African Journal of Biotechnology

Volume 16 Number 51, 20 December, 2017

ISSN 1684-5315

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 16 Number 51 20 December, 2017

ARTICLES

Changes observed in *Pritchardia pacifica* palms affected by a lethal yellowing-type disease in Mexico 2331
María Narváez, Elizabeth Ortíz, Carmen Silverio, Jorge M. Santamaría, Francisco Espadas and Carlos Oropeza

Effects of *Salaha-A* (herbomineral drug) on blood parameters of rats and *in silico* inhibition of P-glycoprotein by it bioactive compounds 2341
Rahma M. Adamu, Magaji G. Taura and Balwant K. Malik

academicJournals

Vol. 16(51), pp. 2331-2340, 20 December, 2017
DOI: 10.5897/AJB2017.16218
Article Number: 754BD4D55235
ISSN 1684-5315
Copyright © 2017
Author(s) retain the copyright of this article
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Changes observed in *Pritchardia pacifica* palms affected by a lethal yellowing-type disease in Mexico

María Narváez[1,2], Elizabeth Ortíz[2], Carmen Silverio[1], Jorge M. Santamaría[1], Francisco Espadas[1] and Carlos Oropeza[1*]

[1]Centro de Investigación Científica de Yucatán, Mérida, Yucatán, México.
[2]Instituto Tecnológico de Mérida, Mérida, Yucatán, México.

Received 18 August 2017; Accepted 16 November, 2017

Lethal yellowing (LY) is a devastating disease, affecting more than 35 palm species. One of them, *Pritchardia pacifica*, was found to be very susceptible in Florida and it is affected by a LY-type syndrome in Yucatan, Mexico. In this study, plants of *P. pacifica* were exposed naturally to feral insects in an area affected by LY in Mexico. All the plants exposed died showing symptoms of leaf decay with death occurring within approximately four months after first symptom. Real-time (polymerase chain reaction) PCR analysis of DNA extracts for phytoplasma detection and *in silico* sequence analysis of nested-PCR amplicons showed an association with 16SrIV-D phytoplasmas in plants studied. Time-course changes were studied for the detection of phytoplasmas, stomatal conductance and leaf temperature by thermography. One month before appearance of the first symptom, changes in all the parameters were observed: positive detection of phytoplasma DNA, a reduction in stomatal conductance that is complete and irreversible, and the appearance of a peak in leaf temperature. These results provide insights into a better understanding of this disease in *P. pacifica*. Moreover, from a practical point of view, the changes described could be useful for developing methods for early detection of the disease.

Key words: Lethal yellowing-type disease, phytoplasmas, *Pritchardia pacifica*, stomatal conductance, thermography.

INTRODUCTION

Lethal yellowing (LY) is a devastating disease associated with phytoplasmas, identified as the causal agent when they were found in phloem cells of coconut plants (*Cocos nucifera*) with symptoms of LY, through observations using transmission electron microscopy (Beakbane et al., 1972; Heinze et al., 1972; Plavsic-Banjac et al., 1972) and observation of differential remission of symptoms in symptomatic plants treated with oxytetracycline antibiotics and penicillin (McCoy, 1972; Hunt et al., 1974). Actually, LY affects more than 35 species of palms (Harrison and Oropeza, 2008). According to McCoy et al. (1983), the most susceptible

*Corresponding author. E-mail: cos@cicy.mx.

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

are coconut, *Phoenix dactylifera* and *Pritchardia* species, including *Pritchardia pacifica*. In studies carried out in Florida (USA) using insect-proof cages, the transmission of LY was achieved with the insect *Haplaxius crudus* (previously known as *Myndus crudus*) to young palms of different species selected for their susceptibility, including coconut and *P. pacifica* (McCoy et al., 1983; Howard, 1984; Harrison and Oropeza, 2008). When these studies were carried out, the molecular techniques to identify taxonomically, the phytoplasmas causing the disease in different palms were not available. At present, the phytoplasmas of 16SrIV group are accepted as the causal agent of LY and similar diseases, based on the detection and identification of their DNA with PCR and amplicon sequencing (Ntushelo et al., 2013). In Mexico, the presence of three sub-groups of 16SrIV were reported: 16SrIV-A in coconut, *Thrinax radiata, Coccothrinax readii* (Narvaez et al., 2006), *Roystonea regia* and *Acrocomia mexicana* (Narvaez et al., 2015); 16SrV-B in coconut (Harrison and Oropeza, 1997); and 16SrV-D in *Carludovica palmata* (Cordova et al., 2000), *Pseudophoenix sargentii* and *Sabal mexicana* (Vázquez et al., 2011).

Since coconut is the most economically important palm species affected by LY, it is also the palm species in which LY studies have been more extensive. The LY symptoms observed in coconut are: first all nuts drop within days, followed by necrosis of inflorescences, then yellowing of leaves starts with the oldest leaves progressing to younger leaves, and finally loss of all leaves leaving the bare trunk (McCoy et al., 1983). During the development of these visual symptoms, related physiological and biochemical changes occur, such as the reduction of stomatal conductance before the appearance of the first symptom which becomes complete and irreversible (León et al., 1996; Maust et al., 2003). These authors proposed that LY-induced stomatal closure is central to the development of LY symptoms in coconut palms and leaf yellowing is part of a leaf senescence process, only that instead of one leaf senescing approximately each month in non-infected palms, this rate is about ten times faster in LY affected palms (Leon et al., 1996). Also, with the closure of the stomata, transpiration and its refreshing effect is reduced, thus, an increase in the temperature of the leaf would be expected, which could be detected by thermography as reported in other species (Jones and Schofield, 2008; Oerke and Steiner, 2010).

Other species have also been important for the study of LY, as it is the case of *P. pacifica* young palms that were used as a model palm for research on LY transmission given its high susceptibility (Howard et al., 1984). Casual observations at the gardens of the Centro de Investigación Científica de Yucatán (CICY) in Merida, Yucatan, Mexico, showed that *P. pacifica* plants could develop an LY-type syndrome and die (CICY, unpublished results). Also, a preliminary analysis of DNA from one of these plants by BLAST suggested that a 16SrIV-D phytoplasma could be associated with this occurrence (Cordova et al., 2014).

The present study was carried out with the objective of extending the understanding of this association, including the evaluation of changes in physiological parameters during the development of LY-type syndrome in *P. pacifica* plants, and show if these could be useful for early detection of infection by 16SrIV phytoplasmas in palms.

MATERIALS AND METHODS

Biological material

Two batches of *P. pacifica* plants were used. The first batch consisted of nine plants in pots (soil/compost 60/40 w:w) of approximately one year of age, located in the nursery "Palma Real" (Temozon Norte Merida, Yucatan, Mexico, 21°3'48.5''N, 89°36'39.7''W), all of which showed symptoms similar to those of LY. These plants remained in the nursery and were used only for taking leaf samples to determine the presence of phytoplasma DNA by real-time PCR and identification of phytoplasmas found. The second batch consisting of 22 asymptomatic plants in pots (soil/compost 60/40 w:w) of approximately one year of age was obtained from another nursery "Xochimilco" (Santa Gertrudis Copo, Merida, Yucatan, Mexico, 21°1'46.8''N, 89°34'28.8''W) and analyzed by real-time PCR to determine if they were infected or not. Once confirmed that they were negative, free of phytoplasma infection, a set of 17 plants were transferred to a single site on the lawns of the gardens of CICY in Yucatan, Mexico, where LY is present. They were kept under natural conditions without a cover and irrigated with tap water twice a week. This way, plants were exposed naturally to feral insects to facilitate phytoplasma infection. These plants were then evaluated every month during the period of September 2013 to February 2014, for symptom development, analysis by real-time PCR, stomatal conductance and thermography. The other five plants of this second batch were placed within an insect-proof cage 1 to avoid contact with insects and kept free of phytoplasma infection. Another set of five plants, those from the first batch that were infected with phytoplasmas and symptomatic were placed within an insect-proof cage 2, with the purpose of keeping them under the same conditions of those plants within cage 1. The cages were kept under natural conditions and plants were irrigated with tap water twice a week. These two sets of caged plants were used for comparative diurnal evaluation of stomatal conductance.

Symptom development

In order to describe the development of symptoms in affected palms in the second batch, visual observations were carried out continuously during the period of the study to define the changes observed and the chronology of appearance.

Extraction of DNA and detection of phytoplasma by real-time PCR

Leaf tissue samples were taken from symptomatic and asymptomatic plants. DNA was extracted from 1 g of each leaf tissue sample using the CTAB protocol (Doyle and Doyle, 1990). Detection of DNA phytoplasma was done using the real-time

PCR/Taqman LY16S assay reported by Cordova et al. (2014) with modifications. The reaction mix was 20 µl of final volume containing 1 µl of DNA sample (50 ng/µl), 1 µl of TaqMan Probe 503Sr (250 nM) and 2 µl of the Universal PCR Master Mix with AmpErase Uracil N-glycosylase (UNG) (Applied Biosystems USA), 1 µl of primer mix containing 900 nM of each primer. The equipment used for the detection was a Rotor-Gene Q - QIAGEN thermal cycler. Program conditions were 50°C for 2 min (1 cycle), 95°C for 10 min (1 cycle), 95°C for 15 s and 61°C for 1 min (40 cycles). All DNA samples including controls were assessed in duplicate. The threshold cycle (Ct) value of each PCR reaction was set manually intersecting the exponential phase of the amplification curves and the baseline was automatically set by Rotor-Gene Q manager software (QIAGEN). The Ct cut-off value was determined by preparing a standard curve by sequential 10-fold dilutions of control DNA sample (extracts positive to Real-time detection), each dilution was tested in triplicate and the curve was carried out six times. Negative controls included were DNA samples (extracts negative to real-time detection) and water.

