) isolates showed susceptibility to Nalidixic acid and Amoxicillin antibiotics (Table 3 and Figure 2). Isolates of verotoxin-producing *E. coli* were found multidrug-resistant.

For maintaining a healthy life there is no alternative to take fresh fruit and vegetables. Because of their low fat and high vitamin, mineral contents, day by day total consumption ratio is increasing (Rekhy and McConchie,

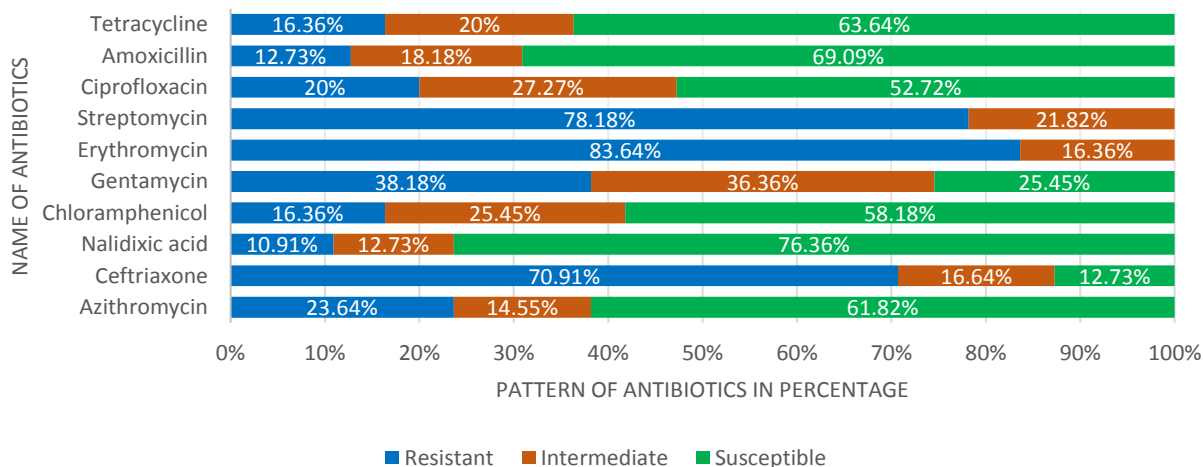


Figure 2. Pattern of antibiotics for the *E. coli* isolates from raw vegetable salad samples.

2014). Moreover, fresh fruit and vegetables are also prescribed to cardiovascular, cancer, diabetes-like diseased patients (Wang et al., 2014). But it must be ensured first that the consumable fruit and vegetables are free from all types of contamination. In this study, it had been found that Vegetable salad is working as a potential source of *E. coli*. It is predicted that foods are primarily contaminated during post-harvest handling and unhygienic processing environment. (Chowdhury et al., 2014; Alam et al., 2015). Moreover, the excess amount of use of antibiotics in farm areas is increasing the number of MDR organisms. It has been found that pesticide residue, which is used for agricultural purposes, may affect the body homeostasis of animals (Kobir et al., 2020).

During this investigation, one hundred nineteen raw vegetable samples were collected from a different market in Jashore, Bangladesh. Out of the 119 samples, *E. coli* was isolated from 55 samples, with a prevalence of 46% for this investigation. Isolation of *E. coli* from raw vegetables is also published in Dhaka city by several researchers (Islam et al., 2015; Kabir et al., 2014). But the prevalence ratio is relatively low. It might be the knowledge gap on the community level in this region, where farmers do not maintain the necessary hygienic measures during the production and harvesting of their products. The use of untreated wastewater and manure as fertilizers for producing fruits and vegetables is a major contributing factor to contamination (Rai and Tripathi, 2007). Also, restaurants, and hotel kitchens' hygienic condition is responsible for being so high prevalence in this area. Another source might be the middle man who is unconscious about the hygienic state, which plays a crucial role in contamination.

