OPEN ACCESS

African Journal of **Biotechnology**

30 May 2018
ISSN 1684-5315
DOI: 10.5897/AJB
www.academicjournals.org

ACADEMIC JOURNALS
expand your knowledge

ABOUT AJB

The African Journal of Biotechnology (AJB) (ISSN 1684-5315) is published weekly (one volume per year) by Academic Journals.

African Journal of Biotechnology (AJB), a new broad-based journal, is an open access journal that was founded on two key tenets: To publish the most exciting research in all areas of applied biochemistry, industrial microbiology, molecular biology, genomics and proteomics, food and agricultural technologies, and metabolic engineering. Secondly, to provide the most rapid turn-around time possible for reviewing and publishing, and to disseminate the articles freely for teaching and reference purposes. All articles published in AJB are peer-reviewed.

Contact Us

Editorial Office: ajb@academicjournals.org

Help Desk: helpdesk@academicjournals.org

Website: http://www.academicjournals.org/journal/AJB

Submit manuscript online http://ms.academicjournals.me/

Editor-in-Chief

George Nkem Ude, Ph.D
Plant Breeder & Molecular Biologist
Department of Natural Sciences
Crawford Building, Rm 003A
Bowie State University
14000 Jericho Park Road
Bowie, MD 20715, USA

Editor

N. John Tonukari, Ph.D
Department of Biochemistry
Delta State University
PMB 1
Abraka, Nigeria

Associate Editors

Prof. Dr. AE Aboulata
Plant Path. Res. Inst., ARC, POBox 12619, Giza, Egypt
30 D, El-Karama St., Alf Maskan, P.O. Box 1567,
Ain Shams, Cairo,
Egypt

Dr. S.K Das
Department of Applied Chemistry and Biotechnology, University of Fukui,
Japan

Prof. Okoh, A. I.
Applied and Environmental Microbiology Research Group (AEMREG),
Department of Biochemistry and Microbiology,
University of Fort Hare.
P/Bag X1314 Alice 5700,
South Africa

Dr. Ismail TURKOGLU
Department of Biology Education, Education Faculty, Fırat University,
Elazığ, Turkey

Prof T.K.Raja, PhD FRSC (UK)
Department of Biotechnology
PSG COLLEGE OF TECHNOLOGY (Autonomous)
(Affiliated to Anna University)
Coimbatore-641004, Tamilnadu,
INDIA.

Dr. George Edward Mamati
Horticulture Department,
Jomo Kenyatta University of Agriculture
and Technology,
P. O. Box 62000-00200,
Nairobi, Kenya.

Dr. Gitonga
Kenya Agricultural Research Institute,
National Horticultural Research Center,
P.O Box 220,
Thika, Kenya

Editorial Board

Prof. Sagadevan G. Mundree
Department of Molecular and Cell Biology
University of Cape Town
Private Bag Rondebosch 7701
South Africa

Dr. Martin Fregene
Centro Internacional de Agricultura Tropical (CIAT)
Km 17 Cali-Palmira Recta
AA6713, Cali, Colombia

Prof. O. A. Ogunseitan
Laboratory for Molecular Ecology
Department of Environmental Analysis and Design
University of California,
Irvine, CA 92697-7070. USA

Dr. Ibrahima Ndoye
UCAD, Faculte des Sciences et Techniques
Departement de Biologie Vegetale
BP 5005, Dakar, Senegal.
Laboratoire Commun de Microbiologie
IRD/ISRA/UCAD
BP 1386, Dakar

Dr. Bamidele A. Iwalokun
Biochemistry Department
Lagos State University
P.M.B. 1087. Apapa – Lagos, Nigeria

Dr. Jacob Hodeba Mignouna
Associate Professor, Biotechnology
Virginia State University
Agricultural Research Station Box 9061
Petersburg, VA 23806, USA

Dr. Bright Ogheneovo Agindotan
Plant, Soil and Entomological Sciences Dept
University of Idaho, Moscow
ID 83843, USA

Dr. A.P. Njukeng
Département de Biologie Végétale
Faculté des Sciences
B.P. 67 Dschang
Université de Dschang
Rep. du CAMEROUN

Dr. E. Olatunde Farombi
Drug Metabolism and Toxicology Unit
Department of Biochemistry
University of Ibadan, Ibadan, Nigeria

Dr. Stephen Bakiamoh
Michigan Biotechnology Institute International
3900 Collins Road
Lansing, MI 48909, USA

Dr. N. A. Amusa
Institute of Agricultural Research and Training
Obafemi Awolowo University
Moor Plantation, P.M.B 5029, Ibadan, Nigeria

Dr. Desouky Abd-El-Haleem
Environmental Biotechnology Department &
Bioprocess Development Department,
Genetic Engineering and Biotechnology Research Institute (GEBRI),
Mubarak City for Scientific Research and Technology Applications,
New Burg-Elarab City, Alexandria, Egypt.

Dr. Simeon Oloni Kotchoni
Department of Plant Molecular Biology
Institute of Botany, Kirschallee 1,
University of Bonn, D-53115 Germany.

Dr. Eriola Betiku
German Research Centre for Biotechnology,
Biochemical Engineering Division,
Mascheroder Weg 1, D-38124,
Braunschweig, Germany

Dr. Daniel Masiga
International Centre of Insect Physiology and Ecology,
Nairobi,
Kenya

Dr. Essam A. Zaki
Genetic Engineering and Biotechnology Research
Institute, GEBRI,
Research Area,
Borg El Arab, Post Code 21934, Alexandria
Egypt

Dr. Alfred Dixon
International Institute of Tropical Agriculture (IITA)
PMB 5320, Ibadan
Oyo State, Nigeria

Dr. Sankale Shompole
Dept. of Microbiology, Molecular Biology and Biochemisty,
University of Idaho, Moscow,
ID 83844, USA.

Dr. Mathew M. Abang
Germplasm Program
International Center for Agricultural Research in the Dry Areas
(ICARDA)
P.O. Box 5466, Aleppo, SYRIA.

Dr. Solomon Olawale Odemuyiwa
Pulmonary Research Group
Department of Medicine
550 Heritage Medical Research Centre
University of Alberta
Edmonton
Canada T6G 2S2

Prof. Anna-Maria Botha-Oberholster
Plant Molecular Genetics
Department of Genetics
Forestry and Agricultural Biotechnology Institute
Faculty of Agricultural and Natural Sciences
University of Pretoria
ZA-0002 Pretoria, South Africa

Dr. O. U. Ezeronye
Department of Biological Science
Michael Okpara University of Agriculture
Umudike, Abia State, Nigeria.

Dr. Joseph Hounhouigan
Maître de Conférence
Sciences et technologies des aliments
Faculté des Sciences Agronomiques
Université d'Abomey-Calavi
01 BP 526 Cotonou
République du Bénin

Prof. Christine Rey
Dept. of Molecular and Cell Biology,
University of the Witwatersand,
Private Bag 3, WITS 2050, Johannesburg, South Africa

Dr. Kamel Ahmed Abd-Elsalam
Molecular Markers Lab. (MML)
Plant Pathology Research Institute (PPathRI)
Agricultural Research Center, 9-Gamma St., Orman, 12619,
Giza, Egypt

Dr. Jones Lemchi
International Institute of Tropical Agriculture (IITA)
Onne, Nigeria

Prof. Greg Blatch
Head of Biochemistry & Senior Wellcome Trust Fellow
Department of Biochemistry, Microbiology & Biotechnology
Rhodes University
Grahamstown 6140
South Africa

Dr. Beatrice Kilel
P.O Box 1413
Manassas, VA 20108
USA

Dr. Jackie Hughes
Research-for-Development
International Institute of Tropical Agriculture (IITA)
Ibadan, Nigeria

Dr. Robert L. Brown
Southern Regional Research Center,
U.S. Department of Agriculture,
Agricultural Research Service,
New Orleans, LA 70179.

Dr. Deborah Rayfield
Physiology and Anatomy
Bowie State University
Department of Natural Sciences
Crawford Building, Room 003C
Bowie MD 20715,USA

Dr. Marlene Shehata
University of Ottawa Heart Institute
Genetics of Cardiovascular Diseases
40 Ruskin Street
K1Y-4W7, Ottawa, ON, CANADA

Dr. Hany Sayed Hafez
The American University in Cairo,
Egypt

Dr. Clement O. Adebooye
Department of Plant Science
Obafemi Awolowo University, Ile-Ife
Nigeria

Dr. Ali Demir Sezer
Marmara Üniversitesi Eczacilik Fakültesi,
Tibbiye cad. No: 49, 34668, Haydarpasa, Istanbul,
Turkey

Dr. Ali Gazanchain
P.O. Box: 91735-1148, Mashhad,
Iran.

Dr. Anant B. Patel
Centre for Cellular and Molecular Biology
Uppal Road, Hyderabad 500007
India

Prof. Arne Elofsson
Department of Biophysics and Biochemistry
Bioinformatics at Stockholm University,
Sweden

Prof. Bahram Goliaei
Departments of Biophysics and Bioinformatics
Laboratory of Biophysics and Molecular Biology
University of Tehran, Institute of Biochemistry and Biophysics
Iran

Dr. Nora Babudri
Dipartimento di Biologia cellulare e ambientale
Università di Perugia
Via Pascoli
Italy

Dr. S. Adesola Ajayi
Seed Science Laboratory
Department of Plant Science
Faculty of Agriculture
Obafemi Awolowo University
Ile-Ife 220005, Nigeria

Dr. Yee-Joo TAN
Department of Microbiology
Yong Loo Lin School of Medicine,
National University Health System (NUHS),
National University of Singapore
MD4, 5 Science Drive 2,
Singapore 117597
Singapore

Prof. Hidetaka Hori
Laboratories of Food and Life Science,
Graduate School of Science and Technology,
Niigata University.
Niigata 950-2181,
Japan

Prof. Thomas R. DeGregori
University of Houston,
Texas 77204 5019,
USA

Dr. Wolfgang Ernst Bernhard Jelkmann
Medical Faculty, University of Lübeck,
Germany

Dr. Moktar Hamdi
Department of Biochemical Engineering,
Laboratory of Ecology and Microbial Technology
National Institute of Applied Sciences and Technology.
BP: 676. 1080,
Tunisia

Dr. Salvador Ventura
Department de Bioquímica i Biologia Molecular
Institut de Biotecnologia i de Biomedicina
Universitat Autònoma de Barcelona
Bellaterra-08193
Spain

Dr. Claudio A. Hetz
Faculty of Medicine, University of Chile
Independencia 1027
Santiago, Chile

Prof. Felix Dapare Dakora
Research Development and Technology Promotion
Cape Peninsula University of Technology,
Room 2.8 Admin. Bldg. Keizersgracht, P.O. 652, Cape Town 8000,
South Africa

Dr. Geremew Bultosa
Department of Food Science and Post harvest Technology
Haramaya University
Personal Box 22, Haramaya University Campus
Dire Dawa,
Ethiopia

Dr. José Eduardo Garcia
Londrina State University
Brazil

Prof. Nirbhay Kumar
Malaria Research Institute
Department of Molecular Microbiology and Immunology
Johns Hopkins Bloomberg School of Public Health
E5144, 615 N. Wolfe Street
Baltimore, MD 21205

Prof. M. A. Awal
Department of Anatomy and Histplogy,
Bangladesh Agricultural University,
Mymensingh-2202,
Bangladesh

Prof. Christian Zwieb
Department of Molecular Biology
University of Texas Health Science Center at Tyler
11937 US Highway 271
Tyler, Texas 75708-3154
USA

Prof. Danilo López-Hernández
Instituto de Zoología Tropical, Facultad de Ciencias,
Universidad Central de Venezuela.
Institute of Research for the Development (IRD),
Montpellier,
France

Prof. Donald Arthur Cowan
Department of Biotechnology,
University of the Western Cape Bellville 7535 Cape Town, South Africa

Dr. Ekhaise Osaro Frederick
University Of Benin, Faculty of Life Science
Department of Microbiology
P. M. B. 1154, Benin City, Edo State,
Nigeria.

Dr. Luísa Maria de Sousa Mesquita Pereira
IPATIMUP R. Dr. Roberto Frias, s/n 4200-465 Porto
Portugal

Dr. Min Lin
Animal Diseases Research Institute
Canadian Food Inspection Agency
Ottawa, Ontario,
Canada K2H 8P9

Prof. Nobuyoshi Shimizu
Department of Molecular Biology,
Center for Genomic Medicine
Keio University School of Medicine,
35 Shinanomachi, Shinjuku-ku
Tokyo 160-8582,
Japan

Dr. Adewunmi Babatunde Idowu
Department of Biological Sciences
University of Agriculture Abia
Abia State,
Nigeria

Dr. Yifan Dai
Associate Director of Research
Revivicor Inc.
100 Technology Drive, Suite 414
Pittsburgh, PA 15219
USA

Dr. Zhongming Zhao
Department of Psychiatry, PO Box 980126,
Virginia Commonwealth University School of Medicine,
Richmond, VA 23298-0126,
USA

Prof. Giuseppe Novelli
Human Genetics,
Department of Biopathology,
Tor Vergata University, Rome,
Italy

Dr. Moji Mohammadi
402-28 Upper Canada Drive
Toronto, ON, M2P 1R9 (416) 512-7795
Canada

Prof. Jean-Marc Sabatier
Directeur de Recherche Laboratoire ERT-62
Ingénierie des Peptides à Visée Thérapeutique,
Université de la Méditerranée-Ambrilia
Biopharma inc.,
Faculté de Médecine Nord, Bd Pierre Dramard,
13916,
Marseille cédex 20.
France

Dr. Fabian Hoti
PneumoCarr Project
Department of Vaccines
National Public Health Institute
Finland

Prof. Irina-Draga Caruntu
Department of Histology
Gr. T. Popa University of Medicine and Pharmacy
16, Universitatii Street, Iasi,
Romania

Dr. Dieudonné Nwaga
Soil Microbiology Laboratory,
Biotechnology Center. PO Box 812,
Plant Biology Department,
University of Yaoundé I, Yaoundé,
Cameroon

Dr. Gerardo Armando Aguado-Santacruz
Biotechnology CINVESTAV-Unidad Irapuato
Departamento Biotecnología
Km 9.6 Libramiento norte Carretera
Irapuato-León Irapuato,
Guanajuato 36500
Mexico

Dr. Abdolkaim H. Chehregani
Department of Biology
Faculty of Science
Bu-Ali Sina University
Hamedan,
Iran

Dr. Abir Adel Saad
Molecular oncology
Department of Biotechnology
Institute of graduate Studies and Research
Alexandria University,
Egypt

Dr. Azizul Baten
Department of Statistics
Shah Jalal University of Science and Technology
Sylhet-3114,
Bangladesh

Dr. Bayden R. Wood
Australian Synchrotron Program
Research Fellow and Monash Synchrotron
Research Fellow Centre for Biospectroscopy
School of Chemistry Monash University Wellington
Rd. Clayton,
3800 Victoria,
Australia

Dr. G. Reza Balali
Molecular Mycology and Plant Pthology
Department of Biology
University of Isfahan
Isfahan
Iran

Dr. Beatrice Kilel
P.O Box 1413
Manassas, VA 20108
USA

Prof. H. Sunny Sun
Institute of Molecular Medicine
National Cheng Kung University Medical College
1 University road Tainan 70101,
Taiwan