Identification of phytoplasma strain

DNA was taken from 7 plants (2 from first batch and 5 from the second batch) that were positive to the detection of phytoplasmas by real-time PCR and used to determine the type of phytoplasma present. Corresponding amplicons were obtained by nested-PCR. In the first reaction, the primers P1 (Deng and Hiruki, 1991) and P7 (Smart et al., 1996) were used and 2 µL of the DNA extract (50 ng) product, was used as template in the second reaction and the primers used were LY16Sf/LY1623Sr (Harrison et al., 2002), finally obtaining a product of approximately 1.7 Kb. Amplification conditions for the first and second reactions for nested-PCR were as previously described by Harrison et al. (2001, 2002). The amplified products were cloned in the PGEM-T Easy Vector (Promega) and *E. coli* top 10 (Invitrogen), and sent for sequencing to the University of California (The College of Biological Sciences UCDNA Sequencing Facility). The sequences obtained were compared in the database of the NCBI-BLAST. F2n/R2 partial sequences (Gundersen and Lee, 1996) from the 1.7 Kb sequences of two *P. pacifica* plants (Acc. KY508691, KY508693) were subjected to virtual RFLP analysis, using iPhyclassifier software and *in silico* digestion with 17 restriction enzymes (AluI, BamHI, BfaI, BstUI, DraI, EcoRI, HaeIII, HhaI, HinfI, HpaI, HpaII, KpnI, Sau3AI, MseI, RsaI, SspI and TaqI). The F2N/R2 sequences obtained in the present study and other equivalent sequences of phytoplasmas associated with LY type diseases in palms were used to construct a phylogenetic tree by the method of neighbor-joining and a bootstrap value of 2000, using MEGA5.05 (Tamura et al., 2011) software. *Acholeplasma palmae* was used as external group.

Stomatal conductance and thermography

Stomatal conductance (mmol m^{-2} s^{-1}) was calculated as the inverse of diffusive stomatal resistance, measured *in situ* with a LI-COR LI-1600 porometer on the surface of the leaves or parts of leaves that were not affected. Two assays were carried out. For the first assay, measurements were taken throughout the day (at 6:00, 9:00,13:00, 18:00 and 20:00 h) in five asymptomatic plants (real-time PCR negative) and five plants with symptoms (real-time PCR positive). These two sets of palms were kept separated, each within insect-proof cages 1 and 2, respectively. For the second assay, the measurements were taken in the morning between 10:00 and 12:00 am, and in monthly determinations throughout the development of the disease in 17 plants. At the beginning of the assay, all the plants were asymptomatic (real-time PCR negative). In this second assay, the temperature of the surface of the canopy foliage was also recorded in the 17 plants with a thermographic camera (ICI Prodigy 640, thermal resolution of 640 x 480) and the environmental temperature was measured independently. For the analysis, the difference between both temperatures was taken into consideration (ΔT = temperature by thermography – environmental temperature). Each ΔT value of the thermography reported for a plant was the average of five points, each one taken from a different leaf. For analysis of the different parameters, the statistical method used to calculate the differences between means of each point in the graph was Holm-Sidak with a test of ANOVA statistical package of SigmaPlot. The set of five in cage 1 (asymptomatic and real-time PCR negative) used for the first assay were kept as control plants (not exposed to feral insects) for the second assay.

Comparative analysis of results of plants in the second batch

Plants were analyzed for different parameters (real-time PCR detection, stomata conductance and thermography) monthly, and once the symptom appeared, follow up continued further for three months. Due to the fact that symptoms started at a different time for each plant, for the purpose of comparative analysis of the parameters studied between all plants, the monthly data obtained for each plant was equated based on alignment of the timeline at the moment of appearance of first symptom. Therefore, the results are presented in the following chronology three months before appearance of first symptom and three months later.

RESULTS

Symptoms observed in the plants

The *P. pacifica* plants exposed naturally to feral insects in the gardens of CICY showed no immediate LY-type symptoms (Figure 1A). The appearance of the first symptom after exposure, took between one and three months depending on the plant, and this was followed by a progressive development of symptoms of leaf decay syndrome. The time lapse between the appearance of the first symptom and death of the plants was from 3 to 4 months. Development of symptoms was in the following manner: the first visual symptom was damage on the spear leaf, hindering subsequent aperture of this leaf in some cases, depending on the degree of damage (Figure 1B), this was followed by yellowing of mature leaves (Figure 1C) and emission of new leaves stopped in the palm, resulting in reduction of foliage (Figure 1 D). In most cases, yellowing occurs first in only one part of the leaf, the area becomes brown and this pattern extends to the rest of the leaf, which subsequently dries up and dies. Eventually, all the leaves are affected and the plant dies (Figure 1E). In some cases, yellowing occurs simultaneously in several leaves, affecting the whole leaf; the leaves then turn brown and die. In the case of plants in cage 1 (asymptomatic and real-time PCR negative) that were unexposed to feral insects throughout the whole study, no symptom was developed.

Detection by PCR of phytoplasma DNA

For the detection of phytoplasma DNA by real-time PCR,

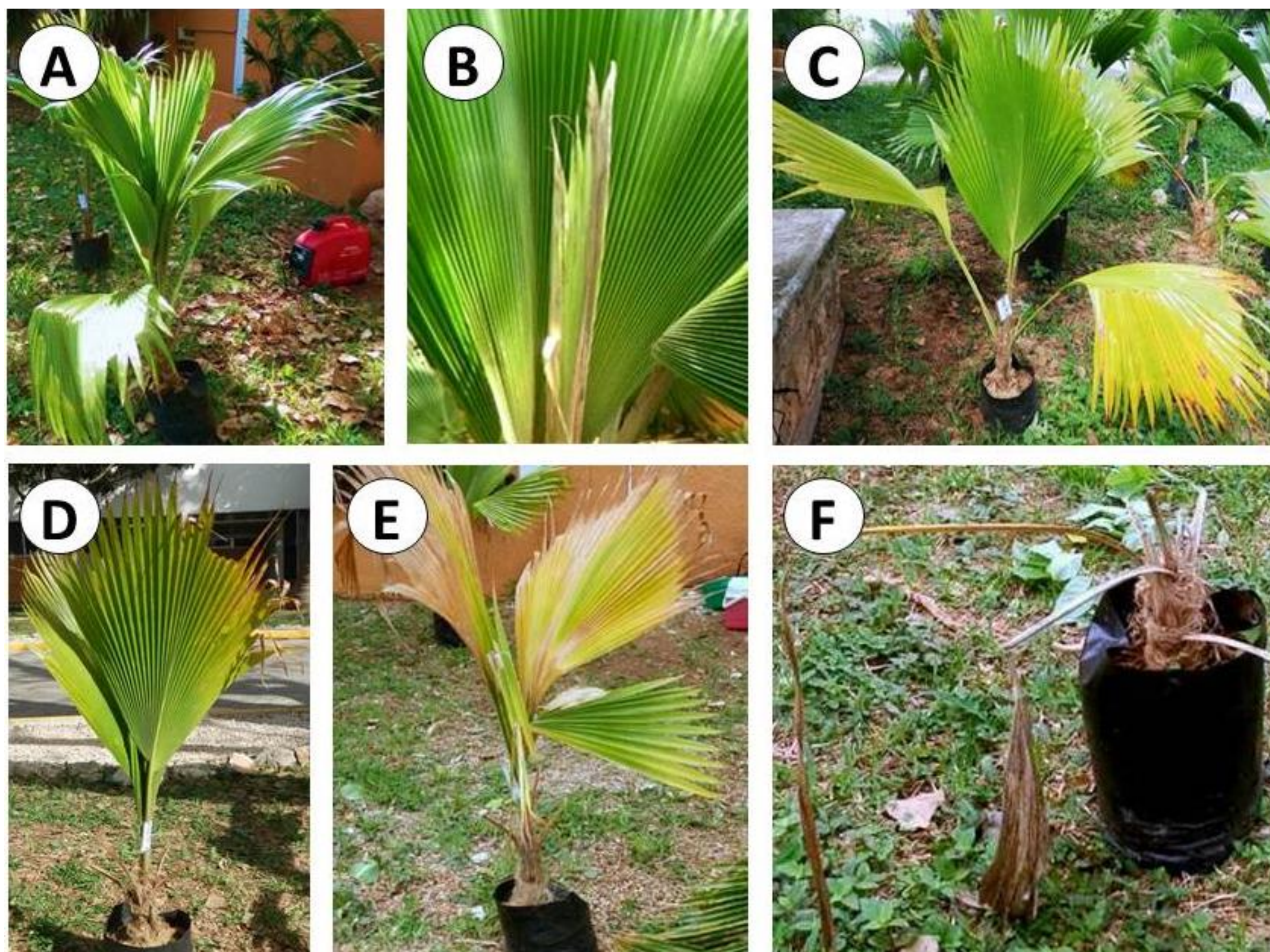

Figure 1. Development of symptoms of a lethal yellowing-type syndrome as observed in *P. pacifica* plants. **(A)** Asymptomatic plant. **(B)** appearance of the first symptom, damage in the spear leaf. **(C)** Yellowing of the older leaves. **(D)** Loss of the older leaves. **(E)** Yellowing of younger leaves. **(F)** Loss of young leaves and death of the palm.

a Ct cut-off value of 32 ± 0.13 was determined and values above were negative. In the first batch of nine plants at Palma Real Nursery, when monitoring started, four were already symptomatic and five were asymptomatic; the latter began to show symptoms within a month, and within four months, all of them died. The symptoms observed were also leaf decay syndrome as described earlier, and all the plants were positive to the detection of phytoplasma DNA by real-time PCR. In the case of the second batch of plants, all were real-time PCR negative and symptomless at the beginning of the study, and were exposed naturally to feral insects in the gardens of CICY. Each of them developed symptoms of leaf decay syndrome. The data presented here is reported within the following time frame: zero time denotes the moment of appearance of the first symptom (to chronologically equating development of symptoms and corresponding measurements); -3, -2 and -1 are months before the appearance of the first symptom and +1, +2 and +3 are months after the appearance of the first symptom. Regarding real-time PCR in month -1, there was a positive detection of LY 16SrIV phytoplasmas with an average Ct value of 29.6 (Figure 2A). This initial detection took place because presumably, the titre of phytoplasmas increases with time to become detectable, and continues increasing as shown by a decrease of Ct value to 25.3 at time zero (time of appearance of first symptom). CT value remained with very little change afterwards (Figure 2A). The difference between average negative Ct values and average positive Ct values was significant.