The *vt* gene was also detected in *E. coli* utilizing a PCR methodology and its molecular characterization in this investigation. In all, 55 *E. coli* isolates were tested for the presence of the *vt* gene using three sets of primers (VT1,

VT2, and Eae). 6 (10.9%) *E. coli* isolates were discovered to possess the *vt1* gene, but no isolates carried the *vt2* and *eaeA* gene. According to these findings, the *vt1* gene is the most frequent verotoxin found in vegetable strains. In bovine isolates, the *vt1* gene is the most frequent toxin, while in porcine isolates, the *vt2* gene is the most prevalent (Gyles, 2007). The presence of just the *vt1* gene in this study might be related to the geographical dispersion of the isolates or members of a single clone that has spread globally.

The antibiotic susceptibility test yielded that the isolates exhibited resistance to Erythromycin 83.64% (46), Streptomycin 78.18% (43), Ceftriaxone 70.91% (39), Nalidixic acid 76.36% (42), Amoxicillin 69.09% (38), Tetracycline 63.64% (35) antibiotics showed the highest susceptibility to the isolates. Antibiotic pattern normally depends on the use of antibiotics on an area. For example, in the Chittagong district, one study says the *E. coli* isolates from salad samples are highly resistant to Gentamycin, Ampicillin, and Streptomycin, where our isolate showed only 38.18% (21) resistant to Gentamycin. Their isolate showed 100% sensitivity to Ciprofloxacin. But in our study, we found only 52.72% (29) susceptible to this antibiotic (Nipa et al., 2011). For any specific region, a class of antibiotics may be successful in any outbreak, whereas it may work as resistant in another region. To eradicate or successfully treat any outbreak, along with genetic information, location-specific information (data containing which antibiotic is mostly resistant or susceptible in any location) is compulsory (Sarker et al., 2021). These developments of antibiotic resistance can be deadly to both humans and other animals. In the investigation, the highest resistance was found against Erythromycin and Streptomycin. Uncontrolled usage of antibiotics in the treatment of animals and their integration in animal feeds has been believed to account for the increase in antibiotic resistance (Kundu et al., 2021; Hassan et al., 2017;

Woolhouse et al., 2015). In Bangladesh, all open markets are more or less in the same arrangement or style. There all types of raw vegetables, fish, meat are sold nearby. In this unhygienic and damp environment of the open market, flies play a vital role in contaminating raw vegetables or other food products. Resistance of *E. coli* to the Penicillin group of antibiotics has been on the higher side and is increasing day by day in different parts of the world. Nalidixic acid, the most efficient antibiotic against the isolates, prevents protein chain elongation by inhibiting the peptidyl transferase activity of the bacterial ribosome.

Conclusion

It might be concluded from this study that the raw salad vegetables collected from different areas are contaminated with antibiotic-resistant *E. coli*. The highest resistance was found against Erythromycin 83.64% (46), Streptomycin 78.18% (43), Ceftriaxone 70.91% (39) drug discs. They also contain the *vt* gene where *vt1* was the most common verotoxin among the *E. coli* strains isolated from vegetables in Jashore, Bangladesh. *vt1* gene locates on *E. coli*, might play an important role in horizontal transfer of *vt* gene. Above all, we must be aware of proper washing of salad vegetables.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

The authors are grateful to hotel and restaurant owners for giving us access to collect samples during the study and this research was funded by R & D (Research and development) project of MOST (Ministry of Science and Technology), Bangladesh.