Prof. Ima Nirwana Soelaiman
Department of Pharmacology
Faculty of Medicine
Universiti Kebangsaan Malaysia
Jalan Raja Muda Abdul Aziz
50300 Kuala Lumpur,
Malaysia

Prof. Tunde Ogunsanwo
Faculty of Science,
Olabisi Onabanjo University,
Ago-Iwoye.
Nigeria

Dr. Evans C. Egwim
Federal Polytechnic,
Bida Science Laboratory Technology Department,
PMB 55, Bida, Niger State,
Nigeria

Prof. George N. Goulielmos
Medical School,
University of Crete
Voutes, 715 00 Heraklion, Crete,
Greece

Dr. Uttam Krishna
Cadila Pharmaceuticals limited ,
India 1389, Tarsad Road,
Dholka, Dist: Ahmedabad, Gujarat,
India

Prof. Mohamed Attia El-Tayeb Ibrahim
Botany Department, Faculty of Science at Qena,
South Valley University, Qena 83523,
Egypt

Dr. Nelson K. Ojijo Olang'o
Department of Food Science & Technology,
JKUAT P. O. Box 62000, 00200, Nairobi,
Kenya

Dr. Pablo Marco Veras Peixoto
University of New York NYU College of Dentistry
345 E. 24th Street, New York, NY 10010
USA

Prof. T E Cloete
University of Pretoria Department of Microbiology and Plant Pathology,
University of Pretoria,
Pretoria,
South Africa

Prof. Djamel Saidi
Laboratoire de Physiologie de la Nutrition et de Sécurité
Alimentaire Département de Biologie,
Faculté des Sciences,
Université d'Oran, 31000 - Algérie
Algeria

Dr. Tomohide Uno
Department of Biofunctional chemistry,
Faculty of Agriculture Nada-ku,
Kobe., Hyogo, 657-8501,
Japan

Dr. Ulises Urzúa
Faculty of Medicine,
University of Chile Independencia 1027, Santiago,
Chile

Dr. Aritua Valentine
National Agricultural Biotechnology Center,
Kawanda
Agricultural Research Institute (KARI)
P.O. Box, 7065, Kampala,
Uganda

Prof. Yee-Joo Tan
Institute of Molecular and Cell Biology 61 Biopolis Drive,
Proteos, Singapore 138673
Singapore

Prof. Viroj Wiwanitkit
Department of Laboratory Medicine,
Faculty of Medicine, Chulalongkorn University,
Bangkok
Thailand

Dr. Thomas Silou
Universit of Brazzaville BP 389
Congo

Prof. Burtram Clinton Fielding
University of the Western Cape
Western Cape,
South Africa

Dr. Brnčić (Brncic) Mladen
Faculty of Food Technology and Biotechnology,
Pierottijeva 6,
10000 Zagreb,
Croatia.

Dr. Meltem Sesli
College of Tobacco Expertise,
Turkish Republic, Celal Bayar University 45210,
Akhisar, Manisa,
Turkey.

Dr. Idress Hamad Attitalla
Omar El-Mukhtar University,
Faculty of Science,
Botany Department,
El-Beida, Libya.

Dr. Linga R. Gutha
Washington State University at Prosser,
24106 N Bunn Road,
Prosser WA 99350-8694

Dr Helal Ragab Moussa
Bahnay, Al-bagour, Menoufia,
Egypt.

Dr VIPUL GOHEL
DuPont Industrial Biosciences
Danisco (India) Pvt Ltd
5th Floor, Block 4B,
DLF Corporate Park
DLF Phase III
Gurgaon 122 002
Haryana (INDIA)

Dr. Sang-Han Lee
Department of Food Science & Biotechnology,
Kyungpook National University
Daegu 702-701,
Korea.

Dr. Bhaskar Dutta
DoD Biotechnology High Performance Computing
Software Applications
Institute (BHSAI)
U.S. Army Medical Research and Materiel Command
2405 Whittier Drive
Frederick, MD 21702

Dr. Muhammad Akram
Faculty of Eastern Medicine and Surgery,
Hamdard Al-Majeed College of Eastern Medicine,
Hamdard University,
Karachi.

Dr. M. Muruganandam
Departtment of Biotechnology
St. Michael College of Engineering & Technology,
Kalayarkoil,
India.

Dr. Gökhan Aydin
Suleyman Demirel University,
Atabey Vocational School,
Isparta-Türkiye,

Dr. Rajib Roychowdhury
Centre for Biotechnology (CBT),
Visva Bharati,
West-Bengal,
India.

Dr Takuji Ohyama
Faculty of Agriculture, Niigata University

Dr Mehdi Vasfi Marandi
University of Tehran

Dr FÜgen DURLU-ÖZKAYA
Gazi Üniversity, Tourism Faculty, Dept. of
Gastronomy and Culinary Art

Dr. Reza Yari
Islamic Azad University, Boroujerd Branch

Dr Zahra Tahmasebi Fard
Roudehen branche, Islamic Azad University

Dr Albert Magrí
Giro Technological Centre

Dr Ping ZHENG
Zhejiang University, Hangzhou, China

Dr. Kgomotso P. Sibeko
University of Pretoria

Dr Greg Spear
Rush University Medical Center

Prof. Pilar Morata
University of Malaga

Dr Jian Wu
Harbin medical university , China

Dr Hsiu-Chi Cheng
National Cheng Kung University and Hospital.

Prof. Pavel Kalac
University of South Bohemia, Czech Republic

Dr Kürsat Korkmaz
Ordu University, Faculty of Agriculture, Department
of Soil Science and Plant Nutrition

Dr. Shuyang Yu
Department of Microbiology, University of Iowa
Address: 51 newton road, 3-730B BSB bldg. Iowa City,
IA, 52246, USA

Dr. Mousavi Khaneghah
College of Applied Science and Technology-Applied Food Science, Tehran, Iran.

Dr. Qing Zhou
Department of Biochemistry and Molecular Biology, Oregon Health and Sciences University Portland.

Dr Legesse Adane Bahiru
Department of Chemistry,
Jimma University,
Ethiopia.

Dr James John
School Of Life Sciences,
Pondicherry University,
Kalapet, Pondicherry

African Journal of Biotechnology

Table of Content: Volume 17 Number 22 30 May, 2018

ARTICLES

Screening for total antioxidant activity, flavonoids and phenolics variability in forty-five accessions of roselle (*Hibiscus sabdariffa* L.) 694
W. Nunekpeku, H. M. Amoatey, S. Agbenyegah, M. Owusu-Ansah, V. Oduro, K. E. Danso, D. K. Asare and E. Achoribo

Isolation and screening of antibiotic producing actinomycetes from rhizosphere and agricultural soils 700
Atsede Muleta and Fassil Assefa

Vol. 17(22), pp. 694-699, 30 May, 2018
DOI: 10.5897/AJB2017.16286
Article Number: 832C86257296
ISSN: 1684-5315
Copyright ©2018
Author(s) retain the copyright of this article
http://www.academicjournals.org/AJB

ACADEMIC JOURNALS
expand your knowledge

African Journal of Biotechnology

Full Length Research Paper

Screening for total antioxidant activity, flavonoids and phenolics variability in forty-five accessions of roselle (*Hibiscus sabdariffa* L.)

W. Nunekpeku[1*], H. M. Amoatey[1,3], S. Agbenyegah[2], M. Owusu-Ansah[1], V. Oduro[4], K. E. Danso[4], D. K. Asare[1] and E. Achoribo[2]

[1]Nuclear Agriculture Research Center, Biotechnology and Nuclear Agriculture Research Institute, Ghana Atomic Energy Commission, P. O. Box LG 80, Legon, Accra, Ghana.
[2]Applied Radiation Biology Center, Radiological and Medical Science Research Institute, Ghana Atomic Energy Commission, P. O. Box LG 80, Legon, Accra, Ghana.
[3]School of Nuclear and Allied Sciences, University of Ghana, P. O. Box AE 1, Atomic Energy, Accra, Ghana.
[4]Biotechnology Center, Biotechnology and Nuclear Agriculture Research Institute, Ghana Atomic Energy Commission, P. O. Box LG 80, Legon, Accra, Ghana.

Received 19 December, 2017; Accepted 7 May, 2018

Build-up of free radicals in the human body can cause oxidative stress which may invariably lead to degenerative diseases and eventual death. Antioxidants such as polyphenols and flavonoids found in fruits and vegetables have been shown to protect the body against the damaging effects of these free radicals. Roselle contains high amounts of antioxidants and its leaves are often used for sauces or in herbal preparations to treat certain ailments. Several landraces of roselle occur in Ghana but have not been screened for antioxidant activity. Leaf samples of roselle acessions were harvested 60 days after sowing, freeze dried and analysed for total phenolic content (TPC), total flavonoid content (TFC) as well as total antioxidant activity (TAA) using UV-VIS Spectrophotometer (Shimadzu, 1201, Japan). No statistically significant differences were observed in TPC which ranged from 20 ± 0.6 µg/g (RNL) to 90 ± 0.6 µg/g (Sob-4). TFC and TAA in the leaf samples; however, showed statistically significant variation and ranged from 10.04 ± 0.31 µg/g (WH-S2V) to 49.79 ± 0.48 µg/g (Don-1) and 37.48% (Don-4) to 58.58% (WNL-HS), respectively. Higher leaf phenolic or flavonoid content did not necessarily translate into a higher antioxidant activity. This suggests that other forms of antioxidants other than phenolics or flavonoids might be responsible free radical scavenging activity in roselle leaves. Nevertheless, very high free radical percentage inhibition observed in the roselle leaves makes it an excellent material for mitigation against the adverse effect of free radicals. Nine promising accessions with high free radical scavenging activity have been identified for further improvement.

Key words: *Hibisicus sabdariffa*, roselle, antioxidant activity, flavonoids, phenolic content.

INTRODUCTION

Free radicals are unstable and highly reactive chemical species containing unpaired electrons in their atomic orbital (Lobo et al., 2010). They occur either naturally in the human body as products of normal biochemical processes or are derived from the effects of ionizing radiation. Their formation causes damaging activity in the

Table 1. Geographical distribution of the roselle accessions assembled for the study.

Location	Landrace/Accession
Greater Accra	L-1, L-2, L-4A, L-4B, L-5B, L-5A, L-5C, L-7, S-1, Sob-1, Sob-2, Sob-3, Sob-4, Sob-5, Sob-6, Sob-7,Sob-8, Sob-9, Sob-10, RNL, RBL , WH-S2, WHN-S2BL, WNL-HLS, WNL-VSH2, WHN-HS, WH-S2VLPro, WNL-STT, WNL-H, WNL-VSHT, WNL-HS, WBL, WH-S2V
Central Region	WH-S2TP, WH-S2VL
Northern Region	Don-1, Don-2, Don-3, Don-4,N1-NL, N1-GP, N-3, N-4, N-5, S-2

body on important components such as carbohydrates, proteins, lipids and DNA. This damaging effect can cause oxidative stress which leads to degenerative diseases such as atherosclerosis, cancers and diabetes (Oboh and Rocha, 2006; Young and Woodside, 2001; Bagchi et al., 2000) which account for about 1.7 million (2.8%) deaths worldwide (WHO, 2016).

These diseases or death cases could be prevented by adequate intake of antioxidants which have been identified as compounds capable of inhibiting or delaying such stress processes. Antioxidants have been reported to provide body cells with significant protection against oxidative damage (Cid-Ortega and Guerrero-Beltrán, 2015; Carvajal-Zarrabal et al., 2012). Maintaining a body balance between them and pro-oxidative species such as peroxides, hydroxyl radicals and singlet oxygen is therefore, critical to ensuring good health status in humans. Important examples of antioxidants include polyphenols, lycopenes and flavonoids which can be obtained from fruits and vegetables (Karadag et al., 2009; Cai et al., 2004).

In Ghana, roselle (*Hibiscus sabdariffa* Linn.) which belongs to the family Malvaceae is one of the most important vegetables, especially popular among rural folks. Both leaves and calyces are prepared into sauces or herbal drinks which are believed to offer tremendous health benefits due to its phytochemical constituents, particularly antioxidants. Elsewhere, roselle extracts are, commonly used in traditional medicine because of their antihypertensive, hepatoprotective, cardio-protective and anti-cancerous properties (Odigie et al., 2003; Tseng et al., 1997).

In spite of its beneficial uses and potential as medicinal crop, not much research has been done on roselle in Ghana. Depending on genotype and environment, the phytochemical composition of roselle has been found to be quite variable (Burkill, 1997). However, no study has been conducted in Ghana to establish the variability among landraces. This study was, therefore, carried out to determine the phenolic content, total flavonoids and antioxidant activity in the leaves of 45 accessions of roselle in Ghana and to identify promising lines for use in future breeding programmes.

MATERIALS AND METHODS

Plant collection

Forty-five accessions of roselle were collected from three geographical regions (Table 1) of Ghana (Greater Accra, Central and Northern regions) between April and June 2013. These locations are areas where the crop is commonly grown and consumed as vegetable or processed into local beverage.

Experimental site

The roselle accessions collected were cultivated at the Biotechnology and Nuclear Agriculture Research Institute (BNARI) which is located 76 m above sea level and situated on latitude 05° 40’ N and longitude 0° 13’ W in the coastal savannah agro-ecological zone.

Cultivation

Seeds of each accession were sown in plastic containers (40 cm x 30 cm x 25cm) filled with a mixture of top soil and decomposed broiler manure in 6:1 ratio, respectively. Four seeds were sown per container and replicated 5 times in randomly complete block design (RCBD). Seedlings were thinned to 2, two weeks after emergence. Watering was done when necessary to prevent plants from wilting.

Sample preparation

For each accession, fresh leaves were harvested from the mid-sections of 5 randomly selected plants at 60 days after emergence (DAE). The leaves were bulked and pulverized after freeze-drying. Pulverized samples were put into air-tight plastic bags and stored at 2°C until needed. For each sample, 0.05 g was weighed into a clean 15 mL Falcon tube and extracted serially with 5 mL of 60 % methanol and extraction was done four times, each time vortexing for 5 min. The supernatants were collected and stored for phytochemical analysis.

Phenolic content determination

Polyphenolic contents were determined by a modified Folin-

*Corresponding author. E-mail: nuswonder1@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

Ciocalteu method using gallic acid as standard (Singleton et al., 1999). A 50 µL portion of each of the extracted sample was mixed with 3 mL of distilled water (dH_2O) and 250 µL of a 1 in 10 diluted Folin-Ciocalteu Phenol Reagent. The mixtures were allowed to stand for 5 min, after which 750 µL of 20% Na_2CO_3 was added. This was thoroughly mixed and incubated for 30 min at room temperature (22 to 25°C) in the dark. Absorbance was measured at 760 nm using a UV-VIS Spectrophotometer (Shimadzu, 1201, Japan). A calibration curve was prepared using serial dilutions of 1 mg/mL gallic acid dissolved in water at the following concentrations: 0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL. A linear regression equation y = 1.1738x – 0.0041 with a regression coefficient of R^2 = 0.9992 was established for the curve. Total phenolic content (TPC) was determined and expressed as milligram gallic acid equivalent per gram of sample according to the formula:

$$\text{TPC (mg/g)} = [(\text{c} \times \text{v})/m]$$

Where, c = concentration of gallic acid (mg/mL); v = volume of sample extract (mL); m = load of sample extract (g).

Flavonoid content determination

Total flavonoid content (TFC) was determined in the samples using the aluminium chloride colorimetric assay method (Zhishen et al., 1999). An aliquot of 500 µL sample extract was mixed with 1500 µL of 99.9% ethanol, 100 µL of 1.0 M potassium acetate, 100 µL of 10% aluminium chloride and 3000 µL of distilled water. After incubating the resulting mixtures for 30 min at room temperature, the corresponding spectrophotometric absorbance was measured at 415 nm. Standard calibration curve using quercetin standard solutions of 12.5, 25, 50, 75 and 100 µg/mL each time the samples were analysed, was constructed. For each standard, 500 µL was treated in the manner as indicated above for the samples. The linear regression equation y = 0.0055x +0.0026 with a regression coefficient, R^2 = 0.99, was derived for the calibration. Total flavonoid content (TFC), expressed as microgram of quercetin equivalent per gram of sample was determined from the expression:

$$\text{TFC (µg/g)} = [(c \times df \times v)/ w]$$

Where, c = concentration derived from standard curve (µg/mL); df = dilution factor; v = volume of stock solution (mL) and w = sample weight (g).

Free radical scavenging assay

The determination of total antioxidant activity (TAA) was done by means of free radical scavenging assay using 2,2-diphenyl-1-picrylhydrazyl (DPPH) as described by Brand-Williams et al. (1995) with modification. Two hundred micro litres (200 µL) of extracts were each added to 3800 µL of 0.004% DPPH in methanol. After 60 min of incubation at room temperature in the dark, the absorbance was measured at 517 nm. The methanolic blank sample was used to set the spectrophotometer reading to zero. Absorbance readings were done in triplicates. The free radical scavenging activity, expressed as percent inhibition (I %) of the DPPH was computed using the equation:

$$I (\%) = [(A_0 – A_c) / A_0] \times 100$$

Where, A_0 = absorbance of the blank and A_c = absorbance of the test sample (Brand-Williams et al., 1995).

ANOVA on TPC, TFC and TAA was performed using Statgraphics Centurion XVI, version 16.1.11. Duncan multiple range test, was used to separate means.

RESULTS AND DISCUSSION

Geographical distribution, phenolic and flavonoid contents of roselle leaves

Thirty-three accessions (73.3%) of roselle were collected from urban and peri-urban vegetable farms around the Greater Accra regional capital, Accra. Diversity in the accessions collected seemed to be greatest in this location probably because there is ready market for the fresh cut-foliage of roselle. As a result, farmers might have collected many accessions across the country and beyond for propagation. Only 12 accessions were collected from central and northern regions combined, where less diversity was observed.

Variations in phenolic and flavonoid contents of leaves of the 45 roselle (*H. sabdariffa* Linn) accessions are presented in Table 2. Total phenolic content (TPC) in the leaf samples ranged from 20 ± 0.6 (RNL) to 90 ± 0.6 µg/g (Sob-4). The highest and lowest contents were detected in accessions collected from the Greater Accra region. Accessions collected from the Central and Northern regions had TPC between 47 ± 0.6 (WH-S2VL) to 54 ± 0.3 (WH-S2TP) and 27 ± 0.4 (N1-NL) to 54 ± 3.4 (DON-1), respectively. Maximal TPC contents of samples from the Central and Northern regions were therefore 40% below the highest TPC obtained in the study. Statistical analysis, however, did not show significant differences ($p \geq 0.05$) among all the accessions in terms of TPC. Total phenolic contents observed in the current study were lower than values obtained in similar studies in ethanolic and aqueous extraction systems of roselle leaves in Brazil (Formagio et al., 2015) or elsewhere (Sirag et al., 2014; Al-Hashimi, 2012). The values are also lower than those obtained for moringa (Owusu-Ansah et al., 2011) and cassava leaves (Quartey et al., 2016) which are also used in preparation of herbal drinks and sauces for similar purposes as roselle leaves.

Total flavonoid content (TFC) of the roselle leaves varied significantly ($p \leq 0.05$) among the accessions. Don-1 recorded the highest value (49.79 ± 0.48 µg/g). The lowest TFC was observed in WH-S2V (10.04 ± 0.31 µg/g). These values are lower than those determined in Brazil by Formagio et al. (2015) in roselle leaves (140.29 ± 3.14 to 148 ± 2.42 mg/g) and calyces (95.85 ±1.85 to 104.52 ± 4.85 mg/g) possibly because the cultivation media were highly enriched with organic substrates or it is simply a factor of inherent genetic attribute of those varieties. Phenolic and flavonoid contents of plant materials have been shown to vary considerably depending on variety and environment (Yang et al., 2006; Burkill, 1997). Since the values reported in the current study are lower than those reported in other countries, it might be necessary to optimise agronomic and cultivation practices aside carrying out genetic improvement of the crop so as to obtain varieties with improved antioxidant potentials in Ghana.

Table 2. Antioxidant activity, total phenolics and flavonoids in the leaves of 45 roselle accessions.

Accession number	Accession	Phenolics		Flavonoids		Antioxidant activity	
		Total content, TPC (μg /g)	Ranking	Total content, TFC (μg/g)	Ranking	Inhibition (%), TAA	Ranking
1	RNL	90±0.6[a]	1	49.27±3.10[y]	2	45.17±0.00[s]	3
2	S-1	88±0.6[a]	2	44.00±0.00[x]	3	41.17±1.17[opqr]	7
3	L-4A	74±1.9[a]	3	30.01±1.35[tu]	8	27.20±0.71[fg]	31
4	RBL	68±1.3[a]	4	31.89±0.54[v]	5	20.52±0.14[ab]	43
5	SOB-1	62±2.4[a]	5	28.06±0.38[rs]	11	20.73±0.21[ab]	42
6	SOB-3	58±0.3[a]	6	29.89±0.74[tu]	9	37.31±2.12[klmn]	15
7	DON-1	54±3.4[a]	7	49.79±0.48[y]	1	37.18±0.18[klmn]	16
8	WH-S2TP	54±0.3[a]	8	19.11±0.15[mn]	19	38.14±4.20[klmnop]	11
9	N1-GP	50±0.4[a]	9	30.89±0.35[uv]	6	32.90±0.35[hij]	24
10	WH-S2VL	47±0.6[a]	10	22.38±0.64[q]	13	37.74±0.74[klmnop]	13
11	N-3	46±1.7[a]	11	26.28±0.48[r]	12	33.07±0.17[hij]	23
12	L-2	46±0.3[a]	12	17.50±0.24[klm]	23	39.20±2.75[nopqr]	8
13	WBL	45±1.4[a]	13	22.10±0.37[pq]	14	27.38±1.83[fg]	30
14	SOB -8	44±0.9[a]	14	20.99±0.25[opq]	15	27.60±1.12[fg]	29
15	DON-2	43±3.5[a]	15	35.60±0.55[w]	4	36.11±3.89[ijklmn]	19
16	L-5B	42±0.0[a]	16	19.21±0.45[mno]	18	22.41±1.47[abcd]	37
17	DON-4	39±2.3[a]	17	30.53±0.67[uv]	7	37.48±1.76[klmno]	14
18	DON-3	39±0.9[a]	18	28.55±0.20[st]	10	23.07±1.26[bcde]	35
19	WHN-S2BL	38±1.1[a]	19	16.05±0.23[jk]	27	21.16±0.36[ab]	41
20	L-7	38±0.8[a]	20	17.75±0.18[klm]	22	38.87±0.94[mnopq]	9
21	N-4	38±0.0[a]	21	17.24±0.53[kl]	24	21.46±0.94[abc]	40
22	S-2	37±1.6[a]	22	17.90±0.07[lm]	20	35.28±0.81[ijklm]	20
23	L-4B	37±0.3[a]	23	14.99±0.28[ij]	28	21.81±0.15[abcd]	39
24	N-5	35±0.0[a]	24	16.88±0.06[kl]	25	22.77±0.66[abcde]	36
25	L-5C	34±0.9[a]	25	19.81±0.12[no]	17	49.75±0.77[t]	2
26	L-1	33±1.0[a]	26	13.64±0.27[efghi]	33	20.24±0.28[ab]	44
27	WH-S2	33±0.9[a]	27	16.21±0.05[jkl]	26	41.28±1.86[pqr]	6
28	WNL-HS	32±1.3[a]	28	20.31±0.91[nop]	16	58.58±1.11[u]	1
29	SOB-9	32±0.5[a]	29	14.64±0.48[hij]	29	34.84±1.07[ijj]	22
30	WNL-HLS	31±0.4[a]	30	13.86±0.27[fghi]	32	35.02±1.07[ijkl]	21
31	SOB-7	31±0.0[a]	31	11.95±0.00[bcde]	40	36.47±1.64[jklmn]	18
32	SOB-6	30±1.5[a]	32	12.76±0.04[cdefg]	36	38.01±0.09[klmnop]	12
33	WNL-H	30±0.0[a]	33	12.30±0.70[cdef]	39	25.41±1.08[def]	33
34	WNL-STT	28±0.2[a]	34	14.18±0.24[ghi]	31	29.63±1.40[gh]	26
35	WNL-VSH2	27±0.5[a]	35	12.84±0.15[defgh]	35	22.10±0.15[abcd]	38
36	N1-NL	27±0.4[a]	36	17.81±0.65[klm]	21	28.55±0.32[fg]	27
37	L-5A	26±0.8[a]	37	11.48±0.40[abcd]	41	32.56±1.03[hi]	25
38	WNL-VSHT	25±1.1[a]	38	11.02±0.44[abc]	42	25.17±0.08[cdef]	34
39	WH-S2VLPro	25±0.2[a]	39	14.58±0.46[hij]	30	28.39±0.32[fg]	28
40	WH-S2V	23±0.5[a]	40	10.04±0.31[a]	45	26.41±0.70[efg]	32
41	SOB-5	22±1.6[a]	41	10.49±0.15[ab]	44	38.77±0.47[lmnopq]	10
42	WHN-HS	22±1.1[a]	42	10.98±0.12[abc]	43	42.94±0.00[rs]	4
43	SOB-2	22±0.2[a]	43	13.31±0.18[efghi]	34	42.24±0.30[qrs]	5
44	SOB-10	22±0.1[a]	44	12.45±0.16[cdefg]	37	37.09±0.46[klmn]	17
45	SOB-4	20±0.6[a]	45	12.45±0.00[cdefg]	38	19.12±0.97[a]	45

Values within a column followed by the same superscript are not significantly ($p \leq 0.05$) different according to Duncan multiple range test.

Table 3. Correlation coefficients and p-values among phyto-chemicals and antioxidant activity in roselle leaf samples of 45 accessions of roselle.

Antioxidant activity	Total phenolic content	Total flavonoids content	Total antioxidant activity
Total phenolic content	-		
Total flavonoid content	0.4495 (0.00)	-	
Total antioxidant activity	0.0681 (0.5235)	0.1856 (0.0798)	-

Figures in parenthesis are p-values.

Free radical scavenging activity in the roselle leaf landraces

The total antioxidant activity (TAA) determined in the roselle accessions are presented in Table 2. The highest TAA detected in the roselle leaf samples was 58.58 ± 1.11% (WNL-HS) which differs significantly ($p \leq 0.05$) from the least value of 19.12 ± 0.97% recorded for Sob-4. Roselle leaves exhibit a considerably high degree of free radical scavenging activity. Of the accessions studied, 15.56% showed free radical scavenging capacities above the highest value detected in 14 accessions of moringa (39.48%). This indicates that roselle leaves could constitute an excellent recipe for making herbal drinks aimed at combating the adverse effects of free radicals in the body via its antioxidant activity.

Indeed, ethanolic and aqueous extracts of roselle leaves showed a reduced lipid peroxidation in rats which confirmed their antioxidant properties (Ochani and D'Mello, 2009). Extracts evaluated for *in vitro* anti-cancerous activity against several cultured human cancer lines also demonstrated significant selective activity against leukemia lines (Formagio et al., 2015). Flavonoids and polyphenols are powerful free radical scavengers which regulate the activities of most enzyme systems as they interact with other biomolecules. Dietary inclusion of antioxidants can therefore prevent oxidative damage to biological molecules which can cause harmful diseases (Essa and Shubramanian, 2006; Rice-Evans et al., 1996). The results of the current study therefore, suggest that roselle leaves as vegetables or herbal drink may be consumed to scavenge free radicals and boost the body's immune system.

Relationship among phenolics, flavonoids and total antioxidant activity in roselle leaves

Correlations among TPC, TFC and TAA are shown in Table 3. A positive correlation (0.4495) was found between TPC and TFC. Positive correlations also existed between TFC and TAA as well as TPC and TAA. However, though not statistically significant, the relationship between TFC and TAA (0.1856) was relatively stronger than what was observed between TPC and TAA (0.0681). Flavonoids are the principal phytochemicals found in roselle (Tsai et al., 2002). This implies that higher proportion of the free radical scavenging activity in the leaf samples could be attributable to flavonoid rather than phenolic antioxidant activity as also suggested by Formagio et al. (2015).

Conclusions

Leave samples of the 45 roselle accessions tested exhibited wide variations in TPC, TFC and TAA. Though TPC and TFC observed were below ranges determined in roselle elsewhere, the roselle leaves showed very high free radical scavenging activity, suggesting that they may be excellent ingredients in anti-aging and therapeutic herbal preparations to combat various forms of cancers and heart related diseases. Total flavonoid content correlated more strongly with TAA (0.1856) than with phenolics (0.0681). Much of the free radical scavenging activity in the leaves could be mainly attributed to its flavonoid content.

A twenty percent selection intensity imposed on the 45 roselle accessions yielded 9 promising lines with high total antioxidant activity ranging from 38.87 ± 0.94 to 58.58 ± 1.11% identified for future genetic improvement. These include two accessions which rank very high for both TPC and TFC (RNL and S-1).

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

Technical support provided by technicians at the Applied Radiation Biology Center, Radiological and Medical Science Research Institute, Ghana Atomic Energy Commission is very much appreciated.

REFERENCES

Al-Hashimi AG (2012). Antioxidant and antibacterial activities of *Hibiscus sabdariffa* L. extracts. African Journal of Food Science, 6(21):506-511.

Bagchi D, Bagchi M, Stoh SJ, Das DK, Ray SD, Kuszynski CA (2000). Free radicals and grape seed pro-anthocyanidin extract: Importance in human health and disease prevention. Toxicology, 148:187-197.

Brand-Williams W, Cuvellier ME, Berset C (1995). Use of a free radical method to evaluate antioxidant activity. LWT-Food science and Technology, 28:25-30.

Burkill HM (1997). The useful plants of west tropical Africa. Royal Botanic Gardens, Kew. 2nd ed. pp. 36-38.

Cai Y, Luo Q, Sun M, Corke H (2004). Antioxidant activity and phenolic compound of 112 traditional Chinese medicinal plants associated with anticancer. Life Science, 74:2157-2184.

Carvajal-Zarrabal O, Barradas-Dermitz DM, Orta-Flores Z, Hayward-Jones PM, Nolasco-Hipólito C, Aguilar-Uscanga MG, Bujang KB (2012). *Hibiscus sabdariffa* L, Roselle calyx, from ethnobotany to pharmacology. Journal of Experimental Pharmacology, 4:25-39.

Cid-Ortega S, Guerrero-Beltrán JA (2015). Roselle calyces (*Hibiscus sabdariffa*), an alternative to the food and beverages industries: a review. Journal of Food Science and Technology, 52(11):6859-6869.

Essa MM, Subramanian P (2006). *Hibiscus sabdariffa* affects ammonium chloride-induced hyper-ammonemic rats. Evidence-Based Complementary and Alternative Medicine, 4:321

Formagio ASN, Ramos DD, Vieira MC, Ramalho SR, Silva MM, Zárate NAH, Foglio MA, Carvalho JE (2015). Phenolic compounds of *Hibiscus sabdariffa* and influence of organic residues on its antioxidant and anti-tumoral properties. Brazilian Journal of Biology, 75(1):69-76.

Karadag A, Ozcelik B, Saner S (2009). Review of methods to determine antioxidant capacities. Food Analytical Methods, 2:41-60.

Lobo V, Patil A, Phatak A, Chandra N (2010). Free radicals, antioxidants and functional foods: Impact on human health. Pharmacognosy Reviews, 4(8):118-126.

Oboh G, Rocha JBT (2006). Antioxidant in foods: A new challenge for food processors In. New Development in Antioxidants Research: Harold V (Ed.). Panglossi. Publisher: Nova Science Publishers, pp. 66-73.

Ochani CP, D'Mello P (2009). Antioxidant and antihyperlipidic activity of *Hibiscus sabdariffa* Linn. Leaves and calyces extracts in rats. Indian Journal of Experimental Biology, 47:276-282.

Odigie IP, Ettarh RR, Adigun SA (2003). Chronic administration of aqueous extract of *Hibiscus sabdariffa* attenuates hypertension and reverses cardiachypertrophy in 2K-1C hypertensive rats. Journal of Ethnopharmacology, 86(2-3):181-185.

Owusu-Ansah M, Achel DG, Adaboro RM, Asare DK, Amoatey HM (2011). Total Phenolic Content and Antioxidant Activity in Leaf Samples of Twelve Accessions of *Moringa oleifera* Lam. Journal of Chemical and Analytical Science, 2(10):1226-1230.

Quartey EK, Amoatey HM, Achoribo E, Owusu-Ansah M, Nunekpeku W, Donkor S, Appiah AS, Ofori ESK (2016). Phytochemical Constituents and Antioxidant Activities in Leaves of 14 Breeding Lines of Cassava (*Manihot esculenta* Crantz). American Journal of Experimental Agriculture, 12(5):1-10.

Rice-Evans CA, Miller NJ, Paganga G (1996). Structure antioxidant activity relationships of flavonoids and phenolic acids. Free Radical Biology and Medicine, 20(7):933-956.

Singleton VL, Orthofer RM, Lamuela-Raventós PL (1999). Methods in Enzymology. Academic Press, pp.152-178.

Sirag N, Elhadi MM, Algaili MA, Hozeifa MH, Mohamed O (2014). Determination of total phenolic content and antioxidant activity of Roselle (*Hibiscus sabdariffa* L.) Calyx ethanolic extract. Standard Research Journal of Pharmacy and Pharmacology, 1(2):034-039.

Tsai PJ, Mcintosh J, Pearce P, Camden B, Jordan BR (2002). Anthocyanin and antioxidant capacity in roselle (*Hibiscus sabdariffa* L.) extract. Food Research International, 35(4):351-356.

Tseng TH, Kao ES, Chu FP, Lin-Wa HW, Wang CJ (1997). Protective effect of dried flower extracts of *Hibiscus sabdariffa L.* against oxidative stress in rat primary hepatocytes. Food and Chemical Toxicology, 35(12):1159-1164.

World Health Organization (WHO) (2016). Global Strategy on Diet, Physical Activity and Health. http://www.who.int/dietphysicalactivity/fruit/en/

Yang RY, Chang L, Hsu J, Brian BCW, Palada MC, Chadha L, Levasseur V (2006). Nutritional and functional properties of moringa leaves− from germplasm, to plant, to food, to health, Paper presented at a workshop on Moringa and other highly nutritious plant resources: Strategies, standards and markets for a better impact on nutrition in Africa, Accra, Ghana, November 16-18, 2006. pp. 3-6.

Young IS, Woodside JV (2001). Antioxidants in health and disease. Journal of Clinical Pathology, 54:176-186.

Zhishen J, Mengcheng T, Jianming W (1999). The determination of flavonoid content in mulberry and their scavenging effects on superoxide radicals. Food Chemistry, 64:555-559.

Vol. 17(22), pp. 700-714, 30 May, 2018
DOI: 10.5897/AJB2017.16080
Article Number: 7A5853857299
ISSN: 1684-5315
Copyright ©2018
Author(s) retain the copyright of this article
http://www.academicjournals.org/AJB

ACADEMIC JOURNALS
expand your knowledge

African Journal of Biotechnology

Full Length Research Paper

Isolation and screening of antibiotic producing actinomycetes from rhizosphere and agricultural soils

Atsede Muleta[1,2*] and Fassil Assefa[1]

[1]Microbial, Cellular and Molecular Biology Department, College of Natural Science, Addis Ababa University, P. O. Box 1176, Addis Ababa, Ethiopia.
[2]Department of Biology, College of Natural and Computational Science, University of Gondar, P.O. Box 176, Gondar, Ethiopia.

Received 24 May, 2017; Accepted 23 March, 2018

Antibiotics are secondary metabolites produced by microorganisms. Actinomycetes are the top producers of antibiotics. The study was designed to isolate and screen antibiotic producing actinomycetes obtained from rhizosphere of plants and agricultural soils. A total of 30 actinomycetes were isolated. Out of the 30 actinomycetes, 18 (60%) showed antimicrobial activities against one of the tested microorganisms of which 9 isolates were selected for their wide spectrum of antibiotic activities. In this study, isolates AAUBA5 and AAUBA30 were found to inhibit all of the test organisms, except *Escherichia coli* ATCC 25922. The latter was found to be the most resistant and was only inhibited by 3/18 (17%) of the isolates. The isolates were identified and characterized by cultural, morphological, physiological, and biochemical characteristics and found to be classified under the genus *Streptomycetes*. In order to extract the active compounds from isolates, fermentation was carried out on 9 isolates and the former was extracted by using ethyl acetate. Finally, isolate AAUBA13 was selected for further study based on spectrum activity from disc diffusion assay. Antimicrobial compound obtained from AAUBA13 isolate was separated and purified with thin layer chromatography (TLC), column chromatography, and preparative chromatography. The finding of this study, in general, showed that, antimicrobial compounds obtained from AAUBA13 demonstrate broad spectrum antimicrobial activity against *Staphylococcus aureus* ATCC25923, *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC25853, *S. aureus* (clinical isolate), *Shigella boydii* (clinical isolate), *Streptococcus pneumonia* ATCC49619, and *Candida albicans* ATCC62376.

Key words: Actinomycetes, antibiotics, antimicrobial compound, disc diffusion assay, soil.

INTRODUCTION

Antibiotics are chemical substances produced by microorganisms, which in small amount selectively inhibit or kill other microorganisms (Aneja, 2005; Tortora et al., 2010). They are secondary metabolites that inhibit other competing cells to give competitive advantage to the microorganisms that produce them (Sanglier et al., 1993; Kumbhar and Watve, 2013).

Antimicrobial agents are natural products and produced

*Corresponding author. E-mail: atsede_muleta@yahoo.com.

Author(s) agree that this article remain permanently open access under the terms of the Creative Commons Attribution License 4.0 International License

by various types of bacteria and fungi. Hundreds of these natural products have been identified and developed as therapeutic agents against many infectious diseases (Berdy, 2005). Microbial natural metabolites still appear as the most promising sources of antibiotic in the future (Fernando, 2006; Wohlleben et al., 2016). Some of the important antibiotic producing microorganisms are of *Streptomyces*, *Bacillus, Cephalosporium,* and *Penicillium* that have been studied continuously for their ability to produce antibiotics (Brock and Madigan, 1991).

From all the known microbes, actinomycetes are the most important source of biologically active microbial products, including many medically and commercially important antibiotics (Dhanasekaran et al., 2009; Adegboye and Babalola, 2013). The broad-spectrum antibiotic, vancomycin, a potent antimicrobial agent against methicillin resistant *Staphylococcus aureus* and rifampcin, and effective drug against tuberculosis and leprosy is derived from several species of actinomycetes (Berdy, 2005).

The genus *Streptomyces* has been widely recognized as industrially important microorganism because of its ability to produce and secrete a large variety of secondary metabolites (Kornwendisch and Kutzner, 1992; Saadoun and Gharaibeh, 2003; Pandey et al., 2004). These include aminoglycosides, macrolides, β lactams, peptides, polyenes, tetracyclines, anthracycline antibiotics, and nucleosides (Vijayakumar et al., 2007; Miyadoh, 1993). It is estimated that more than 80% of the antibiotics are obtained from *Streptomycetes* (Vijayakumar et al., 2007). *Micromonospora* is the runner up with less than one-tenth *Streptomyces* (Saadoun and Gharaibeh, 2003; Arifuzzaman et al., 2010).

Although the introduction of antimicrobials helps to combat many diseases, large numbers of pathogenic bacteria and fungi causing different human diseases have become resistant to antibiotics in use (Livermore, 2003). *S. aureus,* a virulent pathogen, which is responsible for a wide range of infections, including pimples, pneumonia, osteomyelitis, endocarditis and bacteremia, has developed resistance to most classes of antibiotics (Enright, 2003). The increase in antibiotic resistance has been attributed to a combination of microbial characteristics, selective pressure of antibiotic use, and social changes that enhance the transmission of resistant organisms (Okeke et al., 2005).

With increase in misuses of antibiotics, the evolution of emergent and reemerging antibiotic resistant disease is developing at an alarming rate (Demain and Elander, 1999). In recent years, new resistant strains emerge more quickly while the rate of discovery of new antibiotics is slowing down (Oskay et al., 2004; Parungao et al*.,* 2007). This necessitates the screening of micro-organisms for antimicrobial activity for the production of new and novel drugs (Singh et al., 2016). Hence, intensive search for new antibiotics has become imperative worldwide especially from new actinomycetes (Oskay et al., 2004; Parungao et al., 2007). In addition, it has been reported that screening programs using highly selective procedure allows the detection and isolation of effective antibiotic producing microorganisms from soil (Rondon et al., 2000; Oskay et al., 2004; Parungao et al., 2007). Soil is the largest source of microorganisms and a natural reservoir for microorganisms (Dancer, 2004; Hackl et al., 2004).

Although soils have been screened by the pharmaceutical industry for about 50 years, only a small fraction of the surface of the earth has been sampled and only a small fraction of actinomycetes taxa has been discovered (Baltz, 2007). This requires the employment of several strategies to explore new compounds from microorganisms such as actinomycetes from different ecological niches that may yield novel compounds with diverse antimicrobial properties (Pandey et al., 2004; Ningthoujam et al., 2009).

In Ethiopia, a few investigators showed the existence of antibiotic producing microorganisms from different ecosystems. Biniam (2008) isolated antimicrobial producing actinomycetes from southern part of Ethiopian Rift Valley alkaline lakes. The potential of mushroom compost as a good source of antibiotic producing thermophilic actinomycete was also reported by Moges (2009). However, there is a further need to explore indigenous actinomycetes for their potential to produce effective antibiotics from different sources in the country to combat infectious diseases. Hence, this study aimed to isolate, screen, and characterize effective antibiotics producing actinomycetes and evaluate their potential against some test microorganisms.

MATERIALS AND METHODS

Soil sample collection

A total of 15 soil samples were collected from the rhizosphere of plants and agricultural soils from field sites of Deberizit (5 samples) and Holeta (5 samples) and garden soil from the College of Natural Sciences (5 samples) in December 2010. The soils were excavated from depth of 5 to 15 cm by using sterile spatula and collected in clean, dry and sterile polyethylene bags. All samples were labeled, transported to Addis Ababa University, Applied Microbiology Laboratory and stored in the refrigerator at 4°C for further investigations.

Media for the cultivation of actinomycetes

For isolation of actinomycetes, the following two media were used: starch casein agar medium (SCA, g/L) containing soluble starch 10; casein, 0.3; KNO_3 2, NaCl 2, K_2HP0_4 2, $MgSO_4.7H_2O$ 0.05, $CaCO_3$ 0.02, $FeSO_4.7H_2O$ 0.01; agar, 15; and pH was adjusted to 7.0±2 before sterilization (Arifuzzaman et al., 2010) and actinomycetes isolation agar (AIA) medium of g/L containing heart infusion broth, 25.0; casein hydrolysate, 4.0; yeast extract, 5.0; dextrose, 5.0; cysteine HCl, 1.0; soluble starch, 1.0; potassium phosphate, 15.0; ammonium phosphate, 1.0; magnesium sulphate, 0.2; calcium chloride, 0.02; Agar, 20; and pH was adjusted to 7 before sterilization (Awad et al., 2009). The two media were autoclaved at 121°C for 15 min; cooled up to 50°C and supplemented with 50

μg/ml of amphotericin B to minimize fungal growth; 20 to 25 ml of media was poured on sterile Petri dishes allowed to cool at room temperature.

Isolation, maintenance and designation of isolates

Actinomycetes were isolated by serial dilution plate technique (Arifuzzaman et al., 2010). About 1 g of each soil sample was suspended in 10 ml of sterile normal saline (0.85%) and shaken on orbital shaker for about 30 min at 121 rpm; the suspension was left for 30 min in the Laminar Air Flow hood. Consequently, 1 ml of suspension was taken and diluted 7-fold in 9 ml of sterile normal saline (0.85%). The suspensions were agitated with vortex and 0.1 ml of suspension was taken from 10^{-3}, 10^{-5}, and 10^{-7} dilutions and spread on each of the Starch Casein Agar medium and Actinomycetes Isolation Agar medium. The plates were incubated at 30°C for 7 to 10 days (Dhanasekaran et al., 2009). All isolates were identified as actinomycetes based on colony morphology and color of mycelium (Williams and Cross, 1971). The isolates were further sub-cultured to ensure their purity and maintained on starch casein agar medium. The inoculated agar media or slant was incubated at 30°C for 7 days and then stored at 4°C for further use. Code was assigned (AAUBA1 to AAUBA31) for every entity to confirm the source of the organism and the color of aerial mycelium.

Screening of antimicrobial activity of actinomycetes isolates against test microorganisms

Test microorganisms

The test organisms used for antimicrobial screening were: *S. aureus* ATCC25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 25853, *S. aureus* (clinical isolate), *Shigella boydii* (clinical isolate), *Streptococcus pneumonia* ATCC 49619, *Sallmonella typhi* ATCC 6539, *Candida albicans* ATCC 62376 and *Cryptococcus neoformance* (clinical isolate). The test bacteria and fungi were obtained from Ethiopian Health and Nutrition Research Institute (EHNRI) and Biomedical Laboratory, Faculty of Life Sciences, AAU.

Turbidity standard for inoculum preparation

Standardization of the inoculum density of isolates for susceptibility test was done by the method described in Lalitha (2004). In order to determine the active phase of test organisms, each isolate was grown in 100 ml of nutrient broth for bacteria and Sabourouds broth for fungi in 250 ml Erlenmeyer flask on a rotary shaker at 120 r/min and 37°C. Samples were taken every 2 h to measure optical density using spectrophotometer (JENWAY, London) at 660 nm. The optical density values were extra plotted against time to determine the different phases of the growth curve.
Samples from the exponential phase were taken to adjust the inoculum density with 0.5 McFarland Turbidity Standard prepared by adding a 0.5 ml of $BaCl_2$ solution into 99.5 ml of solution H_2SO_4 (Lalitha, 2004). The density of the turbidity standard was determined using spectrophotometer (JENWAY, London) at 660 nm.

Primary screening

A total of 30 isolates were primarily screened for antimicrobial activity against five test microorganisms according to Pandey et al. (2004). Seven day grown isolates were streaked as a straight line across diameter on Nutrient Agar plates (Oxiode) and incubated at 30°C for 6 days. After 6 days, the test microorganisms, namely, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 25853, *S. aureus* clinical isolate and *S. boydii* clinical isolate from overnight culture broth were streaked at right angle, but not touching the streaked isolate and incubated at 37°C for 24 h. Clearing zone formation between the antibiotic producing isolate and the test organisms was considered positive for antibiotic production. The isolates were then selected based on a wide spectrum activity against tested microorganisms for further studies.

Secondary screening

Cultivation and extraction of cultures of actinomycetes for secondary metabolites

Nine isolates were selected for secondary screening in small scale submerged fermentation system. 200 ml of starch casein broth was dispensed into 500 ml Erlenmeyer flask, to which a loop full of seven days grown isolates were inoculated and incubated on a platform shaker (New Brunswick Scientific), at 200 rpm and room temperature for 10 days (Remya and Vijayakumar, 2008; Dhanasekaran et al., 2009). After ten days of incubation, the content of incubated flask was filtered through 0.2 μm pore size Whatman No. 1 filter paper. Equal volume of ethyl acetate (1:1) was then added to the culture filtrates and shaken vigorously for 1 h and solvent phase that presumably contains antibiotics compound was separated from aqueous phase in a separatory funnel (Assistant, Germany). The ethyl acetate phase that contains antibiotics was evaporated and concentrated in vacuum rota-vapor (BUCHI-Germany) at 100 rev/min and 60°C (Fessenden, 1993; Remya and Vijayakumar, 2008). The dry crude extract was weighted using a balance (SCALTEC, Germany) and kept in small vials at 4°C for further test.

Disc diffusion assay

Antimicrobial activity of the dried crude extract of each isolate was evaluated by using disc diffusion assay (Hassan et al., 2001; Ningthoujam et al., 2009). For this purpose, nutrient agar (NA) (Oxiod) for bacteria and Sabouraud Dextrose Agar (SDA, Oxiod) for yeast/fungi were inoculated with 0.2 ml overnight culture of each standardized test organism; *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 25853, *S. aureus* (clinical isolate), *S. boydii* clinical isolate, *S. pneumonia* ATCC 49619, *S. typhi* ATCC 6539, *C. albicans* ATCC 62376 and *C. neoformance* (clinical isolate).

About 20 μl of 50 μg/ml crude extract of each isolate was impregnated with sterile Whatman 0.2 μm pore size antibiotic assay discs (6.0 mm) and placed on the inoculated agar plates. Blank discs impregnated with the solvent were also placed on inoculated plates that served as negative control. This was done in duplicates. The Petri dishes were then kept in a refrigerator at 4°C for 2 h to allow the diffusion of the extracts in the media. The Petri dishes were then incubated at 37°C for 24 h to detect and measure the inhibition diameter around the discs.

Bioassay guided purification of active compound from AAUBA13

The crude extract of AAUBA13 isolate was selected based on inhibition zone diameter and spectrum activity from antimicrobial assay test to detect the antimicrobial component of the crude extract using chromatographic methods (Al-Bari et al., 2006;

Gurung et al., 2009).

Thin layer chromatography (TLC)

The TLC plate was cut into 5 × 10 cm and the penal line was drawn on the white side up using pencil and ruler. Consequentially, 10 µl of the crude extract to be separated was spotted in a single small spot of 1.5 cm from the end of the TLC plate using capillary tube. Before inserting the TLC plate, the developing solvent was poured into the solvent tank to cover the bottom of the tank to a depth of 1.0 cm. When the spot was dried, the plate was immersed into solvent tank containing a 1:9 solvent mixture of chloroform: methanol. The solvent was allowed to get to about 90% of the way up, the plate was taken out of the jar with forcipes, and then the solvent front was marked with a pencil immediately and allowed to dry. Spot was visualized with ultraviolet (black) lamp at 254 and 366 nm (Fessenden, 1993; Gurung et al., 2009). Visualization of the spot was also done with vanillin-sulfuric acid spray reagent (Al-Bari et al., 2006; Selvameenal et al., 2009). The spot was circled with pencil. The distance of the spot moved up the plate and that of the solvent was measured in cm. The retention factor (R_f) values of the antimicrobial compound were calculated by dividing distance travelled by the spot to the distance traveled by the solvent (Fessenden, 1993).

R_f = Distance traveled by the compound / Distance traveled by the solvent

To check the antimicrobial activity of single spot from TLC plates, active bioautography was done on nutrient agar plate inoculated with 0.2 ml (3×10^6) overnight culture of *S. aureus* (clinical isolates). The spots from TLC plates were cut and placed downward on the center of seeded plate. After 1 h at 4°C in refrigerator, the plate was incubated for 24 h at 37°C. The inhibition of the test organism indicated the spot has antimicrobial activity (Pandy et al., 2004).

Column chromatography

For the single spot from TLC plate, active column chromatography was performed to purify and detect the components of the antimicrobial compound based on their polarity with solvents. The column (Sorbisil 60 mesh; column dimensions 2.5 cm inner diameter × 30 cm length) was packed very tightly with silica gel as slurry with the desired solvent (ethyl acetate). 100 mg of the active crude extract was dissolved in a minimum amount of ethyl acetate and applied directly on top of the packed column. Thereafter, 10 ml of the eluting gradient chloroform: methanol solvent was added to the top of the column. The proportion of crude extract to silica gel in the solvent mixture is 1:30. The column was developed and the flow rate of the system was adjusted per minute. The elution process was conducted by adding different solvents, namely, ethanol, petroleum ether, acetone and methanol in the middle of the process (Fessenden, 1993). Thirteen different fractions of the compound were collected (each of 5 ml) from the column chromatography.

Preparative thin layer chromatography

Preparative TLC plate was prepared by suspending 160 g of Merksilca gel GF 254 powder in 260 ml of deionized water that was vigorously shaken for 45 s in 500 ml Erlenmeyer flask, with rubber stopper. The thickened slurry was poured into 10 × 20 cm plate and spread to prepare 1.0 mm trailing edge. The plate was air dried until it turned white for 45 min and activated at 120°C for half an hour. Ten microliters of the fraction to be separated was applied in a single small spot of 1.5 cm from the end of TLC plate using capillary tube. Before inserting the TLC plate, the developing solvent was poured into the solvent tank to cover the bottom of the tank to a depth of 1.0 cm. When the spot was dried, the plate was immersed into solvent tank containing chloroform: methanol system (1:9). The spots on the chromatography were visualized in UV chamber 245 and 366 nm (Al-Bari et al., 2006). The obtained band was then scratched and dissolved with ethyl acetate and centrifuged at 3000 rpm for 15 min. Supernatant was collected in a pre-weighed vial and kept for evaporation (Selvameenal et al., 2009).

***In vitro* antimicrobial activity of purified compound from AAUBA13**

The antimicrobial potential of 13 fractions of the crude extract from AAUBA13 was tested using disc diffusion techniques.

Identification and characterization of selected isolates

Selected actinomycetes isolates: AAUBA1, AAUBA5, AAUBA6, AAUBA8, AAUBA10, AAUBA13, AAUBA19, AAUBA30 and AAUBA31 were described by some cultural, morphological, physiological and biochemical characterization to identify the isolates at genus level based on Bergey's Manual of Systematic Bacteriology, Volume four (Williams et al., 1989a,b).

Cultural (macroscopic) characterization

Cultural characteristics of the isolates were investigated by growing the isolates on starch casein agar, starch nitrate agar, glycerol asparagine agar, glucose asparagine agar and actinomycetes isolation agar. A loop full of each isolate from 7 days old culture was taken and inoculated onto each of the medium by streak plating technique and incubated at 30°C for 7 days. The experiment was done in duplicates and colony morphology was noted with respect to color of aerial mycelium and substrate mycelium, colony diameter and diffusible pigment (Williams and Cross, 1971; Remya and Vijayakumar, 2008). The results were recorded.

Microscopic characterization

The microscopic examination was carried out by cover slip culture and Gram staining methods to study the morphology of the isolates. Cover slip culture method was done according to previous works (Williams and Cross, 1971; Tiwarty, 2009) by inserting sterile cover slip at an angle of 45° in the starch casein agar medium. A loop full of isolates was taken from 7-day old culture and inoculated, at the insertion of the cover slip on the medium and incubated at 30°C for 7 days. The cover slip was carefully removed using sterile forceps and placed upward on a glass slide. The growth on the cover slip was fixed with few drops of absolute methanol for 15 min and washed with tap water and flooded with crystal violet reagent for 1 min followed by washing and blot drying. It was then examined through microscope (Wagtech, England) under magnification of ×1000 in oil immersion. The morphology of spore chains and hyphae of substrate and aerial mycelia were observed and the picture was captured by U-LH100HG Florescent microscope (OLYMPUS BXSI, JAPAN) in the Applied Microbiology Laboratory, Faculty of Life Science, AAU. The observed characteristics were compared with the actinomycetes morphology provided in Bergey's manual of Systematic Bacteriology (Williams et al., 1989a,b).

Physiological characterization

Physiological characters of the isolates were studied on the basis of

pH tolerance, temperature tolerance, resistance towards sodium chloride and utilization of carbon and nitrogen sources.

pH tolerance

A loop full of the test isolate from 7 days old culture was taken and serially diluted from 10^{-1} - 10^{-6} in sterile distilled water; it was agitated with vortex and about 0.1 ml of the suspension was taken and inoculated with spread plate technique onto starch casein agar media which was adjusted to pH levels of 5, 6, 7, 8, 9, 10, 11, and 12. The experiment was done in duplicates and colony was counted with log colony forming unit after incubating the isolates at 30°C for 7 days. The result was recorded (Laidi et al., 2006).

Temperature tolerance

Temperature tolerance of the isolates was determined on nutrient agar plates (Oxide). A loop full of the test isolate from 7 days old culture was taken and serially diluted from 10^{-1} - 10^{6} in sterile distilled water; it was agitated with vortex and 0.1 ml of the suspension was taken and inoculated with spread plate technique. The experiment was done in duplicate and colony was counted with log colony forming unit after incubating the isolates at 15, 25, 30, 37 and 45°C. The results were recorded (Laidi et al., 2006).

Growth with sodium chloride

The isolates were tested for their levels of tolerance to sodium chloride on nutrient agar (Oxide) supplemented with 5, 7 and 10% sodium chloride. Agar plates were inoculated with test isolates with streak plate technique. The experiment was done in duplicates. The plates were incubated at 30°C for 7 days and observations were made to record the highest concentration of salt that allows the growth (Santhi et al., 2010).

Utilization of carbon and nitrogen source

Different carbon and nitrogen sources were tested, in the basal medium consisting of (g/L) K_2HPO_4, 1.0; $MgSO_4.7H_2O$, 0.5; $CaCl_2.2H_2O$, 0.04; $FeSO_4.7H_2O$, 0.005; $ZnSO_4.7H_2O$, 0.0005; 15 g agar and 1.0% of each of the carbon and nitrogen sources such as D-mannose, sucrose, D-galactose, D-glucose, L-arabinose, D-mannitol, cellobiose and D-fructose were used. Nitrogen sources such as L-arganine, yeast extract, peptone, (NH_4) SO_4 and (NH_4) H_2PO_4 and pH were adjusted to 7. The carbon and phosphate sources were sterilized separately and added just prior to inoculation. Each isolate from 7 days culture was inoculated with streak plate technique. The experiment was done in duplicates and the plates were incubated at 30°C together with basal medium as negative control. The growth was read after 7, 14, and 21 days and the results were recorded as abundant (When growth on tested Carbon or Nitrogen in basal medium greater than growth on basal medium), moderate (when growth on tested carbon or nitrogen in basal medium is significantly better than growth on basal medium), and good (when growth on tested carbon or nitrogen in basal medium is less than growth on basal medium) (Shirling and Gottlieb, 1966; Oskay et al., 2004; Pandy et al., 2005).

Biochemical characteristics

Biochemical characteristics of the isolates were studied. They include gelatin hydrolysis, starch hydrolysis, and esculin degradation.

Starch hydrolysis

Starch hydrolysis was done using starch agar plates having a composition of soluble starch, 20 g; beef extract, 3 g; peptone, 5 g; agar, 15 g, and distilled water, 1 L. The isolates were taken from 7 days old culture. They were streaked on the media and incubated at 30°C for 7 days together with uninoculated plates that serve as a control. The iodine solution was flooded onto the plates to see the clear zone of hydrolysis around the colony (Aneja, 2005; Remya and Vijayakumar, 2008).

Gelatin hydrolysis

This test was done on sterile nutrient gelatin media having a composition of beef extract, 3 g; peptone, 5 g, gelatin, 120 g, and distilled water, 1 L. Each isolate was taken from 7 days old culture and stabbed into nutrient gelatin tubes with sterile needle. The tubes were incubated for 10 days at 30°C together with uninoculated tube used as control. After incubation, the tubes were placed in refrigerator, at 4°C for 15 min (Aneja, 2005; Sundaramoorthi et al., 2011). The refrigerated gelatin tubes were examined to see whether the medium was liquid for positive test or solid for negative test.

Esculin degradation

Esculin degradation was determined after Kutzner (1976). The isolates were taken from 7 days old culture and streaked into Esculin agar slants having a composition of yeast extract, 0.3 g; ferric ammonium citrate, 0.05 g, agar, 0.75 g; 0.1% of esculin, and 50 ml distilled water; they were incubated at 30°C for 7 days. Observations were made between the periods of incubation to check the blackening of the medium. Positive tests were confirmed compared to the control which was a dark brown substrate.

Data analysis

Data analysis was made by Statistical Package for Social Sciences (SPSS) windows version 17 in terms of the mean of the growth inhibition zone value obtained from each of the seven bacterial test pathogens and two fungal test pathogens. The data on temperature and pH tolerance were analyzed by comparing the mean growth in log colony forming unit through analysis of variances (ANOVAs). The mean for each group was compared using Tukey test (HSD).

RESULTS AND DISCUSSION

Cultures of actinomycetes isolated from different habitats

A total of 30 different actinomycete isolates were recovered from rhizosphere of different plants and soil samples collected from Debrezit and Holeta farm lands and garden soils of Arat Kilo campus. Sixty seven percent of the actinomycetes were isolated from rhizosphere of plants. Rouatt et al. (1951) and Geetanjali and Jain (2016) also reported that greater percentages of actinomycetes are found in rhizosphere soils, whereas 33% of the isolates were recovered from farm soil by using starch casein agar and actinomyctes isolation agar medium supplemented with Amphotracin B (50 µg/ml)

Table 1. Culture collection of soil actinomycetes isolates.

Codes of isolates	Source of soil samples		Sites	Total number of isolates
	Rhizosphere	Soil		
AAUBA1	+	-	Debrezit	
AAUBA2	-	+	Debrezit	
AAUBA3	+	-	Debrezit	
AAUBA4	-	+	Debrezit	
AAUBA5	+	-	Debrezit	
AAUBA6	+	-	Debrezit	
AAUBA7	-	+	Debrezit	14
AAUBA8	+	-	Debrezit	
AAUBA9	+	-	Debrezit	
AAUBA10	-	+	Debrezit	
AAUBA11	+	-	Debrezit	
AAUBA12	+	-	Debrezit	
AAUBA13	+	-	Debrezit	
AAUBA14	-	+	Debrezit	
AAUBA15	-	+	Holeta	
AAUBA16	-	+	Holeta	
AAUBA17	-	+	Holeta	
AAUBA18	-	+	Holeta	7
AAUBA19	+	-	Holeta	
AAUBA20	-	+	Holeta	
AAUBA21	+	-	Holeta	
AAUBA22	+	-	Aratkillo	
AAUBA23	+	-	Aratkillo	
AAUBA24	+	-	Aratkillo	
AAUBA26	+	-	Aratkillo	
AAUBA27	+	-	Aratkillo	9
AAUBA28	+	-	Aratkillo	
AAUBA29	+	-	Aratkillo	
AAUBA30	+	-	Aratkllo	
AAUBA31	+	-	Aratkillo	
Total (%)	20 (67)	10 (33)		30

(Table 1).

Primary screening of the antimicrobial producing isolates

Out of the 30 actinomycetes isolates subjected for primary screening, 18 (60%) isolates showed varying levels of antimicrobial activities against the five test microorganisms (Table 2). Upon primary screening, nine isolates were identified based on spectrum activity against test organisms, and the potent isolates were isolated from the rhizosphere soils. The present results in agreement with that of Abo-Shadi et al. (2010) reported that microorganisms isolated from rhizosphere soil could be an interesting source of antimicrobial bioactive substance. Ramakrishnan et al. (2009) also reported that rhizosphere soil can serve as an effective source of antimicrobial compounds. This ratio is much higher than the 22 and 34% reported by Abo-Shadi et al. (2010) and Oskey et al. (2004), respectively. This difference may be attributed to the differences in inhibiting antibiotic resistant of test organisms, the genetic differences of the antibiotic producing isolates, and their capacity to produce more than one secondary metabolite.

Out of the different actinomycetes isolates screened for antimicrobial activities, 2 isolates (11%) AAUBA5 and AAUBA30, showed a wide spectrum of antibiosis against

Table 2. Primary screening of antimicrobial activity of actinomycetes isolates.

Isolate	Test microorganisms					Spectrum activity
	S. aureus **ATCC25923**	***E. coli*** **ATCC25922**	***P. aeruginosa*** **ATCC25853**	***S. boydii***	***S. aureus***	
AAUBA1*	+	-	-	+	+	3
AAUBA3	-	-	+	-	-	1
AAUBA5*	+	-	+	+	+	4
AAUBA6*	+	-	+	-	+	3
AAUBA8*	-	+	+	-	+	3
AAUBA9	-	-	-	-	+	1
AAUBA10*	-	+	+	+	-	3
AAUBA11	-	-	-	-	+	1
AAUBA12	-	-	+	-	-	1
AAUBA13*	+	-	+	-	+	3
AAUBA14	-	-	+	+	-	2
AAUBA19*	+	-	+	+	-	3
AAUBA21	-	+	+	-	-	2
AAUBA22	-	-	+	-	+	2
AAUBA26	-	-	+	-	+	2
AAUBA28	+	-	-	-	+	2
AAUBA30*	+	-	+	+	+	4
AAUBA31*	+	-	-	+	+	3

+, Active against test organism; -, inactive against test organism. *Show broad spectrum activity.

4 test organisms except *E. coli* ATCC 25922. Likewise, 7 (39%), 5 (28%) and 4 (22%) of isolates were found to inhibit any 3, 2, and 1 of the test organisms, respectively. Antimicrobial activities of isolates that inhibited 3 test organisms were AAUBA1, AAUBA6, AAUBA8, AAUBA10, AAUBA13, AAUBA19 and AAUBA31. The antimicrobial activities of AAUBA14, AAUBA21, AAUBA22, AAUBA26 and AAUBA28 were found to inhibit 2 test organisms and the rest isolates inhibited 1 test organism (Table 2).

The antimicrobial activities of isolates also showed variations, in inhibiting the test organisms. Consequently, most of the actinomycetes of isolates 13 (72%) inhibited *P. aeruginosa* ATCC 25853, followed by antimicrobial activities of 12 actinomycetes isolates (67%) and antimicrobial activities of 8 actinomycetes isolates (44%) that inhibited *S. aureus* (clinical isolate) and *S. aureus* ATCC25923, respectively. Likewise, antimicrobial activities of 7 actinomycetes isolates (39%) showed a pattern of suppression on *S. boydii* (clinical isolate). The most resistant test isolate was *E. coli* ATCC 25922 that was inhibited by only 3 actinomycetes isolates (17%), namely, AAUBA8, AAUBA10, and AAUBA21 (Table 2).

Many isolates (39%) also showed a wide pattern of dual inhibition of *P. aeruginosa ATCC* 25853 and *S. aureus* (clinical isolate) and *S. aureus* ATCC25923 and *S. aureus* (clinical isolate). Few actinomycetes isolates (17%) had dual inhibition capacity against *E. coli* ATCC 25922 and most of the isolates (28%) had multiple inhibition capacity against S. *aureus* ATCC25923 and *P. aeruginosa* ATCC 25853, *S. aureus* ATCC25923 and *S. boydii* (clinical isolate). Some isolates (17%) had multiple inhibition capacity against *P. aeruginosa* ATCC 25853 and *S. boydii* (clinical isolates).

From 9 actinomycetes isolates that showed a wide spectrum of activity, antimicrobial activities of actinomycetes isolate (AAUBA10) were active

Table 3. Antimicrobial activities of the selected putative actinomycetes isolate crude extract.

Test microorganism	Antimicrobial activity (Zone of inhibition in mm) (Mean)								
	AAUB1	AAUBA5	AAUBA6	AAUBA8	AAUBA10	AAUBA13	AAUBA19	AAUBA30	AAUBA31
S. aureus ATCC25923	11	9	9	14	-	19	-	14	-
E. coli ATCC25922	-	13	11	10	-	20	-	8	-
P. aeruginosa ATCC25853	-	18	8	-	-	18	-	10	-
S. pneumoniae ATCC49619	12	20	11	7	10	20	-	10	-
S. typhi ATCC 6539	10	10	2	-	10	17	-	15	-
S. aureus clinical isolate	12	13	12	9	11	22	10	8	7
S. boydii clinical isolate	-	16	19	3	-	19	-	-	-
C. albicans ATCC62376	-	17	-	-	-	17	-	-	-
C. neoformance clinical isolates	-	-	-	-	-	-	-	-	-

-, No inhibition zones.

against only Gram negative microorganism and antimicrobial activities of eight actinomycetes isolates (AAUBA1, AAUBA5, AAUBA6, AAUBA8, AAUBA13, AAUBA19, AAUBA30, and AAUBA31) were against both Gram positive and Gram negative microorganisms. Of the nine actinomycetes isolates, AAUBA5, AAUBA6, AAUBA8,AAUBA10, AAUBA13, AAUBA19, and AAUBA30 were active against *P. aeruginosa* ATCC 25853; AAUBA1, AAUBA5, AAUBA6, AAUBA13, AAUBA19, AAUBA30 and AAUBA31 were active against *S. aureus* ATCC25923; AAUBA1, AAUBA5, AAUBA6, AAUBA8, AAUBA10, AAUBA13, AAUBA30 and AAUBA31 were active against *S. aureus* (clinical isolate); AAUBA1, AAUBA5, AAUBA10, AAUBA19, AAUBA30 and AAUBA31 were against *S. boydii* (clinical isolate); AAUBA8 and AAUBA10 were active against *E. coli* ATCC 25922 (Table 2).

Secondary screening of selected actinomycetes crude extract by disc diffusion assay

Based on the results of primary screening, 9 active actinomycetes isolates were selected for fermentation. The active actinomycete isolates include AAUBA1, AAUBA5, AAUBA6, AAUBA8, AAUBA10, AAUBA13, AAUBA19, AAUBA30 and AAUBA31. The results of ethyl acetate crude extracts of the actinomycetes for antimicrobial activities are shown in Table 3. The crude extracts of nine actinomycetes isolates showed activities against a minimum of 1 test organism and maximum of 8.

Isolate AAUBA30 crude extract was found to inhibit 6 test organisms while isolate AAUBA8 crude extract inhibited 5 test organisms. Isolate AAUBA1 crude extract was found to inhibit 4 test organisms except *E. coli* ATCC25922, *P. aeruginosa ATCC25853, S. boydii* (clinical isolate), *C. albicans* ATCC62376 and *C. neoformance* (clinical isolates). Isolate AAUBA10 crude extract inhibited *S. pneumonia* ATCC49619, *S. typhi* ATCC and *S. aureus* (clinical isolate). Isolates AAUBA19 and AAUBA31 crude extract inhibited only *S. aureus* (clinical isolate) (Table 3). The crude extract of isolates showed variations in spectrum against test microorganisms. Consequently, AAUBA1, AAUBA5, AAUBA6, AAUBA8, AAUBA10, AAUBA13, AAUBA19, AAUBA30 and AAUBA31 crude extracts were found to inhibit Gram positive bacteria *S. aureus* (clinical isolate) including AAUBA1, AAUBA5, AAUBA6, AAUBA8, AAUBA10, AAUBA13 and AAUBA30; AAUBA1, AAUBA5, AAUBA6, AAUBA8, AAUBA13 and AAUBA30 showed inhibition spectrum against Gram positive *S. pneumoniae* ATCC49619 and *S. aureus* ATCC25923, respectively. Likewise, AAUBA1, AAUBA5, AAUBA6, AAUBA10, AAUBA13 and AAUBA30 suppressed Gram negative *S. typhi* ATCC 6539; AAUBA5, AAUBA6, AAUBA8, AAUBA13 and AAUBA30 and AAUBA5, AAUBA6, AAUBA13 and AAUBA30 isolates inhibited Gram negative *E. coli* ATCC25922 and *P. aeruginosa* ATCC25853; AAUBA5, AAUBA6, AAUBA8 and AAUBA13 inhibited Gram negative *S. boydii* (clinical isolate). Two isolates of crude extract (AAUBA5 and AAUBA13) inhibited *C. albicans* ATCC62376 (Table 3).

Upon the result of primary and secondary screening methods it seems that from the 9 effective isolates, more isolates were active against Gram positive bacteria than Gram

negative bacteria (Tables 2 and 3). This might be due to the morphological differences on cell wall components between those two types of microorganisms. Gram negative bacteria have an outer lipopolysaccharide membrane, besides peptidoglican (Pandey et al., 2002). Hence, their cell wall is impermeable to lipophilic solutes. Porins constitute a selective barrier to the hydrophilic solutes (Nokaido and Vaara, 1985), antimicrobial compound rather than positive bacteria. In the antimicrobial bioassay screening process (Table 3), larger inhibition zone diameter was observed by crude extract of isolate AAUBA13 (22 mm) against *S. aureus* (clinical isolate) followed by *S. pneumoniae* ATCC49619 and *E. coli* ATCC25922 (20 mm) and the least was shown by crude extract of isolate AAUBA6 (2 mm) against *S. typhi* ATCC 6539. The differences in the ability to produce the clear zone were presumably dependent on the secondary metabolites that were produced by test isolates. This assumption was supported by Dharmawan et al. (2009) and Singh et al. (2016) who state there is variation in the diameter of clear zone because every isolate produces different types of secondary metabolites. Different types of secondary metabolites have different chemical structure, compounds and chemical concentration.

The resistance test isolates were *S. aureus* ATCC25923, *E. coli* ATCC25922, *P. aeruginosa* ATCC25853, *S. pneumoniae* ATCC49619, *S. typhi* ATCC 6539 and *S. boydii* (clinical isolate) that were resistant to one of the antagonistic isolates. The most sensitive one was *S. aureus* (clinical isolate) that was inhibited by all isolates crude extract; they vary in inhibition zone diameter. However, the most resistant test isolate was *C. neoformance* (clinical isolates) against all antagonistic isolates, followed by *C. albicans* ATCC62376 except against AAUBA5 and AAUBA13 crude extract (Table 3).

Accordingly, crude extract of isolates AAUBA5 and AAUBA13 showed a wider spectrum of antimicrobial activities. In this study, isolate AAUBA13 crude extract was found to be the best isolate from antimicrobial bioassay method; it is shown by its broad-spectrum activity with high mean zone of inhibition (22 mm) more than AAUBA5 crude extract and others. This isolate crude extract might produce more than one antimicrobial metabolites that make them effective inhibitor to Gram positive and Gram negative bacterial pathogen and also effective inhibitor to *C. albicans* ATCC62376 fungal pathogen (Gurung et al., 2009).

The antimicrobial activity of the crude extract of AAUBA13 isolate against the test pathogenic bacteria was positive with variations in inhibition zone diameter ranging from 17 to 22 mm. Among the Gram positives bacteria, *S. aureus* was found to be more sensitive followed by *S. pneumonia* ATCC49619 and *S. aureus* ATCC25923 in decreasing order. In case of Gram negative bacteria, *E. coli* ATCC25922 was more sensitive followed by *S. boydii* (clinical isolate), *P. aeruginosa* ATCC25853, and *S. typhi* ATCC in decreasing order. However, no activity was observed against the test fungus, *C. neoformance* (clinical isolate) (Table 3).

The antimicrobial activities of the 9 isolates through primary and secondary screening revealed different results. In the primary screening, isolates AAUBA5, AAUBA6, AAUBA8, AAUBA10, AAUBA13, AAUBA19 and AAUBA30 (Table 2) were found to inhibit *P. aeruginosa* ATCC 25853, but the fermented products of ABA8, AAUBA10 and AAUBA19 could not inhibit *P. aeruginosa* ATCC 25853 (Table 3). In the secondary screening, the fermented products of isolates AAUBA5, AAUBA6, AAUBA8, AAUBA13 and AAUBA30 were found to inhibit *E. coli* ATCC 25922, but in the primary screening, AAUBA5, AAUBA6, AAUBA13 and AAUBA30 crude extracts could not inhibit *E. coli* ATCC 25922 (Table 2). This might be due to the difference in the morphology of actinomycetes when grown in solid and liquid media as filamentous mycelia and fragmented mycelia, respectively. The present results are in agreement with those obtained by Pandey et al. (2004) who reported that some of the active actinomycetes isolates in the primary screening did not show any activity; but in the secondary screening some showed little activity and some showed improved activity. Bushell (1993) also reported that during the screening of the novel secondary metabolite, actinomycetes isolates failed in their antibiotic activity in liquid culture.

Detection and purification of the active compound from AAUBA13

Thin layer chromatography and column chromatography

The crude extract of AAUBA13 was run on a TLC plate and a single band (spot) with blue color was detected at R_f value 0.73. The column chromatography experiment showed 13 different fractions. This was already indicated by the color of the bands on the silica gel plate. Fractions were tested for antimicrobial activity. Accordingly, fractions 8 was identified as best active fraction and concentrated to be further characterized as given in Table 5.

Preparative thin layer chromatography

Fraction 8 was further purified using preparative chromatography technique; it resulted in single visible band when visualized with UV light, and upon scratching of these bands out from the glass plate a pure compound was obtained.

The antimicrobial form AAUBA13 showed R_f value of 0.73 in chloroform: methanol (1:9); 0.48 ethanol: petroleum ether (1:1) solvent systems and 0.57

Table 4. R_f values of the pure active fraction 8 in 3 solvent systems.

Solvent system	R values
Chloroform: Methanol, 1:9	0.73
Chloroform: Ethyl acetate, 1:1	0.57
Ethanol: Petroleum ether, 1:1	0.48

Table 5. Inhibition zone of the antimicrobial activity of fractions 4, 8 and 10.

Test microorganism	Zone of inhibition in mm (Mean)		
	F4	F8	F10
S. aureus ATCC25923	6	7	5
E. coli ATCC25922	4	8	4
P. aeruginosa ATCC25853	6	5	0
S. pneumonia ATCC49619	13	14	13
S. typhi ATCC 6539	15	16	12
S. aureus clinical isolate	11	24	5
S. boydii clinical isolates	12	8	10
C. albicans ATCC62376	0	11	0
C. neoformance clinical isolates	0	0	0

Chloroform: ethyl acetate (1:1) solvent system (Table 4).

In vitro antimicrobial activities of the fractions

From the 13 fractions, only 3 were found to be active against all tested microorganisms (Table 5). Accordingly, fraction F8 displayed large inhibition zone than that of fractions F4 and F10. The highest antimicrobial inhibition activity was observed against *S. aureus* clinical isolate with a clear zone diameter of 24 mm; the lowest was observed against *P. aeruginosa* ATCC25853 with diameter of 5 mm. Fraction 4 showed activity against all test bacteria. Fraction F10 showed less activity against all test pathogens and this fraction has no activity against *P. aeruginosa* ATCC25853 compared to fractions F4 and F8. Only F8 has activity against *C. albicans* ATCC 62376. However, all fractions did not show antifungal activity against *C. neoformance* (clinical isolates).

In this work, the antimicrobial compound from isolate AAUBA13 crude extract separated into individual component with chromatography and test confirms the crude extract was a mixture of different compound. The active fraction eluted at fractions 4, 8 and 10. This indicates the active components have polar and non-polar nature. Fraction which moves or elutes faster (F4) and is retained least on the adsorbed silica is most likely non-polar and fraction that moves slower (F10) and is retained more on the adsorbent silica gel is polar (Atta et al., 2009).

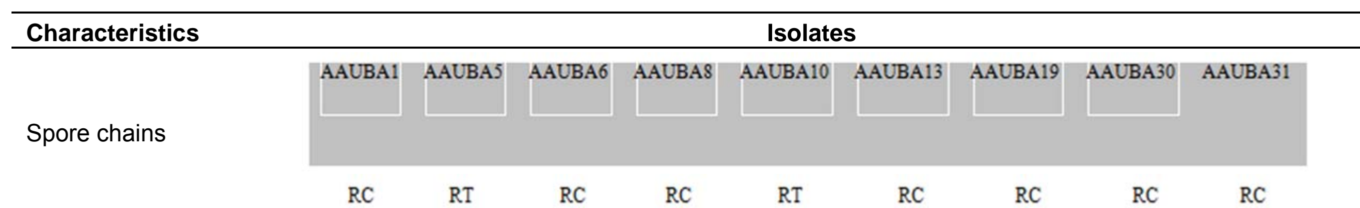
The antimicrobial activity of the purified fraction from isolate AAUBA13 crude extract showed variations in inhibition zone diameter from 5 to 24 mm against test microorganisms. Maximum inhibition zone diameter for crude extract was 22 mm against *S. aureus* (clinical isolate) (Table 3), whereas the purified fraction showed inhibition zone diameter of 24 mm against *S. aureus* clinical isolates (Table 5). This indicates that the activity of antimicrobial compound increases as the compound becomes pure. This agrees with the work of Kavitha and Vijayalakshmi (2007). However, like that of crude extracts, purified compound did not show activity against *C. neoformance*. Therefore, the antimicrobial compound obtained from AAUBA13 crude extract was not effective against *C. neoformance*. On the other hand, the crude extract as well as purified compound obtained from AAUBA13 crude extract showed activity against *C. albicans* ATCC62376.

Identification and characterization of selected isolates

Morphological characterization

Among the isolates, AAUBA5 and AAUBA10 have a hook (retinaculiaperti) like structure, whereas isolates AAUBA1, AAUBA6, AAUBA8, AAUBA13, AAUBA30 and AAUBA31 showed (rectiflexible) spore chains (Table 6), with smooth spore chains of 3 or more and branched mycelium from the cover slip culture. The microscopic examination emphasized that the spore chain with rectiflexible and retinaculiaperti with smooth spore surfaces were typical characteristics of genus

Table 6. Microscopic observation of selected isolates

Characteristics	Isolates								
	AAUBA1	AAUBA5	AAUBA6	AAUBA8	AAUBA10	AAUBA13	AAUBA19	AAUBA30	AAUBA31
Spore chains									
	RC	RT	RC	RC	RT	RC	RC	RC	RC

RC, Rectiflexible; RT, retinaculiaperti.

Streptomycetes (Williams et al., 1989a,b).

Cultural characterization

The isolates were found to grow in all media with some variations. However, abundant growth of the isolates was observed on starch casein agar medium, moderate growth was observed on actinomycetes isolation agar and good growth was observed on glycerol asparagine agar medium. Diffusible pigment was observed only on isolate AAUBA6 on starch casein agar medium and glycerol asparagine agar medium. The isolates were characterized by different colony diameter on starch casein agar medium at pH 7 (Table 7).

The color of the aerial and substrate mycelia varied depending on the type of the media used. The color of the substrate mycelia was also recorded (Table 7). The cultural (Macroscopic) characteristics of aerial and substrate mycelium of each isolate range from white, grey, blue, yellow, brownish, pink to dark grey. No diffusible pigment was produced except in AAUBA6 isolate, where red diffusible pigment was observed. These results reveal that the actinomycetes isolates were related to the genus *Streptomyces* (Cross, 1989; Lechevalier, 1989; Locci, 1989).

Physiological characteristics

pH and temperature tolerance

The isolates were found to grow well relatively in wider range of pH 5 to 12; maximum growth was observed at pH 7 and for isolate AAUBA1 and AAUBA13, it was pH 8. Growth tends to significantly decrease as the pH levels increase and decreases (Table 8). The isolates did grow at temperatures of 15 to 37°C, in which number of colony and colony diameter were higher than the rest of the temperature ranges. However, all isolates did not grow at temperature of 45°C (Table 9).

Growth with NaCl

All isolates were grown at a concentration of salt supplemented with 5 and 7% except isolates AAUBA8 and AAUBA19 (Table 10). However, all isolates did not grow at a concentration of salt supplemented on 10% sodium chloride. AAUBA31 did not grow in a salt concentration supplemented with 7% of sodium chloride (Table 10).

Utilization of carbon and nitrogen sources

The isolates found to grow on a wider range of carbon and nitrogen sources were used with little variations. Table 10 shows that the isolate can utilize different carbon and nitrogen sources.

Consequently, all the isolates utilized D-glucose and L-arganine as a carbon and nitrogen source, respectively, followed by D-galactose, D-fructose, cellobiose, D-mannitol and L- arabinose, and D- mannose and sucrose as a source of carbon in decreasing order; yeast extract, peptone, (NH_4) H_2PO_4 and $(NH_4)_2SO_4$ as a source of nitrogen in decreasing order. Isolate AAUBA6 was found to utilize best both carbon and nitrogen source used, followed by isolate AAUBA13. The least utilization was shown on isolates AAUBA10 and AAUBA31. Isolates AAUBA6, AAUBA13 and AAUBA30 were best in the utilization of the carbon source used (Table 10).

Cultural characteristics of the 9 isolates varied based on the type of the media used most likely due to the nutritional versatility of the isolates. The utilization of a variety of carbon sources by this actinomycete isolates will help in adaptation to a variety of inoculation sites and wide soil types (Yadav et al., 2009). Concerning utilization of a variety of nitrogen source, the isolates produced abundant aerial mycelium on organic nitrogen source medium and good growth was observed on inorganic nitrogen source. This is also in agreement with the report of Petrova and Vlahov (2007).

Biochemical test

All of the isolates showed clear zone when flooded with iodine solution indicating that they are capable of hydrolyzing the starch; they were also found to hydrolyzed gelatin. All isolates showed a dark brown to black complex on esculin medium, which is a positive

Table 7. Cultural characteristics of isolates grown on different media.

Medium	Test isolates								
	AAUBA1	AAUBA5	AAUBA6	AAUBA8	AAUBA10	AAUBA13	AAUBA19	AAUBA30	AAUBA31
SCA									
Aerial mycelium	White	Whitish brown	Pink	Grey	Brown	White	Dark grey	Yellowish white	Dark brown
Substrate mycelium	White	Brown	Reddish brown	Brownish grey	Brown	Light yellow	Dark grey	Yellow	Dark brown
Diffusible pigment	None	None	Red	None	None	None	None	None	None
Growth	+++	+++	+++	+++	+++	+++	+++	+++	+++
SNA									
Aerial mycelium	White	Whitish brown	Pink	Grey	Brown	White	Dark grey	Yellowish white	Dark brown
Substrate mycelium	White	Brown	Reddish brown	Brownish grey	Brown	Light yellow	Grey	Yellow	Dark brown
Diffusible pigment	None	None	None	None	None	None	None	None	None
Growth	++	++	+	+	++	++	+	++	++
GAsA									
Aerial mycelium	White	Brown	Pink	Grey	Brown	White	Dark grey	Yellowish white	Dark brown
Substrate mycelium	White	Brown	Pink	Dark grey	Brownish	Light yellow	Dark grey	Yellow	Brown
Diffusible pigment	None	None	None	None	None	None	None	None	None
Growth	+	+	++	+	+	+	++	++	+
GlyAsA									
Aerial mycelium	White	Brown	Pink	Pale grey	Brown	White light	Dark	Yellow	Brown
Substrate mycelium	Yellow	Brown	Brown	Grey	Brownish	Yellow	Dark grey	Yellow	Brown
Diffusible pigment	None	None	Red	None	None	None	None	None	None
Growth	+	+	+	+	+	+	+	+	+
AIA									
Aerial mycelium	White	Whitish brown	Pink	Grey	Brown	White	Dark grey	Yellowish white	Dark brown
Substrate mycelium	White	Brown	Reddish brown	Brownish grey	Brown	Light yellow	Dark grey	Yellow	Dark brown
Diffusible pigment	None	None	Red	None	None	None	None	None	None
Growth	++	++	++	++	++	++	++	++	++

+, Good growth; ++, moderate growth; +++, abundant growth; SCA, starch casein agar; SNA, starch nitrate agar; GAsA, glucose asparagine agar; GlyAsA, glycerol asparagine agar; AIA,actinomycetes isolation agar.

reaction to esculin degradation.

The biochemical tests, physiological properties of local isolates, as well as its carbon and nitrogen source utilization characteristics can also be compared to those of the actinomycetes described in Bergey's Manual of Determinative

Table 8. pH tolerance of selected isolates.

pH	Growth with Log cfu								
	AAUBA1	AAUBA5	AAUBA6	AAUBA8	AAUB10	AAUBA13	AAUBA19	AAUBA30	AAUBA31
5	4.32±0.005^{f}	5.26±0.005^{f}	4.95±0.000^{e}	4.97±0.010^{f}	5.53±0.005^{e}	5.78±0.005^{f}	4.02±0.005^{g}	6.22±0.005^{e}	5.24±0.005^{ef}
6	4.97±0.005^{d}	5.75±0.005^{c}	4.98±0.015^{e}	4.99±0.005^{f}	5.67±0.005^{d}	5.84±0.005^{e}	5.11±0.005^{e}	6.68±0.005^{cd}	5.55±0.005^{d}
7	5.23±0.005^{ab}	5.88±0.010^{a}	5.57±0.005^{a}	6.12±0.005^{a}	6.71±0.005^{a}	6.24±0.005^{b}	5.55±0.005^{a}	7.00±0.005^{a}	5.99±0.005^{a}
8	5.31±0.005^{a}	5.80±0.010^{b}	5.23±0.005^{b}	5.95±0.005^{b}	6.29±0.005^{b}	6.79±0.005^{a}	5.48±0.005^{b}	6.86±0.005^{b}	5.86±0.005^{b}
9	5.24±0.005^{ab}	5.72±0.005^{c}	5.15±0.005^{c}	5.67±0.005^{c}	5.78±0.005^{c}	5.99±0.005^{c}	5.37±0.005^{c}	6.79±0.005^{c}	5.77±0.005^{c}
10	5.16±0.005^{c}	5.68±0.005^{d}	5.02±0.005^{d}	5.58±0.005^{d}	5.49±0.005^{f}	5.89±0.005^{d}	5.32±0.005^{d}	6.69±0.005^{cd}	5.18±0.005^{ef}
11	5.05±0.055^{cd}	5.59±0.005^{e}	4.54±0.005^{f}	5.34±0.005^{e}	5.29±0.005^{g}	5.04±0.005^{g}	5.12±0.005^{e}	5.87±0.495^{f}	4.87±0.005^{g}
12	4.70±0.005^{e}	5.27±0.005^{f}	4.37±0.005^{g}	4.97±0.005^{f}	4.83±0.005^{h}	4.85±0.005^{h}	5.07±0.005^{f}	5.13±0.005^{g}	4.36±0.005^{h}

Means within the column under a pH parameter, having a common letter do not differ significantly ($p \leq 0.05$).

Table 9. Temperature tolerance of selected isolates.

Temperature (T°C)	Growth with Log cfu								
	AAUBA1	AAUBA5	AAUBA6	AAUBA8	AAUBA10	AAUBA13	AAUBA19	AAUBA30	AAUBA31
15	5.43±0.025^{b}	5.25±0.000^{c}	4.96± 0.005^{d}	5.38±0.005^{b}	4.92±0.005^{d}	5.30±0.005^{d}	5.16±0.005^{c}	5.28±0.005^{d}	4.99±0.005^{d}
25	5.49±0.005^{b}	5.42±0.005^{b}	5.31±0.005^{b}	5.39±0.005^{b}	5.21±0.005^{c}	5.48±0.005^{c}	5.25±0.005^{b}	5.39±0.005^{c}	5.60±0.005^{c}
30	5.59±0.005^{a}	5.57±0.005^{a}	5.53±0.005^{a}	5.49±0.005^{a}	5.48±0.005^{a}	5.84±0.005^{a}	5.66±0.005^{a}	5.67±0.005^{a}	5.92±0.005^{a}
37	5.33±0.005^{c}	5.41±0.005^{b}	5.23±0.005^{c}	5.32±0.010^{c}	5.26±0.005^{b}	5.57±0.005^{b}	5.27±0.005^{b}	5.45±0.005^{b}	5.79±0.005^{b}

Means within the column under a temperature parameter, having a common letter do not differ significantly ($p \leq 0.05$).

Table 10. Utilization of carbon and nitrogen source and NaCl tolerance of selected isolates.

Utilization of carbon source	AAUBA1	AAUBA5	AAUBA6	AAUBA8	AAUBA10	AAUBA13	AAUBA19	AAUBA30	AAUBA31
D-Glucose	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
D-Galactose	+ +	+ +	+ +	+	+	+ +	-	+ +	+
D-Fructose	+	+ +	+	-	-	+	+	+ +	-
D-Mannose	-	+	-	-	+	+ +	-	-	+
D-Mannitol	+	-	-	-	+	-	+ +	+	+
L-Arabinose	+	+	+	+ +	-	-	+	-	-
Cellobiose	-	-	+	+	+ +	+	-	+	+ +
Sucrose	+	-	+ +	-	-	+	-	+	-

Table 10. Contd.

Utilization of nitrogen sources									
L-Arganine	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
$(NH_4)\ H_2PO_4$	+	-	+ +	-	-	+	-	+ +	+
$(NH_4)_2SO_4$	-	-	-	+	+	-	+	-	-
Peptone	-	+	+	+ +	+	+ +	-	-	+
Yeast extract	+	+	+ +	+	-	+	+ +	+	-
Growth with NaCl									
5%	+	+	+	-	+	+	-	+	+
7%	+	+	+	-	+	+	-	+	-
10%	-	-	-	-	-	-	-	-	-

Utilization of carbon and nitrogen sources; ++, abundant (When growth on tested carbon or nitrogen in basal medium greater than growth on basal medium); ++, moderate (when growth on tested carbon or nitrogen in basal medium is significantly better than growth on basal medium); -, good (when growth on tested carbon or nitrogen in basal medium is some growth on basal medium). Growth with NaCl; -, no growth; +, growth.

Bacteriology, showing these isolates were found to be classified under genus Streptomyces (Lechevalier et al., 1989; Williams et al., 1989).

Conclusion

The finding of this study showed that the antimicrobial compound obtained from AAUBA13 crude extract has an antibacterial activity and also an antifungal activity. The data, in general, showed that the antimicrobial compounds obtained from AAUBA13 demonstrate broad spectrum and a remarkable antimicrobial activity against bacterial and C. albicans ATCC62376.
Actinomycetes isolates recovered from rhizosphere samples showed the potential to produce antimicrobial bioactive compounds. It is also suggested that the other isolates should be further processed to fully realize their antibiotic property on different test microorganisms. There is need for further studies to optimize the production conditions of the bioactive compounds from the potent actinomycetes isolates.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

The first author would like to thank Addis Ababa University, University of Gondar, for financial support.

REFERENCES

Abo-Shadi MA, Sidkey NM and Al-Mutrafy AM (2010). Antimicrobial Agent Producing Microbes from some Soils' Rhizosphere in Al-Madinah Al-Munawwarah, KSA. Journal of American Science, 6:915-925.

Adegboye MF, Babalola OO (2013). Actinomycetes: a yet in exhaustive source of bioactive secondary metabolites. Microbial Pathogens and Strategies for Combating Them, Science, Technology and Education (Mendez-Vilas A. Ed.). Formatex, pp. 786-795.

Al-Bari AA, Mohammed AS, Sazedur RM, Ashik MM (2006). Characterization and Antimicrobial activities of a Phthalic Acid Derivative produced by Streptomycetes bangladeshiensis. A Novel Species Collected in Bangladish. Research Journal of Medicine and Medical Sciences, 1:77-81.

Aneja KR (2005). Experiments in microbiology plant Pathology and Biotechnology. 4th ed. New Age International Publishers. New Delhi. pp. 74-128.

Arifuzzaman M, Khatun M R, Rahman H (2010). Isolation and screening of actinomycetes from Sundarbans soil for antibacterial activity. African Journal of Biotechnology, 9:4615-4619.

Atta HM, Dabour SM, Desoukey SG (2009). Sparsomycin Antibiotic Production by Streptomyces Spp. AZ-NIOFD1: Taxonomy, Fermentation, Purification and Biological Activities. American-Eurasian Journal of Agricultural & Environmental Science, 5:368-377.

Awad MH, El-Shahed KY I E-Nakkadi AEM (2009). Isolation, Screening and Identification of Newly isolated soil Streptomycetes (Streptomycetes. NRC-35) for β-Lactamase inhibitor production. World Applied Sciences Journal, 7:637-646.

Baltz RH (2007). Antimicrobials from Actinomycetes: Back to

the future. Microbe, 2:125-131.
Berdy J (2005). Bioactive microbial metabolites. The Journal of Antibiotics, 58:1-26.
Biniam W (2008). Bioactive compounds from alkaliphilic Actinomycetes. M.Sc. Thesis, Department of Biology (Microbiology), Addis Ababa University, Addis Ababa.
Brock TD, Madigan MT (1991). Biology of Microorganisms. 6th ed. Prentice-Hall International Inc., USA. pp. 198-223.
Bushell ME (1993). A method for increasing the success rate of duplicating antibiotic activity in agar and liquid cultures of Streptomycetes isolates in new antibiotics screens. Journal of Fermentation and Bioengineering, 76:89-93.
Cross T (1998). Growth and examination of actinomycetes some guidelines. In. Bergey' manual of systematic bacteriology, vol.4, Williams and Wilkins company, Baltimore. pp. 1120-1126.
Dancer SJ (2004). How antibiotics can make us sick: the less obvious adverse effects of antimicrobial chemotherapy. The Lancet Infectious Diseases, 4:611-619.
Demain AL, Elander R (1999). The β-lactam antibiotics: past, present, and future. Antonie Van Leeuwenhoek, 75(1-2):5-19.
Dhanasekaran D Thajuddin N, Panneerselvam A (2009). Distribution and Ecobiology of Antagonistic Streptomycetes from Agriculture and Coastal Soil in Tamil Nadu, India. Journal of Culture Collections, 6:10-20.
Dharmawan IWE, Retno K, Made SP (2009). Isolation of Streptomyces spp. In Bali Barat National Park and inhibition test to five diarrheagenic *Escherichia coli* strain. Biodiversitas Journal of Biological, 13:1-6.
Enright MC (2003). The evolution of a resistant pathogen-the case of MRSA. Current Opinion in Pharmacology, 3:474-79. Fernando P (2006). The historical delivery of antibiotics from microbial natural products-can history repeat? Biochemical Pharmacology, 71:981-990.
Fessenden JR (1993). Organic Laboratory Techniques. Books/Cole Publishing Company Pacific Grove, California. pp. 189-240.
Geetanjali, Jain P (2016). Antibiotic production by rhizospheric soil microflora - a review. International Journal of Pharmaceutical Sciences and Research, 7(11):4304-4314.
Gurung DT, Sherpa C, Agrawal VP, Lekhak B (2009). Isolation and Characterization of Antibacterial Actinomycetes from Soil Samples of Kalapatthar, Mount Everest Region. Nepal Journal of Science and Technology, 10:173-182.
Hackl E Boltenstern S, Bodrossy L, Sessitsch A (2004). Comparison of diversities and compositions of bacterial populations inhabiting natural forest soils. Applied and Environmental Microbiology, 7:5057-5065.
Hassan MA, El-Naggar MY, Said WY (2001). Physiological factors affecting the production of an antimicrobial substance by Streptomyces violatus in batch cultures. Egyptian Journal of Biology, 3:1-10.
Kavitha and Vijayalakshmi M (2007). Studies on Cultural, Physiological and Antimicrobial activities of Streptomycetesrochei. Journal of Applied Sciences Research, 3(12):2026-2029.
Kornwendisch F, Kutzner HJ (1992). The family Streptomycetaceae. In. The Prokaryotes. (Balows A, Truper HG, Dworkin M, Harder W Schleifer KH Eds). Springer-Verlag, New York. pp. 921-995.
Kumbhar C, Watve M (2013). Why antibiotics: A comparative evaluation of different hypotheses for the natural role of antibiotics and an evolutionary synthesis. Natural Science, 5:26-40.
Laidi RF, Kansoh AL, Elshafi AM, Cheikh B (2006). Taxonomy, identification and biological and activity of a novel isolate of streptomycestendae. Arab Journal of Biotechnology, 9:427-436.
Lalitha MK (2004). Manual on Antimicrobial Susceptibility Testing. Department of Microbiology, Christian medical college, Tamil Nadu. pp. 1-47.
Lechevalier HA (1989). A practical guide to genetic identification of actinomycetes. In. Bergey's manual of Systematic Bacteriology. (Williams, S.T., Sharp, M.E. and Holt, J.P. Eds). The William and Wilkins company, Baltimore. pp. 2344-2347.
Livermore DM (2003). Bacterial resistance: origins, epidemiology and impact. Clinical Infectious Diseases, 36:11-23.
Locci R (1989). Streptomycetes and related genera. In. Bergey's manual of systematic bacteriology. (Williams, S.T., sharp, M.E. and Holt, J.P. Eds). The William and Wilkins company, Baltimore. pp. 2344-2347
Miyadoh S (1993). Research on antibiotic screening in Japan over the last decade: a producing microorganism approach. Actinomycetologica, 7:100-106.
Moges K (2009). Antimicrobial compound from thermophlicactinomycete isolated from mushroom compost. M.Sc. Thesis, Department of Biology (Microbiology), Addis Ababa University, Addis Ababa.
Ningthoujam DS, Sanasam S, Nimaichand S (2009). Screening of Actinomycete Isolates from Niche Habitats in Manipur for Antibiotic Activity. American Journal of Biochemistry and Biotechnology, 5:221-222.
Nokaido H, Vaara M (1985). Molecular basis of bacterial outer membrane permeability. Microbiological Reviews, 49:1-32.
Okeke IN, klugman KP, Bhuta, ZA, Duse AG, Jenkins P, Brien TF, Pablos- mendez A, Laxminarayan R (2005). Antimicrobial resistance in developing countries. Part II: strategies for containment. The Lancet Infectious Diseases, 5:568-580.
Oskay M, Tamer A, Azeri C (2004). Antibacterial activity of some actinomycetes isolated from farming soils of Turkey. African Journal of Biotechnology 3:441-446.
Pandey B, Ghimirel P, Prasad V, Thomas M, Chan Y and Ozanick S (2002). Studies of the antimicrobial activity of the actinomycetes isolated from the Khumby region of Nepal. Department of Bacteriology, University of Wisconsin Madison, Wisconsin.
Pandey B, Grimier P, Agrawal VP (2004). Studies on the antibacterial activity of actinomycetes isolated from the Khumbu region of Nepal. Journal Biology Science, 23:44-53.
Parungao MM, Maceda EBG, Villano MAF (2007). Screening of Antibiotics Producing Actinomycetes from Marine, Brackish and Terrestrial Sediment of Samal Island, Philippines. Journal of Research in Science, Computing and Engineering 4:29-38.
Petrova D, Vlahov, S (2007). Taxonomic characterization of the thermophlicactinomycete strain 21E-produce of termostabel collagenase. Journal of Culture Collections, 5:3-9.
Ramakrishnan J, Shunmugasundaram M, Narayanan M, (2009). Streptomyces sp. SCBT isolated from rhizosphere soil of medicinal plants is antagonistic to pathogenic bacteria. Iranian Journal of Biotechnology, 7:75-81.
Remya M, Vijayakumar R (2008). Isolation and Characterization of marine antagonistic actinomycetes from west coast of India. Medicine and Biology,15:13-19.
Rondon M, August P, Bettermann AD, Brady SF, Grossman TH, Liles MR, Loiacono, KA, Lynch, BA, Macneil C, Minor C, Tiong M, Osborne J, Clardy J, Handelsman J Goodman R (2000). Cloning the soil metagenome: a strategy for accessing the genetic and functional diversity of uncultured microorganisms. Applied and Environmental Microbiology 66:2541-2547.
Rouatt JW, Lechevalier M, Waksman SA (1951). Distribution of antagonistic properties among actinomycetes isolated from different soils. Antibiotics & Chemotherapy (Northfield, Ill.), 1:185-92.
Saadoun I, Gharaibeh R (2003). The Stereptomyces flora of Badia region of Jordan and its potential as a source of antibiotics active against antibiotic resistant bacteria. Journal of Arid Environments, 53:365-371.
Sanglier JJ, Haag TA, Huck T A (1993). Novel bioactive compounds from actinomycetes. A short review (1988-1992). Research in Microbiology, 144:633-642.
Santhi VS, Jose PA, Solomon RDJ (2010). Isolation and characterization of antagonistic actinomycetes from mangrove sediments. International Journal of Current Research, 3:020-023.
Selvameenal L, Radhakishnan M, Balagurunathan R (2009). Antibiotic Pigment from Desert Soil Actinomycetes; Biological Activity, Purification and Chemical Screening. Indian Journal of Pharmaceutical Sciences, 71:499-504.
Shirling EB, Gottlieb D (1966). Methods for characterization of Streptomycetes species. International Journal of Systematic and Evolutionary Microbiology, 16:313-340.
Singh V, Haque S, Singh H, Verma J, Vibha K, Singh R, Jawed A, Tripathi CKM (2016). Isolation, screening and identification of novel

isolates of Actinomycetes from India for Antimicrobial Application. Frontiers in Microbiology, 7:1921-1928.

Sundaramoorthi C, Vengadesh PK Gupta S, Karthic K, Tamilsehli V (2011). Production and characterization of antibiotics from soil–isolated actinomycetes. International Research Journal of Pharmacy, 2:114-118

Tiwarty KB (2009). Protocol for actinomycetes studies in RLABB.2008/9. Central department of microbiology, institution of science and technology. Tribhuven University, kiripur. p. 21.

Tortora GJ, Funke BR, and Case CL (2010). Microbiology: An Introduction, 10th ed. Pearson Benjamin Cummings, USA. pp. 530-589.

Vijayakumar R, Muthukumar C, Thajuddin N, Panneerselvam A, Saravanamuthum R (2007). Studies on the diversity of actinomycetes in the Palk Strait region of Bay of Bengal, Inidia. Actinomycetologica, 21:59-65.

Williams ST, Cross T (1971). Actinomycetes. In. Methods in Microbiology. (Booth, C. Ed.). Academic press, London. pp. 29-65.

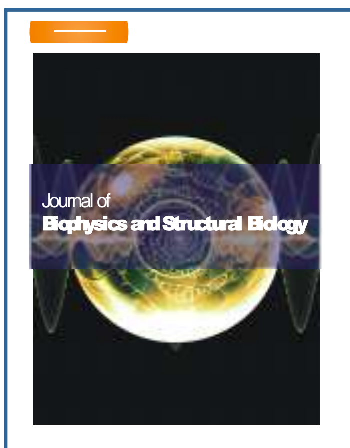
Williams ST, Goodfellow M, Alderson G (1989a). Genus Streptomycetes. In. Bergey's Manual of Systematic Bacteriology. (Williams, S.T., Sharpe, M.E. and Holt, J.P. Eds). The Williams and Wilkins Company, Baltimore. pp. 2452-2490.

Williams ST, Sharpe ME, Holt JG (1989b). Bergey’s Manual of Systematic Bacteriology. Vol. 4, William and Wilkins Co., Baltimore.

Wohlleben W, Mast Y, Stegmann E, Ziemert N (2016). Antibiotic drug discovery. Microbial Biotechnology, 9(5):541-548.

Yadav AK, Kumar R, Saikia, R, Bora TC, Arora DK (2009). Novel copper resistant and antimicrobial Streptomyces isolated from Bay of Bengal, India. Journal of Medical Mycology, 19:234-240.

Related Journals:



www.academicjournals.org