Identification of the phytoplasma affecting *P. pacifica*

Seven sequences were obtained from the products of the

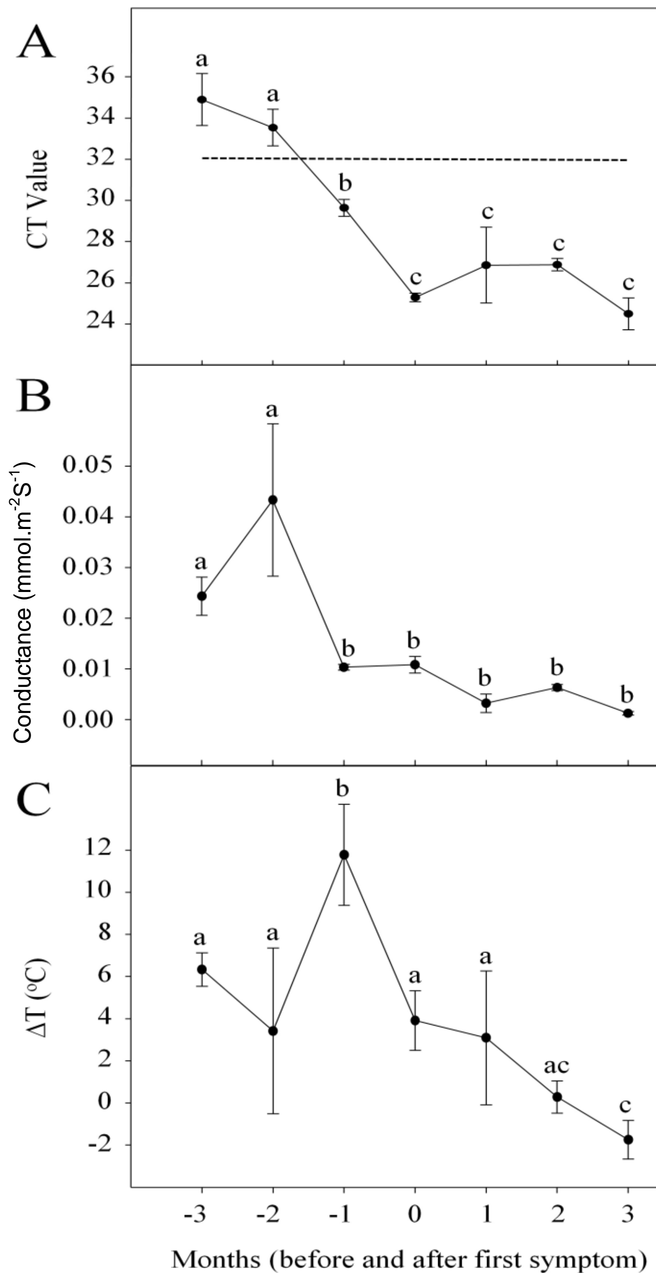

Figure 2. Analysis of three parameters in *Pritchardia pacifica* plants exposed to *Haplaxius crudus* in a site with LY incidence during the different developmental stages of the disease. **(A)** LY detection by real-time PCR. Broken line denotes detection threshold, above CT 32 is negative, and below is positive. **(B)** Leaf stomatal conductance. **(C)** Thermography. Time zero denotes when the first visual symptom was observed. Similar letters have no significant differences, different letters have significant differences (P = 0.001).

amplification of seven DNA extracts from diseased plants (Accessions KY508690, KY508691, KY508692, KY508693, KY508694, KY508695 and KY508696). These sequences were compared in the NCBI-BLAST database and high similarity was obtained (99.56 to 99.94%) with the sequences of the phytoplasmas in *S. palmeto* (Accession HQ613895.1) and *P. dactylifera* (Accession AF434989.1) classified within the subgroup 16Sr-IV-D. Virtual RFLP analysis was carried out and the pattern (Accession KY508691, Figure 3B) was very

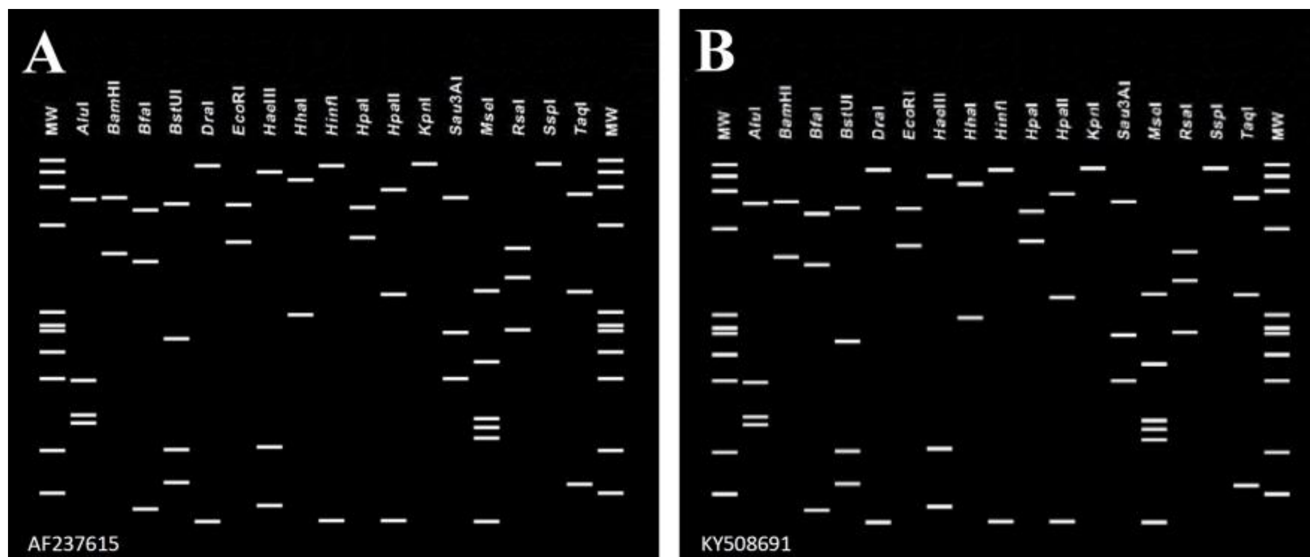

Figure 3. Virtual RFLP patterns derived from *in silico* digestions, using iPhyclassifier software. **(A)** Reference pattern of 16Sr group IV, subgroup D, accession: AF237615. **(B)** Pattern of *P. pacifica*, accession: KY508691.

similar (similarity coefficients of 1.0) to reference pattern of *Carludovica palmata* 16Sr group IV, subgroup D (Acc. AF237615 (Figure 3A). Phylogenetic analysis carried out on the sequences obtained from *P. pacifica*, showed that these are grouped in the clade corresponding to the subgroup 16SrIV-D (Figure 4).

Stomatal conductance

First stomatal conductance was evaluated comparatively between healthy palms (asymptomatic, negative real-time PCR) and diseased palms (symptomatic, positive real-time PCR, one month after the appearance of the first symptom). In healthy palms, conductance showed a typical diurnal fluctuation throughout the day, at 6 am, it was 0.0146 mmol m^{-2} s^{-1}, at 13 pm, it increased to a maximum of 0.038 mmol m^{-2} s^{-1} and at 18 pm, it reduced to 0.00057 mmol m^{-2} s^{-1} (Figure 5). Conductance in diseased palms showed very small diurnal fluctuation with values ranging between 0.01 at (9 am) and 0.0028 mmol m^{-2} s^{-1} (18 pm) (Figure 5).

Also, stomatal conductance was evaluated during the development of the disease, (Figure 2B) and there was a reduction of about 80% from month -2 to month -1 (values dropped from 0.051 to 0.01 mmol m^{-2} s^{-1}). From then on, conductance values remained low (between 0.01 and 0.001 mmol m^{-2} s^{-1}) (Figure 2B). The differences between month -3 and -2 were not significant (P = < 0.001); however, between these and the following months (-1 to +3), they were significant (P = < 0.001).

Thermography

The measurements of ∆T throughout the development of the disease showed a peak (∆T 11.78°C) at month -1, before the appearance of the first symptom, ∆T values after and before month -1 (0.28 to 6.33°C) showed no significant differences between them; in contrast, there was a significant difference with the value for month -1 (P=<0.001) (Figure 2C).

DISCUSSION

In casual observations, it was found that *P. pacifica* plants at CICY's gardens developed a LY-type syndrome and analysis of DNA of one of these plants by BLAST suggested that a 16SrIV-D phytoplasma could be associated with it (Cordova et al., 2014). Further studies to extend the understanding of this association were carried out and are reported in this study.

The first part of the study was on a batch of nine of *P. pacifica* plants in pots of approximately one year of age at Palma Real Nursery that was found to be already showing symptoms of leaf decay. DNA samples from the leaves were analyzed by real-time PCR specific for 16SrIV phytoplasmas and found positive. Then, another batch, in this case 17 *P. pacifica* plants from another site, (Xochimilco nursery) that were asymptomatic and negative for the detection of phytoplasma DNA by real-time PCR, were exposed naturally to feral insects in the gardens of CICY. They developed leaf decay symptoms similar to those of the first batch. Analysis by real-time PCR of DNA from these plants also showed positive detection of 16SrIV phytoplasmas. Amplicons obtained sequenced and after the sequences, were subjected to BLAST; high analogy was found for 16SrIV-D strains and this was further supported by virtual RFLP and phylogenetic analyses. All the sequenced amplicons from