REFERENCES

- Alam M, Chowdhury M, Hossain MS, Rahman MM, Rahman MA, Gan SH, Khalil M (2015). Detection of residual levels and associated health risk of seven pesticides in fresh eggplant and tomato samples from Narayanganj District, Bangladesh. *Journal of Chemistry*. http://eprints.usm.my/38639/1/Detection_of_Residual_Levels_and_Associated_Health_Risk_of_Seven_Pesticides_in_Fresh_Eggplant.pdf
- Cheesbrough M (2006). *District Laboratory Practice in Tropical Countries*. London English Language Book, pp. 100-194.
- Chowdhury AZ, Hasan M, Karim N, Fakhruddin ANM, Hossain S, Chowdhury AA, Alam K (2014). Contamination and health risk assessment of pesticide residues in vegetables from agricultural fields of Gazipur District, Bangladesh. *Sigma* 2(4).
- Clinical Laboratory Standards Institute (CLSI) (2018). Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; approved standard, VET08, Clinical and Laboratory Standards Institute, Wayne, PA.
- Foxman B (2010). The epidemiology of urinary tract infection. *Nature Reviews Urology* 7(12):653-660.
- Gyles CL (2007). Shiga toxin-producing *Escherichia coli*: an overview. *Journal of Animal Science* 85(13):45-62.
- Hamed OM, Sabry MA, Hassanain NA, Hamza E, Hegazi AG, Salman MB (2017). Occurrence of virulent and antibiotic-resistant Shiga toxin-producing *Escherichia coli* in some food products and human stool in Egypt. *Veterinary World* 10(10):1233.
- Hassan J, Nazir KNH, Parvej MS, Kamal T, Rahman MT (2017). Molecular based prevalence of shigatoxigenic *Escherichia coli* in rectal swab of apparently healthy cattle in Mymensingh district, Bangladesh. *Journal of Advanced Veterinary and Animal Research* 4(2):194-199.
- Herold S, Karch H, Schmidt H (2004). Shiga toxin-encoding bacteriophages—genomes in motion. *International Journal of Medical Microbiology* 294(2-3):115-121.
- Islam Z, Sultana S, Rahman MM, Rahman SR, Bari ML (2015). Effectiveness of different sanitizers in inactivating *E. coli* O157: H7 in Tomato and Cucumber. *Journal of Food and Nutrition Sciences* 3(1-2):60-64.
- Kabir A, Das AK, Kabir MS (2014). Incidence of antibiotic resistant pathogenic bacteria in vegetable items sold by local and super shops in Dhaka city. *Stamford Journal of Microbiology* 4(1):13-18.
- Kobir MA, Akther L, Hasan I, Shahid MAH, Haque Z, Karim MR (2020). Effects of Imidacloprid-Contaminated Feed Exposure on Hematological Parameters in Adult Rabbits (*Oryctolagus Cuniculus*). *Research in Agriculture Livestock and Fisheries* 7(3):439-444.
- Kundu T, Rumi NA, Hossain MK, Rahman MS, Hossain MMK, Halder J (2021). Isolation of multidrug-resistant *Escherichia coli* from turkeys in Dinajpur, Bangladesh, and their antibiogram profile. *Journal of Advanced Veterinary and Animal Research* 8(1):64.
- Law D (2000). The history and evolution of *Escherichia coli* O157 and other Shiga toxin-producing *E. coli*. *World Journal of Microbiology and Biotechnology* 16(8):701-709.
- Lindeberg YL, Egedal K, Hossain ZZ, Phelps M, Tulsiani S, Farhana I, Jensen PKM (2018). Can *Escherichia coli* fly? The role of flies as transmitters of *E. coli* to food in an urban slum in Bangladesh. *Tropical Medicine and International Health* 23(1):2-9.
- Mishra P, Mahmud MM, Shahid MAH, Hasan A, Yadav VK, Hasan M (2020). Molecular detection of methicillin resistant staphylococcus aureus (MRSA) from a clinical case of myiasis wound: a case report. *Authorea Preprints*. <http://researcherslinks.com/current-issues/Molecular-Detection-of-Methicillin-resistant-Staphylococcus-aureus-MRSA-from-a-Clinical-Case-of-Myiasis-Wound/18/25/3044/html>.
- Nazmul MHM, Salmah I, Jamal H, Ansary A (2008). Molecular characterization of verotoxin gene in enteropathogenic *Escherichia coli* isolated from Miri Hospital, Sarawak, Malaysia. *Biomedical Research* 19(1):9-12.
- Nazmul MHM, Salmah I, Jamal H, Ansary A (2007). Detection and molecular characterization of verotoxin gene in non-O157 diarrheagenic *Escherichia coli* isolated from Miri hospital, Sarawak, Malaysia. *Biomedical Research* 18(1):39-43.
- Nipa MN, Mazumdar RM, Hasan MM, Fakhruddin MD, Islam S, Bhuiyan HR, Iqbal A (2011). Prevalence of multi drug resistant bacteria on raw salad vegetables sold in major markets of Chittagong city, Bangladesh. *Middle-East Journal of Scientific Research* 10(1):70-77.
- Parvej MS, Mamun M, Hassan J, Mahmud MM, Rahman M, Rahman MT, Nazir KNH (2018). Prevalence and characteristics of Shiga-toxin producing *Escherichia coli* (STEC) isolated from beef slaughterhouse. *Journal of Advanced Veterinary and Animal Research* 5(2):218-225.
- Peattie K (2006). Corporate social responsibility and the food industry—Ken Peattie discusses why developing a CSR agenda is so important. *Food Science Technology-Information Quarterly of the Institute of Food Science and Techn* 20(2):46-48.
- Punom SA, Khan MSR, Pritha ST, Hassan J, Rahman S, Mahmud MM, Islam MS (2020). Isolation and molecular-based identification of bacteria from unhatched leftover eggs of ducks in selected mini-hatcheries of Kishoreganj, Bangladesh. *Journal of Advanced Veterinary and Animal Research* 7(1):164.