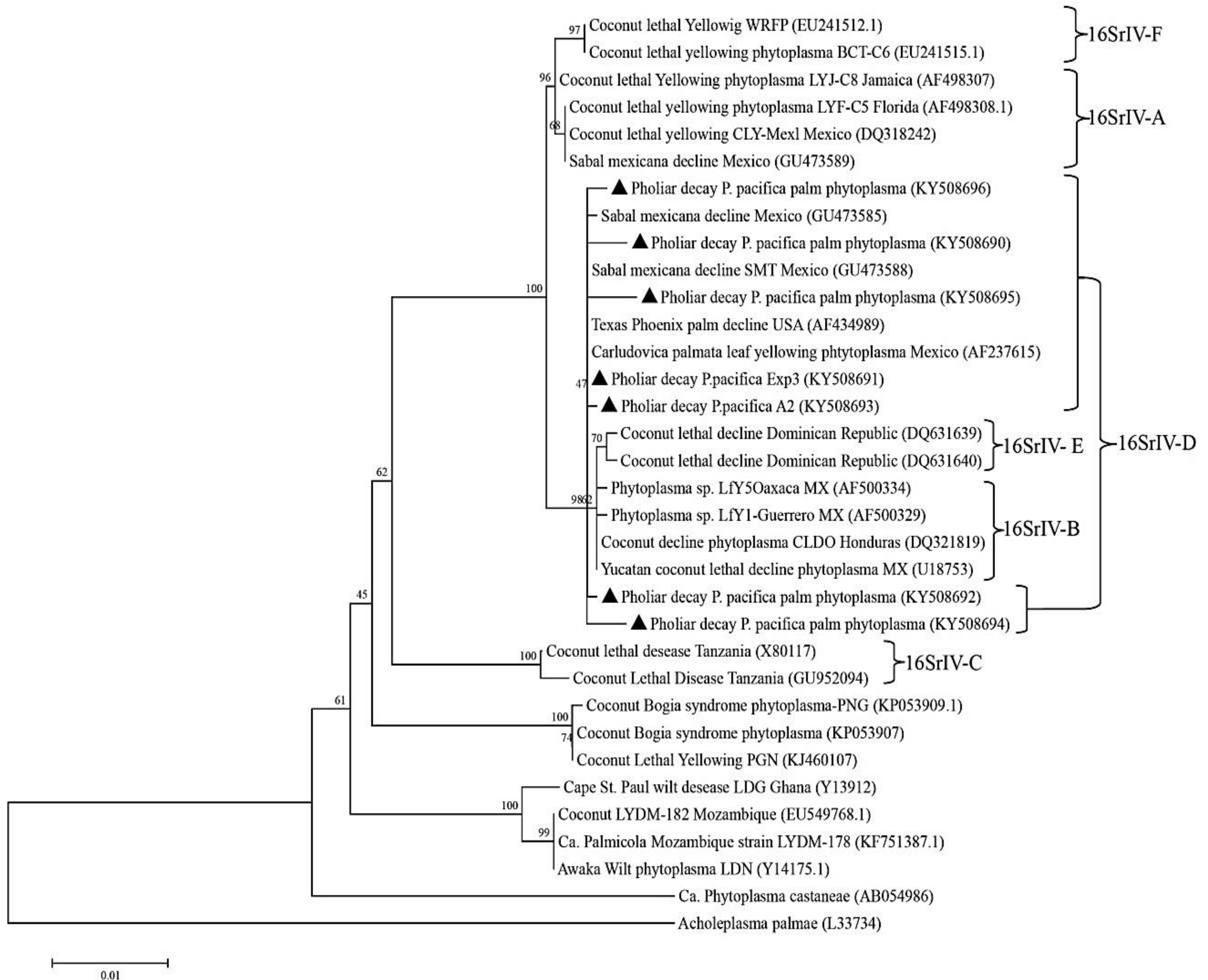

Figure 4. Phylogenetic tree constructed using the method of neighbour-joining (bootstrap: 2000) with 34 sequences of I6Sr RNA gene representative of the phytoplasmas associated with LY in coconut plants and other palm species, including the sequences of phytoplasmas isolated from *Pritchardia pacifica* plants in this study (▲) and from the root of *Acholeplasma palmae*.

DNA samples of plants from both sites (Palma Real Nursery and CICY) were all of the subgroup 16SrIV-D only. This is interesting since the sites are far away (8 km) from each other, and in CICY's gardens, 16SrIV phytoplasmas of subgroups 16SrIV-A and 16SrIV-D were found in different palm species (Vázquez et al., 2011). Thus, suggesting a possible selective interaction between *P. pacifica* plants and 16SrIV-D phytoplasmas, as found for LY-type syndromes in palm species studied in Yucatan, *T. radiata* was found to be associated specifically with 16SrIV-A and *S. mexicana* associated with 16SrIV-D phytoplasmas. However, this occurrence needs to be further studied, for instance, on samples from symptomatic *P. pacifica* plants from more sites and farther.

Follow-up of the development of symptoms of plants in the second batch exposed naturally to feral insects showed that they started appearing after three months of exposure. Chronologically, these symptoms included initial damage of the spear leaf, yellowing of mature leaves, yellowing of younger leaves, yellow leaves turning brown with reduction of foliage and finally, the palm death. As in the case of the first batch, all palms of this second batch also died, suggesting that they are very susceptible, as observed previously in Florida (McCoy et al., 1983; Howard, 1984; Harrison and Oropeza, 2008). According to the literature, the symptoms of palms affected by LY can vary according to the palm species

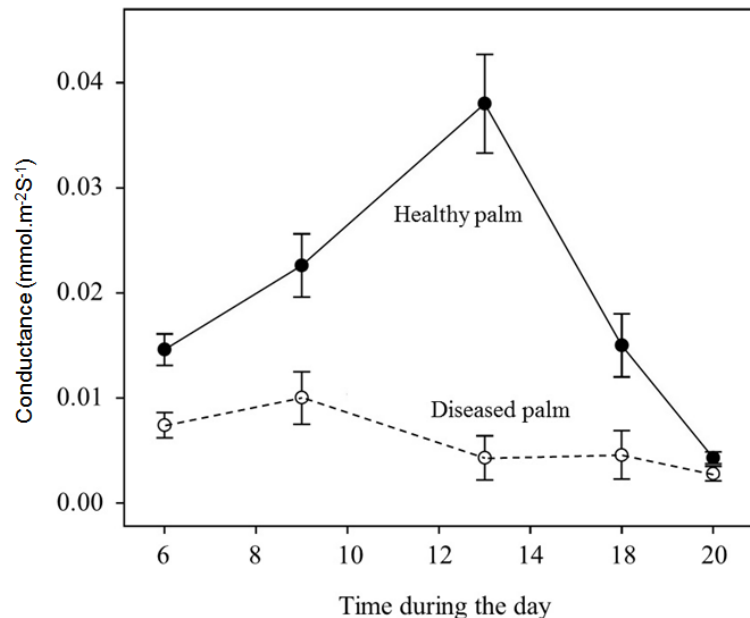

Figure 5. Daytime stomatal conductance in healthy *P. pacífica* plants (asymptomatic, negative real-time PCR) and diseased plants affected by LY (symptomatic, positive real-time PCR, at one month after the appearance of the first symptom).

and strain of phytoplasma affecting it (Harrison and Oropeza, 2008). The leaf decay symptoms observed in the *P. pacifica* plants in the present case, were similar to those reported for *P. dactylifera* associated with 16SrIV-D phytoplasmas (Harrison et al., 1995; Harrison and Oropeza, 2008) and *Washingtonia robusta* associated with 16SrIV-F phytoplasmas (Harrison et al., 2008), in which collapse of the spear leaf preceded yellowing of the leaves. In the case of the leaf decay syndrome of *P. pacifica* in this study, the time lapse between the appearance of the first symptom and death of the plant was from 3 to 4 months, a slightly shorter period of time than that reported for mature coconut palms, 3 to 6 months (Arellano and Oropeza, 1995). Unfortunately, these observations cannot be compared with those of either leaf decay syndrome in adult *P. pacifica* palms, or LY syndrome on young coconut palms, because there are no reports available for such types of cases.

Phytoplasma diseases can affect the physiology of stomata and according to Matteoni et al. (1983), plants with phytoplasma diseases that are lethal show complete closure of stomata even at the time when stomata of healthy plants are open, and in plants with phytoplasma diseases that are not lethal, stomata closure was only partial. In coconut plants affected by LY, there is a reduction of stomatal conductance that starts before the appearance of the first symptom and is complete and irreversible (León et al., 1996; Maust et al., 2003). In the present study, *P. pacifica* plants affected by the leaf decay disease were evaluated to know whether there are changes in stomatal conductance, and leaf temperature. Stomatal conductance in healthy plants was monitored throughout the day and a normal diurnal variation was observed, increasing during the day and decreasing in the afternoon and night, indicating that the stomata are functional. This stomatal behavior is similar to that reported for healthy coconut palms (León et al., 1996). In contrast, in diseased *P. pacifica* plants, no variation were observed in stomatal conductance; remained low throughout the whole day, which indicates a disease-related stomatal closure. Moreover, when stomatal conductance was monitored during the development of the disease symptoms, it decreased in affected plants in a complete and irreversible manner one month before the appearance of the first symptom. These changes are similar to those observed in coconut palms during the development of the LY syndrome, and (together with other biochemical and physiological changes) are believed to be the result of a hormonal imbalance caused by the phytoplasma infection (León et al., 1996; Martinez et al., 2000; Aguilar et al., 2009).

Since the closure of the stomata could reduce plant transpiration and its refreshing effect, leading to an increase in leaf temperature (Jones and Schofield, 2008; Oerke and Steiner, 2010), changes in leaf temperatures (ΔT) in *P. pacifica* plants were evaluated throughout the development of the disease. There was an increase (of about 5°C) one month before the appearance of the first symptom, but it peaked, and decreased again to the previous level by the time of appearance of the first

symptom. Similar changes have been observed in *Nicotiana tabacum* plants infected by the tobacco mosaic virus (Chaerle et al., 1999). The authors reported that the initial increase in temperature was as a result of reduced stomatal conductance and transpiration, but they associated the following decrease in temperature to water loss caused by cell death occurring as part of plant defense. In the case of *P. pacifica* plants infected by 16SrIV-D phytoplasmas, very fast leaf decay took place with noticeable damage in the leaf tissues and its integrity, and probably allowing evaporation to take place. These changes in stomatal conductance and leaf temperature of *P. pacifica* plants occurred before the appearance of the first visual symptom, and at the moment when there was already positive real-time PCR detection of phytoplasmas. Based on these results, it is suggested that changes in stomatal conductance and the resulting changes in leaf temperature in plants of *P. pacifica* with leaf decay syndrome, are result of the presence of pathogen in infected plants, as previously proposed for LY phytoplasmas in coconut (León et al., 1996) and tobacco mosaic virus in tobacco (Chaerle et al., 2007).

In conclusion, the results reported herein supported that the *P. pacifica* diseased plants showing leaf decay syndrome are associated with 16SrIV-D phytoplasmas, contributing in particular, to the understanding of this syndrome affecting *P. pacifica* plants in Mexico, and in general, the mode of action of 16SrIV phytoplasmas in palms. In addition, from a practical point of view, the fact that the changes in the parameters studied occur before the appearance of the first visual symptom, this could be useful for the development of methods for early detection of the disease in *P. pacifica* or 16SrIV phytoplasma diseases in other palm species. In particular, thermography can be helpful in registering increase in temperature in asymptomatic infected plants during surveillance of commercial plantations, for instance, by means of remote sensing equipment mounted on drones (Chen et al., 2015; Mahlein, 2012), and followed by immediate testing of the presence of phytoplasmas by real-time PCR and corresponding actions to stop spread of the disease. Finally, it is very important to extend the studies reported here to a larger population of plants and other palm species to determine how universal they can be.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors thank Rafael Sánchez Borges and Gustavo Rivera for the technical support, and CONACYT (FORDECYT Clave 117315) and CICY for the partial funding.

REFERENCES

Aguilar ML, Espadas F, Maust B, Sáenz L (2009). Endogenous cytokinin content in coconut palms affected by lethal yellowing. J. Plant Pathol. 91(1):141-146

Arellano J, Oropeza C (1995). Lethal yellowing. In *Lethal Yellowing:* Research and Practical Aspects. Springer Netherlands. pp. 1-15.

Beakbane B, Slater CH, Posnette, AF (1972). Mycoplasmas in the Phloem of Coconut, *Cocos Nucifera* L., with Lethal Yellowing Disease. J. Hortic. Sci. 47: 265-265.

Chaerle L, Leinonen I, Jones, HG, Van Der Straeten D (2007). Monitoring and screening plant populations with combined thermal and chlorophyll fluorescence imaging. J. Exp. Bot. 58:773-784.

Chaerle L, Van Caeneghem W, Messens E, Lambers H, Van Montagu M, Van Der Straeten D (1999). Presymptomatic visualization of plant–virus interactions by thermography. Nature Biotechnol. 17: 813-816.

Chen R, Crawford K, Davis C, Ehsani R, Vougioukas S, Upadhyaya S (2015). The drones are coming. Unmanned aerial vehicles for agriculture. Citrograph. Mag. 6:18-23.

Cordova I, Oropeza C, Almeyda H, Harrison NA (2000). First report of a phytoplasma-associated leaf yellowing syndrome of palma jipi plants in southern México. Plant Dis. 84:807.

Córdova I, Oropeza C, Puch-Hau C, Harrison N, Collí-Rodríguez A, Narvaez M, Sáenz L (2014). A real-time PCR assay for detection of coconut lethal yellowing phytoplasmas of group 16SrIV subgroups A, D and E found in the Americas. J. Plant Pathol. 96:343-352.

Deng S, Hiruki C (1991). Amplification of 16S rRNA genes from culturable and non-culturable mollicutes. J. Microbiol. Meth. 14:53-61.

Doyle JJ, Doyle JL (1990). Isolation of plant DNA from fresh tissue. Focus 12:13-15.

Gundersen DE, Lee IM (1996) Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer pairs. Phytopathol. Med. 35:144-151.

Harrison N, Oropeza C (1997). Recent advances in diagnosis and detection of lethal yellowing in the Americas. In: Eden-Green, S.J. and Ofori, F. (Eds.) International Workshop on Lethal Yellowing-like Diseases of Coconut, Elmina, Ghana, November 1995. Chatham, UK, Natural Resources Institute. pp. 221-234.

Harrison NA, Griffiths HM, Carpio ML, Richardson PA (2001). Detection and characterization of an elm yellows (16SrV) group phytoplasma infecting Virginia creeper plants in southern Florida. Plant Dis. 85:1055-1062.

Harrison NA, Helmick EE, Elliott ML (2008). Lethal yellowing-type diseases of palms associated with phytoplasmas newly identified in Florida, USA. Ann. Appl. Biol. 153:85-94.

Harrison NA, Myrie W, Jones P, Carpio ML, Castillo M, Doyle MM, Oropeza C (2002). 16S rRNA interoperon sequence heterogeneity distinguishes strain populations of palm lethal yellowing phytoplasma in the Caribbean region. Ann. Appl. Biol. 141:183-193.

Harrison NA, Richardson P, Tsai JH (1995). Detection and diagnosis of lethal yellowing: conventional methods and molecular techniques. In: Lethal Yellowing: Research and Practical Aspects. C. Oropeza, F. W. Howard & G. R. Ashburner, (eds.), Kluwer Academic Publishers, Dordrecht. The Netherlands. pp. 79-91.

Harrison NA, Oropeza C (2008). Coconut lethal yellowing. In: NA Harrison, GP Rao and C Marcone (Eds.). Characterization, Diagnosis and Management of Phytoplasmas. Studium Press LLC, Houston, USA. pp. 219-248.

Heinze KG, Schuiling M, Romney DH (1972). The possible cause of lethal yellowing disease of coconut. FAO Plant Prot. Bull. 20: 58-68.

Howard FW, Williams DS, Norris RC (1984). Insect transmission of lethal yellowing to young palms. Int. J. Entomol. 26:331-338.

Hunt P, Dabek AJ, Schuiling M (1974). Remission of symptoms following tetracycline treatment of lethal yellowing infected coconut palms. Phytopathology 63:307-312.

Jones HG, Schofield P (2008). Thermal and other remote sensing of

plant stress. Gen. Appl. Plant Physiol. 34:19-32.

Leon R, Santamaria JM, Alpizar L, Escamilla JA, Oropeza C (1996). Physiological and biochemical changes in shoots of coconut palms affected by lethal yellowing. New Phytol. 134:227-234.

Mahlein AK, Oerke EC, Steiner U, Dehne HW (2012). Recent advances in sensing plant diseases for precision crop protection. Eur. J. Plant Pathol.133:197-209.

Matteoni JA, Sinclair WA (1983). Stomatal closure in plants infected with mycoplasma-like organisms. Phytopathology 73:378-402.

Martínez S, Cordova I, Maust B, Oropeza C, Santamaría J (2000). Is abscisic acid responsible for abnormal stomatal closure in lethal yellowing of coconut palms? J. Plant Physiol 156:319-323.

Maust BE, Espadas F, Talavera C, Aguilar M, Santamaría J, Oropeza C (2003). Changes in carbohydrate metabolism in coconut palms infected with the lethal yellowing phytoplasma. Phytopathology 93:976-981.

McCoy RE (1972). Remission of lethal yellowing in coconut palms treated with tetracycline antibiotics. Plant Dis. Rep. 56:1019-1021.

McCoy RE, Howard FW, Tsai JH, Donselman HM, Thomas DL, Basham HG, Atilano RA, Eskafi FM, Britt L, Collins ME (1983). Lethal yellowing of palms. University of Florida Agricultural Experiment Stations, Bulletin 834. Gainesville, Florida, USA. 100p.

Narvaez M, Cordova I, Orellana R, Harrison NA, Oropeza C (2006). First report of a lethal yellowing phytoplasma in Thrinax radiata and Coccothrinax readii palms in the Yucatan Peninsula of Mexico. Plant Pathol. 55:292-292.

Narvaez M, Córdova-Lara I, Reyes-Martínez C, Puch-Hau C, Mota-Narvaez L, Collí A, Camamal G, Harrison N, Oropeza C (2015). Occurrence of 16SrIV Subgroup A Phytoplasmas in *Roystonea regia* and *Acrocomia mexicana* Palms with Lethal Yellowing-like Syndromes in Yucatán, Mexico. J. Phytopathol. 164:1111-1115.

Ntushelo K, Harrison NA, Elliott M. (2013). Palm phytoplasmas in the Caribbean basin. Palms. 57:93-100.

Oerke EC, Steiner U (2010). Potential of digital thermography for disease control. In Precision Crop Protection-the Challenge and Use of Heterogeneity. Springer Netherlands. pp. 167-182.

Plavsic Banjac B, Hunt P, Maramorosch K (1972). Mycoplasmalike bodies associated with lethal yellowing disease of coconut palms. Phytopathology 62:298-299.

Smart CD, Schneider B, Morrer R, Blomquist DJ, Guerra LJ, Harrison NA, Ahrens U, Lorenze KH, Seemüller E, Kirkpatrick BC (1996). Phytoplasma-specific PCR primers based on sequences of the 16S-23S rRNA spacer region. App. Environ. Microbiol. 62:2988-2993

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 10:2731-2739.

Vázquez-Euán R, Harrison N, Narvaez M, Oropeza C (2011). Occurrence of a 16SrIV group phytoplasma not previously associated with palm species in Yucatan, Mexico. Plant Dis. 95:256-262.

academicJournals

Vol. 16(51), pp. 2341-2348, 20 December, 2017
DOI: 10.5897/AJB2017.16219
Article Number: 05E624D55241
ISSN 1684-5315
Copyright © 2017
Author(s) retain the copyright of this article
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Effects of *Salaha-A* (herbomineral drug) on blood parameters of rats and *in silico* inhibition of P-glycoprotein by it bioactive compounds

Rahma M. Adamu[1*], Magaji G. Taura[2] and Balwant K. Malik[3]

[1]Department of Plant Biology, Bayero University, Kano, Nigeria.
[2]Department of Anatomy, Bayero University, Kano, Nigeria.
[3]Department of Biotechnology, School of Engineering and Technology, Sharda University, Greater Noida, India.

Received 26 August, 2017; Accepted 9 October, 2017

The drug under study is a herbomineral drug containing tortoise shell ash and ground seeds of *Piper nigrum* used in the treatment of cancer. *Salaha-A* was found to contain essential minerals that could have medicinal importance to human. Calcium was found to be the major element in the tortoise shell ash and piperine is the pioneer alkaloid found in *Piper nigrum*. This work evaluates the effects of *Salaha-A* at different doses on blood parameters and spleen tissues of rats which result in significant increase in erythrocytes, lymphocytes and granulocytes. P-glycoprotein (P-gp) over expression is found in many types and many stages of cancer cells which impaired the delivery of anticancer drug to target site of action thereby leading to ineffective treatment. Hence, an inhibition of P-gp function is an attractive strategy toward multidrug resistance. It was further postulated that the conjugation of piperine and calcium can inhibit the function of P-gp, thus, molecular docking studies were carried out to predict 3D structure of P-gp and piperine-Ca conjugate. The *in silico* analysis shows higher binding affinity of piperine-Ca conjugate to P-gp model (-9.54 kcal/mol) in comparison with piperine alone (-8.77 kcal/mol). Piperine-Ca conjugate has shown good pharmacokinetic properties and therefore may be co-administered with anticancer drugs as efflux modulator after undergoing further *in-vitro* and *in-vivo* studies.

Key words: P-glycoprotein, herbomineral, calcium, *Piper nigrum*, piperine, pharmacokinetic.

INTRODUCTION

The medicinal use of tortoise/turtles shells has a very long tradition in the Asian countries. There are many reports related to the use of herbomineral therapeutics and were found to be one of the most promising areas of treating diseases like cancer (Sheikh et al., 2012). The drug under study, *Salaha-A*, is a combination of tortoise shell ash and powdered white pepper (*Piper nigrum* Linn.) at a ratio of 90:10, respectively. The powdered drug is administered orally with honey as vehicle, at a dose of 500 mg four times a day. It is locally prescribed to

*Corresponding author. E-mail: turanjoji84@gmail.com.

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

patients suffering from cancer and some degenerative diseases and described to possess anti-inflammatory and immunomodulatory effect. Herbal and mineral formulations possessing such a combination of preventive and therapeutic effects are described as *Rasayana* in ayurveda and have been widely used by ayurvedic physicians. The uses of herbs and minerals as integral parts of traditional systems of medicine are unique to the ayurvedic and Siddha systems of Indian Traditional Medicine (Kumar et al., 2006). These systems of medicine preparation obtained minerals from natural products following a series of purification and incineration.

Cancer has become one of the biggest challenges to the scientific community all over the world despite development of drugs and other modalities for it treatment. Cancer cells often become simultaneously resistant to multiple drugs. The molecular basis of multi drug resistance is the over expression of gene encoding P-glycoprotein (P-gp) which effectively extrudes hydrophobic drugs out of cancer cells, effectively precluding their activity (Ondieki et al., 2017). P-gp plays an important role in drug disposition and distribution. Several studies to enhance oral bioavailability have demonstrated the possible use of P-gp inhibitors that reverse P-gp-mediated efflux in an attempt to improve the efficiency of drug transport across the epithelia. P-gp inhibitor influences metabolism, absorption, distribution, and elimination of P-gp substrates in the process of modulating pharmacokinetics. Recently, *Salaha-A* is being prescribed by traditional medicine practitioners in Kano State, Nigeria and claimed to be effective in the treatment of cancer. Tortoise shell ash contains certain minerals with calcium being the most abundant. Several studies have shown that *P. nigrum* has antimicrobial (Dorman and Deans, 2000), antimutagenic (El-Hamss et al., 2003), antioxidant and radical scavenging property (Gulcin, 2005). Piperine being the main constituents of *P. nigrum* is known to exhibit a variety of biological activities and is anti-metastatic (Pradeep and Kuttan, 2002). Also, piperine has high immunomodulatory and antitumor activity (Sunila and Kuttan, 2004). Piperine also increases the bioavailability of certain drugs in the organism (Karan et al., 1999).

This study was therefore undertaken to ascertain the effect of *Salaha-A* at different doses on hematological parameters of rats. An examination of the histology of the spleen tissues of the rats given *Salaha-A* at different doses was also observed. Postulation of the conjugation of piperine with calcium can inhibit active functional site of P-gp using *in silico* analysis.

MATERIALS AND METHODS

Samples collection and handling

Pieces of tortoise shell were purchased from the local traditional medical center (Sangarib) packed in an airtight bottle, packed powdered white pepper (*P. nigrum* fruit) and bottle sealed pure natural honey were purchased from Jifatu store in Kano and were all stored in a dry place at room temperature. Twenty Wistar rats of both sexes weighing between 100 and 120 g (aged 6 to 7 weeks) were used in this study. They were procured from the animal house of Department of Biological Sciences, Bayero University, Kano. The rats were maintained in the animal room of the department. They were allowed to acclimatize for one week and fed on standard laboratory food pellets and water throughout the experiment.

Quantitative analysis of tortoise shell

Quantitative analysis of minerals like calcium, magnesium, iron, sodium, potassium and zinc of the tortoise shell ash was performed using Atomic Absorption Spectrometer (AAS). Six grams of the samples were weighed approximately in a 250 ml beaker; 50 cm^3 HCl solution (50% v/v) was added and kept for 1 h, then filtered to remove insoluble material. The samples were then transferred to a volumetric flask, volume adjusted to 50 ml and mixed. All precautions were taken to avoid contamination. The samples were then aspirated in atomic absorption spectrometer (AAS) against standard solution and the absorbance was measured.

Atomic Absorption spectrometer (AAS, mg/kg): Sample concentration × Volume made up / Weight of sample

Dosage schedule and drug administration

The dose for experimental animals was calculated by extrapolating the human dose (2000 mg/day) to animals based on the standard table of Paget and Barnes (1964). The therapeutic dose was calculated to be 180 mg/kg weight of rat. Honey was diluted with distilled water at a ratio of 2:3 in accordance with the guidelines for toxicity/safety profile evaluation of Bhasma/Raskalpas.

Conversion formula: Total clinical dose (a) × Conversion factor 0.018 (b) = (c) / 200 g weight of rat

2000 mg × 0.018= 36 mg/200 g weight of rat

36 × 1000/200 = 180 mg/kg

For the administration of the test drug at different doses, 20 rats were randomly assigned into four groups of five rats each. The first group served as control and were orally given 1 ml of honey solution. The remaining three experimental groups were orally given *Salaha-A* at 180, 280 and 380 mg/kg in 1 ml honey solution each for 15 consecutive days.

Determination of blood parameters

After the mentioned duration, the animals were weighed, sacrificed on 16th day and blood samples were withdrawn by cardiac collection into labeled EDTA bottles. For the complete blood count, the procedure followed was based on the instruction manual of Haematology Analyzer (Sysmex XP-300). Total red blood cell (RBC) count, hemoglobin content (HGB), haematocrit (HCT), granulocytes count (GR) total white blood cells (WBC), lymphocyte count (LYM), and platelet (PLT) were assessed. Mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red blood cell distribution width (RDW), platelet distribution width (PDW), mean platelet volume (MPV), and platelet larger cell ratio (P-LCR) were also calculated.

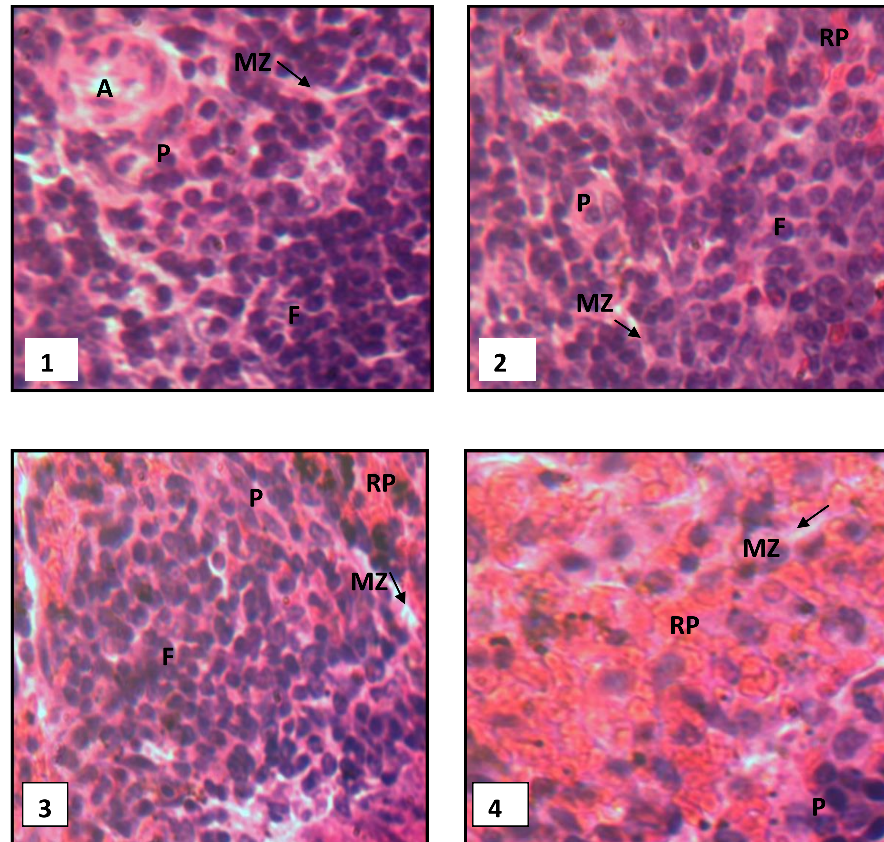

Figure 1. Histology of spleen tissues of rats given Salaha-A at different doses. A, Central artery; RP, Red pulp; MZ, Marginal zone; F, follicles; P; periarteriolar lymphoid sheath (PALS).

Determination of blood calcium level

Blood samples were centrifuged and by the method of Barnett et al. (1973), the resulting plasma was analyzed for total calcium using a colorimetric method (Randox, RX Monza CA kit no 590).

Reaction principle

Complex with O-Cresolphthalein complexone in an alkaline medium.

Sample material

Serum heparinized plasma diluted in 1+1 in 0.9% NaCl × 2

Concentration × 2.50 (mmol/l) = Sample / Standard

Histological examination of spleen tissues

The anterior abdominal wall muscle was incised to expose the gut in the abdomen. The spleen was carefully dissected out using a sharp sterilized scapel knife. The tissues were removed and were fixed in 10% formal saline in specimen bottles. Following fixation, the tissues were further processed by dehydration through ascending grades of alcohol. The first grade of alcohol used was 70% alcohol for a day followed by 90% alcohol overnight and finally two changes of absolute alcohol the following day. After dehydration, the tissues were treated with xylene (70% xylene/30% absolute alcohol) for a day followed by infiltration in three changes of paraffin at 60°C for two days, using an oven. Lastly, the tissues were transferred into an embedding medium (fresh paraffin wax) followed by blocking. Sections of about 5 microns thick were cut using a rotary microtome.

Haematoxylin and Eosin staining

Method of Drury and Wallington (1967) were adopted for Haematoxylin and Eosin staining. Sections were dewaxed for 2 min in each of the two changes of xylene and were transferred into absolute alcohol for removal of xylene for a minute and were stained with iron haematoxylin for 20 min. They were washed in running tap water for 2 to 3 min. Sections were differentiated in 1% acid alcohol for a few second, blued in running tap water for 5 min and counter-stained with 1% eosin for 3 min, then rinsed in water. They were dehydrated through ascending grades of alcohol (70, 90 and 100%) for 1 min each, cleared in xylene for a minute and mounted in distrene, plasticizer and xylene (DPX).

Photomicrography

Records of the histological results were obtained by photomicrography using a microscope with a camera at the Department of Anatomy, Bayero University Kano as shown in Figure 1.

Table 1. Minerals present in the tortoise shell ash.

Mineral	Weight (mg/kg)
Calcium	314.87
Iron	35.28
Magnesium	32.19
Sodium	3.09
Potassium	35.33
Zinc	20.34

Statistical analysis

Results are presented as mean ± standard error of mean (SEM) and total variation present in a set of data was analyzed through one way analysis of variance (ANOVA). Comparison between groups was done using least significant difference (LSD). Differences were considered statistically significant at $p<0.05$.

Homology modeling of P-glycoprotein (P-GP)

The sequence of P-glycoprotein (NP_001335874.1) was retrieved from National Center for Biotechnology Information (NCBI) and subjected to protein-protein Basic Local Alignment Search Tool (BLASTp) search against the Protein Database for the identification of a suitable template. Structures with higher identity, lowest E-value query coverage and good resolution (PDB ID 4Q9H, 4F4C) were selected as best templates. Multiple alignments of the target sequence and templates were generated by running the Advanced Modeling script in MODELLER 9v17 (Sali et al., 1995) based on a dynamic programming algorithm; it takes into account structural information from the templates when constructing an alignment. Five 3D structures were developed and the one with the low molpdf score was chosen as the best model. The developed model of P-gp was further evaluated by Ramachandran plot using Rampage server to assess the quality of the predicted model.

Binding site prediction

Binding site of the P-gp model was predicted by submitting the sequence to the COACH (Yang and Zhang, 2013) server. Residues from the group with the highest confidence score (C-score) and cluster size was selected as our functional active site for setting of x, y, and z grid size.

Piperine-calcium conjugate and retrieval of piperine

The structure of the piperine-Ca conjugate was drawn in the ACD-chemsketch software and then saved in sdf format. The sdf format of the piperine-Ca was converted to PDB and SMILES format using the Open BaBel Software (O'Boyle et al., 2011). The 3-Dimensional structure of piperine (PubChem CID: 638024) in sdf format was downloaded from PubChem database and also converted to PDB format in Open BaBel Software. The SMILES of the piperine-Ca conjugate and that of piperine obtained from PubChem Database were submitted to SwissADME server (Daina et al., 2017) for evaluation of their molecular properties and pharmacokinetic properties.

Molecular docking simulations

Docking simulations of piperine and piperine-Ca conjugate into the binding site of P-gp predicted model was performed using Autodock 4.2 (Norgan et al., 2011), the hydrogen polar atoms were added to the receptor molecules and Lamarckian algorithm was applied. Autogrid with a size of 60×60×60 points in X (-27.030), Y (28.281), and Z (-11.219) directions was built with a grid spacing of 0.375 Å.

RESULTS AND DISCUSSION

Quantitative analysis of tortoise shell ash

The quantitative analysis of the tortoise shell ash reveals that it contains minerals that are essential for maintenance of good health. Calcium, nutritionally important alkaline mineral was found to be the main active mineral found in the tortoise ash (Table 1).

Hematological parameters

The examination of blood gives the opportunity to investigate the presence of several metabolites and other constituents in the body of animals and it plays a vital role in the physiological nutrition and pathological status of an organism (Aderemi, 2004; Doyle, 2006). The significant increase of white blood cells (Table 2) in the rats indicates their capability of generating antibodies in the process of phagocytocis and have high degree of resistance to diseases (Soetan et al., 2013) and enhance adaptability to local environmental and disease prevalent conditions (Kabir et al., 2011; Iwuji and Herbert, 2012; Isaac et al., 2013). Lymphocytes are the main effectors cells of the immune system. They are formed and released from lymphoid tissue such as lymph nodes, spleen, etc. They are unable to eat or engulf organisms, but fulfill their function of defending the body against invading microorganisms, foreign macromolecules, and cancer cells (Junqueira and Carneiro, 2003).

Junqueira and Carneiro (2003) stated that in some cancers, e.g. melanoma and colorectal cancer, lymphocytes can migrate into and attack the tumor; this can sometimes lead to regression of the primary tumor.

The significant increase in red blood cells, haemoglobin and hematocrit, in all treated groups (Table 3) indicates the capacity of *Salaha-A* in the increase of delivering oxygen to the body tissues via blood flow through the circulatory system (Maton et al., 1997). The non-significant change in the Mean Platelets volume (MPV), Platelets distribution width (PDW) in therapeutic and high dose given groups indicates the reduction in production of platelets in the blood. The increase in proportion of whole blood occupied by platelets (PCT) in group that received the test drug at therapeutic dose was not highly significant. These results has showed the ability of Salaha-A in blood clotting was not achieved (Table 4).

In the adult human body, 99% of calcium is found in mineralized tissues (bones and teeth), in which it is present as calcium phosphate or calcium carbonate (Bedi et al., 2006). The remaining 1% is found in the blood, extracellular fluid, and various tissues. The maintenance

Table 2. Leukocytes indices of rats given at different doses of Salaha-A.

Parameter	WBC ($\times10^9$/L)	LYM (%)	MID (%)	GR (%)
Control	3.38±0.1	53.08±0.5	25.16±0.9	10.8±0.68
180 mg/kg	3.52±0.4	58.4±0.7	28.05±0.5	13.3±0.3
280 mg/kg	5.8±0.5	70.9±0.64	23.2±0.64	6.11±0.2
380 mg/kg	3.94±0.35	86.2±0.8	11.51±0.44	2.99±0.28
LSD	0.93	1.75	1.62	1.05

Data represented as mean±SEM. LSD, Least significant difference.

Table 3. Erythrocytes indices of rats given Salaha-A at different doses.

Parameter	MCV (fL)	MCH (Pg)	MCHC (g/dl)	RBC ($\times10^{12}$/L)	HGB (g/dL)	HCT (%)	RDW (%)	P-LCR (%)
Control	60.5±0.7	20.2±0.4	31.8±0.7	3.5±0.5	6.16±1.1	19.9±0.3	25.4±0.6	52.7±0.7
180 mg/kg	60.3±0.5	19.9±0.7	31.8±0.7	4.7±0.3	8.5±0.27	27.4±0.44	25.1±0.5	47.5±0.7
280 mg/kg	62.0±0.5	21.5±0.6	32.0±0.7	5.97±0.3	11.62±0.2	33.68±1.0	24.0±0.4	33.9±1.0
380 mg/kg	62.3±1.3	21.5±0.8	32.0±0.8	4.89±0.5	10.87±0.4	28.66±0.3	24.3±0.6	48.0±0.6
LSD	NS	NS	NS	1.15	1.8	1.07	NS	1.98

Data represented as mean±SEM, LSD, Least significance difference.

Table 4. Platelets indices of rats given at different doses of Salaha-A.

Parameter	MPV (FL)	PDW (%)	PCT (%)
Control	13.1±0.3	7.52±0.3	0.29±0.02
180 mg/kg	13.2±0.4	8.29±0.5	0.62±0.02
280 mg/kg	10.5±0.3	10.9±0.4	0.51±0.07
380 mg/kg	13.01±0.5	8.44±0.4	0.34±0.01
LSD	1.0	1.13	0.1

Data represented as mean±SEM. LSD, Least significant difference.

Table 5. Blood Ca^2 level at different doses.

Group	Ca^2 level (mmol/l)
Control	2.18±0.03
180 mg/kg	2.20±0.78
280 mg/kg	2.23±0.12
380 mg/kg	2.25±0.62

Data represented as mean±SEM.

of normal blood calcium level in rats given higher doses of Salaha-A (Table 5) may be achieved by well intestinal calcium absorption. The concentration of calcium in the blood, in which it is present as ionized calcium is maintained dynamically within a tightly regulated range through intestinal calcium absorption (Bedi et al., 2006). It was therefore concluded that the plant constituent of *Salaha-A* (Piperine) plays a significant role in enhancing the absorption of mineral contents of the test drug.

Histological results of spleen tissues

The spleen is responsible for initiating immune reactions to blood-borne antigens and for filtering the blood of foreign material and old or damaged red blood cells (Cesta, 2006). It comprised 2 functionally and morphologically distinct compartments, the red pulp and the white pulp (periarteriolar lymphoid sheath, follicles, and marginal zone). The white pulp which surrounds the central arterioles contains lymphocytes and initiates immune responses to blood-borne antigens. The white pulp is abundantly seen in groups 2 (180 mg/kg) and 3 (280 mg/kg) and red pulp in group 4 that received the test drug at high dose of 380 mg/kg. The red pulp is anatomically well suited for its blood-filtering function and also contains macrophages that have special properties for fighting bacteria and facilitating iron metabolism (Mebius and Kraal, 2005).

In silico studies results

The multiple templates homology modeling of the P-gp resulted in five models and the best model among them was selected and evaluated using Ramachandran plot (Figure 2) with 96.3% in most favoured regions (1298 residues), 3.0% in allowed regions (40 residues) and only 0.7% in the outlier region (10 residues). The piperine-Ca conjugate was drawn in ACD-chemsketch (Figure 3) with molecular weight of 309.39 kcal/mol and the elemental analysis confirmed the composition of the piperine-Ca conjugate as $C_{13}H_{11}Ca_2NO_3$. The molecular docking simulation results (Figure 4) reveal Piperine-Ca to have

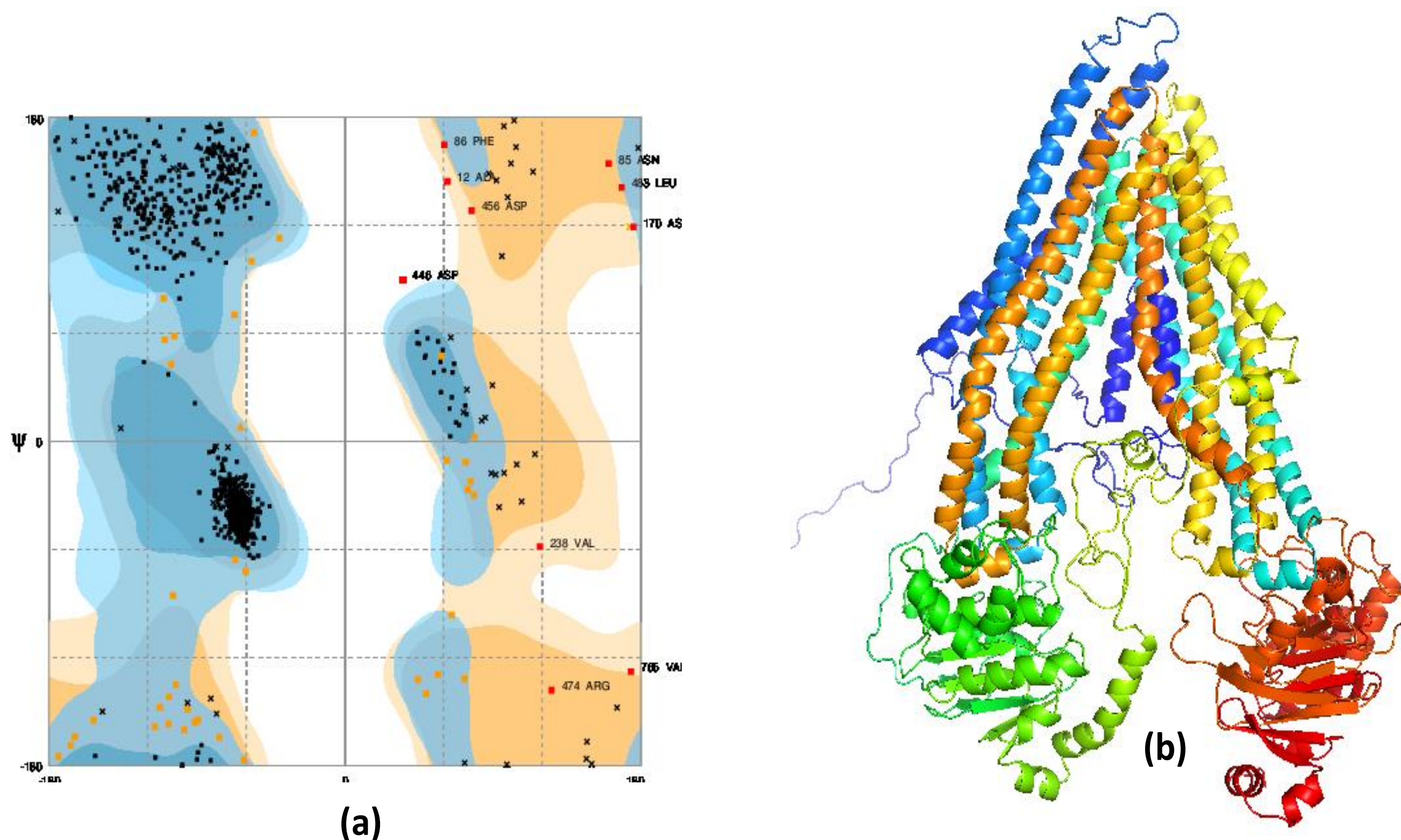

Figure 2. (a) Ramachandran plot showing distribution of residues in phi (Φ) and psi (ψ) torsion angles. **(b)** Predicted 3D structure of P-gp.

Table 6. Binding energy and pharmacokinetic properties of Piperine and Piperine-Ca.

Parameter	Piperine-Ca	Piperine
Binding energy	-9.54 kcal/mol	-8.77 kcal/mol
Molecular weight	309.39 g/mol	285.34 g/mol
WlogP	1.68	2.51
TPSA	38.77 Å²	38.77 Å²
LogS Ali	-2.98 (soluble)	-3.96 (soluble)
Log K_p (skin permeation)	-6.40 cm/s	-5.58 cm/s
GI Absorption	High	High
BBB Permeant	Yes	Yes
P-gp Substrate	No	No
Lipinkin's Rule of 5	Yes 0 violation	Yes 0 violation
Leadlikeness	Yes	Yes

WlogP, Water partition coefficient; TPSA, topological polar surface area; LogS, aqueous solubility; GI, gastrointestinal; BBB, blood brain barrier.

high binding affinity than piperine (Table 6). The piperine-Ca has better pharmacokinetic properties than piperine (Table 6).

Conclusion

From the outcome of the study, it could be concluded that *Salaha-A* contains compounds which have intrinsic importance to human health and could result in the increase of blood oxygen level. Conjugate of the bioactive compounds (piperine + calcium) could inhibit the function of P-glycoprotein and therefore can be efflux modulator that can be co-administered for the treatment of multidrug-resistant cancers.

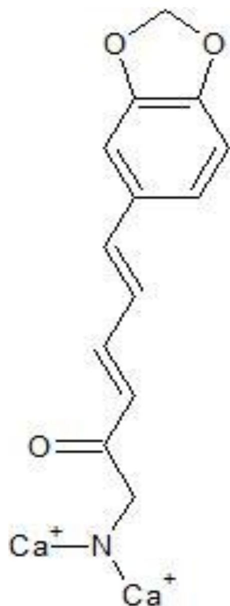

Figure 3. Piperine-Ca conjugates drawn using ACDchemsketch Software.

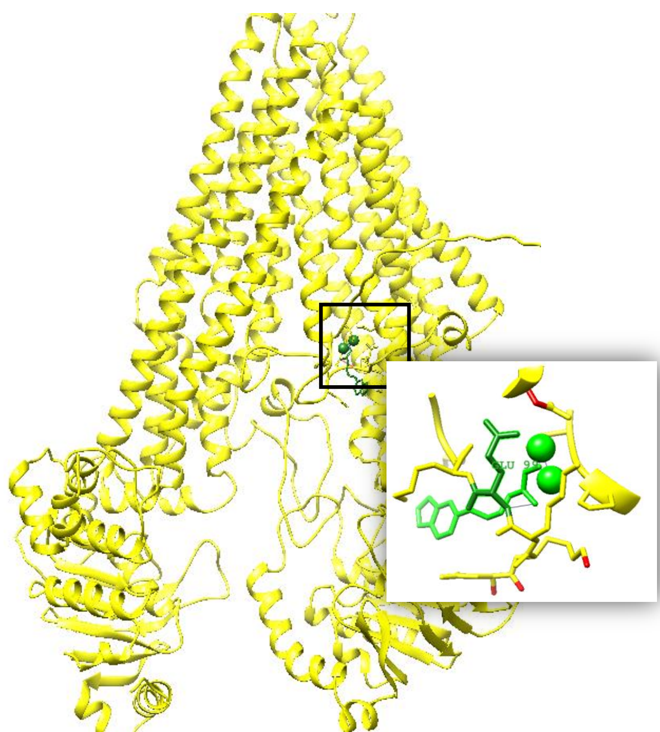

Figure 4. Piperine-calcium conjugate docked inside the binding site of P-gp.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors will like to thank Mal. Yakubu (Animal house), Usman Aliyu (Biochem. Lab.), Mal. Idris (Histology Lab) and Mal Hassan Lawan (Anatomy Dept.) for their technical supports. A big thanks to the HOD of Plant Biology, Dr. Ibrahim Lawan for his excellent contribution to the success of this work.

REFERENCES

Aderemi FA (2004). Effects of replacement of wheat bran with cassava root sieviate supplemented or unsupplemented with enzyme on the haematology and serum biochemistry of pullet chicks. Trop. J. Anim. Sci. 7:147-153.

Bedi K, Gupta BD, Rakesh KJ, Khan IA, Qazi GN, Johri RK (2006). Use of herbal agents for potentiation of bioefficacy of anti infectives. U.S. patents US 7119075.

Cesta MF (2006). Normal structure, function, and histology of the spleen. Toxicol. Pathol. 34(5):455-465.

Daina A, Michielin O, Zoete V (2017). SwissADME: A free web tool to evaluate pharmacokinetics, drug-likeness and medicinal chemistry friendliness of small molecules. Sci. Rep. 7:42717.

Dorman HJD, Deans SG (2000). Antimicrobial agents from plants: antibacterial activity of plant volatile oils. J. Appl. Microbiol. 88(2):308-316.

Drury RAB, Wallington EA (1967). Carleton's Histological Technique, 4th ed. London: Oxford University Press.

El-Hamss R, Idaomar M, Alonso-Morag A, Munos SA (2003). Antimutagenic Properties of Bell and Black Pepper. Food Chem. Toxicol. 41(1):41-47.

Guidelines for toxicity/safety profile evaluation Of Bhasma/raskalpas: Standard Operating Procedure (SOP) for Administration of Bhasma/Rasakalpas with Honey as Vehicle. Available from: http://studylib.net/doc/7712073/guidelines-for-toxicity---safety-profile-evaluation-of-bhasma/raskalpas.

Gulcin I (2005). The antioxidant and radical scavenging activities of black pepper (Piper nigrum) seeds. Int. J Food Sci. Nutr. 56(7):491-499.

Isaac LJ, Abah G, Akpan B, Ekaette IU (2013). Haematological properties of different breeds and sexes of rabbits. Proceedings of the 18th Annual Conference of Animal Science Association of Nigeria. pp. 24-27.

Iwuji TC, Herbert U (2012). Haematological and serum biochemical characteristics of rabbit bucks fed diets containing garcimiola kola seed meal. Proceedings of 37th Annual Conference of Nigerian Society for Animal Production. pp. 87-89.

Junqueira LC, and Carneiro J (2003). Basic Histology text and atlas, 10th edition. Lange Medical Books McGraw-Hill. pp. 233-270.

Kabir M, Akpa GN, Nwagu BI, Adeyinka IA, Bello UI (2011). Sexual dimorphism, breed and age characteristics of rabbits in Zaria, Nigeria. Proceedings of the 16th Annual Conference of Animal Science Association of Nigeria. pp.133-137.

Karan RS, Bhargava VK, Garg SK (1999). Effect of trikatu, an Ayurvedic prescription, on the pharmacokinetic profile of rifampicin in rabbits. J. Ethnopharmacol. 64(3):259-264.

Kumar A, Nair AGC, Reddy AVR, Garg AN (2006). Availability of essential elements in bhasmas: Analysis of Ayurvedic metallic preparations by INAA. J. Radioanal. Nucl. Chem. 270(1):173-180.

Maton D, Hopkins J, McLaughlin CW, Johnson S, Warner MQ, LaHart D, Wright JD, Kulkarni V (1997). Human Biology and Health. Englewood Cliffs, New Jersey, USA: Prentice Hall.

Mebius RE, Kraal G (2005). Structure and function of the spleen. Nature reviews. Immunology 5(8):606.

Norgan AP, Coffman PK, Koche JPA, Katzmann DJ, Sosa CP (2011). Multilevel parallelization of AutoDock 4.2. J. Cheminform. 3(1):12.

O'Boyle NM, Banck M, James CA, Morley C, Vandermeersch T, Hutchison GR (2011). Open Babel: An open chemical to olbox. J.

Cheminform. 3(1):33.
Ondieki G, Nyagblordzro M, Kikete S, Liang R, Wang L, He X (2017). Cytochrome P450 and P-Glycoprotein-Mediated Interactions Involving African Herbs Indicated for Common Noncommunicable Diseases. Evid. Based Complement. Alternat. Med. Jan 31; 2017.
Paget GE, Barnes JM (1964). Toxicity tests, Evaluation of drug activities: pharmacometrics. New York: Academic Press 1:135-65.
Pradeep CR, Kuttan G (2002). Effect of piperine on the inhibition of lung metastasis induced B16F-10 melanoma cells in mice. Clin. Exp. Metastasis 19(8):703-708.
Sali A, Potterton L, Yuan F, van Vlijmen H, Karplus M (1995). Evaluation of comparative protein modeling by MODELLER. Proteins 23(3):318-326.
Sheikh S, Srivastava A, Tripathi R, Tripathi S, Trivedi VP, Saxena RC (2012). Toxicity of a novel herbomineral preparation Las01 on human cancer cell lines and its safety profile in humans and animals. Evid. Based Complement. Alternat. Med. Aug 30; 2012.
Soetan KO, Akinrinde AS, Ajibade TO (2013). Preliminary studies on the haematological parameters of cockerels fed raw and processed guinea corn (*Sorghum bicolor*). Proceedings of 38th Annual Conference of Nigerian Society for Animal Production. pp. 49-52.

Related Journals Published by Academic Journals

- *Biotechnology and Molecular Biology Reviews*
- *African Journal of Microbiology Research*
- *African Journal of Biochemistry Research*
- *African Journal of Environmental Science and Technology*
- *African Journal of Food Science*
- *African Journal of Plant Science*
- *Journal of Bioinformatics and Sequence Analysis*
- *International Journal of Biodiversity and Conservation*

academicJournals