- Rai PK, Tripathi BD (2007). Microbial contamination in vegetables due to irrigation with partially treated municipal wastewater in a tropical city. *International Journal of Environmental Health Research* 17(5):389-395.
- Ranjbar R, Masoudimanesh M, Dehkordi FS, Jonaidi-Jafari N, Rahimi E (2017). Shiga (Vero)-toxin producing *Escherichia coli* isolated from the hospital foods; virulence factors, o-serogroups and antimicrobial resistance properties. *Antimicrobial Resistance and Infection Control* 6(1):1-11.
- Rekhy R, McConchie R (2014). Promoting consumption of fruit and vegetables for better health. Have campaigns delivered on the goals?. *Appetite* 79:113-123.
- Sarker MSA, Shahid MAH, Hoque MN, Sarker MA, Rahman MB, Islam SS (2021). The The Rich Mapping: Be a Supplementary Approach for Anthrax Control at Community Level. *Journal of Advanced Veterinary Research* 11(1):41-46.
- Shahid MAH, Nazir KHMNH, Zowalaty MEE, Kabir A, Sarker MSA, Siddique MP, Ashour HM (2021). Molecular Detection of Vancomycin Resistance and Methicillin Resistance in *Staphylococcus aureus* from Food Processing Environments. *One Health* 100276.
- Wang X, Ouyang Y, Liu J, Zhu M, Zhao G, Bao W, Hu FB (2014). Fruit and vegetable consumption and mortality from all causes, cardiovascular disease, and cancer: systematic review and dose-response meta-analysis of prospective cohort studies. *British Medical Journal* P 349.
- World Health Organization (WHO) (2002). WHO global strategy for food safety: safer food for better health. WHO, Geneva, Switzerland, 2002.
- Woolhouse M, Ward M, van Bunnik B, Farrar J (2015). Antimicrobial resistance in humans, livestock and the wider environment. *Philosophical Transactions of the Royal Society B: Biological Sciences* 370(1670):20140083.
- Younus MI, Sabuj AAM, Haque ZF, Sayem SM, Majumder S, Parvin MS, ... Saha S (2020). Microbial risk assessment of ready-to-eat mixed vegetable salads from different restaurants of Bangladesh Agricultural University campus. *Journal of advanced veterinary and animal research* 7(1):34.

Related Journals:

