THE UNIVERSITY OF YAOUNDE I

CENTRE FOR RESEARCH AND TRAINING IN GRADUATE STUDIES IN LIFE, HEALTH AND ENVIRONMENTAL SCIENCES

RESEARCH AND DOCTORATE TRANING UNIT IN LIFE SCIENCES

UNIVERSITE DE YAOUNDE I

CENTRE DE RECHERCHE ET DE FORMATION DOCTORALE EN SCIENCES DE LA VIE ET ENVIRONNEMENT

UNITE DE RECHERCHE ET DE FORMATION DOCTORALE EN SCIENCES DE LA VIE

DEPARTMENT OF BIOCHEMISTRY

DEPARTEMENT DE BIOCHIMIE

LABORATORY FOR PHYTOBIOCHEMISTRY AND MEDICINAL PLANTS STUDIES

LABORATOIRE DE PHYTOBIOCHIMIE ET D'ETUDE DES PLANTES MEDICINALES

ANTIMICROBIAL AND BIOCONTROL AGENTS UNIT

Antibacterial and modes of action of some endophytic fungal secondary metabolites against the causative agents of pneumonia

Thesis presented in partial fulfillment of the requirements for the award of a Doctorat /PhD Degree in Biochemistry

by

MBEKOU KANKO Michèle Inès

Registration Nº 10R0948

M.sc in Biochemistry

The jury

President: **PENLAP BENG Véronique**, *Professor* University of Yaoundé I

Supervisor: **FEKAM BOYOM Fabrice**, *Professor* University of Yaoundé I

Examiners: **KORO KORO FRANCIOLI**, *Associate professor* University of Douala

NYEGUE Maximilienne Ascension, *Professor* University of Yaoundé I

LENTA NDJAKOU Bruno, *Professor* University of Yaoundé I

Academic year 2023-2024

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NYEGUE Maximilienne Ascension, *Professor* University of Yaoundé

LENTA NDJAKOU Bruno, *Professor* University of Yaoundé

Academic year 2023-2024

THE UNIVERSITY OF YAOUNDE I UNIVERSITÉ D

Faculty of Science Faculté d

THE UNIVERSITY OF YAOUNDE I		UNIVERSITÉ DE YAOUNDÉ I
Faculty of Science		**Faculté des Sciences**
Division of Programming and Follow-up of Academic Affairs		Division de la Programmation et du Suivi des Activités Académiques

LIST OF PERMANENT TEACHING STAFF LISTE DES ENSEIGNANTS PERMANENTS

ACADEMIC YEAR 2022/2023

(By Department and by Grade)

UPDATE : 31 May 2023

ADMINISTRATION

DEAN : TCHOUANKEU Jean- Claude, Associate Professor

VICE-DEAN / DPSAA : ATCHADE Alex de Théodore, Professor

VICE-DEAN/ DSSE : NYEGUE Maximilienne Ascension, Professor

VICE-DEAN / DRC : ABOSSOLO Monique, Associate Professor

Head of Administrative and Financial Division: NDOYE FOE Marie C. F., Associate Professor

Head of Division of Academic affairs, Research and corporation: AJEAGAH Gideon AGHAINDUM, Professor

1- DEPARTMENT OF BIOCHEMISTRY (BC) (43)			
N°	**NAMES AND SURNAMES**	**GRADE**	**OBSERVATIONS**
1.	BIGOGA DAIGA Jude	Professor	On duty
2.	FEKAM BOYOM Fabrice	Professor	On duty
3.	KANSCI Germain	Professor	On duty
4.	MBACHAM FON Wilfred	Professor	On duty
5.	MOUNDIPA FEWOU Paul	Professor	**Head of Department**
6.	NGUEFACK Julienne	Professor	On duty
7.	NJAYOU Frédéric Nico	Professor	On duty
8.	OBEN Julius ENYONG	Professor	On duty
9.	ACHU Merci BIH	Associate Professor	On duty
10.	ATOGHO Barbara MMA	Associate Professor	On duty
11.	AZANTSA KINGUE GABIN BORIS	Associate Professor	On duty
12.	BELINGA née NDOYE FOE F. M. C.	Associate Professor	**Chief DAF / FS**
13.	DJUIDJE NGOUNOUE Marceline	Associate Professor	On duty
14.	DJUIKWO NKONGA Ruth Viviane	Associate Professor	On duty
15.	EFFA ONOMO Pierre	Associate Professor	**Vice Dean/FS/Univ Ebolowa**
16.	EWANE Cécile Annie	Associate Professor	On duty
17.	KOTUE TAPTUE Charles	Associate Professor	On duty
18.	LUNGA Paul KEILAH	Associate Professor	On duty
19.	MBONG ANGIE M. Mary Anne	Associate Professor	On duty
20.	MOFOR née TEUGWA Clotilde	Associate Professor	**Dean FS / UDs**
21.	NANA Louise épouse WAKAM	Associate Professor	On duty
22.	NGONDI Judith Laure	Associate Professor	On duty
23.	TCHANA KOUATCHOUA Angèle	Associate Professor	On duty
24.	AKINDEH MBUH NJI	Senior Lecturer	On duty
25.	BEBEE Fadimatou	Senior Lecturer	On duty
26.	BEBOY EDJENGUELE Sara Nathalie	Senior Lecturer	On duty
27.	DAKOLE DABOY Charles	Senior Lecturer	On duty
28.	DONGMO LEKAGNE Joseph Blaise	Senior Lecturer	On duty

29.	FONKOUA Martin	Senior Lecturer	On duty
30.	FOUPOUAPOUOGNIGNI Yacouba	Senior Lecturer	On duty
31.	KOUOH ELOMBO Ferdinand	Senior Lecturer	On duty
32.	MANANGA Marlyse Joséphine	Senior Lecturer	On duty
33.	OWONA AYISSI Vincent Brice	Senior Lecturer	On duty
34.	Palmer MASUMBE NETONGO	Senior Lecturer	On duty
35.	PECHANGOU NSANGOU Sylvain	Senior Lecturer	On duty
36.	WILFRED ANGIE ABIA	Senior Lecturer	On duty
37.	BAKWO BASSOGOG Christian Bernard	Assistant lecturer	On duty
38.	ELLA Fils Armand	Assistant lecturer	On duty
39.	EYENGA Eliane Flore	Assistant lecturer	On duty
40.	MADIESSE KEMGNE Eugenie Aimée	Assistant lecturer	On duty
41.	MANJIA NJIKAM Jacqueline	Assistant lecturer	On duty
42.	MBOUCHE FANMOE Marceline Joëlle	Assistant lecturer	On duty
43.	WOGUIA Alice Louise	Assistant lecturer	On duty
2- DEPARTMENT OF ANIMAL BIOLOGY AND PHYSIOLOGY(ABP) (52)			
1.	AJEAGAH Gideon AGHAINDUM	Professor	*DAARS/FS*
2.	BILONG BILONG Charles-Félix	Professor	**Head of Department**
3.	DIMO Théophile	Professor	On duty
4.	DJIETO LORDON Champlain	Professor	On duty
5.	DZEUFIET DJOMENI Paul Désiré	Professor	On duty
6.	ESSOMBA née NTSAMA MBALA	Professor	**DC and Vice dean/FMSB/UYI**
7.	FOMENA Abraham	Professor	On duty
8.	KEKEUNOU Sévilor	Professor	On duty
9.	NJAMEN Dieudonné	Professor	On duty
10.	NJIOKOU Flobert	Professor	On duty
11.	NOLA Moïse	Professor	On duty
12.	TAN Paul VERNYUY	Professor	On duty
13.	TCHUEM TCHUENTE Louis Albert	Professor	**Insp. Serv. Coord. Progr. in HEALTH**

14.	ZEBAZE TOGOUET Serge Hubert	Professor	On duty
15.	ALENE Désirée Chantal	Associate Professor	**Vice Dean /Univ Ebwa**
16.	BILANDA Danielle Claude	Associate Professor	On duty
17.	DJIOGUE Séfirin	Associate Professor	On duty
18.	GOUNOUE KAMKUMO Raceline épse FOTSING	Associate Professor	On duty
19.	JATSA BOUKENG Hermine épse MEGAPTCHE	Associate Professor	On duty
20.	LEKEUFACK FOLEFACK Guy B.	Associate Professor	On duty
21.	MAHOB Raymond Joseph	Associate Professor	On duty
22.	MBENOUN MASSE Paul Serge	Associate Professor	On duty
23.	MEGNEKOU Rosette	Associate Professor	On duty
24.	MOUNGANG LucianeMarlyse	Associate Professor	On duty
25.	NOAH EWOTI Olive Vivien	Associate Professor	On duty
26.	MONY Ruth épse NTONE	Associate Professor	On duty
27.	NGUEGUIM TSOFACK Florence	Associate Professor	On duty
28.	NGUEMBOCK	Associate Professor	On duty
29.	TAMSA ARFAO Antoine	Associate Professor	On duty
30.	TOMBI Jeannette	Associate Professor	On duty
31.	ATSAMO Albert Donatien	Senior Lecturer	On duty
32.	BASSOCK BAYIHA Etienne Didier	Senior Lecturer	On duty
33.	ETEME ENAMA Serge	Senior Lecturer	On duty
34.	FEUGANG YOUMSSI François	Senior Lecturer	On duty
35.	FOKAM Alvine Christelle Epse KENGNE	Senior Lecturer	On duty
36.	GONWOUO NONO Legrand	Senior Lecturer	On duty
37.	KANDEDA KAVAYE Antoine	Senior Lecturer	On duty
38.	KOGA MANG DOBARA	Senior Lecturer	On duty
39.	LEME BANOCK Lucie	Senior Lecturer	On duty
40.	MAPON NSANGOU Indou	Senior Lecturer	On duty
41.	METCHI DONFACK MIREILLE FLAURE EPSE GHOUMO	Senior Lecturer	On duty

42.	MVEYO NDANKEU Yves Patrick	Senior Lecturer	On duty
43.	NGOUATEU KENFACK Omer Bébé	Senior Lecturer	On duty
44.	NJUA Clarisse YAFI	Senior Lecturer	**Head Div. Univ. Bamenda**
45.	NWANE Philippe Bienvenu	Senior Lecturer	On duty
46.	TADU Zephyrin	Senior Lecturer	On duty
47.	YEDE	Senior Lecturer	On duty
48.	YOUNOUSSA LAME	Senior Lecturer	On duty
49.	AMBADA NDZENGUE GEORGIA ELNA	Assist. Lecturer	On duty
50.	KODJOM WANCHE Jacguy Joyce	Assist. Lecturer	On duty
51.	NDENGUE Jean De Matha	Assist. Lecturer	On duty
52.	ZEMO GAMO Franklin	Assist. Lecturer	On duty

3- DEPARTMENT OF PLANT PHYSIOLOGY AND BIOLOGY (PPB) (34)			
1.	AMBANG Zachée	Professor	**Head of Department**
2.	DJOCGOUE Pierre François	Professor	On duty
3.	MBOLO Marie	Professor	On duty
4.	MOSSEBO Dominique Claude	Professor	On duty
5.	YOUMBI Emmanuel	Professor	On duty
6.	ZAPFACK Louis	Professor	On duty
7.	ANGONI Hyacinthe	Associate Professor	On duty
8.	BIYE Elvire Hortense	Associate Professor	On duty
9.	MAHBOU SOMO TOUKAM. Gabriel	Associate Professor	On duty
10.	MALA Armand William	Associate Professor	On duty
11.	MBARGA BINDZI Marie Alain	Associate Professor	**DAAC /Univ , Douala**
12.	NDONGO BEKOLO	Associate Professor	On duty
13.	NGALLE Hermine BILLE	Associate Professor	On duty
14.	NGODO MELINGUI Jean Baptiste	Associate Professor	On duty
15.	NGONKEU MAGAPTCHE Eddy L.	Associate Professor	**CT / MINRESI**
16.	TONFACK Libert Brice	Associate Professor	On duty
17.	TSOATA Esaïe	Associate Professor	On duty

18.	ONANA JEAN MICHEL	Associate Professor	On duty
19.	DJEUANI Astride Carole	Senior Lecturer	On duty
20.	GONMADGE CHRISTELLE	Senior Lecturer	On duty
21.	MAFFO MAFFO Nicole Liliane	Senior Lecturer	On duty
22.	NNANGA MEBENGA Ruth Laure	Senior Lecturer	On duty
23.	NOUKEU KOUAKAM Armelle	Senior Lecturer	On duty
24.	NSOM ZAMBO EPSE PIAL ANNIE CLAUDE	Senior Lecturer	**In détachment /UNESCO MALI**
25.	GODSWILL NTSOMBOH NTSEFONG	Senior Lecturer	On duty
26.	KABELONG BANAHO Louis-Paul-Roger	Senior Lecturer	On duty
27.	KONO Léon Dieudonné	Senior Lecturer	On duty
28.	LIBALAH Moses BAKONCK	Senior Lecturer	On duty
29.	LIKENG-LI-NGUE Benoit C	Senior Lecturer	On duty
30.	TAEDOUNG Evariste Hermann	Senior Lecturer	On duty
31.	TEMEGNE NONO Carine	Senior Lecturer	On duty
32.	MANGA NDJAGA JUDE	Assistant lecturer	On duty
33.	DIDA LONTSI Sylvere Landry	Assistant lecturer	On duty
34.	METSEBING Blondo-Pascal	Assistant lecturer	On duty

4- DEPARTMENT OF INORGANIC CHEMISTRY (IC) (28)			
1.	GHOGOMU Paul MINGO	Professor	**Minister in charge of mission. P.R.**
2.	NANSEU NJIKI Charles Péguy	Professor	On duty
3.	NDIFON Peter TEKE	Professor	**TC MINRESI**
4.	NENWA Justin	Professor	On duty
5.	NGAMENI Emmanuel	Professor	**Dean FS Univ. Ngaoundere**
6.	NGOMO Horace MANGA	Professor	**Vice Chancelor/Univ. Buea**
7.	NJOYA Dayirou	Professor	On duty
8.	ACAYANKA Elie	Associate Professor	On duty

9.	EMADAK Alphonse	Associate Professor	On duty
10.	KAMGANG YOUBI Georges	Associate Professor	On duty
11.	KEMMEGNE MBOUGUEM Jean C.	Associate Professor	On duty
12.	KENNE DEDZO GUSTAVE	Associate Professor	On duty
13.	MBEY Jean Aime	Associate Professor	On duty
14.	NDI NSAMI Julius	Associate Professor	**Head of Department**
15.	NEBAH Née NDOSIRI Bridget NDOYE	Associate Professor	**Senator/SENAT**
16.	NJIOMOU C. épse DJANGANG	Associate Professor	On duty
17.	NYAMEN Linda Dyorisse	Associate Professor	On duty
18.	PABOUDAM GBAMBIE AWAWOU	Associate Professor	On duty
19.	TCHAKOUTE KOUAMO Hervé	Associate Professor	On duty
20.	BELIBI BELIBI Placide Désiré	Associate Professor	**Head of division/ ENS Bertoua**
21.	CHEUMANI YONA Arnaud M.	Associate Professor	On duty
22.	KOUOTOU DAOUDA	Associate Professor	On duty
23.	MAKON Thomas Beauregard	Senior Lecturer	On duty
24.	NCHIMI NONO KATIA	Senior Lecturer	On duty
25.	NJANKWA NJABONG N. Eric	Senior Lecturer	On duty
26.	PATOUOSSA ISSOFA	Senior Lecturer	On duty
27.	SIEWE Jean Mermoz	Senior Lecturer	On duty
28.	BOYOM TATCHEMO Franck W.	Assistant Lecturer	On duty

5- DEPARTMENT OF ORGANIC CHEMISTRY (OC) (37)			
1.	Alex de Théodore ATCHADE	Professor	**Vice-Dean/PSAA**
2.	DONGO Etienne	Professor	**Vice Dean/CSA/ F. SED**
3.	NGOUELA Silvère Augustin	Professor	**Head of Department UDs**
4.	PEGNYEMB Dieudonné Emmanuel	Professor	**Director MINESUP/ Head of Department**
5.	WANDJI Jean	Professor	On duty
6.	MBAZOA née DJAMA Céline	Professor	On duty

7.	AMBASSA Pantaléon	Associate Professor	On duty
8.	EYONG Kenneth OBEN	Associate Professor	On duty
9.	FOTSO WABO Ghislain	Associate Professor	On duty
10.	KAMTO Eutrophe Le Doux	Associate Professor	On duty
11.	KENMOGNE Marguerite	Associate Professor	On duty
12.	KEUMEDJIO Félix	Associate Professor	On duty
13.	KOUAM Jacques	Associate Professor	On duty
14.	MKOUNGA Pierre	Associate Professor	On duty
15.	MVOT AKAK CARINE	Associate Professor	On duty
16.	NGO MBING Joséphine	Associate Professor	**Head of cell MINRESI**
17.	NGONO BIKOBO Dominique Serge	Associate Professor	**Study charge Ass. n°3/MINESUP**
18.	NOTE LOUGBOT Olivier Placide	Associate Professor	**DAAC/Univ. Bertoua**
19.	NOUNGOUE TCHAMO Diderot	Associate Professor	On duty
20.	TABOPDA KUATE Turibio	Associate Professor	On duty
21.	TAGATSING FOTSING Maurice	Associate Professor	On duty
22.	TCHOUANKEU Jean-Claude	Associate Professor	**Dean /FS/ UYI**
23.	YANKEP Emmanuel	Associate Professor	On duty
24.	ZONDEGOUMBA Ernestine	Associate Professor	On duty
25.	MESSI Angélique Nicolas	Senior Lecturer	On duty
26.	NGNINTEDO Dominique	Senior Lecturer	On duty
27.	NGOMO Orléans	Senior Lecturer	On duty
28.	NONO NONO Éric Carly	Senior Lecturer	On duty
29.	OUAHOUO WACHE Blandine M.	Senior Lecturer	On duty
30.	OUETE NANTCHOUANG Judith Laure	Senior Lecturer	On duty
31.	SIELINOU TEDJON Valérie	Senior Lecturer	On duty
32.	TCHAMGOUE Joseph	Senior Lecturer	On duty
33.	TSAFFACK Maurice	Senior Lecturer	On duty
34.	TSAMO TONTSA Armelle	Senior Lecturer	On duty
35.	TSEMEUGNE Joseph	Senior Lecturer	On duty
36.	MUNVERA MFIFEN Aristide	Assistant lecturer	On duty

37.	NDOGO ETEME Olivier	Assistant lecturer	On duty
6- DEPARTMENT OF COMPUTER SCIENCE (CS) (22)			
1	ATSA ETOUNDI Roger	Professor	**Chief Div. MINESUP**
2	FOUDA NDJODO Marcel Laurent	Professor	**Head of department HTTC/Chief IGA. MINESUP**
3	NDOUNDAM Réné	Associate Professor	On duty
4	TSOPZE Norbert	Associate Professor	On duty
5	ABESSOLO ALO'O Gislain	Senior Lecturer	**Head of cell MINFOPRA**
6	AMINOU HALIDOU	Senior Lecturer	**Head of Department**
7	DJAM Xaviera YOUH - KIMBI	Senior Lecturer	On duty
8	DOMGA KOMGUEM Rodrigue	Senior Lecturer	On duty
9	EBELE Serge Alain	Senior Lecturer	On duty
10	HAMZA Adamou	Senior Lecturer	On duty
11	JIOMEKONG AZANZI Fidel	Senior Lecturer	On duty
12	KOUOKAM KOUOKAM E. A.	Senior Lecturer	On duty
13	MELATAGIA YONTA Paulin	Senior Lecturer	On duty
14	MESSI NGUELE Thomas	Senior Lecturer	On duty
15	MONTHE DJIADEU Valery M.	Senior Lecturer	On duty
16	NZEKON NZEKO'O ARMEL JACQUES	Senior Lecturer	On duty
17	OLLE OLLE Daniel Claude Georges Delort	Senior Lecturer	**C/D ENSET Ebolowa**
18	TAPAMO Hyppolite	Senior Lecturer	On duty
19	BAYEM Jacques Narcisse	Assistant lecturer	On duty
20	EKODECK Stéphane Gaël Raymond	Assistant lecturer	On duty
21	MAKEMBE. S . Oswald	Assistant lecturer	**Director CUTI**
22	NKONDOCK. MI. BAHANACK.N.	Assistant lecturer	On duty
7- DEPARTMENT OF MATHEMATICS (MA) (33)			
1.	AYISSI Raoult Domingo	Professor	**Head of Department**
2.	KIANPI Maurice	Associate Professor	On duty
3.	MBANG Joseph	Associate Professor	On duty

4.	MBEHOU Mohamed	Associate Professor	On duty
5.	MBELE BIDIMA Martin Ledoux	Associate Professor	On duty
6.	NOUNDJEU Pierre	Associate Professor	**Chief Service of Programs & Diploms/FS/UYI**
7.	TAKAM SOH Patrice	Associate Professor	**On duty**
8.	TCHAPNDA NJABO Sophonie B.	Associate Professor	**Director/AIMS Rwanda**
9.	TCHOUNDJA Edgar Landry	Associate Professor	On duty
10.	AGHOUKENG JIOFACK Jean Gérard	Senior Lecturer	**Chief Cell MINEPAT**
11.	BOGSO ANTOINE Marie	Senior Lecturer	On duty
12.	CHENDJOU Gilbert	Senior Lecturer	On duty
13.	DJIADEU NGAHA Michel	Senior Lecturer	On duty
14.	DOUANLA YONTA Herman	Senior Lecturer	On duty
15.	KIKI Maxime Armand	Senior Lecturer	On duty
16.	LOUMNGAM KAMGA Victor	Senior Lecturer	On duty
17.	MBAKOP Guy Merlin	Senior Lecturer	On duty
18.	MBATAKOU Salomon Joseph	Senior Lecturer	On duty
19.	MENGUE MENGUE David Joël	Senior Lecturer	**Head department / ENS** Maroua
20.	MBIAKOP Hilaire George	Senior Lecturer	On duty
21.	NGUEFACK Bernard	Senior Lecturer	On duty
22.	NIMPA PEFOUKEU Romain	Senior Lecturer	On duty
23.	OGADOA AMASSAYOGA	Senior Lecturer	On duty
24.	POLA DOUNDOU Emmanuel	Senior Lecturer	**In training course**
25.	TCHEUTIA Daniel Duviol	Senior Lecturer	On duty
26.	TETSADJIO TCHILEPECK M. Eric.	Senior Lecturer	On duty
27.	BITYE MVONDO Esther Claudine	Assistant lecturer	On duty
28.	FOKAM Jean Marcel	Assistant lecturer	On duty
29.	GUIDZAVAI KOUCHERE Albert	Assistant lecturer	On duty
30.	MANN MANYOMBE Martin Luther	Assistant lecturer	On duty
31.	MEFENZA NOUNTU Thiery	Assistant lecturer	On duty
32.	NYOUMBI DLEUNA Christelle	Assistant lecturer	On duty

33.	TENKEU JEUFACK Yannick Léa	Assistant lecturer	On duty
8- DEPARTMENT OF MICROBIOLOGY (MIB) (24)			
1.	ESSIA NGANG Jean Justin	Professor	**Head of Department**
2.	NYEGUE Maximilienne Ascension	Professor	**Vice Dean/DSSE**
3.	ASSAM ASSAM Jean Paul	Associate Professor	On duty
4.	BOUGNOM Blaise Pascal	Associate Professor	On duty
5.	BOYOMO ONANA	Associate Professor	On duty
6.	KOUITCHEU MABEKU Epse KOUAM Laure Brigitte	Associate Professor	On duty
7.	RIWOM Sara Honorine	Associate Professor	On duty
8.	NJIKI BIKOÏ Jacky	Associate Professor	On duty
9.	SADO KAMDEM Sylvain Leroy	Associate Professor	On duty
10.	ESSONO Damien Marie	Senior Lecturer	On duty
11.	LAMYE Glory MOH	Senior Lecturer	On duty
12.	MEYIN A EBONG Solange	Senior Lecturer	On duty
13.	MONI NDEDI Esther Del Florence	Senior Lecturer	On duty
14.	NKOUDOU ZE Nardis	Senior Lecturer	On duty
15.	TAMATCHO KWEYANG Blandine Pulchérie	Senior Lecturer	On duty
16.	TCHIKOUA Roger	Senior Lecturer	**Head of school division**
17.	TOBOLBAÏ Richard	Senior Lecturer	On duty
18.	NKOUE TONG Abraham	Assistant lecturer	On duty
19.	SAKE NGANE Carole Stéphanie	Assistant lecturer	On duty
20.	EZO’O MENGO Fabrice Télésfor	Assistant lecturer	On duty
21.	EHETH Jean Samuel	Assistant lecturer	On duty
22.	MAYI Marie Paule Audrey	Assistant lecturer	On duty
23.	NGOUENAM Romial Joël	Assistant lecturer	On duty
24.	NJAPNDOUNKE Bilkissou	Assistant lecturer	On duty
9- DEPARTMENT OF PHYSICS (PY) (43)			
1	BEN- BOLIE Germain Hubert	Professor	On duty
2	DJUIDJE KENMOE spouse ALOYEM	Professor	On duty

3	EKOBENA FOUDA Henri Paul	Professor	**Vice-Rector Univ. Ngaoundéré**
4	ESSIMBI ZOBO Bernard	Professor	On duty
5	HONA Jacques	Professor	On duty
6	NANA ENGO Serge Guy	Professor	On duty
7	NANA NBENDJO Blaise	Professor	On duty
8	NDJAKA Jean Marie Bienvenu	Professor	**Head of Department**
9	NJANDJOCK NOUCK Philippe	Professor	On duty
10	NOUAYOU Robert	Professor	On duty
11	SAIDOU	Professor	**Chief of centre /IRGM/MINRESI**
12	TABOD Charles TABOD	Professor	**Dean FS Univ. Bamenda**
13	TCHAWOUA Clément	Professor	On duty
14	WOAFO Paul	Professor	On duty
15	ZEKENG Serge Sylvain	Professor	On duty
16	BIYA MOTTO Frédéric	Associate Professor	**General director /HYDRO Mekin**
17	BODO Bertrand	Associate Professor	On duty
18	ENYEGUE A NYAM épse BELINGA	Associate Professor	On duty
19	EYEBE FOUDA Jean sire	Associate Professor	On duty
20	FEWO Serge Ibraïd	Associate Professor	On duty
21	MBINACK Clément	Associate Professor	On duty
22	MBONO SAMBA Yves Christian U.	Associate Professor	On duty
23	MELI'I Joelle Larissa	Associate Professor	On duty
24	MVOGO ALAIN	Associate Professor	On duty
25	NDOP Joseph	Associate Professor	On duty
26	SIEWE SIEWE Martin	Associate Professor	On duty
27	SIMO Elie	Associate Professor	On duty
28	VONDOU DerbetiniAppolinaire	Associate Professor	On duty
29	WAKATA née BEYA Annie Sylvie	Associate Professor	**Director/ENS/UYI**
30	WOULACHE Rosalie Laure	Associate Professor	**In training course**
31	ABDOURAHIMI	Senior Lecturer	On duty

32	AYISSI EYEBE Guy François Valérie	Senior Lecturer	On duty
33	CHAMANI Roméo	Senior Lecturer	On duty
34	DJIOTANG TCHOTCHOU Lucie Angennes	Senior Lecturer	On duty
35	EDONGUE HERVAIS	Senior Lecturer	On duty
36	FOUEJIO David	Senior Lecturer	**Chief of Cell MINADER**
37	KAMENI NEMATCHOUA Modeste	Senior Lecturer	On duty
38	LAMARA Maurice	Senior Lecturer	On duty
39	OTTOU ABE Martin Thierry	Senior Lecturer	**Director of reagents production Unit IMPM**
40	TEYOU NGOUPO Ariel	Senior Lecturer	On duty
41	WANDJI NYAMSI William	Senior Lecturer	On duty
42	NGA ONGODO Dieudonné	Assistant lecturer	On duty
43	SOUFFO TAGUEU Merimé	Assistant lecturer	On duty

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39	FEUMBA Roger	Senior Lecturer	On duty

40	MBANGA NYOBE Jules	Senior Lecturer	On duty
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42	NGO’O ZE ARNAUD	Assistant lecturer	On duty
43	TENE DJOUKAM Joëlle Flore, spouse KOUANKAP NONO	Assistant lecturer	On duty

DISTRIBUTION OF PERMANENT LECTURERS IN THE FACULTY OF SCIENCE OF THE UNIVERSITY OF YAOUNDE I ACCORDING TO DEPARTMENTS

NUMBER OF LECTURERS					
DEPARTMENT	**Professor**	**Associate Professor**	**Senio lecturer**	**Assistant lecturer**	**Total**
BCH	8 (01)	15 (11)	13 (03)	7 (05)	**43 (20)**
ABP	14 (01)	16 (09)	18 (04)	4 (02)	**52 (16)**
PBP	6 (01)	12 (02)	13 (07)	3 (00)	**34 (10)**
IC	7 (01)	15 (04)	5 (01)	1 (00)	**28 (06)**
OC	6 (01)	18 (04)	11 (04)	2 (00)	**37 (09)**
CS	2 (00)	2 (00)	14 (01)	4 (00)	**22 (01)**
MAT	1 (00)	8 (00)	17 (01)	7 (02)	**33 (03)**
MIB	2 (01)	7 (03)	8 (04)	7 (02)	**24 (10)**
PHY	15 (01)	15 (04)	11 (01)	2 (00)	**43 (06)**
ES	8 (00)	17 (03)	15 (04)	3 (01)	**43 (08)**
Total	**69 (07)**	**125 (40)**	**125 (30)**	**40 (12)**	**359 (89)**

A total of **359 (89)** including

- Professors **69(07)**
- Associate professors **125 (40)**
- Senior lecturers **125 (30)**
- Assistant lecturer **40 (12)**

( ) = Number of women **89**

DEDICATION

I dedicate my thesis to

My family ***KANKO***

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I give the honour and glory to God Almighty for keeping me alive and giving me the opportunity, strength, and patience to realize and compile this piece of work.

I am particularly grateful to **Professor Fabrice FEKAM BOYOM,** who, inspired by his enormous responsibilities, kept a keen eye on and ensured that this work was done strictly according to the scientific norms. I have gained much knowledge and scientific methods from you over the past years. I hope that you will find the expression of my gratitude in this work.

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LIST OF ABREVIATIONS

ATCC: American Type Culture Collection

BHT: Buthyl hydroxyl toluene

CC_{50}: Cytotoxic Concentration 50

CLSI: Clinical Laboratory Standard Institute

DAD: Diode Array Detector

DMSO: Dimethylsulfoxide

DNA: Deoxyribonucleic Acid

DPPH: 1,1-diphenyl-2-picrylhydrazyle

ESI: Electrospray Ionization

EtOAC Ethyl acetate

FRAP: Ferric Reducing Antioxidant Power

HCA Hierarchical Cluster Analysis

HPLC: High Performance Liquid Chromatography

IC_{50}: Inhibitory Concentration 50

ITS: Internal Space Transcriber

MBIC Minimal Biofilm Inhibitory Concentration

MeOH Methanol

MIC: Minimal Inhibitory Concentration

MS: Mass Spectrometry

MTT: 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide

NA: Nutrient Agar

NaCl Sodium chloride

NB: Nutrient Broth

NMR: Nucleic Magnetic Resonance

OD: Optical Density

PDA: Potatoes Dextrose agar

PDB: Potatoes Dextrose Broth

RC_{50}: Radical Concentration 50

sp. Species (singular)

TLC: Thin Layer Chromatography

UPLC: Ultra-Performance Liquid Chromatography

UV: Ultraviolet

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ABSTRACT

Pneumonia is still a public health concern in sub-Saharan Africa and South Asia and the emergence of multidrug-resistant bacteria is worsening the situation. Searching for new antibiotics with novel mechanisms of action is of paramount importance. The reputation of endophytic fungi as a source of structurally novel and bioactive secondary metabolites is well established. Therefore, the investigation of endophytic fungi from Cameroonian medicinal plants could lead to new drug discovery. The present study aims to investigate the antibacterial, antioxidant, the cytotoxicity potential, modes of action of secondary metabolites and induced the production of secondary metabolites from some endophytic fungi inhabiting *Terminalia mantaly*, *Terminalia catappa* and *Cananga odorata* against causative agents of pneumonia.

Crude ethyl acetate extracts from 56 endophytic fungi were screened using the broth microdilution method against seven causative agents of pneumonia including *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 43300, *Staphylococcus aureus* BAA-977, *Streptococcus pneumoniae* ATCC 49619, *Klebsiella pneumonia* ATCC 13883, *Pseudomonas aeruginosa* HM 601 and *Haemophilus influenzae* ATCC 49247. The antibacterial modes of action of the most active extracts (04) were evaluated using *Escherichia coli* ATCC 25922 and *Haemophilus influenzae* ATCC 49247 strains. Both the DPPH and FRAP assays were used to determine the antioxidant capacity of the potent extracts. The cytotoxicity of potent extracts against the Vero cell line was evaluated using the MTT assay. One of the most active extract was subjected to bioguided fractionation using column chromatography and the potent sub-fractions analyzed by UPLC coupled to mass spectrometry. The induction of the production of antibacterial secondary metabolites by the most potent fungal strain was performed using small chemical elicitors.

Out of the 56 crude ethyl acetate extracts tested, about 13% were considered very active, 66% were considered partially active, and 21%were considered non-active against all tested bacterial strains with MIC values ranging from 0.32 µg/mL to 25 µg/mL. The four more potent extracts (MIC<5µg/mL) (from *Aspergillus* sp. N454, *Aspergillus* sp. N13, *Curvularia* sp. N101, and *Aspergillus* sp. N18) significantly induced bacteria cells lysis, increased outer membrane permeability, reduced salt tolerance, and inhibited bacterial catalase activity. They exhibited a DPPH free radical scavenging activity with

IC_{50}values ranging from 150.71 to 936.08 µg/mL. Three of the four potent organic extracts (*Aspergillus* sp. N454, *Curvularia* sp. N101, and *Aspergillus* sp. N18) were non cytotoxic against the Vero cells line (CC_{50} > 100 µg/mL). The antibacterial-guided fractionation of *Aspergillus* sp. N454 extract led to 10 fractions with the most potent being fraction F2 (MIC ranged from 0.39 to12.5 µg/mL). The subsequent fractionation of F2 led to 17 subfractions with subfraction KIMS4 (25 % hexane-ethyl acetate) being the most active (MIC ranged from 0.078 to 10 µg/mL). Fractionation improved the antibacterial potency from 1.24 to 40 folds depending on the tested bacteria. The compositional analysis of the most active sub-fractions revealed the presence of 10 known antimicrobial compounds including, N-Acetyl tryptamine, Usnic acid, Sinapic acid, Kaurane-1,18-dioic acid, 9,10-Epoxyoctadecenoic acid, 1 Hexadecanoyl glycerol, Gibberellin A4, cyclo (Leu-Pro), cyclo (L-Leu-L-Pro), and Methyl 3,4,5 trimethoxy cinnamate. On the other hand, the culture of *Aspergillus* sp. N454 in the presence of acetone, chloroform, ethanol, and nicotine increased the number of metabolites produced by 1.05 to 2.54 folds and improved the antibacterial activity by 2 and 128.20 times depending on the tested bacteria and to the elicitor.

This investigation demonstrated that endophytic fungi from *C. odorata*, *T. catappa*, and *T. mantaly* produce of secondary metabolites with potential activity against agents of pneumonia. Moreover, the culture of these endophytes using elicitors could increase not only their antibacterial activity but also stimulate the production of new secondary metabolites with improves antibacterial activity.

Keywords: Cameroonian medicinal plants, *Terminalia mantaly*, *Terminalia catappa*, *Cananga odorata*, Endophytic fungi, *Aspergillus* sp. N454, Antibacterial activity, Antioxydant activity, Cytotoxicity, Bioguided fractionation, Elicitors.

RESUME

La pneumonie demeure un problème majeur de santé publique en Afrique subsaharienne et en Asie du sud en raison de l'émergence de souches de bactéries résistantes. De ce fait, la recherche de nouveaux antibiotiques dotés de nouveaux mécanismes d'action est d'une importance capitale. La réputation des champignons endophytes en tant que source de métabolites secondaires structurellement nouveaux et bioactifs est bien établie. Par conséquent, l'étude des champignons endophytes des plantes médicinales camerounaises pourrait conduire à la découverte de nouveaux médicaments. La présente étude vise à étudier le potentiel antibactérien, antioxydant, cytotoxique, les modes d'action des métabolites secondaires ainsi que l'induction de la production de métabolites secondaires en présence d'éliciteurs par quelques champignons endophytes isolés de *Terminalia mantaly*, *Terminalia catappa* et *Cananga odorata*.

L'activité antibactérienne des extraits bruts à l'acétate d'éthyle provenant de 56 champignons endophytes a été évaluée par la méthode de microdilution en milieu liquide contre sept agents responsables de la pneumonie, notamment *Escherichia coli ATCC* 25922, *Staphylococcus aureus* ATCC 43300, *Staphylococcus aureus* BAA-977, *Streptococcus pneumoniae* ATCC 49619, *Klebsiella pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* HM 601 et *Haemophilus influenzae* ATCC 49247. Les modes d'action antibactériens des extraits les plus actifs (04) ont été évalués sur les souches d'*Escherichia coli* ATCC 25922 et *Haemophilus influenzae* ATCC 49247. Les tests DPPH et FRAP ont été utilisés pour déterminer le pouvoir antioxydant des extraits prometteurs. La cytotoxicité des extraits prometteurs contre la lignée cellulaire Vero a été évaluée à l'aide du test au MTT. L'un des extraits les plus actifs a été soumis à un fractionnement bioguidé par chromatographie sur colonne et les sous-fractions les plus actives analysées par UPLC couplées à la spectrométrie de masse. L'induction de la production de métabolites secondaires par la souche fongique la plus active a été réalisée à l'aide des éliciteurs chimiques de faibles poids moléculaires.

Sur les 56 extraits bruts à l'acétate d'éthyle testés, environ 13 % ont été considérés comme très actifs, 66 % partiellement actifs et 21 % non actifs contre toutes les souches bactériennes testées avec des valeurs de CMI allant de 0,32 µg/mL à 25 µg/mL. Les quatre extraits plus actifs (MIC<5µg/mL) (*d'Aspergillus* sp. N454, *Aspergillus* sp. N13, *Curvularia* sp. N101 et *Aspergillus* sp. N18) ont significativement induit la lyse des cellules bactériennes, augmenté la perméabilité de la membrane externe, réduit la tolérance au sel et inhibé l'activité de la catalase bactérienne. Ils ont présenté une activité de piégeage des radicaux libres de DPPH avec des IC_{50} allant de 150,71 à 936,08 µg /mL. Trois des quatre extraits les plus actifs (*Aspergillus* sp. N454, *Curvularia* sp. N101, and *Aspergillus* sp. N18) étaient non cytotoxiques contre la ligné cellulaire Vero (CC_{50}> 100µg/mL). Le fractionnement bioguidé de l'extrait d'*Aspergillus* sp. N454 a conduit à 10 fractions, la plus active étant la fraction F2 (MIC variait de 0,39 à 12,5 µg / mL). Le fractionnement subséquent de F2 a conduit à 17 sous-fractions, la sous-fraction KIMS4 (25 % d'hexane-acétate d'éthyle) étant la plus active (la CMI variait de 0,078 à 10 µg/mL). Le fractionnement a permis l'amélioration de l'activité antibactérienne de 1,24 à 40 fois en fonction des bactéries testées. L'analyse de la composition de la plupart des sous-fractions actives a révélé la présence de 10 composés antimicrobiens connus, notamment la N-acétyl tryptamine, l'acide usnique, l'acide sinapique, l'acide kaurane-1,18-dioïque, l'acide 9,10-époxyoctadécénoïque, le 1-hexadécanoyl glycérol, la gibbérelline A4, le cyclo (Leu-Pro), le cyclo (L-Leu-L-Pro) et le 3,4,5-triméthoxycinnamate de méthyle. D'autre part, la culture *d'Aspergillus* sp. N454 en présence d'acétone, de chloroforme, d'éthanol et de nicotine a augmenté la quantité de métabolites produits de 1,05 à 2,54 fois et amélioré l'activité antibactérienne de 2 et 128,20 fois selon les bactéries testées et l'éliciteur.

Cette étude a démontré que les champignons endophytes de *C. odorata*, *T. catappa* et *T. mantaly* produisent des métabolites secondaires ayant une activité potentielle contre les agents responsables de la pneumonie. De plus, la culture de ces endophytes à l'aide de eliciteurs organiques pourrait non seulement augmenter leur activité antibactérienne, mais aussi stimuler la production de nouveaux métabolites secondaires avec une activité antibactérienne améliorée.

Mots-clés : Plantes médicinales camerounaise, *Terminalia mantaly*, *Terminalia catappa*, *Cananga odorata*, Champignons endophytes, *Aspergillus* sp. N454, Activité antibactérienne, Activité antioxydante, Cytotoxicité, Fractionnement bioguidé, Eliciteurs.

Introduction

INTRODUCTION

Pneumonia is the leading cause of death among children under five worldwide, making it one of today's major public health concerns. The World Health Organization (WHO) reports an estimated 740 180 cases of pneumonia death among children under the age of 5 in 2019 **(WHO, 2021)**. Moreover, 99% of all pneumonia deaths occur in low- and middle-income countries **(Rudan *et al*., 2013).** The most significant incidence was recorded in South Asia (2 500 cases per 100.000 children) and West and Central Africa (1620 cases per 100 000 children) **(UNICEF, 2021)**. According to the **UNICEF (2021),** a child dies of Pneumonia every 39 seconds. In Cameroon, pneumonia was responsible for approximately 11.5% of deaths of children under five in 2018, highlighting the need to design practical and efficient control strategies to counteract this deadly infection **(Abdul-Aziz, 2019)**.

Accordingly, the use of antibiotics and the development of a range of vaccines comprising the pneumococcal conjugate vaccines, the 23-valent pneumococcal polysaccharide vaccine and the Hib vaccine gave hope of complete eradication of pneumonia. However, the costly treatment and its inaccessibility to the indigenous population coupled with the emergence of antibiotic-resistant bacterial strains make formulating an effective universal treatment protocol against pneumonia very challenging **(WHO, 2019a; WHO, 2019b; Leung *et al*., 2016).** To fight against this disease, new lead molecules having good antibacterial potential and multiple modes of action are urgent in fighting the emergence of resistance (**Balachandran *et al.*, 2015)**.

Historically, microbial natural products have been the most consistent source of antibiotic lead compounds. Moreover, about 60% of all new chemical entities develop in the field of antibacterial over the last 40 years were based on or derived from natural products (Newman and Cragg 2020, Miethke et al., 2021). However, their ability to reveal useful novelty is limited by both a high rediscovery rate of already known molecules associated with pre-existing resistance mechanisms. Therefore, the investigation of microbes living in unusual or underexplored habitats is now a new trend. In this respect, endophytic fungi living inside plants tissues without causing any symptoms of diseases have proven over the past decades to be an outstanding source of a great diversity of structurally novel and complex metabolites with exceptional biological activities with potential to be developed as new antibiotics (Toghueo 2019; Toghueo and Boyom, 2020). Therefore, the exploration of these symbiotic fungi could lead to the identification of potent molecules active against the causative agents of pneumonia.

However, metabolite biosynthesis in microbes is tightly controlled by regulatory mechanisms which often limit the discovery of novel metabolites. Thus, a plethora of secondary metabolites encoded in the fungi genomes remain undiscovered (Bode et al., 2002; Marmann et al., 2014; Wiemann and Keller, 2014). Many approaches have been proposed to stimulate the production of new metabolites in-vitro. An effective screening process can be achieved through systematic manipulation of culture conditions for a small number of promising organisms. In fact, culture conditions have a major impact on the growth of microbes and the production of microbial products (Bode et al., 2002). In addition, recent studies have indicated that various low molecular weight compounds are able to stimulate novel secondary metabolites in fungi (Pettit et al., 2011; Guo et al; 2014).

Therefore, bioactive metabolites isolated from endophytic fungi possessing antibacterial potential may be used to identify new targets or compose the basis for the synthesis of novel drugs to fight against causative agents of human pneumonia. Hence, we hypothesized that endophytic fungi inhabiting Cameroonian medicinal plants could produce secondary metabolites with antibacterial activity against pneumonia-related bacteria. This study was designed to investigate the antibacterial properties and modes of action of secondary metabolites from endophytic fungi isolated from *C. odorata*, *T. catappa* and *T. mantaly* and study the impact of elicitors on the production of antibacterial metabolites.

More specifically, this study aimed to:

- Determine the biological potential of some endophytic fungal ethyl acetate extracts from *Terminalia mantaly*, *Terminalia catappa* and *Cananga odorata* against two Gram negative and five Gram positive bacteria that cause pneumonia;
- Conduct a bioguided fractionation of promising crude extract;
- Evaluate the antibacterial secondary metabolites production using small chemical elicitors.

Literature review

CHAPTER I: LITERATURE REVIEW

I.1 Overview on pneumonia

I.1.1 Definition, symptoms, classification and risks factors

Pneumonia is a pathogen-initiated acute inflammation of the lower respiratory tract, characterized by inflammation of the lung parenchyma (the respiratory unit comprising the alveoli, alveolar ducts, and the interstitial tissues) **(Tong, 2013)**. **Stein and Marostica (2006),** also defined it**,** as inflammation of the lung parenchyma due to an infectious agent(s) causing a response that damages the lung tissue.

The illness is primarily characterized by the following:

- High fever with tachycardia and sweats;
- Cough that may be either non-productive or productive with mucoid, purulent or blood-tinged sputum;
- Shortness of breath;
- Pleuretic chest pain.

Other symptoms include fatigue, headache, myalgia and, in cases of complications, lung abscesses, empyema, metastatic infection, pleural effusion, and death **(Huijskens *et al*., 2014)**.

Pneumonia is classified into three types based on the way the inflammatory cells infiltrate the lung tissue or the appearance of the affected tissue **(Singh, 2012)**.

- **Bronchopneumonia:** affects areas throughout both lungs, causing scattered, patchy infiltrates of inflammation in the air sacs throughout the lungs.
- **Lobar pneumonia:** causes inflammation of one lobe of a lung and typically involves all the airspaces in a single lobe.
- **Lipoid pneumonia:** characterized by the accumulation of fats within the airspaces. It can be caused by the aspiration of oils or associated with airway obstruction.

Several risk factors are implicated in the development of pneumonia. We can have malnutrition, antibiotic therapy that alters the normal bacterial flora, concomitant diseases (e.g., diarrhea, asthma, diabetes), immunosuppression, parental smoking, zinc deficiency, low birth weight, lack of measles immunization, indoor air pollution and crowding **(Tong,**

2013). Possible environmental risk factors such as humidity, high altitude and outdoor air pollution can increase the risk **(Simonetti *et al*., 2014).**

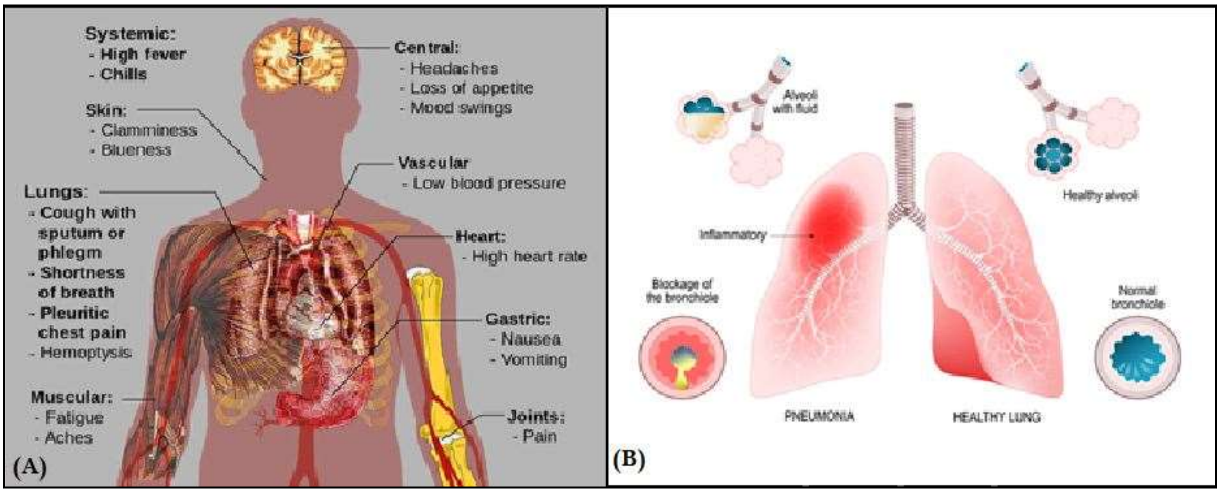


Figure 1: Main symptoms of pneumonia (A) and lungs affected by bacterial pneumonia (B).

I.1.2 Causes and etiology of pneumonia

I.1.2.1 Causes of pneumonia

Pneumonia is caused by several infectious agents, including parasites, viruses, fungi, and bacteria (Figure 2) **(Tong, 2013)**. The identification of the causal germ is very important for an appropriate treatment of the disease.

- Parasitic causes: The parasitic pneumonia is uncommon because they occurred almost exclusively on immuno-compromised patients. Its scale is dependant to the type of parasites involved and the parasitic charge. The most common parasitic agents are *Ascaris* spp, *Schistosoma* spp, *Toxoplasma gondii* **(Cheepsattayakorn and Cheepsattayakorn, 2014)**.

- Fungal causes: some fungi are also implicated in pneumonia in immunocopromised patients or patients with organs transplant. The most encounter fungi are *Aspergillus*, *Blastomyces*, *Candida*, *Coccidioides*, *Cryptococcus*, *Histoplasma* and *Pneumocystis* that could induced pneumonia in immunocopromised or immunocompetent patients **(Hage et al., 2012)**.

- Viral causes: some viral species are settled in nasopharyngeal space. They are gripp virus, syncytial respiratory virus, para-influenzae, *Haemophilus influenzae* type A and B, adenovirus, rhinovirus, and coronavirus **(Jain et al., 2015; Jain et al., 2020)**.

- Bacterial causes: bacteria responsible of pneumonia can be Gram-positive or Gram-negative bacteria. According to their prevalence, the most common Gram-negative bacteria are *Streptococcus pneumoniae, Haemophilus influenzae* type b, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Bordetella pertussis*as. As Gram-positive bacteria we have *Staphylococcus aureus* and *Bacillus anthracis*). Atypic bacteria species like *Legionella* spp, *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, *Chlamydia psittaci* and *Coxiella burnetii* are also encountered in clinical exams (**Sattar et Sharma, 2020).**

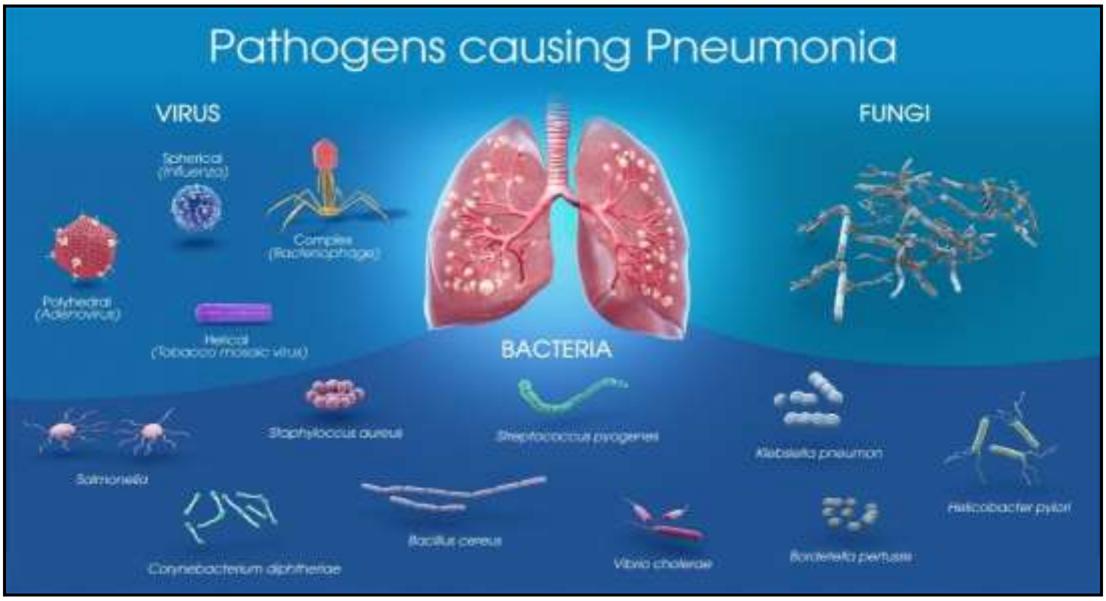


Figure 2: Pathogenic microorganisms causing pneumonia

I.1.2.2 Etiology of bacterial pneumonia

Two groups of bacteria are involved in pneumonia: the cocci Gram-positive bacteria and the Gram-negative bacteria.

a) Cocci gram positive bacteria

Cocci Gram-positive bacteria such as *Staphylococcus aureus*, whether they are sensitive or resistant to methycillin are commensally bacteria that colonized the skin, nasal tract and armpit **(Weiner et al., 2016)**. They are responsible of various infections like abscess and cutaneous lesions, osteomyelitis, endocarditis and infections of the urinary and respiratory tracts **(Jones, 2010)**. They are also implicated in septicemia and toxic shock syndrome by the liberation of super antigens in blood flow **(Foster and Mc Devitt, 1994)**.

b) Gram-negative bacteria

The most encounter bacteria are *Streptococcus pneumoniae, Escherichia coli*, *Haemophilus influenzae*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* **(Jones, 2010)**.

- ***Escherichia coli*****:** is a type of bacteria that lives in the intestines of humans and animals. Most strains of E. coli are harmless but some strains can cause serious illnesses such as diarrhea, urinary tract infections, blood infections, or pneumonia **(Mostapha, 2024)**. They produced Shiga toxin that damage the cells of the body. Some studies have reported that isolates of *E. coli* had a resistance rates to amoxicillin-clavulanic acid (94%) and erythromycin (94%) which are antibiotics that are often used for treating respiratory and skin infections **(Ammar et al., 2016)**.

- ***Haemophilus influenzae*** **type b:** is an encapsulated bacterium which infects mostly children and immunocompromised individuals and common etiological agent of lower respiratory tract infections such as pneumonia. It can also cause many other types of infections such as meningitis, epiglottitis, empyema and cellulitis **(khattak and Ajunm, 2023)**. *H. influenzae* isolated showed resistance to ampicillin and erythromycin antibiotics **(Maddi et al., 2017)**.

- ***Streptococcus pneumoniae*****:** is a gram positive, lancet-shaped bacterium implicated in community acquired pneumonia. *Streptococcus pneumoniae* has several virulence factors that allow it to cause infection in humans such as a polysaccharide capsule, the IgA1 protease and neuramidase. Penicillin resistant *Streptococcus pneumoniae* have become more common. This resistance is the result of alteration in the penicillin binding proteins and affects the binding penicillin but not that of all the β-lactams **(Dion and Ashurst, 2023)**.

- ***Klebsiella pneumoniae*****:** is an ubiquist Gram negative bacterium mostly encountered in faecal flora of animal and human. It is and encapsulated enterobacteria frequently responsible of nosocomial pneumonia in immunocompromised patients **(Boughachiche, 2016)**. Its pathogenic factors include adhesines, siderophores, capsular polysaccharides, surface cell lipopolysaccharides and toxins that play a crucial role in the infection mode **(Janda et Abbott, 2006; Boughachiche, 2016)**. *Klebsiella pneumoniae* possess a gene that code for a chromosomic penicillinase which give him a natural resistance to penicillines and carbenicilline. Therefore, she plays a key role in the spread of resistance mechanisms against β-lactamines. She is also sensitive to aminosides, carbapenems, cotrimoxazole and fluoroquinolones **(Courvalin et al., 2006)**.

- ***Pseudomonas aeruginosa*****:** is an aerobic bacterium that is capable of causing a variety of infections in both immunocompetent and immunocompromised hosts. It can cause infections like osteomyelitis, otitis externa, and pneumonia. Pseudomonas is sensitive to ciprofloxacin, ceftazidim, gentamycin and carbapenem **(CDC, 2014)**. Pseudomonas aeruginosa displays resistance to a variety of antibiotics, including aminoglycosides, quinolones andβ-lactams **(Hancock and Speert, 2000)**. Generally, the major mechanisms of *P. aeruginosa* used to counter antibiotic attack can be classified into intrinsic, acquired and adaptive resistance. The intrinsic resistance of *P. aeruginosa* includes low outer membrane permeability, expression of efflux pumps that expel antibiotics out of the cell and the production of antibiotic in activating enzymes. The acquired resistance of *P. aeruginosa* can be achieved by either horizontal transfer of resistance genes or mutational changes **(Breidenstein *et al*., 2011)**. The adaptive resistance of *P. aeruginosa* involves formation of biofilm in the lungs of infected patients where the biofilm serves as a diffusion barrier to limit antibiotic access to the bacterial cells **(Drenkard, 2003)**.

I.1.3 Physiopathology and diagnosis of pneumonia

I.1.3.1 Physiopathology of pneumonia

The microorganisms enter the lungs by one of the following four routes: inhalation of the microbes, aspiration of organisms, hematogenous spread from a distant focus and direct spread from an adjoining site of infection. When bacteria enter the upper respiratory tract in sufficiently high numbers, they can overwhelm macrophages and result in a full-scale activation of systemic defense mechanisms. These mechanisms include the release of multiple chemical mediators of inflammation, infiltration of white blood cells, and activation of the immune response resulting in typical symptoms such as fever, chills and fatigue. Mucus plugs are released from the leaky capillaries into the blood. White blood cells accumulate in the lungs to clear the plugs, and eventually, cell debris accumulates in the alveoli and makes it solid, called consolidation, a feature of bacterial pneumonia **(Wootton *et al*., 2014)**.

c) Diagnosis

The diagnosis of pneumonia is based on the association of clinical symptoms and thorax radiology. The main diagnostic tests are:

- **Chest X-ray** consist to look for signs of inflammation in the chest;
- **Blood culture and sputum culture** are used firstly to confirm the effectiveness of the infection and secondly to identify the cause of the infection. These methods used differential and selective culture media for identification. Its helps also to determine the sensitivity of germs to antibiotics **(Bradley et al., 2011)**;
- **Bronchoscopy:** consist to look the airway in lungs. A positive diagnostic is made by the observation of opacity in the alveolar parenchyma of the patient;
- **Clinical diagnosis:** symptoms that characterized pneumonia are check on patient like fever that is supposed to be greater than 38.5°C, tachypnia, ausculatory anomalies **(Margolis et Gadomski, 1998)**;
- **Pulse oxymetry** consist to measure the amount of oxygen in the blood of patient **(Cilloniz *et al*., 2016)**.

I.1.3 Epidemiology of pneumonia

Pneumonia is a common lower respiratory infection accounting for 2.7 million deaths worldwide **(GBD, 2017)**. In 2019, Pneumonia accounted for 14% of all deaths of children less than 5 years old and 22% of all deaths in children aged 1 to 5, killing 740 180 children **(WHO, 2021)**. There was an estimated 808694 pediatric pneumonia-associated deaths in 2017 and more than 100 million childhood pneumonia episodes estimated worldwide **(WHO, 2019a)**. The mortality rate due to Pneumonia in developed countries is low (<1 per 1000 per year) (**Jain *et al*., 2015**) compared to low and middle-income countries which are disproportionately affected, particularly those in South Asia and sub-Saharan Africa (**WHO, 2021; UNICEF, 2021)**.

In low- and middle-income countries, it is classified as the first leading cause of death before heart disease, diarrhea, malaria, HIV/AIDS and stroke **(WHO, 2012; UNICEF, 2012)**. It was founded that 3370000 children encounter pneumonia annually, which contributes to 20 % of all causes of death, killing over 40,000 under five children every year **(UNICEF 2014)**. **Kasundriya *et al*., 2020** reported an incidence of 64% of severe pneumonia. In sub-Saharan Africa, estimates suggest 4 million episodes of pneumonia each year, resulting in 200,000 deaths **(Scott *et al*., 2012)**. The last report on pneumonia at Wondo Genet district in Ethiopia has shown a prevalence of 33.5% among under-five children **(Teshome, 2017)**. In 2018, pneumonia led to the death of 17,624 children below five years old in Tanzania **(IVAC, 2018)**.

In Cameroon in 2018, 9955 deaths among children under five were attributed to pneumonia for a prevalence of 11.5% **(Abdul-Aziz, 2019**). Pneumonia, considered as forgotten disease is the second cause of hospitalization in Jamot hospital in Yaoundé after tuberculosis **(HJY, 2018)**. Additionally, 54.7% of children under five were affected by respiratory tract infection, among which 25 % had moderate pneumonia and 16% had severe pneumonia **(Tazinya *et al*., 2018)**.

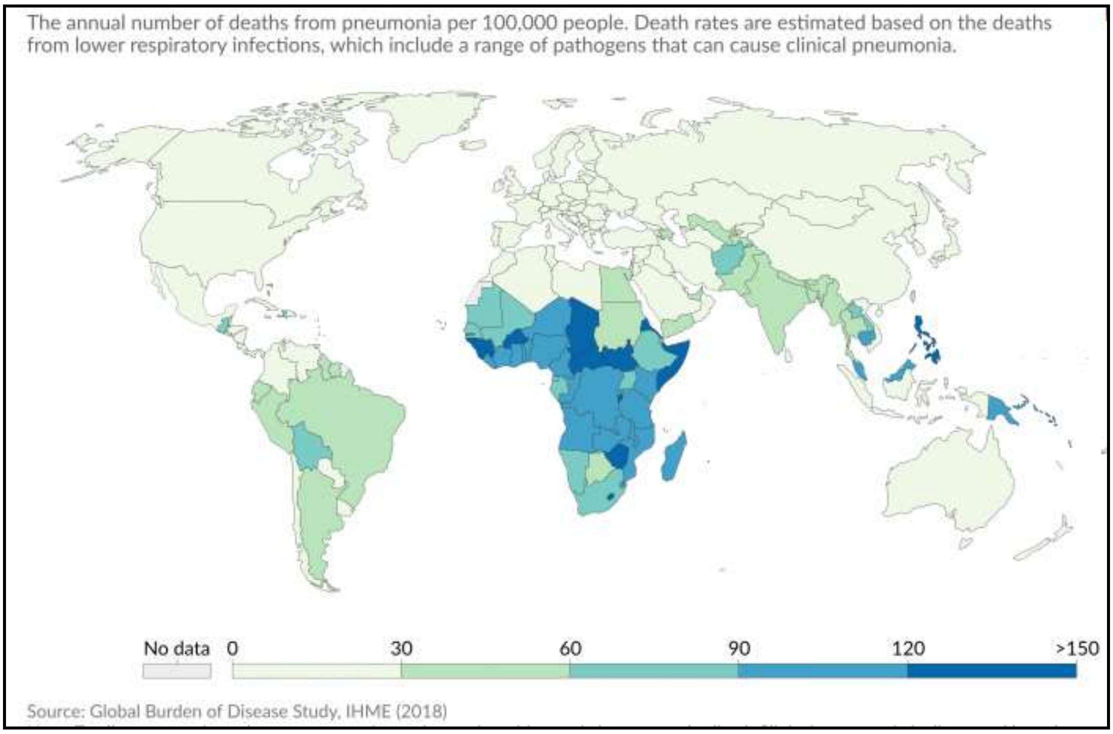


Figure 3: Death rate from pneumonia in 2017**(Global Burden Disease, 2018)**

I.1.4 Management of pneumonia and limits

Measures to prevent and treat pneumonia in children are available, but reducing morbidity and mortality remains an enormous challenge **(Leung *et al*., 2016)**.

a) Prevention of pneumonia and limits

To reduce the incidence and the severity of pneumonia, three main vaccines were developed.

- The *Haemophilus infuenzae* type b vaccine gives 90% of protective efficacy against *Haemophilus influenzae* type b **(WHO, 2012b)**.
- The pneumococcal conjugate vaccines (PCV 7, PCV 10 and PCV 13) trade under the name Prev(e)nar ®, Synflorix ® and Prev(e)nar-13 ®, respectively, elicits a T cell-dependent response and produces an anamnestic reaction that makes the vaccine more effective in infants and children younger than two years of age. Unfortunately, the PCV 7 vaccine did not contain most virulent serotypes encountered in developing countries; and the other vaccines protect against 10 and 13 different serotypes of *Streptococcus pneumoniae* strain **(WHO, 2012b)**.
- The 23-valent pneumococcal polysaccharide vaccine (PPV23), a T-cell-independent vaccine, does not enhance the reaction of the body's immunologic memory, and immunity may not be long lasting **(Zimmerman and Middleton, 2011)**. Therefore, PPV 23 is ineffective in children younger than two years old, but it is approved for individuals two and older at risk of developing pneumonia. It is deemed more appropriate for adults (mainly those aged 50 years and older) **(Cohen et *al*., 2016)**. The vaccine efficacy of PPV23 ranged from 45% to 70% efficacy and therefore cannot prevent all cases of pneumonia in adults **(Lucero *et al*., 2009; Moberley *et al*., 2013)**.

Globally, despite existing Hib, pneumococcal conjugate and pneumococcal polysaccharide vaccines, disparities in access to these vaccines exist within countries, which reduce vaccine impact as cost-effective interventions against childhood pneumonia and impede efforts to close the 'rich-poor' gap in vaccine introduction **(UNICEF, 2012)**. Additionally, numerous studies show that routine vaccination in developing countries with Hib and PCV vaccines are highly cost-effective health interventions over a range of plausible assumptions related to efficacy, price and disease burden **(Madhi *et al*., 2008; Laxminarayan *et al*., 2006)**.

b) Treatment of pneumonia and limits

The treatment of pneumonia relies on the use of antibiotics. Third-generation cephalosporins are the most common class of antibiotics used. Antibiotics used as first-line drugs in the treatment of pneumonia are cotrimoxazole, amoxicillin, cephalosporins, and macrolides. To treat non-severe pneumonia in children, oral amoxicillin is currently prescribed while for very severe pneumonia, ampicillin, ceftriaxone, and gentamicin are recommended. For infants between 2 months up to 1 year, cotrimoxazole is the drug recommended for severe cases of pneumonia **(WHO, 2019b)**.

Despite good access to antibiotics, *Streptococcus pneumoniae* is still a significant cause of illness and mortality globally **(European Center for Disease Prevention and Control, 2011)**. These treatments are facing many other problems, among which the high cost (70166 FCFA) **(WHO 2019a)**, the diversity of causative agents and a diagnostic deficit of approximately 30% make the formulation of an effective universal treatment challenging **(WHO, 2019a)**. Moreover, many antibiotics have side effects (diarrhea, nausea, vomiting, lower gastrointestinal irritation reactions, headache) **(Cilloniz *et al.*, 2016)** in addition to the development and rise of pathogenic resistant bacteria

Figure 4: Main vaccines against pneumonia

I.1.5 Mechanisms of action of available antibiotics

For bacteria to grow, they need to make all the parts necessary for building new bacterial cells. DNA must be copied, new RNA, ribosomes, and proteins must be made, and the cell walls must be built. Membranes must be synthesized. Then, the cells must divide. Many antibiotics act by inhibiting the events necessary for bacterial growth.

Available antibiotics possess different modes of action owing to the nature of their structure and degree of affinity to specific target sites within bacterial cells **(Thenmozhi *et al*., 2014)**.

- **Inhibitors of cell wall synthesis**: The cell wall is a critical structure for the life and survival of bacterial species. Therefore, a drug that targets cell walls can selectively kill or inhibit bacterial organisms. This is the case of penicillin, cephalosporins, and vancomycin.
- **Inhibitors of cell membrane function**: Cell membranes are essential barriers that segregate and regulate intra- and extracellular substance flow. A disruption or damage to this structure could leak important solutes essential for the cell's survival. Examples: polymixin B and colistin.
- **Inhibitors of protein synthesis**: Protein synthesis is an essential process necessary for the multiplication and survival of all bacterial cells. Several antibacterial agents target bacterial protein synthesis by binding to either the 30S or 50S subunits of the intracellular ribosomes. This activity then results in the disruption of the normal cellular metabolism of the bacteria. Consequently, it leads to the organism's death or the inhibition of its growth and multiplication. Examples: Aminoglycosides, chloramphenicol, and tetracyclines.
- **Inhibitors of nucleic acid synthesis**: DNA and RNA are keys to the replication of bacteria. Some antibiotics work by binding to components involved in DNA or RNA synthesis, which causes interference of the normal cellular processes, ultimately compromising bacterial multiplication and survival. Examples: quinolones, metronidazole, and rifampin.
- **Inhibitors of other metabolic processes**: Other antibiotics act on selected cellular processes essential for the survival of bacterial pathogens. For example, sulfonamides and trimethoprim disrupt the folic acid pathway, which is necessary for bacteria to produce precursors essential for DNA synthesis by inhibiting dihydropteroate synthase and dihydrofolate reductase, respectively.

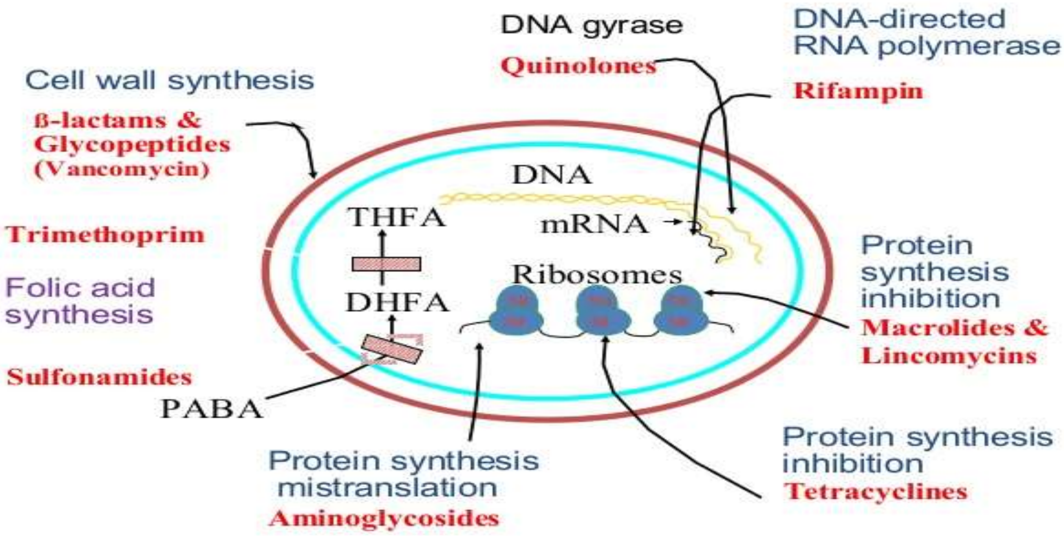


Figure 5: Sites of antibacterial action **(Cohen *et al*., 1992)**.

I.1.6 Mechanisms of resistance to current drugs

The development of drugs resistance is nowadays the primary way microorganisms escape the action of antibiotics. This resistance to antibiotics can be through four main types (**Cesur and Demiröz, 2013)**:

- **Natural (intrinsic) resistance**: the microorganisms not including the structure of the target antibiotic or antibiotics not reaching their target due to its characteristics. For example, vancomycin does not pass in the outer membrane of Gram-negative bacteria, making them naturally resistant to vancomycin.
- **Acquired resistance**: This resistance can result from structural changes in bacterial cells. The result may be reduced permeability of bacterial drugs or changes in the target of the drug in the cell.
- **Cross-resistance**: Some microorganisms are resistant to a specific drug that acts with the same or similar mechanism and other drugs. This condition is usually observed in antibiotics whose structures are similar. Examples include resistance to erythromycin, neomycin-kanamycin, cephalosporins and penicillin.
- **Multidrug resistance and panresistance**: here, a drug can no longer kill or control the bacteria. Inappropriate use of antibiotics for therapy resulted in selecting pathogenic bacteria resistant to multiple drugs. Multidrug resistance in bacteria can occur by one of two mechanisms. First, these bacteria may accumulate multiple genes, each coding for resistance to a single drug. This type of resistance typically occurs on

resistance plasmids. Second, it may also occur by the increased expression of genes that code for multidrug efflux pumps, enzymatic inactivation, and changes.

Several mechanisms are implicated in those resistance types (**Cesur and Demiröz, 2013)**:

- Poor drug influx or excessive efflux;
- drug inactivation by enzymes or lack of activation;
- Alterations such as changes in expression levels of the drug target or its modification;
- Activation of adaptive pro-survival responses and a lack of cell death induction due to dysfunction.

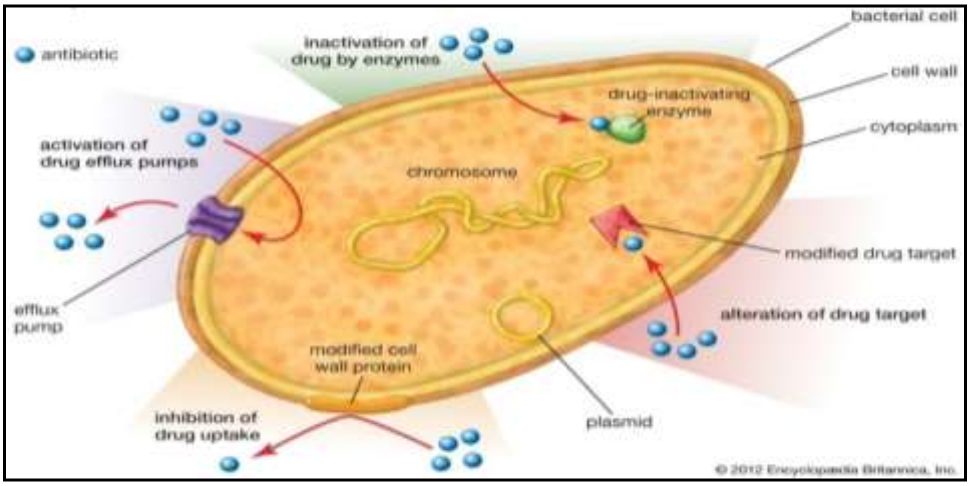


Figure 6: Mechanisms of antibiotic resistance in bacteria **(Encyclopaedia britanica, 2012)**

I.1.7 Oxidative stress and pneumonia

I.1.7.1 Oxidative stress

Oxidative stress is the state of inability of an organism to defend itself against attack of oxygenated or non oxygenated free radicals, following an imbalance linked either to an increased production of reactive oxygen species, or to a reduction in the capacity antioxidant defense of said organism **(Nyegue, 2006)**. Oxidative stress could result from the presence of xenobiotics, the activation of the immune system in response to invading microorganisms (inflammation), and radiation, which makes oxidative stress a common denominator of toxicity or stress. Excess of free radicals damage essential functional molecules of the cell (DNA, proteins, lipids, carbohydrates), leading to multiples consequences: abnormal gene expression, disturbance in receptor activity, proliferation or cell dye, immunity perturbation, mutagenesis, and protein or lipofuscin deposition **(Favier, 2006, Djova, 2019)**. Depending to their structures, we have several classes of reactive oxygen species.

I.1.7.2 Free radicals and their types

Free radicals are highly reactive and unstable molecules that are produced in the body naturally as a derivative of metabolism, or by exposure to toxins in the environment such as tobacco smoke and ultraviolet light **(Kunming, 2022)**. Free radicals are constantly formed in the body. They are used by immune cells to fight infections. There are many types of free radicals, though, in humans, the most significant are oxygen free radicals (reactive oxygen species). Examples include singlet oxygen, hydrogen peroxide, superoxide, and hydroxyl anions **(Kunming, 2022)**. As second predominant free radicals we have nitrogen reactive species that are produced during the oxidation of the cell **(Pincemail, 1998)**.

I.1.7.3 Oxidative stress in infectious pneumonia

Oxidative stress plays a crucial role in the development and progression of pneumonia. It was revealed that five times more H_2O_2 is released in exhaled air of patients suffering from pneumonia than control, and the amount decreases with treatment. This H_2O_2 is synthesized by activated leukocytes, monocytes, and macrophages. The development of oxygen species leads to the activation of neutrophils and other effector cells with the generation of excess active oxygen forms in the lungs of patients **(Majewska *et al*., 2004)**. These reactive oxygen species (ROS) migrate through the alveolar-capillary membrane in gas exchange and induce oxidative stress development in erythrocytes **(Ugurlu, 2016)**. ROS does not kill some bacteria such as *Streptococcus pneumoniae* as the bacterium has developed several mechanisms to escape oxygen species-mediated killing. They are eliminated by nitrogen monoxide and peroxynitrite produced by macrophages in response to the pneumococcal cell wall and the toxin pneumolysin **(Mac Micking *et al*., 1997)**. **Chen *et al*., (2014)** demonstrated an increase in OS and cytokines such as TNF-α and IL-6 in the lung and peripheral blood with the severity of pneumonia.

The emergence of multidrug-resistant bacterial strains, the high cost of treatment, and the side effects associated with available antibiotics have reinforced the exploration of novel, more effective, and less toxic antibiotic drugs that could be the potential solutions to pathogen resistance **(Balachandran *et al.*, 2015)**. Over the past two decades, endophytic fungi have been reported as an outstanding source of structurally novel and bioactive secondary metabolites, which can constitute a good starting point for developing new potential antibiotic drugs **(Bacon *et al*., 2000)**.

I.1.7.4 Antioxidants and their mechanism of action

Antioxidants are the compounds that can stabilize ROS. These molecules are the scavengers of free radicals and get easily oxidized. Vitamins are the most important class of non-enzymatic antioxidants. There is two classes water soluble like vitamin C and fat soluble vitamin A (retinoic acid or retinol) and vitamin E. Vitamin E (α -tocopherol) is a predominant scavenger that has its significant activity in the protection of biomolecules of biomembranes, which are attacked by free radicals **(Irshad and Choudhary, 2002)**. Antioxidants donate their electron to stabilize free radical and make it a stable compound so as to minimize the harmful effect of free radicals. Antioxidants have been classified on the bases of their location and their nature and action

- On the basis of their location we have

  - Plasma antioxidants: are antioxidants that are able to give electrons to the antiradical oxygen to trap him so he will no more be able to attacks biological cells. We can have uric acid, ascorbic acid, bilirubin, transferrin, caeruloplasmin.
  - Cell membrane antioxidants; α- tocopherol (membranous chain breaking antioxidant)
  - Intracellular antioxidants; superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase.

On the basis of their nature and action

- **Enzymatic antioxidants**: the principals are superoxydes dismutases (SOD), catalase, glutathione peroxidase and glutathione reductase **(Favier, 2003)**.

- superoxydes dismutases are cytosolic copper dependent enzyme or manganese dependent enzymes, that stabilize superoxide molecule **(Sheng et al., 2014)**. They catalyzed the dismutation of the radical superoxide in hydrogen peroxide

$$2O_2^{\circ -} + 2H^+ \xrightarrow{\textit{Superoxyde dismutase}} H_2O_2 + O_2$$

- Catalase is an enzyme from peroxisome that converts the acidic hydrogen peroxide to water and molecular oxygen **(Lobo et al., 2010)**. It action prevent the formation of hydroxyls radicals

$$2O_2^{\circ -} + 2H^+ \xrightarrow{\textit{Catalase}} H_2O_2 + O_2$$

- Glutathione peroxidase is a selenium dependent enzyme. Selenium is a cofactor one of the enzymes for glutathione peroxidase, is considered the major detoxification enzyme for H_2O_2. In this process, the disulfide (GSSG) format by the oxidation of reduced glutathione (GSH). More clearly, it catalyses the oxidation of glutathione (GSH) and release a hydrogen that will attached on a hydroxyl radical or hydrogen peroxide to form water **(Lobo *et al*., 2010)**.

$$2GSH + H_2O_2 \xrightarrow{\textit{Glutathione peroxydase}} GSSG + H_2O_2$$

- Non-enzymatic antioxidants and nutrient antioxidants: Beta-carotene, α-tocopherol, ascorbic acid **(Haleng *et al*., 2007)**.
- Metabolic antioxidants: glutathione, bilirubin, uric acid, transferrin, caeruloplasmin, albumin, haptoglobin **(Haleng *et al*., 2007)**.

I.1.7.5 In vitro antioxidants tests

Several tests have been developed to trigger the in vitro antioxidant potential of a molecule.

- **The DPPH (2, 2'-diphényl-1-picryl hydrazine) test**: this test is made of to react the tested molecule with a stable and free radical like the 2,2'-diphényl-1-picryl hydrazine (DPPH$^{\bullet}$). The DPPH in its radical form absorb light between 515 and 517 nm but after reduction with and antiradical species (R$^{\bullet}$) this absorption disappear **(Brand-Williams et al., 1995)**.
- **The FRAP test**: it measure the capacity of an antioxidant to reduced ferric complex 2,4,6 tripyridyl-s-triazine (Fe^{3+} - TPTZ) into ferrous ion 2,4,6-tripyridyl-s-triazine (Fe^{2+} - TPTZ). The reduction is expressed by the increase of the absorbance of the blue coloration and its absorbance can be measure at 593 nm **(Benzie et Strain, 1999)**.
- **The ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonique acid) test**: is used to measure the capacity of an antioxidant substance to increase the life expectancy of anion ABTS. This capacity is proportional to capacity of the antioxidant to decrease the coloration of ABTS radical (ABTS$^{+\bullet}$) **(Ré et al., 1999)**.

I.1.8 Cytotoxicity and pneumonia

Cytotoxicity is a term relatively referred to a compound or substance that is toxic on cells. The cytotoxicity assessment is a fundamental biological measurement and screening test applied on tissue cells as in vitro sample to distinguish the cell proliferation rate, reproduction as well as the morphological effects of substances **(Tolosa *et al*., 2015)**. It is a crucial preliminary method necessary to develop drugs and to predict the starting doses that is able to treat diseases without affecting normal cells **(Hussein *et al*., 2016; Gosa *et al*., 2019**). In vitro cell culture has always been prioritized to assess the biological materials or active compounds at the cellular level cytotoxicity test on cell culture is often defined as the quality of a compound to be toxic through cell growth inhibition that destroy the living cells **(Liu *et al*., 2018; Li *et al*., 2015)**.

The constant establishment of cytotoxicity tests leads to several well-defined methods such as determination and measurement of cell damage and cell growth as well as observing the morphological changes which can be analyzed through qualitative and quantitative methods. Among those methods we can have:

- **Crystal violet staining assay**

Crystal violet, or Tris(4-(dimethylamino)phenyl)methylium chloride, is a triarylmethane dye which is used to investigate cell viability responses. Crystal violet staining is used mainly in living cell membranes, because this dye is able to bind to proteins and DNA in cells. Cells that die will lose adherence and then disappear from the cell population. Losses from the population of cells reduce the amount of dye staining in a culture, which enables the researcher to count the number of cells in a monolayer culture via absorption of dye by the cells. This test is a simple, fast method of cell viability screening and is useful for acquiring information about relative cell density. Crystal violet can also be used to measure the cytotoxicity of a compound **(Feoktistova *et al.*, 2016; Castro-Garza** et al., **2007)**.The Crystal violet test has major advantages over other cytotoxic tests: in a Crystal violet test, after staining, changes in cell morphology can be observed and stored for a long time.

- **The Cell Titer-Glo luminescent cell viability assay:**

Cell Titer-Glo luminescent cell viability assay is a homogeneous method used to determine the number of active cells in cell cultures. This method quantifies cells based on the presence of adenosine triphosphate (ATP) nucleotides in cells. In this method, the presence of ATP is interpreted as an indicator of cell proliferation and an indicator of energy changes in the cell's biological system. ATP is commonly found in living cells because it plays a role in catabolic and anabolic cell processes. The measurement of ATP is fundamental in the study of cells, as the quantities of ATP directly correlate with cell populations **(Terry *et al*., 2016)**. This method uses the enzyme luciferase, which uses ATP to produce luminescence. This luminescence is then measured by the amount of light, or signal, produced, which is strongly correlated to the amount of ATP in the cell population. ATP quantities are highly correlated with the number of living cells **(Terry *et al*., 2016)**.

- **The XTT and Hoechst staining assay methods,**

The principle of the XTT test is the breakdown of the tetrazolium salt into formazan by succinate-tetrazolium reductase in the mitochondria, involving electron transfer by mitochondrial and non-mitochondrial enzymes. Compared to MTT, the XTT test is faster, more reproducible, and gives more sensitive results. The viable cells in the XTT test were measured based on the activity of mitochondrial enzymes in reducing tetrazolium salt **(Roehm, 1991)**.

The Trypan Blue assay

Trypan Blue assay is a method used to determine the viability of a cell. The basic principle of this method is that normal cells have intact cell membranes that are able to bind to foreign substances, such as the Trypan Blue dye. In abnormal cells, however, the cell membrane does not have the ability to bind foreign substances onto the cell **(Shapiro, 1988)**. In this test, a cell suspension is mixed with Trypan Blue dye, then the cell is observed visually and the viability is calculated using a microscope. Observations are made by examining whether cells would repel or uptake the dye. If we observed that viable cells exhibit clear cytoplasm it mean their cell membranes cannot be penetrated by the dye, while if nonviable cells show blue cytoplasm, it mean there are damages on their cell membranes that allow them to be easily penetrated by foreign substances such as Trypan Blue.

The MTT test is a colorimetric

This assay is an enzyme-based method commonly used to test mitochondrial dehydrogenase activity in cells. This method is frequently used because it is easy, safe, and has a high sensitivity. It is the most commonly used method for testing cell toxicity and viability **(Berridge *et al.*, 2005)**. The MTT test is used to evaluate the ability of cells to reduce tetrazolium salt or 3-(4, 5-dimethylthiazole-2-yl) 2, 5diphenyltetrazolium bromide to form insoluble formazan violet crystals. Colored tetrazolium salt, when interacting with cells, will turn purple (formazan). This color is caused by cells undergoing metabolic reduction by the enzyme dehydrogenase to form NADH or NADPH. It is the absorbance value of this purple color that is measured. This absorbance value is used to determine the cell viability. If the absorbance value observed is smaller than the absorbance value of the control, the cell is undergoing reduction; in other words the cell’s ability to proliferate is low. However, on the contrary, if the absorbance produced is higher than the control, the cell’s ability to proliferate is very high. If the level of proliferation is too high, however, this can result in cell death, due to potential changes in cell morphology.

I.2 Endophytic fungi

I.2.1 Definition

Endophytic fungi are fungal microorganisms that live inside plant tissues for at least part of their life cycle without causing any disease symptoms in the host (**Petrini, 1991)**. Endophytes are ubiquitous in terrestrial plants and are found within the healthy tissues of plants **(Schulz *et al*., 1993)**.

The transmission of endophytes to their host may occur either horizontally or vertically through airborne spores or seeds respectively **(Hartley and Gange, 2009)**. Endophytes can be found in the internal tissues of leaves, stems, twigs, petioles, roots, barks, fruits and flowers of plants (**Strobel and Daisy, 2003**), and some are seed-borne. The population of endophytes is variable from plant to plant and may even differ according to the climatic conditions of the same region **(Nair and Padmavathy, 2014)**.

I.2.2 Relationship between endophytic fungi and the host

Concerning the role of endophytes, they are thought to interact with their host in a mutualistic or symbiotic to antagonistic relationship **(Arnold, 2007)**. Fungal endophytes increase the resistance of host plants against pathogens, herbivores, drought, and plant diseases or even promote plant growth, enhance resistance to biotic and abiotic stresses **(Hardoim *et al*., 2015; Rodriguez *et al*., 2009)**and accumulate bioactive secondary metabolites **(Kusari *et al*., 2012)**. Studies have shown that these metabolites are involved in the deterrence of herbivores **(Pannaccione *et al*., 2014)**, protection against fungal or bacterial pathogens **(Soliman *et al*., 2015)** and amelioration of plant abiotic stress **(Hamayum *et al*., 2017)**. Indeed, grass-inhabiting endophytes are known to produce alkaloids to protect the plant against herbivores **(Siegel and Bush, 1997)**. Non-grass inhabiting endophytes increase the resistance of host plants to herbivores, pathogens, drought, and plant diseases or even enhance plant growth **(Sieber, 2007**). Similarly, host plants provide endophytes with spatial structure, nutrition, and protection (**Clay and Schardl, 2002)**.

It is assumed that endophytic fungi adapted to microenvironments through genetic variation during the long period of coevolution. Their possible incorporation of plant's DNA segments into their genomes or the insertion of their DNA into the plant genome has given them the ability to synthesize similar compounds as their hosts and *vice versa* **(Zhao *et al.*, 2010)**. Therefore, many compounds first isolated from plants were also found to be produced by endophytic fungi, the latter having the advantage of large-scale production.

I.2.3 Endophytic fungi metabolites as antibacterial agents in the drug discovery process

Fungal endophytes have been reported to produce a wide range of metabolites belonging to several classes, including alkaloids, lignans, terpenoids, flavonoids, tannins, steroids, benzopyranones, tetralones, cytochalasins, perylene derivatives, furandiones, depsipeptides, and enniatines and exhibit multiple biological activities including antibacterial, antiradical, antiproliferative and antifungal activities **(Radic and Strukelj, 2012; Alfaro and Boyman, 2011; Toghueo, 2019; Toghueo and Boyom, 2020; Toghueo, 2020)**.

Atrovenetinone, a metabolite of *Phoma* sp., an endophytic fungus in *Senecio kleinii* showed good antibacterial activity towards *Eurotium repens* **(Hussain *et al*., 2015)**. Colletotric acid, a metabolite of *Colletotrichum gloeosporioides*, an endophytic fungus in *Artemisia mongolica*, displays antimicrobial activity against bacteria and *Helminthsporium sativum* **(Zou *et al.*, 2000)**. **Hoffman *et al*., (2008**isolateda dibenzofuran compound, usnic acid, from the broth culture of *Phoma pinodella*, a fungal endophyte from *Saurauia scaberrinae* with strong inhibitory activity against several pathogens, including S*taphylococcus aureus*. The antimicrobial compound 7-Amino-4-methylcumarin was isolated from *Xylaria* sp., an endophyte in *Ginkgo biloba* **(Liu *et al.*, 2008).** Javanicin, a highly functionalized napthaquinone exhibiting an intense antibacterial activity against *Pseudomonas* spp., was isolated from *Chloridium* sp. **(Kharwar *et al.*, 2009).** Citrinin, emodin, 1,6,8-trihydroxy-3 hydroxymethyl anthraquinone, and janthinone produced by endophytic fungus*Penicillium janthinellum* from *Melia azedarach* showed strong antibacterial potency **(Marinho *et al.*, 2005)**. Hypericin and Emodin were produced by *Hypericum perforatum*. Both compounds possessed antimicrobial activity against several bacteria, including *S. aureus*, *K. pneumoniae*, *P. aeruginosa*, *S. enterica*, and *E. coli* **(Kusari *et al.*, 2008)**. Three steroids namely, ergosta-5,7,22-trienol, 5α,8α-epidioxyergosta-6,22- dien- 3β-ol, ergosta-7,22-dien-3β,5α,6β-triol and one nordammarane triterpenoid helvolic acid, with strong antibacterial activity were isolated from the endophytic fungus *Pichia guilliermondii* Ppf9 from the medicinal plant *Paris polyphylla* var. Yunnanensis **(Jianglin *et al.*, 2010)**. Preaustinoid A and B isolated from *Penicillium* sp. exhibited a moderate bacteriostatic effect on *E. coli, S. aureus, P. aeruginosa*, *Bacillus* sp. **(Dos Santos and Rodrigues-Fo, 2003)**. **Kim *et al.*, (2004)** isolated antibacterial compounds periconicins A and B from *Periconia* sp. hosted by *Taxus cuspidate*. Guanacasterpene, a novel diterpenoid produced by the unidentified fungus CR115 from *Daphnopsis americana* was detected to show strong antibacterial activity against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium* **(Singh *et al*., 2000)**. Chaetoglobosin U is a cytochalasin-based alkaloid isolated from *Chaetomium globosum*, an endophytic fungus residing within the stem of healthy *Imperata cylindrical* **(Ding *et al*., 2006)**. Chaetoglobosins A and C were characterized from the culture of an endophytic *Chaetomium globosum* isolated from the leaves of *Ginkgo biloba* with antibacterial activity. 3-O-Methylalaternin, and altersolanol A are anthraquinones from the endophytic fungus *Ampelomyces* sp. isolated from the medicinal plant, *Urospermun picroides* exhibited antimicrobial activity against the Gram-positive bacteria *Staphylococcus aureus, S. epidermidis* and *Enterococcus faecalis* **(Aly *et al*., 2008)**. **Brady *et al*., (2000)** isolated two antibacterial active trihydroxybenzene lactones cytosporones D and E, from two endophytes *Cytosproa* sp. CR200 and *Diaporthe* sp. CR146, respectively.

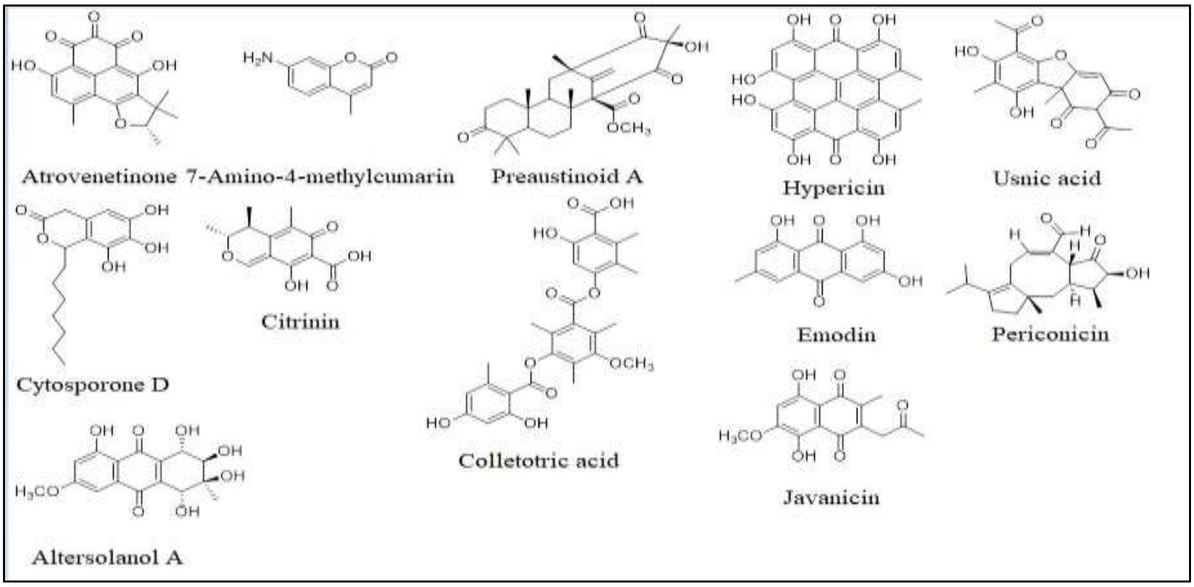


Figure 7: Structures of some antimicrobial compounds from endophytes

The discovery of endophytic fungi capable of producing a wide range of bioactive metabolites has raised the expectation that these compounds could be produced on a large scale through fermentation processes. Thus, scientists devised several methods to trigger the production of new secondary metabolites and the accumulation of known compounds **(Ola *et al.*, 2013)**. These methods are grouped into two types, including molecular-based techniques and cultivation-based approaches **(Reen *et al.*, 2015)**.

I.2.4 Induction of bioactive secondary metabolite production through chemical elicitation

The cultivation-based methods are the application of small-molecule elicitors to specifically affect the transcription of secondary metabolite gene clusters **(Pettit *et al.*, 2011)**. Non-nutrient additives, or elicitors, were shown to affect microbial growth rates and the production of biologically active secondary metabolites **(Akiyama *et al.*, 1992; Benhamou, 1992)**.

I.2.4.1 Effect of organic solvents and organic chemicals on secondary metabolite biosynthesis

Various small organic chemicals belonging to diverse structurally different chemical groups with diverse mechanisms of action are currently used to elicit secondary metabolite biosynthesis.

I.2.4.1.1 Effect of organic solvents on secondary metabolite biosynthesis

Organic solvents such as dimethyl sulfoxide (DMSO), acetone, 1-butanol, chloroform, ethanol, acetonitrile, and methanol have been reported to induce a shift in the metabolic profile of fungi **(Pettit *et al.*, 2011; Toghueo *et al.*, 2016a; Toghueo *et al.*, 2018)**. Indeed, the addition of organic solvents to a given microorganism's growing cultures can quantitatively and qualitatively change the metabolism.

- **Effect of ethanol:** Ethanol was proven to cause mistranslation **(So and Davie, 1965)** and induced the stress response **(Chen *et al.*, 2000)**. Several authors have used ethanol to elicit metabolites production. Indeed, Ethanol (1% v/v) elicited synthesis of a new chlorinated benzophenone antibiotic, pestalone, by the marine fungus Pestalotia **(Cueto *et al.*, 2001)**. Pestalone has potent activity against methicillin-resistant

Staphylococcus aureus and vancomycin-resistant *Enterococcus faecium.* The antibiotic is undetectable in non-elicited Pestalotia cultures. Similarly, the quantity of 3,3-dimethyl-5-oxo-cyclohexane carboxaldehyde significantly increased with the addition of ethanol (1% v/v) in cultures of the endophyte *Aspergillus niger* **(Toghueo *et al*., 2016b)**. The addition of ethanol (0.2%) to cultures of *Phaffia rhodozyma* resulted in increased carotenoid production, apparently due to the activation of oxidative metabolism and induction of HMG-CoA reductase **(Gu *et al*., 1997)**.

- **DMSO:** DMSO also proceeds by mistranslation and stress response to induce secondary metabolite production as ethanol **(Chen *et al*., 2000)**. Although the precise mechanism of inducing secondary metabolite synthesis is unknown, they are included due to their ease of use, inexpensive nature and dramatic results. **Guo *et al.* (2014)** reported the production of the citreo isocoumarin isomer 6,8-dihydroxy-3-(4-hydroxy-2-oxopentyl)-1H-isochromen-1-one by *Eupenicillium* sp. after the addition of dimethyl sulfoxide (DMSO) to the fermentation system. Thiostrepton production increased approximately twofold in *S. azureus* treated with 3% DMSO. When treated and untreated cultures were compared, no significant differences in biomass were observed **(Chen *et al*., 2000)**. Metabolite production increased approximately threefold in *Streptomyces venezuelae* and *S. glaucescens* in the presence of 3% DMSO, chloramphenicol and tetracenomycin C **(Chen *et al*., 2000)**.
- **Effect of acetone, 1-butanol, and acetonitrile:** The culture of *Phomopsis* sp. N114 in the presence of 1-butanol (1% v/v) significantly improved the antiplasmodial potency of the afforded extract by 36.3- and 8.72-fold against Pf3D7 and PfINDO strains, respectively. In contrast, the untreated control vs treated comparative HPLC profiles of crude extracts showed some peaks of enhanced intensities in the butanol-treated sample without any qualitative change in the chromatograms. Similarly, the cultures of *Xylaria* sp. N120 upon treatment with acetonitrile led to the induction of a new metabolite as compared to untreated culture **(Toghueo *et al*., 2018)**. Likewise, the culture of *Aspergillus niger* in the presence of 1% acetone or 1% ethanol also affected the profile of metabolites produced, as revealed by the qualitative HPLC analysis of extracts **(Toghueo *et al*., 2016a)**.
- **Effect of hydrogen peroxide:** Polyphenol production (and biomass) by the medicinal fungus *Inonotus obliquus* is enhanced by the continuous addition of a low concentration of hydrogen peroxide (1 mM) to batch cultures **(Zheng *et al*., 2009)**. Superoxide dismutase and catalase activities were also enhanced.

I.2.4.1.2. Effect of organic chemicals on secondary metabolite biosynthesis

Nicotinamide and quercetin are reported as histone deacetylases inhibitors of class three (HDAC of class III) **(Moore *et al*., 2012)**.

- **Effect of nicotinamide:** The potential of nicotinamide (HDAC of class III inhibitor) to induce the production of cancrolides A and B, and chaetophenol G by *Chaetomium cancroideum* have been reported by **Asai *et al*., (2015)**. Samely, **El-Hawary *et al*., (2018)** supported this observation by inducing the production of nine compounds, including p-anisic acid, p-anisic acid methyl ester, benzyl anisate, syringic acid, sinapic acid, acetosyringone, phenylacetic acid, gentisaldehyde and p-hydroxy benzaldehyde by treating *Penicillium brevicompactum* with nicotinamide. The use of nicotinamide induced a dramatic change in the specialized metabolite profile in the culture medium from endophytic fungi *Chaetomium cancroideum* (NA 50 µM), compared to those of the negative control **(Asai *et al*., 2013a, 2013b)**.
- **Effect of quercetin:** The flavonoid quercetin is known to act as an activator of class three histone deacetylases **(Moore *et al.*, 2012)** and an inhibitor of DNA topoisomerases also regulates gene expression **(Moskaug *et al*., 2004),** as reported by **González-Menéndez *et al*., (2016)**.

Treatment of microbes with one of the many small-molecule elicitors is a simple, inexpensive method to more thoroughly exploit the metabolic potential of microbes. Small-molecule elicitation allows the identification of new secondary metabolites and enhances synthesis of low-yield secondary metabolites in a broad spectrum of microorganisms. Based on these advantages, this approach was applied in this investigation to induce the production of antibacterial secondary metabolites.

I.3 Previous works on endophytic fungi from *Cananga odorata, Terminalia catappa* and *Terminalia mantaly*

Endophytic fungi associated with these plants were isolated and identified by **Toghueo *et al*. (2017)**. A previous study showed that ethyl acetate extracts from some endophytic fungi isolated from *Terminalia catappa*, *Terminalia mantaly*, and *Cananga odorata*, possess antibacterial, antiyeast and antiradical activities **(Toghueo *et al*., 2016a).**The work of **Toghueo *et al*.(2016b)** revealed that small chemical elicitors (1µM

5-Azacytidine, 1% DMSO, 1% ethanol, 1% methanol, 1% Acetone, 1% acetonitrile, 1% 1-butanol and 1% chloroform) were able to induce the production of volatiles metabolite production by *Phomopsis* sp. isolated from the leaves of *T. mantaly* and *Fusarium* sp. from the bark of *C. odorata* in terms of amount and diversity. Additionally, **Toghueo *et al*., (2016c)** highlighted the potential of small organic chemicals to increase the yield and to stimulate the production of new secondary metabolites by *Aspergillus niger* isolated from *T. catappa*. Two endophytic fungi of *Trichoderma* species from *Terminalia catappa* have been shown to promote plant growth for enhanced crop productivity and to manage root rot disease (**Toghueo *et al*., 2016b)**.The capacity of some endophytic fungi from those medicinal plants to produce various enzymes, such as laccase, amylase, lipase, and cellulase, with industrial importance was shown by **Toghueo *et al*., (2017a)**. In the same way, they revealed the potential of endophytic fungi from *T. catappa* and *T. mantaly* to produce cellulolytic enzyme diseases (**Toghueo *et al*., 2017b)**. The production of potent antiplasmodial metabolites by some endophytic fungi from *Cananga odorata* and T*erminalia mantaly* was underpinned in varied culture conditions and by the used of small molecular weight elicitors **(Toghueo *et al*., 2018a).**

Material and Methods

CHAPTER II: MATERIAL AND METHODS

II.1 Material

II.1.1 Endophytic fungal strains

A total of fifty-six isolates of endophytic fungi (Table 1) was obtained from the mycothec bank of the antimicrobial and biocontrol agent units of the University of Yaoundé 1. They were isolated from the stem bark, the bark, the root, the flowers, the root bark and the leaves of *Terminalia catappa* (51244/HNC), *Terminalia mantaly* (64212/HNC), and *Cananga odorata* (42250/HNC) on 16th October 2014 and kept at -80^{o}C in a mixture of glycerol/PDB (50%).

Table 1: Endophytic fungi identities and their site of isolation.

Plant name	Site of isolation	Fungi isolate	Fungal name
	Petals	N431	*Unidentified fungal*
		N422	*Unidentified fungal*
		N399	*Unidentified fungal*
		N419	*Unidentified fungal*
		N401	*Unidentified fungal*
	Flowers	N478	*Unidentified fungal*
Cananga odorata	Ribs	N454	*Aspergillus* sp.
	Leaves	N330	*Diaporthe phaseolorum*
	Stem	N284	*Unidentified fungal*
		N283	*Unidentified fungal*
		N298	*Diaporthaceae* sp.
		N289	*Unidentified fungal*
		N241	*Fungal* sp.

		N262	*Unidentified fungal*
		N263	*Unidentified fungal*
		N266	*Unidentified fungal*
	Bark	N251	*Unidentified fungal*
		N276	*Unidentified fungal*
		N256	*Unidentified fungal*
		N2541	*Unidentified fungal*
		N240	*Fusarium* sp.
		N268	*Fungal* sp.
	Root	N441	*Unidentified fungal*
		N448	*Unidentified fungal*
		N445	*Unidentified fungal*
	Root bark	N368	*Unidentified fungal*
Terminalia catappa	Ribs	N32	*Corynespora* sp.
	Leaves	N89	*Fungal endophyte* sp.
		N114	*Phomopsis* sp.
		N108	*Guignardia* sp.
	Stem	N75	*Pestalotiopsis* sp.
		N74	*Xylaria* sp.
		N44	*Unidentified fungal*
		N781	*Paraconiothyrium variabile*
		N51	*Pestalotiopsis* sp.
		N58	*Unidentified fungal*
		N44	*Unidentified fungal*

	Bark	N101	*Curvularia* sp.
		N97	*Trichoderma* sp.
		N81	*Phomopsis* sp.
	Root bark	N18	*Aspergillus* sp.
		N13	*Aspergillus* sp.
		N23	*Unidentified fungal*
		N15	*Unidentified fungal*
Terminalia mantaly	Ribs	N201	*Colletotrichum* sp.
		N200	*Fungal* sp.
		N191	*Unidentified fungal*
		N196	*Unidentified fungal*
		N221	*Unidentified fungal*
		$N230_2$	*Unidentified fungal*
		$N229_1$	*Diaporthales* sp.
	Leaves	N127	*Diaporthales* sp.
		N116	*Phoma* sp.
		N120	*Xylaria laevis*
	Stem	N162	*Diaporthe* sp.
	Bark	$N190_1$	*Unidentified fungal*
		N178	*Unidentified fungal*

II.1.2 Bacterial strains and culture conditions

The bacterial strains used in this investigation were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and included two Gram positive bacteria: *Staphylococcus aureus* BAA-977, *Staphylococcus aureus* ATCC 43300 and five Gram negative bacteria: *Streptococcus pneumoniae* ATCC 49619, *Klebsiella pneumoniae*

ATCC 13883, *Haemophilus influenzae* ATCC 49247, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* HM 601. Their features are listed in Appendix 3. Twenty-four hours before each experiment, bacteria were sub-cultured on nutrient agar tubes at 37°C.

II.1.3 Chemicals and reagents used for elicitation

Phosphate-buffered saline (PBS), trypsin, peptone, amoxicillin and ciprofloxacin were purchased from Gibco (Gibco, Waltham, MA, USA); 3-(4,5-dimethyl-2-thiazolyl) -2,5- diphenyltetrazolium bromide (MTT), Silica gel 60–120 mesh, and DMSO were purchased from Sigma Chemica (Sigma-Aldrich, New Delhi, India); Acetone (HPLC grade), acetonitrile (HPLC grade), Ethanol (HPLC grade), methanol (HPLC grade), hexane (HPLC grade), and ethyl acetate (HPLC grade) were purchased from Merck (Merck, New Delhi, India).

II.1.4 Cell lines

Normal monkey kidney Vero cells ATCC CRL1586 were cultured in complete medium containing 13.5 g/L DMEM (Gibco, Waltham, MA USA), 10% fetal bovine serum (Gibco, Waltham, MA, USA), 0.21% sodium bicarbonate (Sigma-Aldrich, New Delhi, India), and 50 µg/mL gentamicin (Gibco, Waltham, MA, USA). The cells were obtained from the Centre Pasteur du Cameroun (CPC, Yaoundé, Cameroon).

II.2 Methods

II.2.1 Antibacterial screening and mode of action of bio-active endophytic fungi extracts

II.2.1.1 Culture and extraction of fungi secondary metabolites

Each fungal isolate was cultured on potatoes dextrose broth for seven days and extracted using ethyl acetate. Briefly, one 200 mL Erlenmeyer flask containing 100 mL of sterile potatoes dextrose broth (PDB) medium (Sigma Aldrich, USA) was inoculated with four agar blocks of actively growing pure culture (10 mm diameter) and incubated at 25°C for 7 days. After the incubation period, 100 mL of ethyl acetate was added to each culture, shaken, and kept overnight at room temperature. The mixture was then transferred to a separatory funnel, the organic phase was collected, and the solvent was then removed at 70°C using a rotary vacuum evaporator (Heidolph, Germany). The extraction yield of endophytic fungi was expressed in mg per 200 ml of PDB medium.

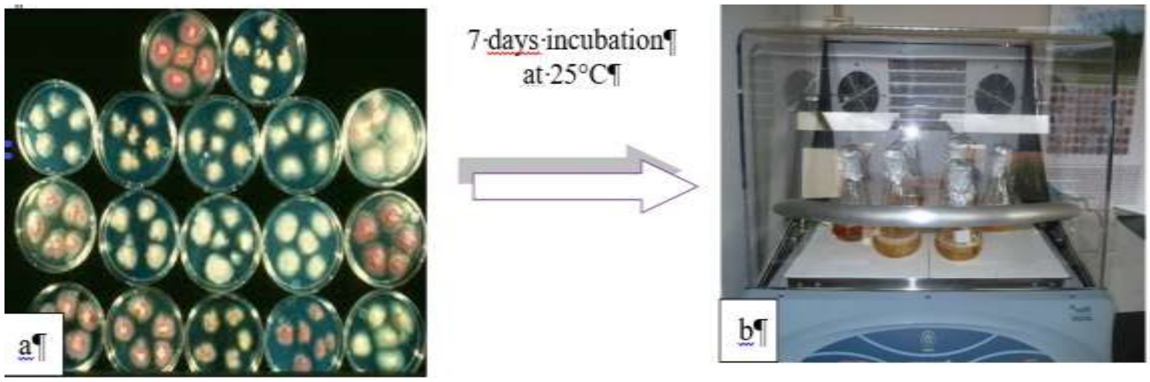


Figure 8: Culture of fungi for secondary metabolite production; (a): fungal culture on PDA medium; (b): broth fermentation of fungi on PDB medium.

II.2.1.2 Preparation of stocks solution of extracts and reference drugs

The stock solution of extracts was prepared by dissolving 25 mg of each crude extract in 1 mL of dimethyl sulfoxide (DMSO) 100% (LobaChemie, India) to yield 25 mg/mL. The prepared solutions were kept at 4°C before the experiment. Amoxicillin and Ciprofloxacin (Sigma Aldrich, USA) were prepared at 2 mg/mL in sterile distilled water for the positive antibacterial controls.

II.2.1.3 Inoculum preparation

A 18 hours-old bacterial culture colonies growing on Nutrient agar was collected with inoculating loop, suspended in sterile saline solution (0.9% NaCl) and homogenized. The turbidity of the solution was compared to a 0.5 Mc Farland standard and adjusted to a final density of 1.5×10^{8} CFU/mL.

II.2.1.4 Intermediary plate preparation

Extracts were prepared in 96 wells microplates. Briefly, 195 µL of nutrient broth medium were introduced in the wells of the first line of the plate (from A1 to G1) and 100 µL into other wells. Then, 5 µL of stocks solution of extracts (25 mg/mL) were added in the wells of the first line followed by a 2 folds geometric dilution. The extract concentration ranged from 625 to 19.53 µg/mL. Plates were sealed and store at 4°C for later use.

II.2.1.5 Determination of minimum inhibitory concentration (MIC) of extracts against bacteria

The minimum inhibitory concentration (MIC) of fungal extracts was determined according to the Clinical Laboratory Standards Institute M07-A9 microdilution method **(CLSI, 2012)** using 96-wells microtiter plates with slight modifications. Briefly, 4 µL of extracts and reference drugs (Amoxicillin and Ciprofloxacin) from intermediary plates were introduced into the wells, followed by the addition of 96 µL of bacteria inoculums standardized at 10^6 CFU/mL. A blank column was included for sterility control, while bacterial strains in the culture medium without any inhibiting substance were used as a negative control. The concentrations of the extracts ranged from 0.195 µg/mL to 25 µg/mL and those of ciprofloxacin and amoxicillin ranged from 0.562 µg/mL to 128 µg/mL. After 24 hours of incubation at 37°C, the turbidity was observed as an indication of growth. The MIC was defined as the lowest concentration inhibiting the visible growth of bacteria. All tests were performed in triplicate.

The criteria for the activity of extracts against bacteria were defined as follow: very active (MIC <5 µg/mL), partially active (MIC 5-15 µg/mL) and non-active (MIC >15 µg/mL).

II.2.1.6 Determination of possible modes of action of promising extracts

The most potent endophyte extracts (*Aspergillus* sp. N454, *Aspergillus* sp. N13, *Aspergillus* sp. N18, and *Curvularia* sp. N101) were used for the determination of their mode of action on *Escherichia coli* ATCC25922 and *Haemophilus influenzae* ATCC 49247 the two most sensitive strains.

II.2.1.6.1 Measurement of the lytic activity of extracts

This method enables the determination of a hypothetical lytic action of the extract by measuring the absorbance at 620 nm. Indeed, a normal bacteria cell absorbs light at 620 nm; therefore, bacteriolysis occurs if the absorbance at 620 nm decreases with time **(Carson *et al*., 2002)**.

The determination of the lytic activity of extracts was performed as described by **Limsuwan and Voravuthikunchai (2013)** with slight modifications. An overnight bacterial culture was used to prepare bacterial suspension at 0.5 Mc Farland in NaCl

0.9%. The bacterial suspension was treated with extracts at MIC, 2 MIC and 4 MIC and incubated at 37°C. Then, the optical density (OD) at 620 nm was measured at four different periods, including 0h, 1h, 2h, and 4h using the microplate reader Infinite M200 (TECAN). Corresponding dilutions of the extract were used as blanks. Ciprofloxacin at 2 µg/mL was used as a positive control. The results were expressed as a ratio of the OD at each time interval versus the OD at 0 min (in %). All assays were carried out in triplicate.

II.2.1.6.2 Integrity of the Cell Membrane

The integrity of the cell membrane of *Haemophilus influenzae* ATCC 49247 and *Escherichia coli* ATCC 25922 was carried out as previously described by **Carson *et al*. (2002)** with slight modifications. Briefly, the test bacteria in the exponential growth phase were washed and suspended in sterile peptone water (0.1 g/100 mL). The bacterial strains (5.10^7 CFU/mL) were incubated with extracts at 4 MIC for different periods (0, 30, 60, 90, and 120 min). The mixtures were then centrifuged at 5000 rpm for 10 minutes, after which the UV absorbance of the supernatant was measured at 260 nm using an Infinite M200 microplate reader (TECAN). The untreated bacterial cultures in sterile peptone water served as the negative control. Ciprofloxacin at 2 µg/mL was used as a positive control, and each test was performed in triplicate. The results were expressed in terms of the optical density of 260 nm absorbing materials in each interval for the ultimate time.

II.2.1.6.3 Outer membrane permeability assay

The outer membrane (OM) permeability of *H. influenzae* ATCC 49247 and *E. coli* ATCC 25922 was determined according to the method of**Oliveira *et al*., (2015)** with minor modifications. An overnight culture (5.10^7 CFU/mL) was inoculated into a Nutrient broth containing the extracts at MIC, 2MIC, and 4 MIC. The media was then poured into sterilized 96-well microplates (100 µL) and incubated at 37°C for 24 h. After the incubation time, the growth of *H. influenzae* ATCC 49247 and *E. coli* ATCC 25922 was measured at 450 nm using the Microplate Reader Infinite M200 (TECAN). The bacterial growth parameter (OD/450 nm) as a function of extract concentration (µg/mL) was plotted. Ciprofloxacin (concentration ranged from 1 to 4 µg/mL) was used as the positive control, and each test was conducted in triplicate.

II.2.1.6.4 Salt Tolerance assay

The effect of active extracts on the osmotic potential of the bacterial cells was studied by their ability to grow on nutrient agar (NA) supplemented with different concentrations of potassium chloride (KCl) **(Mikusanti *et al*., 2008)**. Twenty-four prior to the experiment, bacterial strains were cultured on a nutrient agar medium and incubated at 37°C. The overnight culture was treated with extracts at the MIC and further incubated for 60 min at 37°C. Then, the samples were serially diluted (Dilution Factor =100) and inoculated on nutrient agar plates supplemented with different concentrations of KCl (0%, 2.5%, 5.0%, and 10.0%). Each plate was incubated for 24 hours at 37°C. Ciprofloxacin at 4 µg/mL was used as a positive control. After the incubation period, both the controls and treated plates were compared, the colonies were counted, and the results were expressed in Log10 CFU/mL. The experiment was performed in triplicate.

II.2.1.6.5 Inhibition of bacterial catalase activity

This assay is based on the capacity of a substance to inhibit the activity of the bacterial catalase responsible for the degradation of hydrogen peroxide, which absorbs light at 232 nm into water and oxygen.

The catalase inhibitory activity of the extracts was evaluated using the protocol described by **Weydert and Cullen (2010)** with slight modifications. Extracts at the MIC concentration were added to a test tube containing 400 µL of hydrogen peroxide (40 mM) and 400 µL of PBS. The mixture was then transferred to another tube containing 200 µL of a bacterial suspension (1.5×10^8 UFC/mL). The samples were incubated at 37°C for 30 min after which they were centrifuged at 1200 rpm for 10 min. The supernatants were collected, and their optical density (OD) was read at 232 nm. The phosphate buffer constituted the blank, while bacterial strains in phosphate buffer without any inhibiting substance were used as a negative control. The mixture made the positive control of ciprofloxacin, phosphate buffer, and bacterial strain. The percentage of remaining hydrogen peroxide (H_2O_2) was determined according to the following formula.

$$\%\ \text{of remaining Hydrogen peroxyde} = \frac{(\text{Asample} - \text{Anegative control}) \times 100}{\text{Anegative control}}$$

where $A_{negative\ control}$ is the absorbance of H_2O_2 without the extract and A_{sample} is the absorbance of H_2O_2 with the extract.

II.2.1.7 Evaluation of the *invitro* antioxidant potential of promising extracts

II.2.1.7.1 DPPH radical reduction assay

The DPPH (1, 1-diphenyl-2-picrylhydrazyn) radical scavenging assay was performed according to the method described by **Scherer and Godoy (2009)**. Briefly, 25 µL of extracts dissolved in methanol was added to wells of a microtiter plate in triplicate followed by 75 µL of DPPH solution (0.01%) to yield extract solution with concentrations ranging from 1000 to 1.95325 µg/mL. The content was mixed and incubated for 30 min in the dark at 25 ± 2°C, after which the absorbance was measured at 517 nm using a Microplate Reader Infinite M200 (TECAN). Ascorbic acid was used as a standard antioxidant with final concentrations ranging from 25 to 0.195 µg/mL. The results were expressed through the calculation of the DPPH• inhibition percentage according to the following formula.

$$\text{Inhibition of DPPH (\%)} = \frac{(\text{Acontrol} - \text{Asample}) \times 100}{\text{Acontrol}}$$

where $A_{control}$ is the DPPH• radical absorbance without the extract and A_{sample} is the DPPH• absorbance with the extract.

The concentration of extract proportional to a 50% inhibition of DPPH• radical (IC_{50}) was obtained through the analysis of the extract solution concentration versus inhibition percentage graphic. Thus, lower extract concentrations (µg/mL) mean greater antioxidant capacity provided by the analyzed extract.

II.2.1.7.2 Ferric Ion Reducing Antioxidant Power (FRAP) Assay

The ferric reducing antioxidant power of potent extracts was determined using the method described by **Benzie *et al*., (1996)**. Twenty-five microliters of extracts at different concentrations (7.8125 - 4000 µg/mL) were introduced into a microtiter plate, and 25 µL of a solution of Fe^{3+} at 1.2 mg/mL was added. The plates were pre-incubated for 15 min at room temperature, and 50 µL of 0.2% ortho-phenanthrolin was then added to obtain a final extract concentration ranging from 1000 to 1.95325 µg/mL. The reaction mixtures were further incubated for 15 min at room temperature, and the absorbance was measured at 505 nm using a microplate reader Infinite M200 (TECAN) against the blank (made of 25 µL methanol + 25 µL Fe^{3+} + 50 µL ortho-phenanthrolin). Ascorbic acid was used as a positive control and tested at concentrations ranging from 0.103 to 6.60 µg/mL. The assay was performed in triplicate.

II.2.1.8 Evaluation of the cytotoxicity of promising extracts

The cytotoxic effect of antibacterial extracts was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-bromure de 2,5diphénytétrazolium) assay **(Mosmann, 1983)**, targeting normal monkey kidney Vero cells ATCC CRL1586 cultured in a complete medium containing 13.5 g/L DMEM (Gibco, Waltham, MA USA), 10% fetal bovine serum (Gibco, Waltham, MA, USA), 0.21% sodium bicarbonate (Sigma-Aldrich, New Delhi, India), and 50 µg/mL gentamicin (Gibco, Waltham, MA, USA).

This method is based on the evaluation of Vero cells to survival in a medium containing an inhibitory substance. The viability of cells is determined by measuring the absorbance of a complex blue formazan obtained after reducing MTT initially yellow by dehydrogenase enzymes of the living cells (**Sauter *et al*., 2011)**.

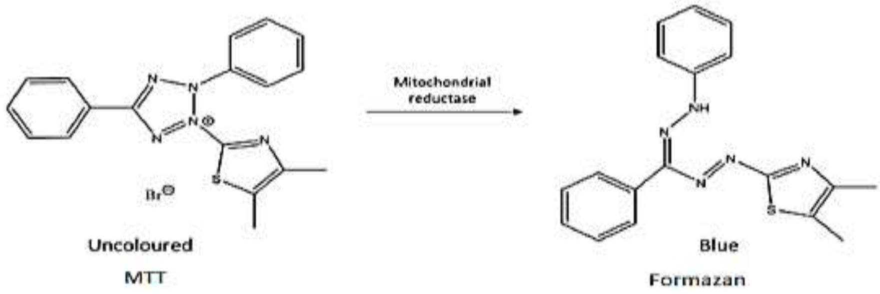


Figure 9: Reduction of MTT into formazan by mitochondrial enzymes

Essentially, Vero cells at 5×10^3 cells / 200 µL/ well were first seeded into 96-well flat-bottomed tissue culture plates (Corning, USA) in complete medium. Second, fifty microliters of serially diluted extract solutions (≤ 200 µg/mL) were added to the plates. After 24 h of seeding, the cells and test substance were incubated for 48 h in a humidified atmosphere at 37°C and 5% CO_2. DMSO (0.4% (v/v) was added as negative control (100% growth). Third, twenty microliters of a stock solution of MTT (5 mg/mL in 1× phosphate-buffered saline) were added to each well, gently mixed, and incubated for an additional 4 hour. After spinning the plate at 1500 rpm for 5 min, the supernatant was carefully removed, and 100 µL of 100% DMSO (v/v) was added to dissolve the formazan. Finally, the plate was read on a microtiter plate reader (Infinite M200

(TECAN)) at 570 nm. The 50% cytotoxic concentrations (CC_{50}) of extracts were determined by analysis of the dose-response curves.

Extract from *Aspergillus* sp. N454, was selected for the bio-guided fractionation based on the antibacterial activity, the multiple modes of action, the non-cytotoxicity and the radical scavenging potential.

II.2.2 The bioguided fraction of ethyl acetate extract from *Aspergillus* sp. N454

II.2.2.1 Large scale cultivation and extraction of *Aspergillus* sp. N454 metabolites

The fungal strain was cultivated onto PDA medium. Subsequently, 1 cm^2 agar pieces from each culture were used to inoculate 500 mL of 30 individual culture broths in 1.5L Erlenmeyer flasks. The fungus isolate was grown in PDB for 7 days under static conditions at room temperature before extraction. Then, 500 mL of ethyl acetate were added to each culture, shaken, and kept overnight at room temperature. This mixture was then transferred to a separatory funnel, and the organic phase was collected. This process was repeated thrice, and the ethyl acetate was evaporated at 40°C in a Labconco Rapid Vap parallel evaporation system. To optimize the extraction, the aqueous phase was acidified (pH 3) using hydrochloric acid (5N) followed by the addition of NaOH paste to obtain pH 12. The resulting mixture was macerated in ethyl acetate for 24 hours, and the organic phase was collected and evaporated as previously described. The crude residue obtained from the two extractions was weighed and kept at 4°C for further analysis.

II.2.2.2 Phytochemical analysis of the crude extract

A qualitative phytochemical screening of crude extract was conducted to determine the specific classes of phytochemical compounds present in the crude by colorimetric methods described by **Roghini and Vijayalakshmi, (2018)**.

- **Test for Phenols**

Two milliliters of distilled water followed by a few drops of 10% ferric chloride was added to 1ml of the extract. The formation of blue or green color indicates the presence of phenols **(Roghini and Vijayalakshmi, 2018)**.

- **Test for flavonoids**

To 2 ml of ethanolic extract, 1ml of 2N sodium hydroxide was added. The occurrence of yellow color indicates the presence of flavonoids **(Roghini and Vijayalakshmi, 2018)**.

- **Test for Alkaloids**

Two milliliters of extract was added to 2 ml of concentrated hydrochloric acid. Then, few drops of Mayer's reagent were added. The presence of green color or white precipitate indicates the presence of alkaloids **(Roghini and Vijayalakshmi, 2018)**.

- **Test for Glycosides**

To two milliliters of extract, 3ml of chloroform and 10% ammonia solution was added. The formation of pink color indicates the presence of glycosides **(Roghini and Vijayalakshmi, 2018)**.

- **Test for saponins**

Ten milliliters of distilled water were mixed with 1 g of powdered dry extract and then boiled and filtered. The filtrate was mixed again with 3 ml of distilled water and shaken for 5 min. The appearance of foam after shaking designates the existence of saponins **(Roghini and Vijayalakshmi, 2018)**.

- **Test for Steroids**

To 1 ml of fruit extract, an equal volume of chloroform was added. A few drops of concentrated sulphuric acid were also added. The appearance of the brown ring indicates the presence of steroids. The appearance of the bluish brown ring indicates the presence of phytosterols **(Roghini and Vijayalakshmi, 2018)**.

- **Test for Coumarins**

One milliliter of 10% sodium hydroxide was added to 1ml of the extract. The formation of yellow color indicates the presence of coumarins **(Roghini and Vijayalakshmi, 2018)**.

- **Anthocyanin**

To 1 ml of the extract was added 1 mL 2N sodium hydroxide and heated for 5 min at 100 °C. The formation of a bluish-green color indicates the presence of anthocyanin **(Roghini and Vijayalakshmi, 2018)**.

Test for tannins

Five hundred milligrams of the powdered dry extract were added to 10 ml of distilled water, the mixture was then filtered, and a few drops of 1% ferric chloride solution were added to the filtrate. The occurrence of a blue-black, green or greenish black precipitate designates the existence of tannins **(Roghini and Vijayalakshmi, 2018)**.

II.2.2.3 Fractionation of ethyl acetate extract from *Aspergillus* sp. N454

II.2.2.3.1 Column chromatography

For the initial fractionation, 15 g of crude extract (MIC: 0.78–6.25 µg/mL) was subjected to silica gel (60 g, silica gel 60–120 mesh) column chromatography separation using solvent systems of increasing polarities, *n*-hexane/EtOAc (1:0–0:1), and EtOAc/MeOH (9:1–0:1). One hundred and twenty-nine (129) fractions of 100 mL each were collected and subsequently pooled based on their thin layer chromatography (TLC) profiles into ten (10) pools designated F1-F10. Furthermore, 730 mg of F2 (fractions 34-59) (MIC: 0.39–12.5 µg/mL) was chromatographed using silica gel (30 g) column with a gradient system of *n*-hexane/EtOAc (1:1–0:1) and EtOAc/MeOH (9:1–0:1). One hundred and ninety-six (196) fractions of 100 mL each were collected and subsequently pooled based on their thin layer chromatography (TLC) profiles into seventeen pools designated KIMS (KIMS1-KIMS17). Each pool was dried, weighed and analyzed for antibacterial activity as described above. Only active pools were screened for the identification of their active substances.

Thin-layer chromatography (TLC) was performed on aluminum silica gel 60 F254 (Merck) pre-coated plates (0.2 mm layer thickness). Spots were visualized on TLC either by UV lamp (254 and 365 nm) or by heating after spraying with 20% H_2SO_4 (v/v) solution.

II.2.2.3.2 Analysis of the chemical composition of the extract by analytical ultra-performance liquid chromatography (UPLC-MS)

Pools KIMS4, KIMS5, KIMS4, and KIMS17 to 1 mg/mL were dissolved with LC-MS grade MeOH and filtered through 0.2 µm, 4 mm PTFE chromatography syringe filters. High-resolution mass spectra were obtained on QTOF Bruker electrospray ionization mass spectrometer with a Synergy MAX-RP 100A reverse-phase column (50 ×

2 mm), using acetonitrile and water as the components of the solvent system. The spectrometer operates in positive mode (mass range: 100-1500, with a scan rate of 1.00 Hz) with automatic gain control to provide high-accuracy mass measurements within 0.40 ppm deviation using Na Formate as calibrant. The following parameters were used for experiments: spray voltage of 4.5 kV and capillary temperature of 200°C. Nitrogen was used as sheath gas (10 L/min). The spectrometer was attached to an Ultimate 3000 (Thermo Fisher, Germany) UPLC system consisting of a LC-pump, Diode Array Detector (DAD) (λ: 190-600 nm), autosampler (injection volume 10 mL) and column oven (40°C). The separations were performed using a Synergy MAX-RP 100A (50x2 mm, 2.5μ particle size) with an H_2O (+0.1 % HCOOH) (A)/acetonitrile (+0.1 % HCOOH) (B) gradient (flow rate 500 μL/min, injection volume 5 μl). Samples were analyzed using a gradient program as follows: 95 % A isocratic for 1.5 min, linear gradient to 100 % B over 6 min, after 100 % B isocratic for 2 min, the system returned to its initial condition (90 % A) within 1 min and was equilibrated for 1 min.

The chromatograms obtained were analyzed with Data Analysis software and the plausible mass of the molecular ions of compounds present in each active fraction were determined. Possible known compounds in active fractions were identified by comparing the UV and MS spectra and fragmentation of the peaks in the samples with those of compounds from plants and fungi reported in databases (Scifinder, NIST/EPA/NIH Mass Spectral Library (NIST 14) and MassBank of North America (MoNA)).

II.2.3 Induction of antibacterial metabolite production using small chemical elicitor

II.2.3.1 Culture of fungi in medium supplemented with small chemical elicitors and extraction

To trigger the production of bioactive metabolites by *Aspergillus* sp. N454, the fungal strain was first cultivated on potato dextrose agar for seven days. Mycelia pieces of 1x1 cm from the culture were used to inoculate 100 mL of potato dextrose broth (potatoes in infusion 200g/L, dextrose 20g/L, pH 5.1±0.2) (PD; HiMedia) in 250 mL flasks supplemented with different organic chemicals elicitors: 1% dimethyl sulfoxide (DMSO) (Sigma Aldrich); 1% ethanol (HPLC grade, Merck); 1% methanol (HPLC grade, Merck), 1% acetone (HPLC grade, Merck), 1% hexane (HPLC grade, Merck), 1% chloroform (HPLC grade, Merck), 1% nicotine (Sigma Aldrich), 1% caffeine (Sigma Aldrich), 1%

quercetin (Sigma Aldrich), 1% trisodium citrate (Sigma Aldrich), 1% BHT, 1% gallic acid (Sigma Aldrich), 1% toluene (Sigma Aldrich), and a control without any supplementation. Liquid cultures were grown for 6 days in a static conditions at room temperature for fermentation. For each treatment, the culture was performed in duplicate.

After the fermentation period, 100mL of ethyl acetate were added to each culture, shaken, and kept overnight at room temperature. Afterwards, the cultures were transferred into a separatory funnel, and the organic phase was collected. The ethyl acetate was evaporated at 40°C in a Labconco RapidVap parallel evaporation system. The residues were dissolved in 0.2-0.4 mL of methanol, transferred to pre-weighed microfuge tubes and evaporated to dryness. Before antibacterial tests, the dry residues were dissolved in DMSO (2 mg/mL).

The antibacterial analysis was carried out using the M07-A9 protocol of the **CLSI (2012)** on five bacteria strains as previously described.

II.2.3.2 Analysis of metabolite profiles by high-performance liquid chromatography coupled to mass spectrometry (HPLC-MS)

The analysis of metabolite profiles was performed on an Agilent 1260 series HPLC system equipped with an autosampler and diode array detector (DAD) using a Synergy 4µL Polar-RP 80A column (250 mm × 4.6 mm) (Shimadzu Company). The mobile phase consisted of methanol and water, increasing linearly from 5% methanol at the injection time to 100% at 90 min. The flow rate was 1 mL/min, and the column temperature was 40°C. Chromatograms were recorded at 214 nm (σ-σ* transitions shown by several aliphatic molecules) and 254 nm (π-π* transition shown by aromatic molecules).

II.2.3.3 Identification of bioactive compounds

Interpretation of HPLC-MS mass spectra was conducted using the database of the National Institute of Standards and Technology (NIST), which has more than 62,000 patterns. Spectra of unknown components were compared with those of known components of the NIST library. The name and molecular formula of the compounds identified were ascertained.

II.2.4 Statistical analysis

Data collected from at least three independent experiments were analyzed using One-Way ANOVA with GraphPad Prism 5.03 for Windows. Microsoft Excel for windows helped to plot graphs and to calculate standard deviation. Data are expressed as the mean ± SD of experiments performed in triplicate. Error bars represent the SD, and a, b, c $p<0.05$, $**p<0.001$, $***p<0.0001$ indicate significant differences compared to the untreated sample. Principal component analysis and hierarchical cluster analysis were performed using the XLSTAT software.

Results and Discussion

CHAPTER III: RESULTS AND DISCUSSION

III.1 Results and interpretations

III.1.1 Antibacterial potencies of endophytic fungal extracts

III.1.1.1 Extraction yield and Minimal Inhibitory Concentration (MIC) of the fungal extracts

The extraction yield of endophytic fungi ranged from 52 to 200 mg for 200 mL of culture medium, with the *Unidentified fungal* N445 from *C. odorata* roots producing high number of metabolites (200 mg) (Tableau 2).

The MIC of crude extracts were ranged from 0.39 to 25 µg/mL According our criteria of activity, out of the 56 tested extracts, eight were very active (13%) (Tableau 2), with the more potent (MIC < 5 µg/mL) being extracts from *Aspergillus* sp. N454, *Aspergillus* sp. N18, *Curvularia* sp. N101, and *Aspergillus* sp. N13 isolated from *C. odorata* and *T. catappa* (Figure 10) respectively. Thirty-seven extracts (66 %) showed moderate potency with MIC between 5 and 15 µg/mL, while 12 extracts (21%) with MIC > 15 µg/mL were considered inactive. Overall, endophytic *Aspergillus* spp. exhibited the best activity, with MIC ranging from 0.78 to 6.25 µg/mL. *E. coli* ATCC 25922 and *H. influenzae* ATCC 49247 were the more sensitive pathogenic strains.

Table 2: Extraction yield (mg/200mL), and MIC (μg/mL) of ethyl acetate extracts from endophytic fungi (Mean ±SD)

Plant name	Fungal name	Yield	EC ATCC 25922	SA ATCC 43300	SA BAA-977	SP ATCC 49619	HI ATCC 49247	PA HM 601	KP ATCC 13883
Cananga odorata	*Unidentified fungal* N431	104	3.125 ± 0.00	12.5 ± 0.00	3.125 ± 0.00	6.25 ± 0.00	0.78 ± 0.00	3.125 ± 0.00	3.125 ± 0.00
	Unidentified fungal N422	100	2.343 ± 1.11	23.14 ± 0.00	5.787 ± 0.00	3.125 ± 0.00	25 ± 0.00	3.125 ± 0.00	25 ± 0.00
	Unidentified fungal N399	128	0.39 ± 0.00	12.5 ± 0.00	3.125 ± 0.00	3.125 ± 0.00	2.343 ± 1.11	12.5 ± 0.00	12.5 ± 0.00
	Unidentified fungal N419	132	0.78 ± 0.00	5.787 ± 0.00	5.787 ± 0.00	3.125 ± 0.00	>25	1.56 ± 0.00	25 ± 0.00
	Unidentified fungal N401	100	1.17 ± 0.55	6.25 ± 0.00	6.25 ± 0.00	3.125 ± 0.00	1.56 ± 0.00	25 ± 0.00	25± 0.00
	Unidentified fungal N478	120	12.5 ± 0.00	25 ± 0.00	25 ± 0.00	3.125 ± 0.00	25 ± 0.00	12.5 ± 0.00	25 ± 0.00
	Aspergillus sp. N454	100	1.56 ± 0.00	1.56 ± 0.90	3.125 ± 0.00	3.125 ± 0.00	0.78 ± 0.00	3.125 ± 0.00	6.25 ± 0.00
	Diaporthe phaseolorum N330	80	1.56 ± 0.00	5.787 ± 0.00	5.787 ± 0.00	3.125 ± 0.00	25 ± 0.00	1.56 ± 0.00	12.5 ± 0.00
	Unidentified fungal N284	106	3.125 ± 0.00	6.25 ± 0.00	6.25 ± 0.00	3.125 ± 0.00	3.125 ± 0.00	6.25 ± 0.00	6.25 ± 0.00
	Unidentified fungal N283	69	0.78 ± 0.00	2.343 ± 1.11	3.125 ± 0.00	3.125 ± 0.00	1.56 ± 0.00	12.5 ± 0.00	12.5 ± 0.00
	Diaporthaceae sp. N298	130	1.56 ± 0.00	5.787 ± 0.00	5.787 ± 0.00	3.125 ± 0.00	12.5 ± 0.00	1.56 ± 0.00	12.5 ± 0.00
	Unidentified fungal N289	111	0.78 ± 0.00	12.5 ± 0.00	3.125 ± 0.00	3.125 ± 0.00	2.343 ± 1.11	12.5± 0.00	12.5 ± 0.00

Fungal sp. N241	110	1.56 ± 0.00	23.14 ± 0.00	23.14 ± 0.00	25 ± 0.00	12.5 ± 0.00	1.56 ± 0.00	12.5 ± 0.00
Unidentified fungal N262	165	>25	>25	>25	>25	>25	>25	>25
Unidentified fungal N263	94	3.125 ± 0.00	6.25 ± 0.00	3.125 ± 0.00	3.125 ± 0.00	0.78 ± 0.00	6.25 ± 0.00	12.5 ± 0.00
Unidentified fungal N266	70	3.125 ± 0.00	6.25 ± 0.00	6.25± 0.00	6.25 ± 0.00	3.125 ± 0.00	6.25 ± 0.00	6.25 ± 0.00
Unidentified fungal N251	110	12.5 ± 0.00	12.5 ± 0.00	25 ± 0.00	6.25 ± 0.00	25 ± 0.00	12.5 ± 0.00	12.5 ± 0.00
Fungal sp.N268	70	ND	23.14 ± 0.00	11.57 ± 0.00	6.25 ± 0.00	25 ± 0.00	1.56 ± 0.00	25 ± 0.00
Unidentified fungal N276	60	0.78 ± 0.00	3.125 ± 0.00	25 ± 0.00	3.125 ± 0.00	25 ± 0.00	25 ± 0.00	25 ± 0.00
Unidentified fungal N256	152	6.25 ± 0.00	6.25 ± 0.00	6.25 ± 0.00	3.125 ± 0.00	3.125 ± 0.00	6.25 ± 0.00	12.5 ± 0.00
Unidentified fungal $N254_1$	52	4.688 ± 0.00	12.5 ± 0.00	12.5 ± 0.00	6.25 ± 0.00	3.125 ± 0.00	6.25 ± 0.00	25 ± 0.00
Fusarium sp. N240	160	1.56 ± 0.00	23.14 ± 0.00	11.57 ± 0.00	6.25 ± 0.00	6.25 ± 0.00	1.56 ± 0.00	12.5 ± 0.00
Unidentified fungal N441	109	6.25 ± 0.00	6.25 ± 0.00	6.25 ± 0.00	3.125 ± 0.00	3.125 ± 0.00	12.5 ± 0.00	12.5 ± 0.00
Unidentified fungal N448	70	0.78 ± 0.00	23.14 ± 0.00	5.787 ± 0.00	3.6875 ± 0.00	25 ± 0.00	0.78 ± 0.00	12.5 ± 0.00
Unidentified fungal N445	200	3.125 ± 0.00	23.14 ± 0.00	11.57 ± 0.00	6.25 ± 0.00	25 ± 0.00	6.25 ± 0.00	25 ± 0.00
Unidentified fungal N368	123	12.5 ± 0.00	25 ± 0.00	25 ± 0.00	6.25 ± 0.00	25 ± 0.00	12.5 ± 0.00	12.5 ± 0.00

	Corynespora sp.N32	70	1.56 ± 0.00	23.14 ± 0.00	11.57 ± 0.00	6.25 ± 0.00	12.5 ± 0.00	1.56 ± 0.00	12.5 ± 0.00
	Fungal endophyte sp. N89	80	1.56 ± 0.00	23.14 ± 0.00	11.57 ± 0.00	6.25 ± 0.00	12.5 ± 0.00	1.56 ± 0.00	12.5 ± 0.00
	Phomopsis sp. N114	140	3.125 ± 0.00	23.14 ± 0.00	11.57 ± 00	6.25 ± 0.00	12.5 ± 0.00	1.56 ± 0.00	12.5 ± 0.00
	*Guignardia*sp. N108	110	2.343 ± 1.11	11.57 ± 0.00	5.787 ± 0.00	3.125 ± 0.00	12.5± 0.00	1.56 ± 0.00	12.5 ± 0.00
	Pestalotiopsis sp. N75	100	3.125 ± 0.00	5.787 ± 0.00	5.787 ± 0.00	3.125 ± 0.00	25 ± 0.00	1.56 ± 0.00	25 ± 0.00
	Xylaria sp. N74	80	4.688 ± 0.00	23.14 ± 0.00	5.787 ± 0.00	6.25 ± 0.00	12.5 ± 0.00	6.25 ± 0.00	6.25 ± 0.00
Terminalia catappa	*Paraconiothyrium variabile* N78$_1$	170	1.56 ± 0.00	23.14 ± 0.00	11.57 ± 0.00	6.25 ± 0.00	12.5 ± 0.00	1.56 ± 0.00	25 ± 0.00
	Pestalotiopsis sp. N51	70	1.56 ± 0.00	11.57 ± 0.00	5.787 ± 0.00	3.125 ± 0.00	12.5 ± 0.00	1.56 ± 0.00	6.25 ± 0.00
	Unidentified fungal N58	60	3.125 ± 0.00	23.14 ± 0.00	5.787 ± 0.00	6.25 ± 0.00	25 ± 0.00	1.56 ± 0.00	12.5 ± 0.00
	Unidentified fungal N44	70	1.17 ± 0.00	23.14 ± 0.00	11.57 ± 0.00	6.25 ± 0.00	25 ± 0.00	1.56 ± 0.00	25 ± 0.00
	Curvularia sp. N101	102	1.56 ± 0.00	4.69 ± 1.80	3.125 ± 0.00	3.125 ± 0.90	1.56 ±0.90	6.25 ± 0.00	6.25 ± 0.00
	Trichoderma sp. N97	60	0.78 ± 0.00	23.14 ± 0.00	11.57 ± 0.00	6.25 ± 0.00	25 ± 0.00	1.56 ± 0.00	25 ± 0.00
	Phomopsis sp. N81	40	1.56 ± 0.00	11.57 ± 0.00	5.787 ± 0.00	3.125 ± 0.00	25 ± 0.00	25 ± 0.00	25 ± 0.00
	Aspergillus sp. N18	103	0.78 ± 0.45	1.56 ± 0.90	3.125 ± 0.00	3.125 ± 0.00	2.343 ± 1.08	6.25 ± 0.00	6.25 ± 0.00

	Aspergillus sp. N13	99	1.56 ± 0.00	6.25 ± 0.00	3.125 ± 0.00	3.125 ± 0.00	0.78 ± 0.00	1.17± 0.27	12.5 ± 0.00
	Unidentified fungal N23	75	1.56 ± 0.00	6.25 ± 0.00	6.25 ± 0.00	3.125 ± 0.00	3.125 ± 0.00	12.5 ± 0.00	12.5 ± 0.00
	Unidentified fungal N15	60	1.56 ± 0.00	23.14 ± 0.00	11.57 ± 0.00	6.25 ± 0.00	3.125 ± 0.00	1.56 ± 0.00	12.5 ± 0.00
	Colletotrichum sp. N201	10	6.25 ± 0.00	23.14 ± 0.00	11.57 ± 0.00	6.25 ± 0.00	25 ± 0.00	6.25 ± 0.00	25 ± 0.00
	Fungal sp. N200	80	3.125 ± 0.00	23.14 ± 0.00	11.57 ± 0.00	6.25 ± 0.00	6.25 ± 0.00	1.56 ± 0.00	6.25 ± 0.00
	Unidentified fungal N191	80	0.39 ± 0.00	5.787 ± 0.00	5.787 ± 0.00	3.125 ± 0.00	12.5 ± 0.00	3.125 ± 0.00	12.5 ± 0.00
	Unidentified fungal N196	58	>25	25 ± 0.00	25 ± 0.00	25 ± 0.00	25 ± 0.00	25 ± 0.00	>25
	Unidentified fungal N221	163	>25	>25	>25	>25	>25	>25	>25
Terminalia mantaly	*Unidentified fungal* $N230_2$	100	1.56 ± 0.00	12.5 ± 0.00	4.688 ± 2.21	4.688 ± 2.21	3.125 ± 0.00	12.5 ± 0.00	12.5 ± 0.00
	Diaporthales sp. $N229_1$	119	3.125 ± 0.00	6.25 ± 0.00	3.125 ± 0.00	6.25 ± 0.00	0.78 ± 0.00	6.25 ± 0.00	6.25 ± 0.00
	Diaporthales sp. N127	80	1.56 ± 0.00	11.57 ±0.00	5.787 ± 0.00	6.25 ± 0.00	12.5 ± 0.00	1.56 ± 0.00	12.5 ± 0.00
	Phoma sp. N116	90	1.56 ± 0.00	23.14 ± 0.00	5.787 ± 0.00	3.125 ± 0.00	6.25 ± 0.00	1.56 ± 0.00	12.5 ± 0.00
	Xylaria laevis N120	57	>25	25 ± 0.00	25 ± 0.00	25 ± 0.00	25 ± 0.00	25 ± 0.00	25 ± 0.00
	Diaporthe sp. N162	159	>25	>25	>25	12.5 ± 0.00	>25	>25	>25

	Unidentified fungal N190$_1$	120	0.78 ± 0.00	23.14 ± 0.00	5.787 ± 0.00	3.125 ± 0.00	6.25± 0.00	1.56 ± 0.00	6.25 ± 0.00
	Unidentified fungal N178	134	>25	>25	>25	>25	>25	>25	>25
Reference drugs	Amoxicillin	/	4 ± 00	16 ± 0.00	64 ± 0.00	16 ± 0.00	4 ±0.00	32 ± 0.156	32 ± 0.00
	Ciprofloxacin	/	2 ± 0.00	4 ± 0.00	1 ± 0.00	32 ± 0.00	1 ± 0.00	1 ± 0.00	8 ± 0.00

SA :*Staphylococcus aureus* ;EC: *Escherichia coli*; SP: *Streptococcus pneumoniae*; HI: *Haemophilus influenzae*; PA: *Pseudomonas aeruginosa*; KP: *Klebsiella pneumoniae*

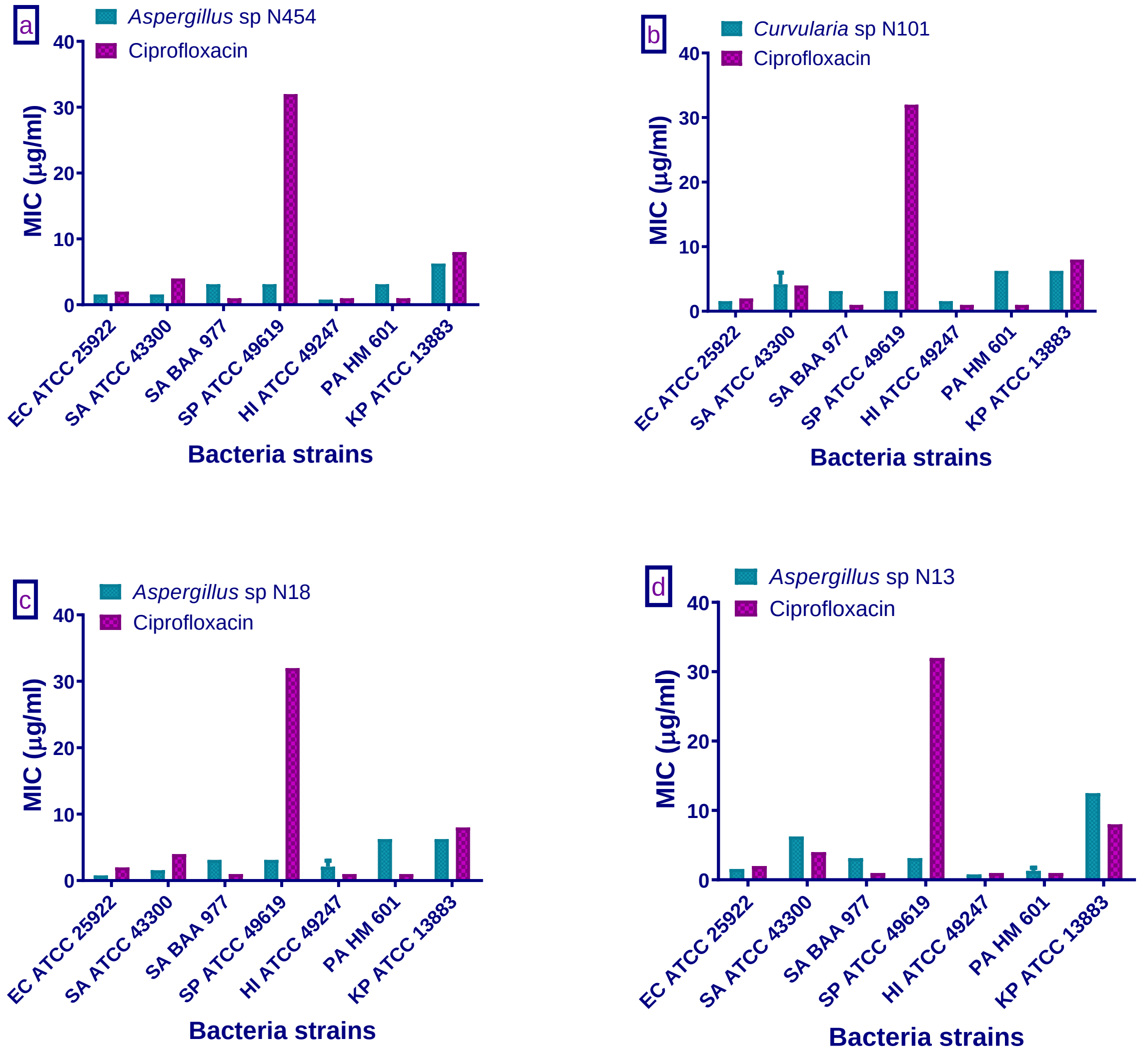


Figure 10: Minimal Inhibitory Concentration (MIC) of the most potent extracts on bacterial strains. The error bars represent the standard deviation of measurement of a sample in three separate runs. (a): *Aspergillus* sp. N454; (b): *Curvularia* sp. N101; (c): *Aspergillus* sp. N18; (d): *Aspergillus* sp. N13. SA: *Staphylococcus aureus*; EC: *Escherichia coli*; SP: *Streptococcus pneumoniae*; HI: *Haemophilus influenzae*; PA: *Pseudomonas aeruginosa*; KP: *Klebsiella pneumoniae*.

III.1.1.2 Mode of action of promising extracts

The results of the mode of action and antioxidant studies of the four most active crude extracts (*Aspergillus* sp. N454, *Aspergillus* sp. N18, *Curvularia* sp. N101, and *Aspergillus* sp. N13) are as follows.

III.1.1.2.1 Bacteriolytic effect of selected extracts

The cell lysis activities exhibited by fungal extracts on *H. influenzae* ATCC 49247 and *E. coli* ATCC 25922 are summarized in Figure 11. Globally, the treatment of bacterial cells with fungal extracts caused gram-negative bacteria cell lysis. This cell lysis was more significant than 50% and 55% for *E. coli* and *H. influenzae*, respectively, after 4 hours of incubation at 2 MIC and 4 MIC. The reduction in the bacterial population was more drastic in the case of *H. influenzae* than *E. coli*. Overall, bacteriolysis was higher with an extract from *Aspergillus* sp. N18 (71%) on *E. coli* at 4 MIC and *Curvularia* sp. N101 (75%) on *H. influenzae* at 2 MIC, respectively.

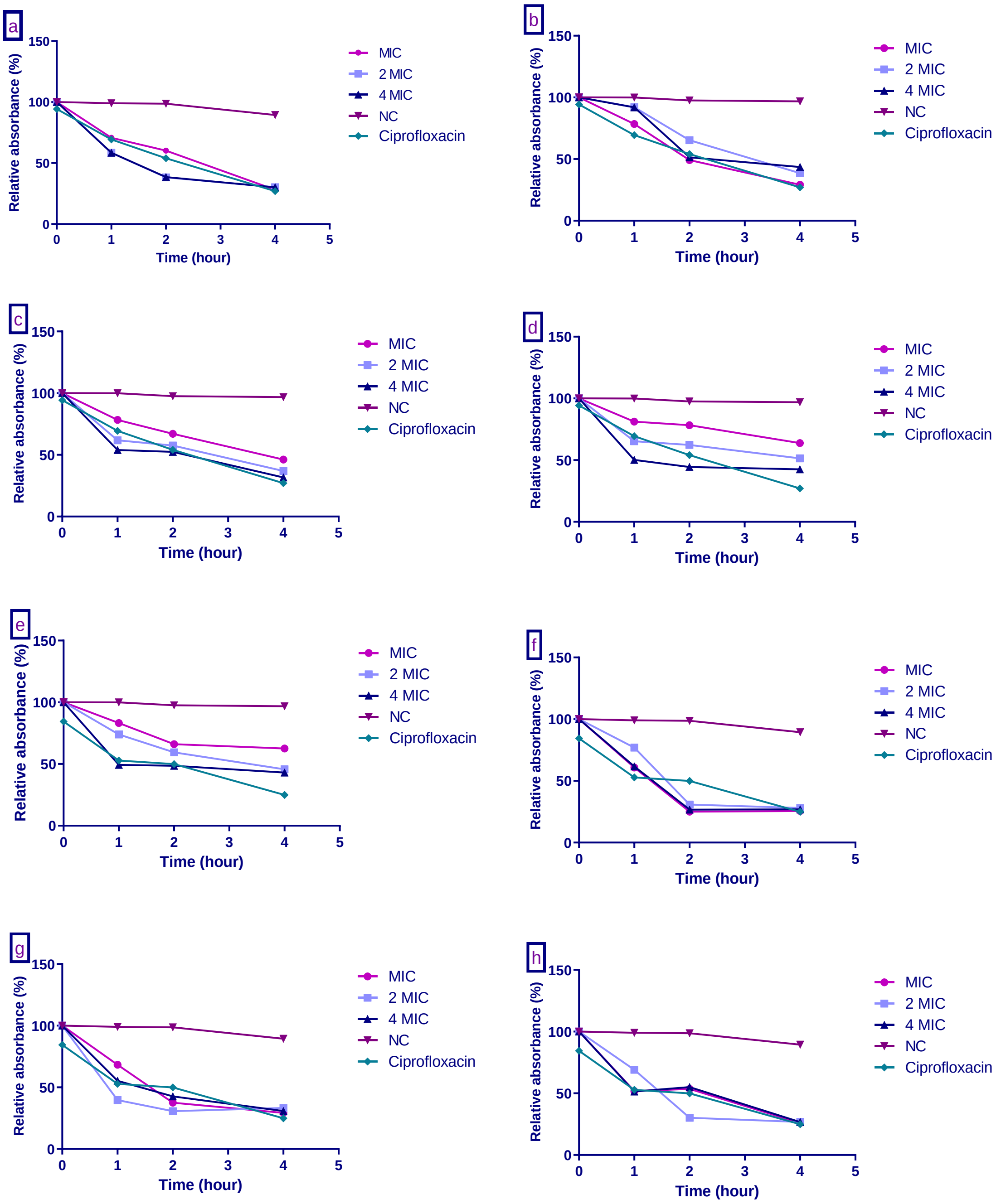


Figure 11: Bacteriolytic activity of endophytic fungal extracts on *E. coli* ATCC25922 and *H. influenzae* ATCC 49247. (a) *Aspergillus* sp. N454 on *E. coli*, (b) *Aspergillus* sp. N18 *on E. coli*, (c) *Aspergillus* sp. N13 on *E. coli*, (d) *Curvularia* sp. N101 on *E. coli*, (e) *Aspergillus* sp. N454 on *H. influenzae*, (f) *Aspergillus* sp.N18 on *H. influenzae*, (g) *Aspergillus* sp. N13 on *H. influenzae*, (h) *Curvularia* sp. N101 on *H. influenzae*. MIC: minimal inhibitory concentration; NC: negative control.

III.1.1.2.2 The effect of extract on the integrity of the cell membrane

The integrity of the cytoplasmic membrane was analyzed by determining the release of cellular materials, including nucleic acids, proteins, metabolites, and ions, which were absorbed at 260 nm into the bacterial suspensions. Treatment of *H. influenzae* ATCC 49247 and *E. coli* ATCC 25922 with potent fungal extracts at the MIC concentrations indicated no significant cell leakage of 260 nm absorbing material in a time-dependent manner. This absence of nucleotide leakage was observed until the 120th minute of incubation (Figure 12a and b). The same result was obtained in the control group treated with ciprofloxacin, revealing its disability to damage the cytoplasmic membrane of the tested bacterial strains.

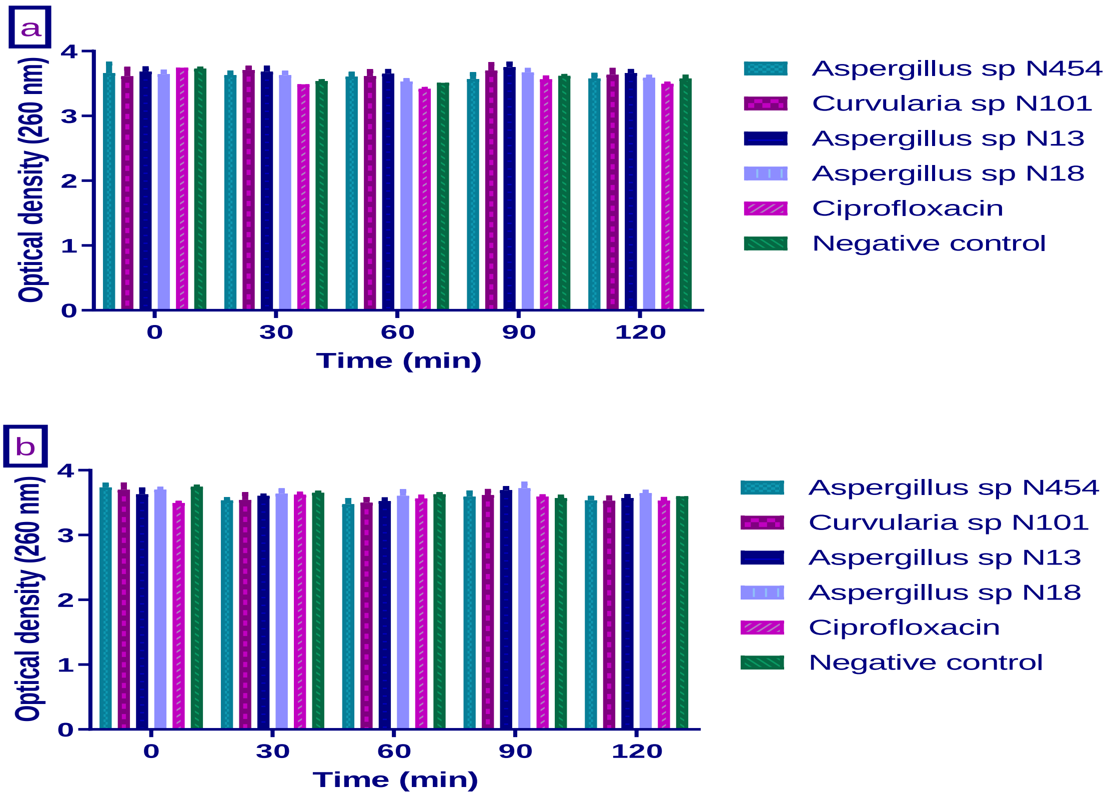
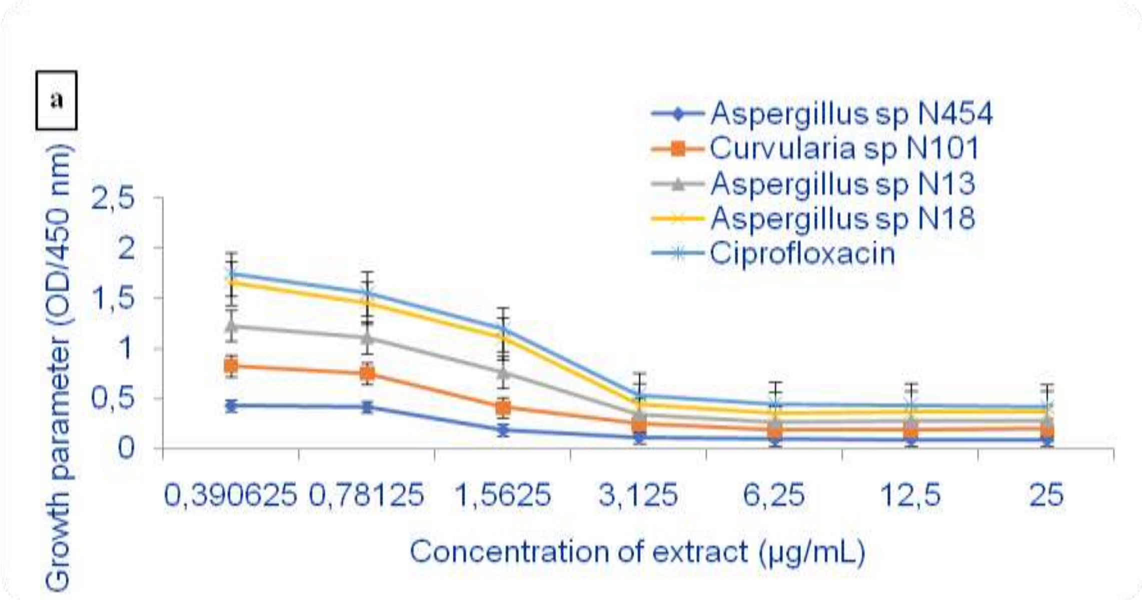


Figure 12: Total nucleotide leakage from (a) *Escherichia coli* ATCC 25922 and (b) *Haemophilus influenzae* ATCC 49247 treated with different endophytic fungal extracts at their MIC concentrations.

III.1.1.2.3 The effect of extracts on the permeability of outer cell membrane

Bacterial cell membrane permeability was determined by measuring the optical density at 450 nm (Figures 13a and 13b). Measurement of the optical density of bacterial cells treated with endophyte extracts demonstrated that *Aspergillus* sp. extracts. N454, *Aspergillus* sp. N18, *Aspergillus* sp. N13 and *Curvularia* sp. N101 affects the permeability of *E. coli* ATCC 25922 and *H. Influenzae* ATCC 49247. When the concentration of extracts in the medium was increased we noticed the decrease of the optical density at 450 nm indicative of the leakage of intracellular components, including electrolytes, from the cells. Furthermore, in both treated bacteria, with the increasing concentration of extracts from 0.39 to 3.125 µg/mL, a sharp decrease in the optical density was observed corresponding to the loss of viability of tested bacteria (Figures 13a and 13b). The inhibition of the growth of *H. influenzae* and *E. coli* was more intense when treated with an extract from *Aspergillus* sp. N454 compared to the antibiotic ciprofloxacin at almost all the concentrations tested.



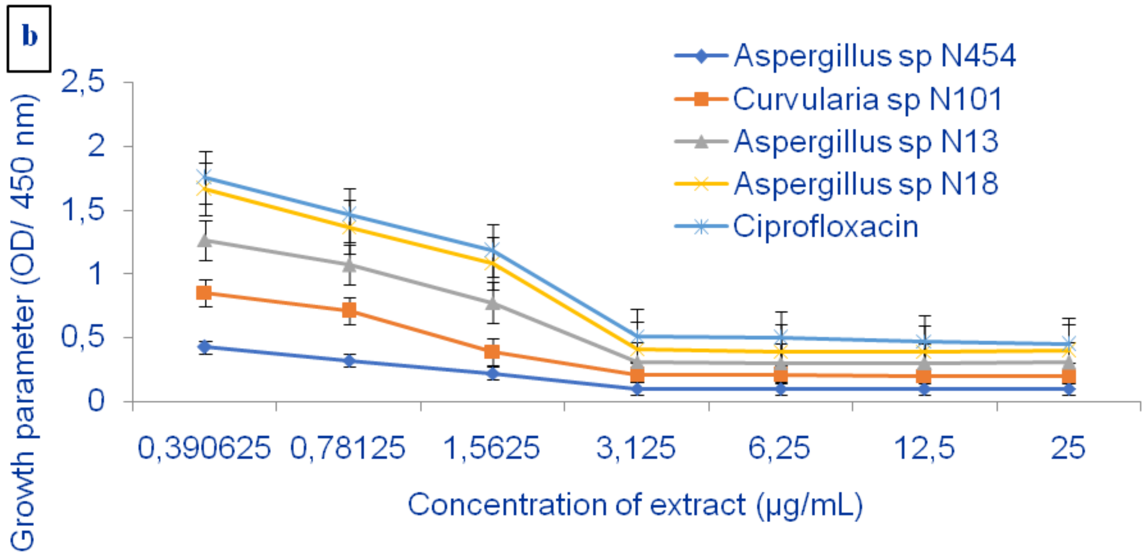


Figure 13: Effect of endophytic fungal extracts on the membrane permeability of (a) *H. influenzae* ATCC 49247 and (b) *E. coli* ATCC 25922. Data are expressed as the mean ± SD.

III.1.1.2.4 The effect of extracts on the loss of salt tolerance capacity

The effects of the extracts on salt tolerance of bacteria are summarized in Figure 14 (14a and 14b). The addition of KCl to the Nutrient Agar (NA) medium significantly reduced the colony-forming units of treated *E. coli* ATCC 25922 and *H. influenzae* ATCC 49247. *H. influenzae* treated with KCl and fungal extract revealed that the number of bacteria able to form colonies on NA-KCl was not significantly reduced at 2.5% and 5% of KCl concentrations compared to the control (0% KCl). At 10 % of KCl, minimal bacterial growth was recorded. However, regarding *E. coli*, the proportion of bacteria forming colonies on KCl nutrient Agar was significantly reduced when KCl was used at 5% and 10% g/L. Overall, when the KCl concentration contained in the medium was increased; there was an increase in the reduction of colony formation.

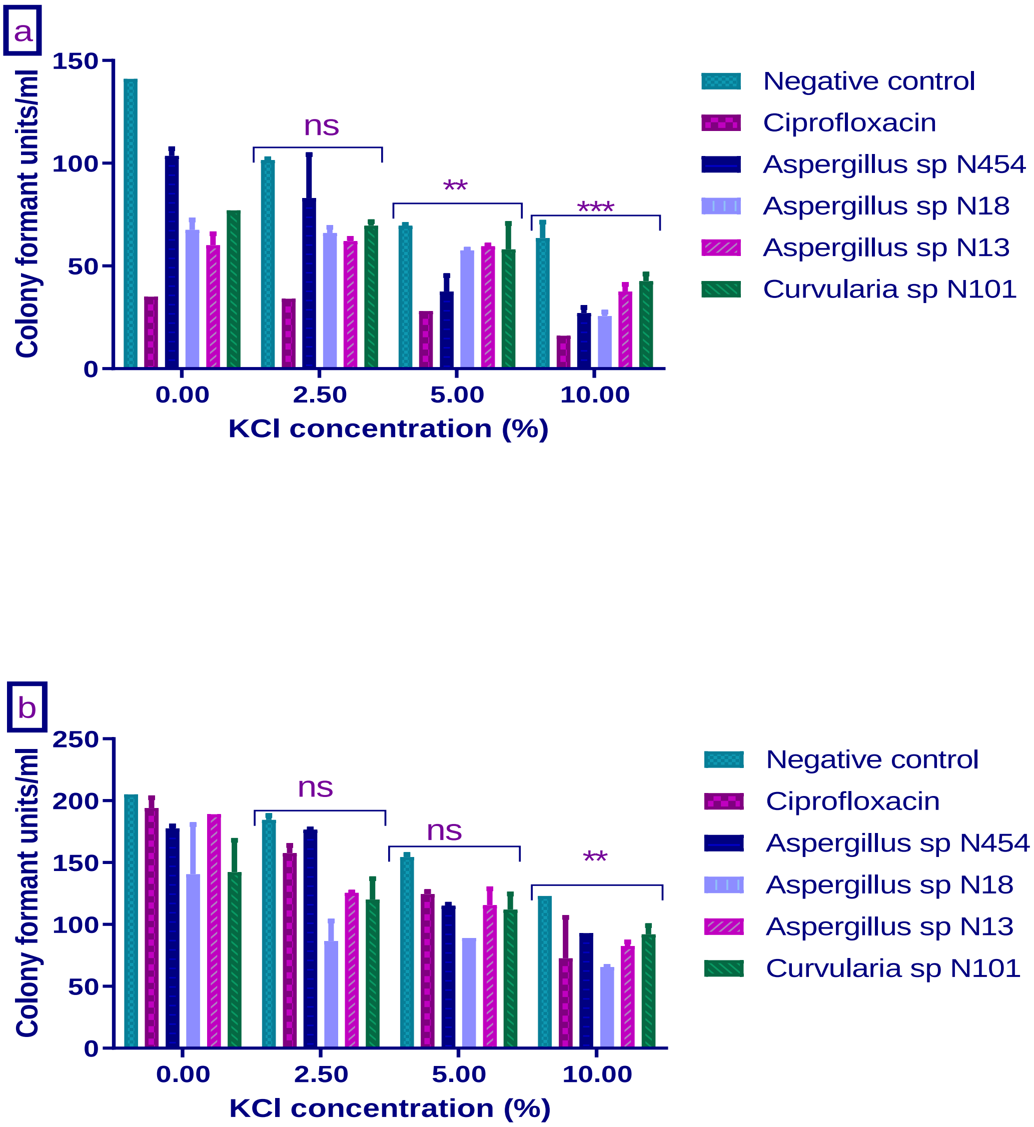


Figure 14: Effect of potent extracts on the reduction of salt tolerance of (a) *E. coli* ATCC 25922 and (b) *H. influenzae* ATCC 49247 at the MIC concentration. The error bars represent the standard deviation of measurement of a sample in three independent sample. ns: no significant, ***: Significantly different compared to the untreated samples ($p < 0.001$), **: Significantly different compared to the untreated samples ($p < 0.01$).

III.1.1.2.5 The effect of extracts on catalase activity

The inhibition of the catalase activity is summarized in figure 15 below. The percentages of remaining H_2O_2 in bacteria culture treated with extracts ranged from 28.48 to 47.63 % for *E. coli* and from 55.28 to 76.67 % for *H. influenzae*, highlighting the tested extracts' ability to exert a certain degree of inhibition against the activity of the bacterial catalase enzyme. The crude extract from *Aspergillus* sp. N18 exerted the highest degree of inhibition against *H. influenzae* (% of remaining H_2O_2 = 47.63) and *E. coli* (% of remaining H_2O_2 =76.67). The catalase inhibition activity exhibited by *Aspergillus* sp. N18 is comparable to that of the positive control; ciprofloxacin showed a percentage of remaining H_2O_2 of 45.09 and 81.57 % on *H. influenzae* and *E. coli*, respectively.

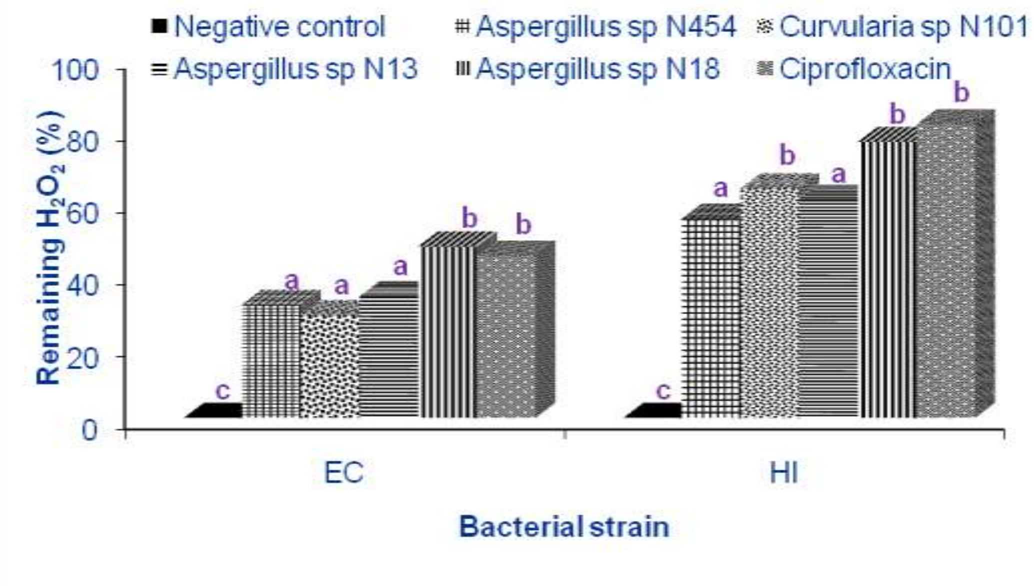


Figure 15: Effect of endophytic fungi extracts on the catalase activity of *Escherichia coli* and *Haemophilus influenzae* at the MIC concentration. Values with different letters express significant differences at $p < 0.05$. EC: *Escherichia coli* ATCC 25922; HI: *Haemophilus influenzae* ATCC 49247.

III.1.1.2.6 Principal component analysis of modes of action exhibited by potent extracts

Principal component analysis of the mode of action exhibited by potent extracts was performed to determine the preferential mode of action by which each active extract exerts its antibacterial activity. The results obtained are presented in Figure 16. The analysis showed that the extract from *Aspergillus* sp. N454 preferentially inhibited both *E. coli* and *H. influenzae* growth through bacteriolysis action. Extract from *Curvularia* sp. N101 preferentially act on *H. influenzae* by reducing it salt tolerance ability, while on *E. coli* it favors outer membrane permeability and salt tolerance. In the case of extract from *Aspergillus* sp. N13 it preferentially acts by altering the inner membrane integrity of *E. coli* and inducing bacteriolysis in *H. influenzae*. Extract from *Aspergillus* sp. N18 preferentially altered the inner membrane integrity and increased membrane permeability of *H. influenzae,* while against *E. coli*, act by inhibiting catalase activity. Overall, the most potent extract possesses different modes of action on bacterial cell. The preferential use of one mode of action instead of another is highly dependent on the extract and the pathogen involved.

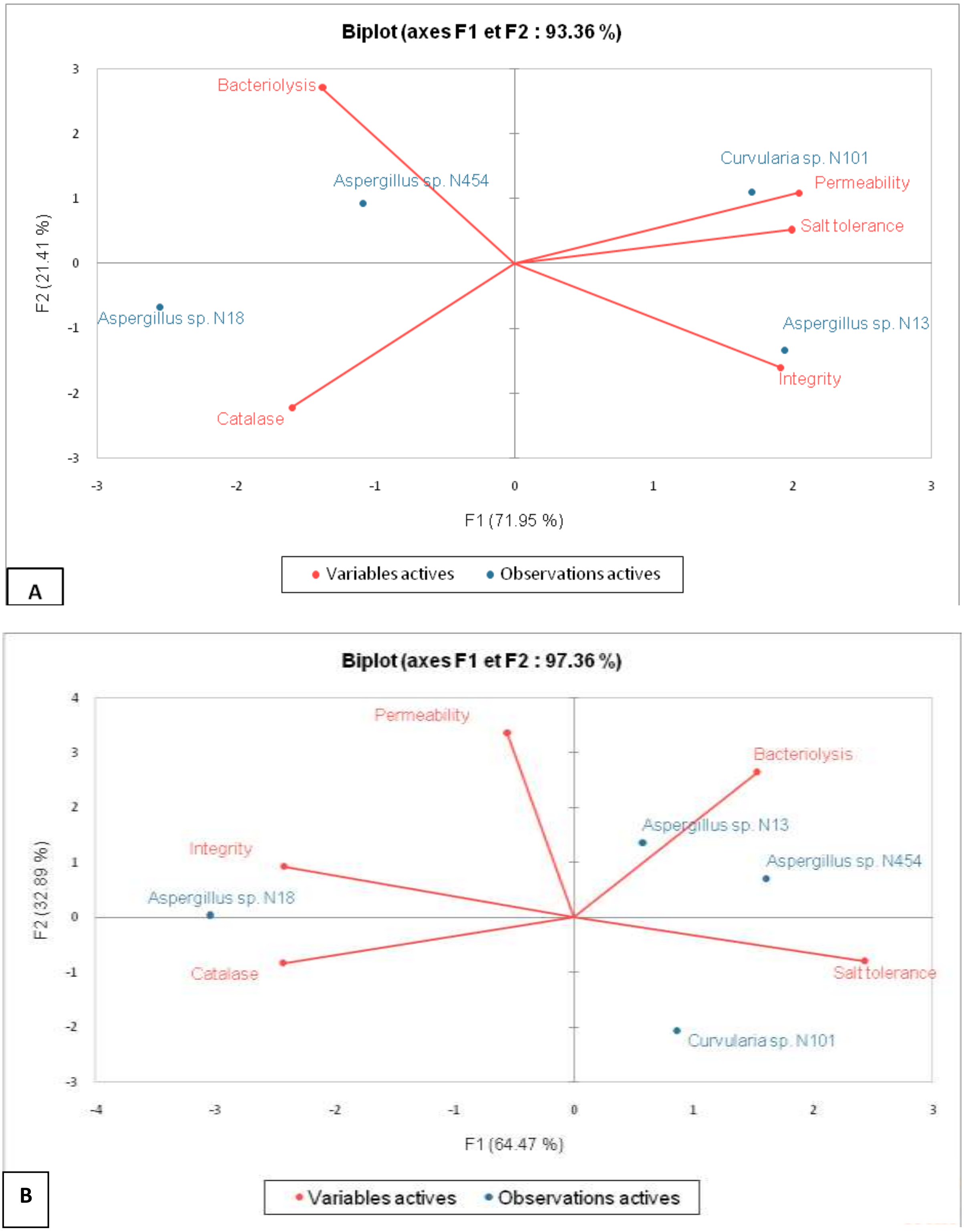


Figure 16: Principal component analysis of modes of action of the potent extracts on *E. coli* (A) and *H. influenzae* (B).

III.1.1.3 Antioxidant potential of selected extracts

The results show that the inhibition concentration 50 (IC_{50}) of DPPH ranged from 150.71 to 936.08 µg/mL depending on the tested extracts (Table 3). Extract of *Aspergillus* sp. N454 showed a high scavenging activity with an IC_{50} of 150.71 µg/mL, whereas the extract from *Curvularia* sp. N101 showed minor antioxidant activity (IC_{50}>1000 µg/mL). Concerning the Fe^{3+}reducing power assay, only the ethyl acetate extract from *Aspergillus* sp. N13 exhibited activity with a 50% reduced concentration (RC_{50}) of 760.96 µg/mL (Table 2).

Table 3: DPPH radical (IC_{50}) and RC_{50} of fungal extracts Means ± SDs.

Fungi name	**IC 50 (µg/mL)**	**RC 50 (µg/mL)**
Aspergillus sp. N13	250 ± 0.350^{c}	760 ± 0.07^{b}
Curvularia sp. N101	>1000	>1000
Aspergillus sp. N454	150.71± 0.02^{b}	>1000
Aspergillus sp. N18	936.08 ±1.93^{d}	>1000
Ascorbic acid	2.71 ± 0.08^{a}	13.94 ± 0.27^{a}

Values carrying the same letter superscripts across a column are not significantly different (P>0.05), IC_{50}: Inhibitory concentration 50 of DPPH radical, RC_{50}:Fe^{3+} Reducing Concentration 50. CC_{50}: Cytotoxic Concentration 50, SD: standard deviation. Data are presented as the mean values ± standard deviations of triplicate experiments.

III.1.1.4 Cytotoxicity potential of selected extracts

The cytotoxicity of the extracts was tested against Vero cells ATCC CRL1586. As shown in Table 3, the extract from *Aspergillus* sp. N13 was cytotoxic against Vero cells ATCC CRL1586 (CC_{50} of 14.285 µg/mL), while no cytotoxic activity was observed with other tested extracts (CC_{50}>100 µg/mL).

Tableau 4: Cytotoxicity (CC_{50}) of fungal extracts Mean ± SD.

Fungal extract	*Aspergillus* sp. N13	*Curvularia* sp. N101	*Aspergillus* sp. N454	*Aspergillus* sp. N18
CC 50 (µg/mL)	14.28 ± 5.86	>100	>100	>100

Being the most active extract, presenting several mode of action and a non-cytotoxicity against Vero cell line, the ethyl acetate extract from *Aspergillus* sp. N454 was selected for phytochemical analysis.

III.1.2 Antibacterial-guided fractionation of *Aspergillus* sp. N454 ethyl acetate extract

III.1.2.1 Phytochemical composition of crude extract

The qualitative analysis results obtained showed the presence of phenolic compounds, flavonoids, glucosides, tannins, alkaloids, anthocyanins and steroids. Ethyl acetate extract from *Aspergillus* sp. N454 did not contain coumarins and saponins (Table 3).

Table 5: Phytochemical constituents of ethyl acetate extract from *Aspergillus* sp. N454

Test	***Aspergillus* sp. N454**
Alkaloids	+
Anthocyanins	+
Coumarins	-
Flavonoids	+
Glucosides	+
Phenolic compounds	+
Saponins	-
Steroids	+
Tannins	+

+: presence of secondary metabolite, -: absence of secondary metabolite

III.1.2.2 Column chromatography of *Aspergillus* sp. N454 crude extract

The large-scale fermentation of *Aspergillus* sp. N454 in liquid medium yielded 17 g of crude EtOAc extract, out of which 15 g was subjected to column chromatographic separation using 60 g of silica gel (60–120 mesh), eluting with solvent systems of increasing polarities, *n*-hexane/EtOAc (1:0–0:1), and EtOAc/MeOH (9:1 – 0:1). The resulting 129 fractions were pooled into 10 fractions according to their thin layer chromatography (TLC) profile (Table 4).

The mass of fractions from *Aspergillus* sp. N454 crude extract varying from 7.46 to 346.10 mg. It was highly dependent on the polarity of the solvent system used. The total yield of the fractions was equal to 854 mg. This yield represented only 17.55% of the total amount of crude extract used for fractionation, showing that 82.44% of the extract was lost during fractionation. A large amount of extract was filling in the silica gel, probably due the polarity of compounds presents in extract. Globally, all fractions were screened against seven pathogenic bacteria at 25 µg/mL, and the MIC values were determined.

The antibacterial activity displayed by the fractions was not proportional to the fractionation yields obtained. Out of the ten fractions tested, two (F7 and F4) exhibited no activity against all tested pathogens. In contrast, eight exhibited antibacterial potencies against at least one bacterium with MICs ranging from 0.39 to 25µg/mL. Two fractions, F1 and F3, inhibited five bacterial species with MIC values ranging from 6.25-25µg/mL and 1.57-25µg/mL, respectively, with distinct activity profiles. Both inhibited *S. aureus* and *H. influenzae*, only F1 also inhibited *S. pneumoniae* and *E. coli,* and only F3 was active against *K. pneumoniae* and *P. aeruginosa*. The fraction F10 inhibited only three bacteria, including *K. pneumoniae*, *S. aureus* and *H. influenzae*, with MIC values ranging from 3.125-12.5µg/mL. Interestingly, F10 inhibited the resistant strain of *S. aureus* ATCC 43300 (MIC 12.5µg/mL) but was inactive against the sensitive *S. aureus* BAA-977 strain. Finally, fraction F2 was active against all tested bacteria with MIC values ranging from 0.39-12.5 µg/mL.

Compared to the parent extract, fractions F1, F4, F5, F6, F7, F8 and F9 did not improve the antibacterial activity of the extract against all tested bacterial strains; in contrast, the activity decreased by 2- to 16-folds for fraction F1, 16-folds for fraction F5 and F6 on *H. influenzae*, 32-fold for fraction F8 and F9 on *H. influenzae*. Contrary to these fractions, fraction F2 showed 2-folds improved antibacterial potential against *H. influenzae* (MIC 0.3906 µg/mL), *P. aeruginosa* (MIC 1.57µg/mL), and *K. pneumoniae* (MIC 3.125 µg/mL) compared to the crude extract. Fractions F3 and F10 improved the activity by 2-folds against *S. aureus* BAA-977 and *K. Pneumoniae,* respectively.

Overall, this first fractionation of the crude ethyl acetate extract from *Aspergillus* sp. N454 did not significantly improve the antibacterial activity against some pathogenic bacteria. Nevertheless, according to its wide range of activity and its improvement of the antibacterial activity on at least three of the seven tested pathogenic bacteria, fraction F2 was selected for further fractionation.

Table 2: Yield (mg) and antibacterial MIC (µg/mL) of fractions from silica gel chromatography of crude ethyl acetate extract of *Aspergillus* sp. N454.

Pools	**Fractions**	**Yield**	**SP ATCC 49619**	**KP ATCC 13883**	**SA BAA-977**	**SA ATCC 43300**	**EC ATCC 25922**	**PA HM 601**	**HI ATCC 49247**	**PI**
Crude extract		15000	3.125 ± 0.00	6.25 ± 0.00	3.125 ± 0.00	1.56 ± 0.00	1.56 ± 0.00	3.125 ± 0.00	0.78 ± 0.00	/
F1	1-12	17.72	25 ± 00	>25	25 ± 00	6.25 ± 00	12.5 ± 00	>25	12.5 ± 00	NA
F2	13-25	730`	12.5 ± 00	3.125 ± 00	6.25 ± 00	12.5 ± 00	3.125 ± 00	1.57 ± 00	0.390 ± 00	2
F3	26 – 29	23.28	>25	12.5 ± 00	1.57 ± 00	12.5 ± 00	>25	25 ± 00	3.125 ± 00	2
F4	30 – 42	83.32	>25	>25	>25	>25	>25	>25	>25	NA
F5	43- 67	13	>25	>25	>25	>25	>25	>25	12.5 ± 00	NA
F6	68 -72	7.46	>25	>25	>25	>25	>25	>25	12.5 ± 00	NA
F7	73-86	64	>25	>25	>25	>25	>25	>25	>25	NA
F8	87 – 97	172	>25	>25	>25	>25	>25	>25	25 ± 00	NA
F9	98 -112	127.36	>25	>25	>25	>25	>25	>25	25 ± 00	NA
F10	113-129	346.10	>25	3.125 ± 00	>25	12.5 ± 00	>25	>25	12.5 ± 00	2
Amoxicillin	/	/	4 ± 00	16 ± 0.00	64 ± 0.00	16 ± 0.00	4 ± 0.00	32 ± 0.00	32 ± 0.00	

F: Silica gel 1 pool and their respective fractions; SA: *Staphylococcus aureus*; EC: *Escherichia coli*; SP: *Streptococcus pneumoniae*; HI: *Haemophilus influenzae*; PA: *Pseudomonas aeruginosa*; KP: *Klebsiella pneumoniae*; Total yield of fractions: 854.24 mg; PI: potential improvement.

III.1.2.3. Second chromatography of fraction F2

Fraction F2 (730 mg) was chromatographed on silica gel (30 g), eluting with solvent systems of increasing polarities, *n*-hexane/EtOAc (1:0–0:1), and EtOAc/MeOH (9:1–0:1). As shown in Table 5, one hundred and ninety-six (196) fractions of 100 mL each were collected and then pooled based on their thin layer chromatography (TLC) profiles into 17 pool sub-fractions with masses ranging from 3 to 20 mg. Sub-fraction KIMS4 (20mg) was the most abundant. All sub-fractions were submitted to antibacterial activity.

Out of the 17 sub-fractions screened at 10 µg/mL against the seven pathogenic bacterial strains, 11 were inactive against all tested pathogens. The six-displaying activity against at least one pathogen was submitted to dose-response assay for MIC determination. Sub-fractions KIMS2 and KIMS3 were only active against *H. influenzae* (MIC 0.312 µg/mL) and *K. pneumoniae* ATCC 13883 (MIC 10 µg/mL), respectively. KIMS5 (MIC 0.156-10µg/mL) and KIMS14 (2.5-10µg/mL) inhibited four and three pathogens, respectively. While both were active against the *S. aureus* (BAA-977 and ATCC 43300) and *E. coli*, KIMS5 was also active against *S. pneumoniae*. Finally, the broad-spectrum antibacterial sub-fractions were KIMS17 (MIC 1.25-5µg/mL) and KIMS4 (MIC 0.078-5µg/mL), which are active against six and seven bacteria, respectively.

Compared to fraction F2, sub-fraction KIMS2 showed improved antibacterial activity against *H. influenzae* by 1.24-fold, while KIMS3 showed decreased activity against *K. pneumoniae* by 3.2 fold. Sub-fractions KIMS4 and KIMS5 showed improved activity by 20- and 40-fold, respectively, against *S. aureus* (BAA – 977 and ATCC 43300); KIMS4 also improved the antibacterial activity against *S. pneumoniae* and *E. coli* by 10- and 40- folds respectively. Sub-fraction KIMS14 improved the activity by 1.25-fold against *E. coli* and 2.5 folds against *S. aureus* ATCC 43300 but was not against *S. aureus* BAA-977. KIMS17 showed improved activity (1.25 to 10 folds) against *E. coli*, *S. pneumoniae* and *S. aureus*.

Overall, the fractionation from the crude extract to sub-fractions led to an improvement of the antibacterial activity ranging from 1.24 to 20 folds. The fractionation of fraction F2 to led to 17 sub-fractions among which fractions KIMS4, KIMS5, KIMS14 and KIMS17 exhibited a broad antibacterial spectrum (MIC 0.078-10 31 µg/mL) with the improvement of antibacterial potency ranging from 1.25 to 40 folds. Therefore, they were selected for compositional analysis using UPLC-MS.

Table 3: Yield (mg), antibacterial MIC (µg/mL) of fractions from silica gel re-chromatography of F2.

Pools	Fractions	Yield	SP ATCC 49619	KP ATCC 13883	SA BAA-977	SA ATCC 43300	EC ATCC 25922	PA HM 601	HI ATCC 49247	PI
F2		730`	12.5 ± 00	3.125 ± 00	6.25 ± 00	12.5 ± 00	3.125 ± 00	1.57 ± 00	0.390 ± 00	-
Sub-fractions										
KIMS1	1- 15	3	>10	>10	>10	>10	>10	>10	>10	-
KIMS2	16-19	4.53	>10	>10	>10	>10	>10	>10	0.312 ± 0.00	1.24
KIMS3	20 – 30	5	>10	10 ± 0.00	>10	>10	>10	>10	>10	-
KIMS4	31- 59	20	1.25 ± 0.00	5± 0.00	0.312 ± 0.00	0.625 ± 0.00	0.078 ± 0.00	5 ± 0.00	5 ± 0.00	10 - 40
KIMS5	60- 63	6	2.5 ± 0.00	>10	0.156 ± 0.00	0.312 ± 0.00	10 ± 0.00	>10	>10	5-40
KIMS6	64 -111	7.7	>10	>10	>10	>10	>10	>10	>10	-
KIMS7	112 -119	4.9	>10	>10	>10	>10	>10	>10	>10	-
KIMS8	120 -124	12	>10	>10	>10	>10	>10	>10	>10	-

KIMS9	125 – 127	3.87	>10	>10	>10	>10	>10	>10	>10	**-**
KIMS10	128 **-**134	6.6	>10	>10	>10	>10	>10	>10	>10	**-**
KIMS11	135 **-**140	2.34	>10	>10	>10	>10	>10	>10	>10	**-**
KIMS12	141 – 146	2.71	>10	>10	>10	>10	>10	>10	>10	**-**
KIMS13	147 – 159	13	>10	>10	>10	>10	>10	>10	>10	**-**
KIMS14	160 – 174	9	>10	>10	10 ± 0.00	5 ± 0.00	2.5 ± 0.00	>10	>10	1.25
KIMS15	174 – 177	4	>10	>10	>10	>10	>10	>10	>10	**-**
KIMS16	178 – 186	3.3	>10	>10	>10	>10	>10	>10	>10	**-**
KIMS17	187- 196	12	5 ± 0.00	5 ± 0.00	1.25± 0.00	1.25± 0.00	2.5± 0.00	5 ± 0.00	>10	1.25 **-** 10
	Ciprofloxacin	/	2 ± 0.00	4 ± 0.00	1 ± 0.00	32 ± 0.00	1 ± 0.00	1 ± 0.00	8 ± 0.00	/

KIMS : Silica gel 2 pools and their respective fraction ; SA : *Staphylococcus aureus* ; EC : *Escherichia coli* ; SP : *Streptococcus pneumoniae* ; HI: *Haemophilus influenzae*; PA: *Pseudomonas aeruginosa*; KP: *Klebsiella pneumoniae*; Total yield of fractions: 119.95 mg.

III.1.2.4 Chemical composition of potent sub-fractions

The four active sub-fractions (KIMS4, KIMS5, KIMS14, and KIMS17) exhibiting broad antibacterial activity were submitted to UPLC-MS for chemical profiling.

The UPLC-MS profile of the selected potent sub-fractions (Figure 17) showed a great variation in metabolites contents. The analysis of sub-fractions KIMS4, KIMS5, KIMS14 and KIMS17 revealed 13, 7, 6, and 6 compounds respectively (Figure 17). Of those, the unidentified compound 15 ($1.6*10^6$) was the more abundant in KIMS4 sub-fraction. Methyl 3,4,5-trimethoxycinnamate was the more abundant compound in KIMS5 ($1.6*10^6$) and KIMS17 ($2.4*10^6$). 9,10-Epoxyoctadecenoic acid was found to be the major compound in KIMS14 ($4.4*10^6$) and was also the second most abundant compound in KIMS17. (Table 6).

Comparative analysis of the chemical compositions showed that compounds 9,10-Epoxyoctadecenoic acid (RT ≈5.3 min) was present in varied proportions in all the tested sub-fractions while Methyl 3,4,5-trimethoxycinnamate (RT≈6.2 min), was present in a relatively constant amount in all sub-fractions excepted in sub-fraction KIMS14. Their simultaneous presence might otherwise justify the antibacterial activity of all these sub-fractions. On other hand, several compounds were found to be specific to each of the tested sub-fractions. The numbers of unique compounds found in different sub-fractions were 7 (KIMS4), 5 (KIMS5), 2 (KIMS14), and 3 (KIMS17). The compound identified in KIMS4 at retention time 6.4 min is a hitherto unknown compound. Sub-fraction KIMS4 contained most various compounds in terms of diversity than any other tested sub-fractions.

The hierarchical cluster analysis (HCA) was used to understand the correlation between antibacterial potency of sub-fractions and their chemical uniqueness (Figure 18). While looking to the dendrogram we may noticed 2 main clusters of sub-fractions based on their UPLC-MS depicted number of constituents viz. Cluster I [KIMS4 (13 components, sensitive bacteria strain:7) and KIMS17 (7 components, sensitive bacteria strain:6)], Cluster II [KIMS5 (06 components, sensitive bacteria strain:4) and KIMS14 (6 components, sensitive bacteria strain:3)]. Cluster I constituted of KIMS4 and KIMS17 was dissimilar from the two other promising sub-fractions, mainly due to their content of peptides, phenolic compound (phenylpropanoids), terpenes, and alkaloids classes. As shown in Figure 19, those two fractions may act on any of the tested bacteria without preference. Additionally, the activity profile indicates that the potency of the sub-fractions analyzed was most consistently on drug resistant *S. aureus* (ATCC 43300) compared to sensitive *S. aureus* (BAA-977). Thus, as shown in Table 7, sub-fractions analyzed in this study were closely related to their individual partially described number of secondary metabolites.

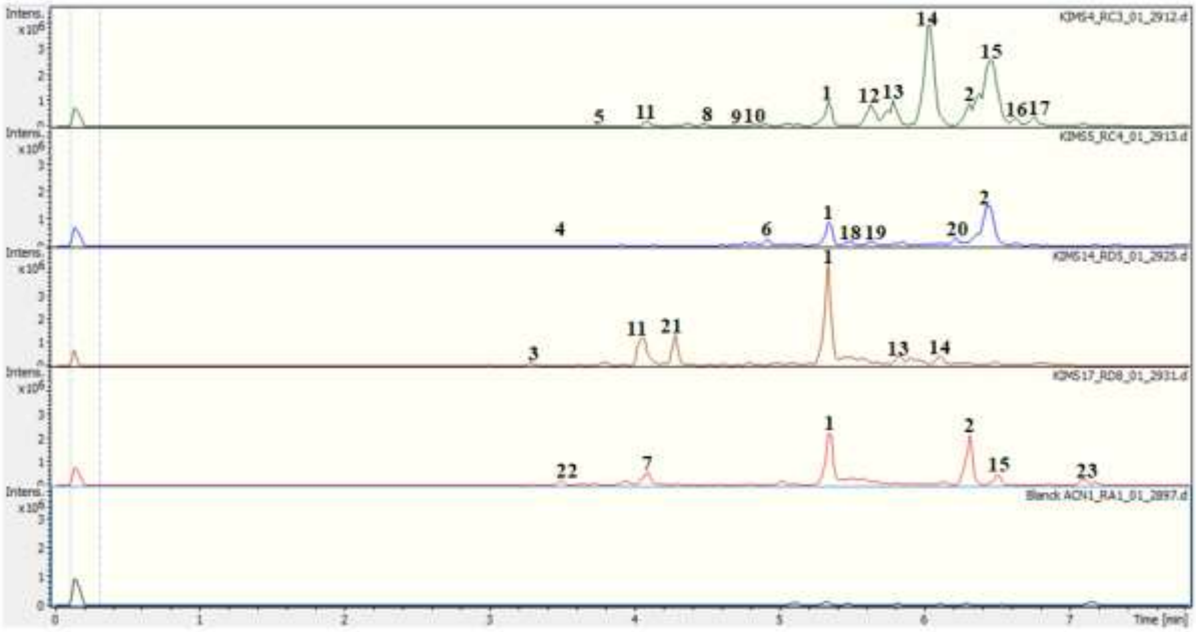


Figure 17: Chromatographic profiles of active sub-fractions from *Aspergillus* sp. N454; 1-10: identified compound present in one or more sub-fraction; 11-23 unidentified compounds present in one or more sub-fraction.

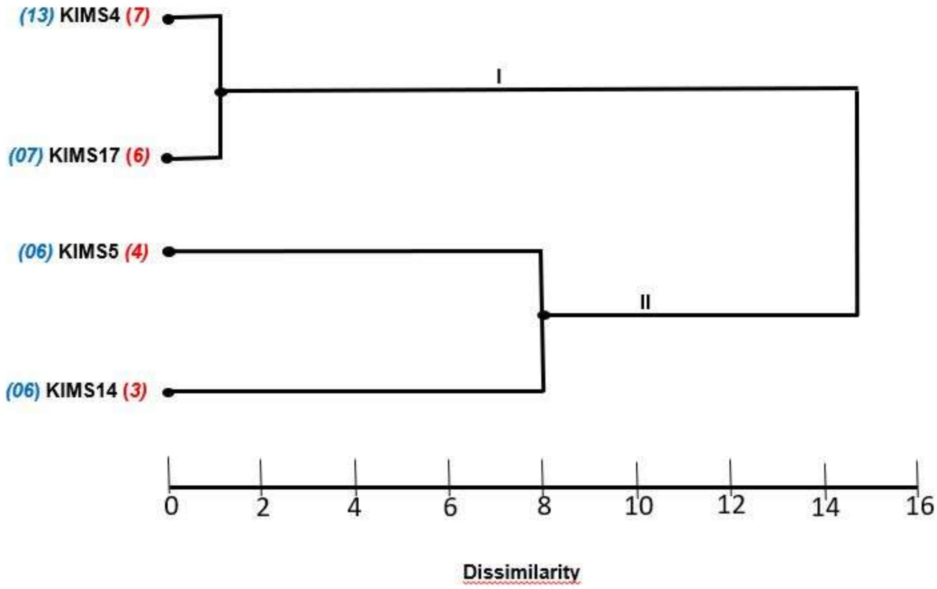


Figure 18: Dendrogram of the chemical relationship among potent antibacterial sub-fractions.

The Euclidean distance was used for similarity measurement, and Ward's linkage was used as the clustering algorithm. Flanking *Aspergillus* sp. N454 (KIMS) sub-fraction numbers indicate the number of compounds in blue and number of sensitive bacteria to the sub-fraction in red. Clusters I-II have been indicated in Roman letters.

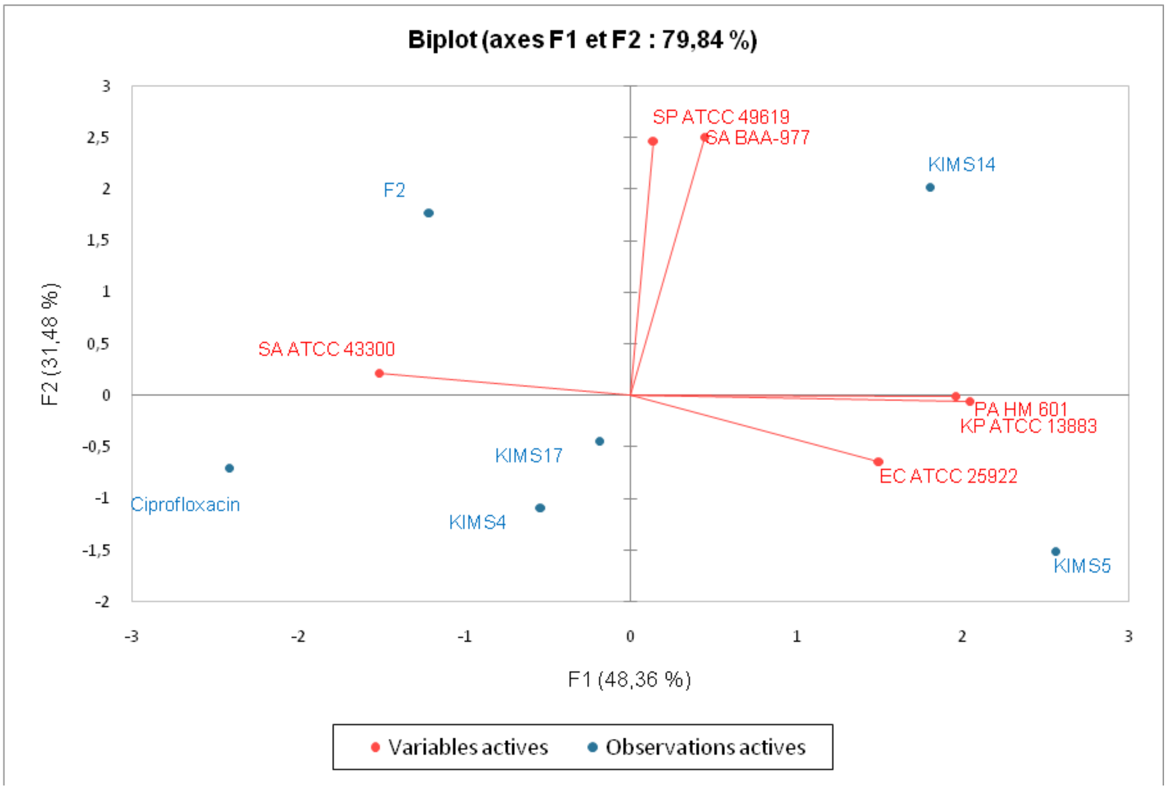


Figure 19: Principal component analysis of potent sub-fractions. *KIMS: Silica gel 2 pools and their respective fraction; SA:Staphylococcus aureus; EC: Escherichia coli; SP: Streptococcus pneumoniae; HI: Haemophilus influenzae; PA: Pseudomonas aeruginosa; KP: Klebsiella pneumoniae.*

Table 4: Metabolites identified by UPLC-MS in four selected highly potent sub-fractions.

Compound Number	Name	Mass (m/z)	Chemical formula	[1]RT (min)	[2]Intensity of compound in each sub-fraction 10^6			
					KIMS4	KIMS5	KIMS14	KIMS17
1	9,10-Epoxyoctadecenoic acid	319.2244	$C_{18}H_{32}O_3$	5.3	1	0.95	4.4	2.2
2	Methyl 3,4,5-trimethoxycinnamate	275.0895	$C_{13}H_{16}O_5$	6.2	0.8	1.6	-	2.4
3	N-Acetyl tryptamine	225.1004	$C_{12}H_{13}N_2O$	3.3	-	-	0.2	-
4	Usnic acid	367.0794	$C_{11}H_{12}O_5$	3.4	-	0.1	-	-
5	Sinapic acid	225.0767	$C_{11}H_{12}O_5$	3.6	0.1	-	-	-
6	Kaurane-17,18-dioic acid	335.2193	$C_{20}H_{30}O_4$	4.9	-	0.4	-	-
7	1 Hexadecanoyl glycerol	353.2668	$C_{19}H_{38}O_4$	4.1	-	-	-	0.6
8	Gibberellin A4	341.1365	$C_{18}H_{22}O_5$	4.5	0.2	-	-	-
9	Cyclo (Leu-Pro)	233.1266	$C_{11}H_{18}N_2O_2$	4.6	0.1	-	-	-
10	Cyclo(L-Leu-L-Pro)	233.1266	$C_{11}H_{18}N_2O_2$	4.7	0.2	-	-	-
11	Unidentified compound		***	4.0	0.4	-	1.2	-
12	Unidentified compound		***	5.3	0.8	-	-	-
13	Unidentified compound		***	5.4	1	-	-	0.4
14	Unidentified compound		***	6.0	4	-	0.4	-
15	Unidentified compound		***	6.4	1.6	-	-	0.4
16	Unidentified compound		***	6.6	0.4	-	-	-
17	Unidentified compound		***	6.8	0.4	-	-	-

18	Unidentified compound	***	5.6	-	0.2	-	-
19	Unidentified compound	***	5.7	-	0.2	-	-
20	Unidentified compound	***	6.2	-	0.4	-	-
21	Unidentified compound	***	4.3	-	-	1.4	-
22	Unidentified compound	***	3.5	-	-	-	0.2
23	Unidentified compound	***	7.1	-	-	-	0.2

KIMS: Silica gel 2 pools and their respective fraction; Compounds were identified through comparison of their spectra with those of known components of the NIST library; [1]Retention time; [2]Relative abundance of sub-fraction components was automatically generated from electronic integration of individual pics of the chromatogram; *** Unknown compound.

III.1.3 Antibacterial metabolites production by using small chemical elicitors

III.1.3.1 Effect of elicitors on dry mass of extract and antibacterial activity

Most potent endophytic fungi were cultured in media supplemented with chemical elicitors to see whether they can increase the production of antibacterial metabolites by endophytes. The results obtained revealed that the presence of small chemicals elicitors in the cultured medium affects this fungus's growth and production of antibacterial metabolites (Table 7).

The dry mass ranged from 5.19 to 21.15 mg depending to the elicitor used. The culture of *Aspergillus* sp. N454 with ethanol, methanol, n-hexane, chloroform, acetone, trisodium citrate, gallic acid, quercetin, BHT, caffeine and nicotine have considerably increased the secondary metabolites production from 1.055 to 2.548 folds. Other elicitor such as DMSO and toluene have down regulated the metabolites production of *Aspergillus* sp. N454 by 1.53 to 1.59 folds respectively. Overall, the presence of nicotine (Dry mass=21.15mg) in the culture medium have shown best improvement of metabolites production by 2.548 times.

The antibacterial activity of crude extracts from *Aspergillus* sp. N454 in the presence and absence of elicitors was assessed using the broth microdilution method. The MICs obtained are given in Table 7. The activities obtained were highly dependent on the extract. The MIC ranged from 0.039 to 5 µg/mL. Compared to the untreated culture, extract obtained with toluene and caffeine did not exhibit any antibacterial activity against all tested microorganisms.

Regarding the antibacterial activity among Gram positive bacteria tested, we may notice that the presence of elicitors ethanol, acetone, BHT and nicotine have increase by 2 to 64 folds the antibacterial potential of *Aspergillus* sp. N454 on *S. aureus* BAA-977 compared to the untreated culture. On *S. aureus* ATCC 43300 which is a methicillin and oxacyclin resistant strain, we observed a down regulation of the activity (from 2 to 4 folds) in presence of almost all elicitors used excepted nicotine which increased the activity by 4 folds. Overall, elicitors used have mostly increased the antibacterial potential of gram-positive sensitive strains than resistant strain.

Concerning gram negative bacteria, ethanol chloroform and nicotine have increased the antibacterial potential of *Aspergillus* sp. N454 on *K. pneumoniae* by 32.05, 128.20 and 4 folds respectively. Acetone and n-hexane did not induce any change in the endophyte antibacterial potential while other elicited cultures have decreased the activity on *K. pneumoniae*. The antibacterial potential of elicited cultures of *Aspergillus* sp. N454 was also alter against *E. coli* and *H. influenzae*. In fact, compared to the untreated culture, only chloroform, acetone and nicotine have enhanced the activity by 16.025, 4 and 8 folds respectively against *E. coli*. Methanol, n-hexane, acetone, trisodium citrate, gallic acid, quercetin, DMSO, toluene, caffeine and BHT have deeply inhibited the antibacterial potential of the endophyte extract by 4 to 8 folds on *H. influenzae* while it was increased by 2 folds in presence of ethanol, chloroform and nicotine.

Globally, extracts from elicited cultures were most active against gram negative bacteria than gram positive bacteria. Among gram positive bacteria tested elicited extract were most potent on sensitive strain of *S. aureus* than methicillin and oxacyclin resistant *S. aureus*. The best amount of secondary metabolites production and wide range of antibacterial potential was obtained in presence of nicotine followed by ethanol, chloroform and acetone. Therefore, they were selected for chemical analysis.

Table 5: Effect of organic chemicals on dry mass of extract (mg) and minimum inhibitory concentration (MIC) in µg/mL of extracts produced by *Aspergillus* sp. N454.

Elicitors	Dry mass	SA BAA 977	SA ATCC 43300	KP ATCC 700603	EC ATCC 25922	HI ATCC 49247	PI
Untreated *Aspergillus* sp. N454	8.30	5 ± 0.00	1.25± 0.00	5 ± 0.00	1.25 ± 0.00	0.625 ± 0.00	-
Ethanol	12.65	0.625 ± 0.00	2.5 ± 0.00	0.156 ± 0.00	1.25 ± 0.00	0.312 ± 0.00	2 - 32.05
Methanol	10.79	5 ± 0.00	>5	>5	2.5 ± 0.00	2.5± 0.00	NA
n-hexane	8.76	1.25 ± 0.00	>5	5 ± 0.00	2.5 ± 0.00	2.5 ± 0.00	4
Chloroform	9.28	>5	>5	0.039 ± 0.00	0.078 ± 0.00	0.312 ± 0.00	2 - 128.20
Acetone	9.35	2.5 ± 0.00	1.25 ± 0.00	5 ± 0.00	0.312 ± 0.00	2.5 ± 0.00	2 – 4
DMSO	5.40	>5	5 ± 0.00	>5	5 ± 0.00	>5	NA
Toluene	5.19	>5	>5	5	>5	>5	NA
Trisodium citrate	8.90	>5	>5	>5	>5	5 ± 0.00	NA
Gallic acid	14.23	>5	5 ± 0.00	>5	5 ± 0.00	5 ± 0.00	NA
Quercetin	10.12	>5	>5	>5	>5	5 ± 0.00	NA

BHT	10.32	2.5 ± 0.00	5 ± 0.00	>5	>5	2.5 ± 0.00	2
Cafein	14.06	>5	>5	>5	>5	>5	NA
Nicotine	21.15	0.078 ± 0.00	0.312 ± 0.00	1.25 ± 0.00	0.156 ± 0.00	0.312 ± 0.00	2 - 64.10
Ampicillin	/	4	4	2	1	4	-
PI fold	1.055 – 2.548	2 – 64	4	4- 128.20	4 – 16.025	2	

SA: *Staphylococcus aureus*; EC: *Escherichia coli*; HI: *Haemophilus influenzae*; KP: *Klebsiella pneumoniae*; NA: no amelioration of the activity; PI: Potency improvement folds corresponding to the ratio of the MIC of extract without elicitor / MIC of extract+ elicitor or the ratio of the dry mass of extract without elicitor / dry mass of extract + elicitor.

III.1.3.2 Comparative HPLC profile of metabolites from *Aspergillus* sp. N454

The analysis of the ethyl acetate extracts from *Aspergillus* sp. N454 cultured in the presence and the absence of small organic chemicals was performed using high-performance liquid chromatography coupled to mass spectrometry (HPLC-MS). Figure 20 shows the metabolic profile obtained in the absence of organic chemical (Figure 20A) and the presence of ethanol (Figure 20B), nicotine (Figure 20C), chloroform (Figure 20D), and acetone (Figure 20E).

The HPLC-MS profile showed a variation in metabolites contents due to exposure to different organic chemicals. In general, LC-MS analysis of potent elicited and non-elicited extracts, led to the detection of approximately 152 components whereas 27, 32, 42, 28, and 23 compounds in untreated culture (Aneg), culture treated with ethanol (Aetoh), culture treated with nicotine (Anico), culture treated with chloroform (Achlo), and culture treated with acetone (Aace) respectively. Of those, compound 9 ($4*10^5$) was the most abundant in untreated extract (Aneg) while compounds 2 ($4.1*10^5$) and 10 ($4*10^5$) were more abundant in extract treated with ethanol (Aetoh). Compound 9 ($4*10^5$), 10 ($4*10^5$) and 30 ($4*10^5$) were most abundant in extract from nicotine treatment (Anico) when compound 23($3.8*10^5$) and 27 ($3.7*10^5$) was most abundant in chloroform treated extract (Achlo) (Table 8). Compound 17 ($2.8*10^5$ to $3.1*10^5$) was present in constant amount in all potent elicited extracts.

A synoptic comparison of the chemical composition revealed that compounds 1 (RT ≈0.313 min), 4 (RT ≈7.156 min), 5 (RT ≈7.431 min), 9 (RT ≈8.018 min), 11 (RT ≈8.723 min), 12 (RT ≈8.958 min), 16 (RT ≈10.720 min), 17 (RT ≈11.111 min), 18 (RT ≈11.425 min), 23 (RT ≈14.048 min), 24 (RT ≈14.713 min), 25 (RT ≈14.831 min), and 26 (RT ≈15.575 min), were present in varied proportions in all activated extracts when compound 27 (RT ≈16.987 min) were present in relative constant amount in all treated and untreated extracts. Additionally, several compounds were extract specific. The number of unique compounds found in the different activated extracts were 0 for untreated extract (Aneg), 4 for extract treated with ethanol (Aetoh), 8 for extract from nicotine treatment (Anico), 3 for extract treated with chloroform (Achlo), and 2 for extract treated with acetone (Aace).

The cultures with elicitors have in certain cases increased the production of some compounds and in other reduced or totally inhibited their production. Compound 1

(RT≈0.313 min) initially produced in untreated culture with the intensity of 0.4×10^5 was increased in presence of nicotine ($1.7*10^5$). Also, compound 2 (RT≈0.548 min) initially produced in untreated culture at 0.2×10^5 was increased by culture with ethanol and totally inhibited by the presence of acetone. In comparison to untreated fungus, ethanol has inhibited the production of 3 compounds (compound 15 (RT≈10.524 min), 19 (RT≈11.777 min) and 20 (RT≈12.012 min)). Nevertheless, the production of compounds 23 (RT≈14.048 min) and 27 (RT≈16.985 min) was not affected by the culture additives.

A global view of the dendrogram (Figure 21) clearly indicate 3 clusters of extracts based on their number of compounds namely, Cluster I [Aneg (27 components, sensitive bacteria strain:5) and Aace (23 components, sensitive bacteria strain:5)], Cluster II [Achlo (28 components, sensitive bacteria strain:3), and Cluster III [Aetoh (32 components, sensitive bacteria strain:5) and Anico (42 components, sensitive bacteria strain:5)]. Antibacterial potency was directly linked to the number of phytochemicals detected in a giving extract using LC-MS approach. Indeed, Aneg (MIC: 0.625-5 µg/mL) and Aace (MIC: 0.312-5µg/mL) exhibited comparable potencies, while phytochemical profiling indicating non-significant number of components. Thus, as shown in figure 21, Cluster II constituted of Achlo was dissimilar from the four other promising activated extracts. Also, Anico (MIC: 0.078-1.25 µg/mL) and Aetoh (MIC: 0.156-2.5 µg/mL) profiles showed no significant difference in terms of number of components and antibacterial activities exhibited (Figure 21).The principal component analysis of potent sub-fractions recorded in Figure 22 shown that extracts from the treatment with ethanol and nicotine can act on any of the five tested bacteria while other elicited extract has preferential bacteria on which they can act. For instance, extract from culture with chloroform preferentially act on Gram positive S. aureus whereas the untreated extract and the extract from acetone treatment preferred to act on Gram negative bacteria (*H. influenzae*, *E. coli*, *K. pneumoniae*).

Overall, among the 51 different identified compounds 27 was obtained in untreated culture (Aneg). Compared to the untreated extract of *Aspergillus* sp. N454, the presence of ethanol, nicotine, chloroform and acetone in PDB induced the production of 4, 8, 3, and 2 new compounds, respectively. The small chemical compound which mostly induced the production of compounds in term of diversity and number was nicotine. Potent activated extracts contain specific or common components that exhibit high antibacterial activity singly or synergistically. Further detailed investigation of these extracts is expected to enable the isolation and characterization of potent active principles.

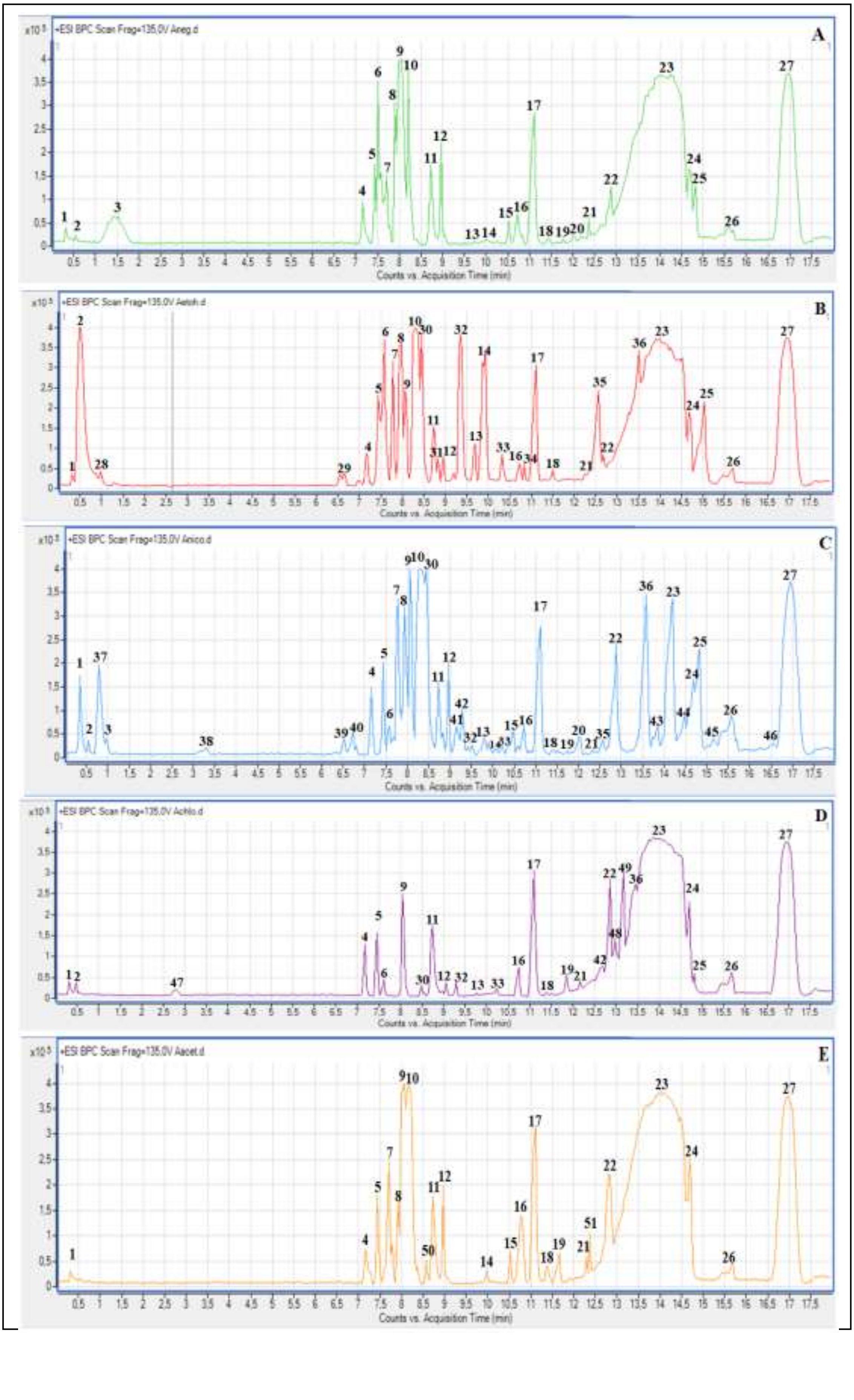


Figure 20: Effect of small chemicals on secondary metabolites production by *Aspergillus* sp. N454 detected at +ESI BPC Scan Frag =135 V. (A): untreated culture; (B): effect of ethanol; (C): effect of nicotine; (D): effect of chloroform; (E): effect of acetone.

Table 6: Metabolites detected by LC-MS in five selected highly potent elicited ethyl acetate extract.

Compound Number	[1]RT (min)	[2]Intensity of compound in each sub-fraction 10^5				
		Aneg	**Aetoh**	**Anico**	**Achlo**	**Aace**
1	0.313	0.42	0.4	1.7	0.4	0.3
2	0.548	0.25	4-	0.8	0.4	-
3	1.527	0.65	-	0.8	-	-
4	7.156	0.98	0.9	1.5	1.35	0.7
5	7.431	1.75	2.4	2	1.6	1.8
6	7.509	3.505	3.7	0.7	0.5	-
7	7.705	1.51	3.2	3.4	-	2.5
8	7.901	3.15	3.6	3.2	-	1.7
9	8.018	4	2.4	4	2.55	4
10	8.214	3.75	4	4	-	4
11	8.723	1.75	1.51	1.6	1.75	1.8
12	8.958	2.4	0.8	2	0.5	2
13	9.741	0.1	1.1	0.4	0.2	-
14	10.015	0.2	3.5	0.2	-	0.3
15	10.524	0.6	-	0.59	-	0.7
16	10.720	1.7	0.7	0.7	0.8	1.4
17	11.111	2.8	3.1	2.8	3.1	3.2
18	11.425	0.2	0.4	0.2	0.2	0.4
19	11.777	0.2	-	0.2	0.6	0.7
20	12.012	0.3	-	0.4	-	-
21	12.365	0.4	0.4	0.2	0.5	0.6
22	12.874	1.3	0.8	2.4	2.4	2.2
23	14.048	3.7	3.7	3.4	3.8	3.8
24	14.713	1.7	1.9	1.7	2.3	2.5
25	14.831	1.35	2.2	2.3	0.6	-
26	15.575	0.4	0.5	0.9	0.6	0.4
27	16.985	3.7	3.7	3.75	3.75	3.7
28	0.985	-	0.5	-	-	-
29	6.568	-	0.4	-	-	-
30	8.451	-	3.9	4	0.3	-
31	8.814	-	0.75	-	-	-
32	9.352	-	3.8	0.3	0.5	-
33	10.348	-	0.9	0.25	0.3	-
34	10.923	-	0.6	-	-	-
35	12.506	-	2.4	0.4	-	-
36	13.495	-	3.5	3.5	2.6	-

37	0.874	-	-	2	-	-
38	3.309	-	-	0.3	-	-
39	6.510	-	-	0.4	-	-
40	6.714	-	-	0.51	-	-
41	9.156	-	-	0.7	-	-
42	9.329	-	-	1	0.7	-
43	13.843	-	-	0.7	-	-
44	14.450	-	-	0.9	-	-
45	15.213	-	-	0.4	-	-
46	18.508	-	-	0.3	-	-
47	2.842	-	-	-	0.2	-
48	12.945	-	-	-	1.4	-
49	13.215	-	-	-	3	-
50	8.143	-	-	-	-	0.5
51	12.406	-	-	-	-	1.1

Compounds were identified through comparison of their spectra with those of known components of the NIST library;[1]Retention time;[2] Relative intensity of extract components was automatically generated from electronic integration of individual pics of the chromatogram relative to the total pics area; Aneg: untreated *Aspergillus* sp. N454 ; Aetoh: *Aspergillus* sp. N454 treated with ethanol; Anico: *Aspergillus* sp. N454 treated with nicotine; Achlo: *Aspergillus* sp. N454 treated with chloroform; Aace: *Aspergillus* sp. N454 treated with acetone.

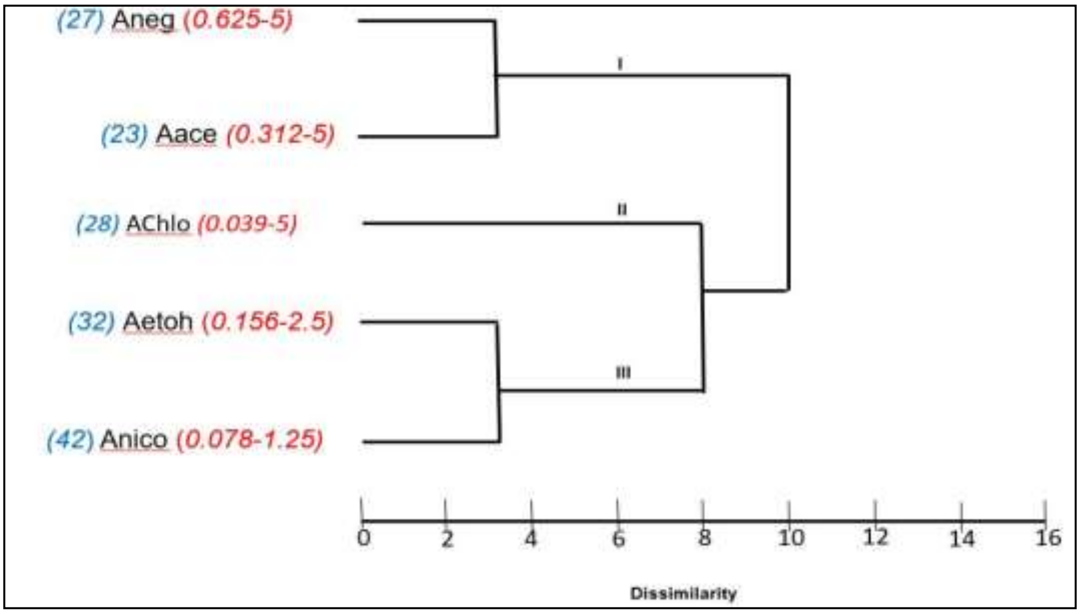


Figure 21: Dendrogram of the chemical relationship among potent antibacterial activated extracts. The Euclidean distance was used for similarity measurement, and Ward's linkage was used as the clustering algorithm. Dendrogram of the chemical relationship among potent antibacterial activated extracts. The Euclidean distance was used for similarity measurement, and

Ward's linkage was used as the clustering algorithm. Flanking *Aspergillus* sp. N454 (A) activated extracts numbers indicate the number of compounds in blue and range of activity in red. Clusters I-III have been indicated in Roman letters; Aneg : extract from culture without elicitor; Achlo : extract from culture with chloroform; Aace: extract from culture with acetone; Anico: extract from culture with nicotine; Aetoh: extract from culture with ethanol.

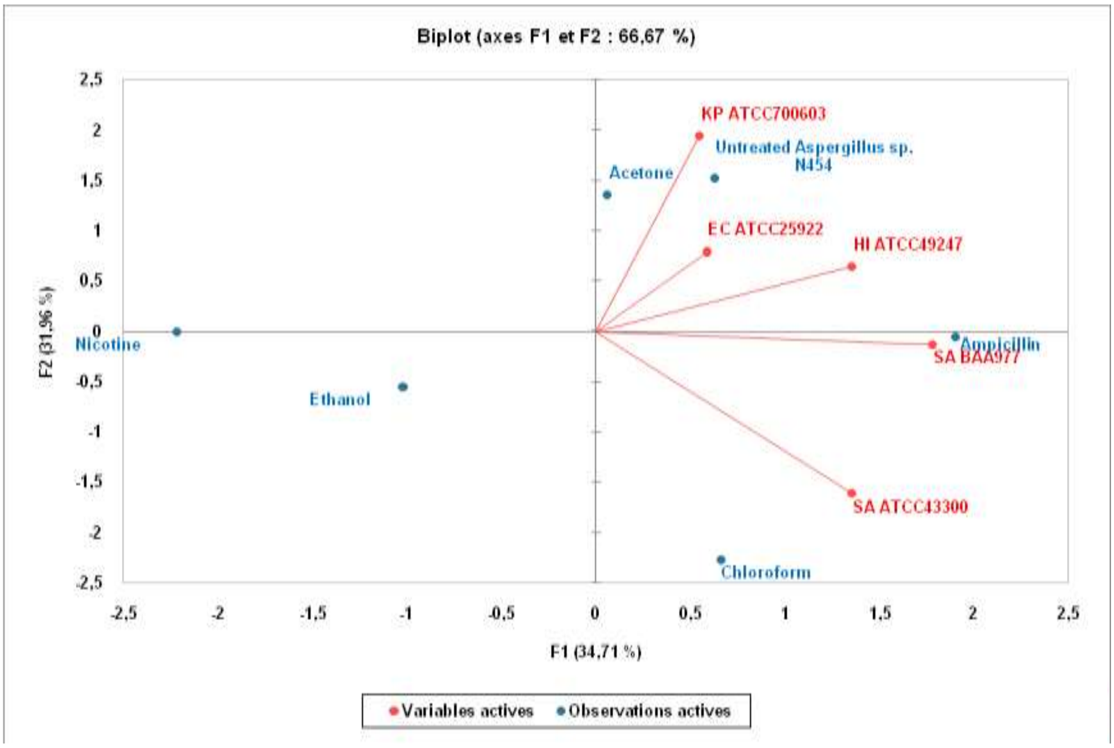


Figure 22: Biplot matrices of elicited extracts from *Aspergillus* sp. N454; *SA:Staphylococcus aureus; EC: Escherichia coli; HI: Haemophilus influenzae; KP: Klebsiella pneumoniae.*

III.2 Discussion

Bacteria resistance to antibiotics has enhanced dramatically, especially in the past few years (**Fair and Tor, 2014)**. Therefore, mining for novel natural compounds is of great importance **(Taylor, 2013)**. The antibacterial activity of the crude ethyl acetate extracts of different endophytic fungi isolated from *Cananga odorata*, *Terminalia mantaly*, and *Terminalia catappa* was evaluated to search for potential secondary metabolites that can be further investigated for the discovery of active principle against pneumonia. Endophytic fungi have enormous potential to produce an extensive range of bioactive secondary metabolites with therapeutic potential like antibacterial, antifungal, and antioxidant molecules **(Toghueo *et al*., 2019; Toghueo, 2020; Toghueo *et al*., 2020)**. In the present study, a total of 8 (13%) strains showed excellent antibacterial activity with a broad spectrum, among which extracts from endophytic fungi *Aspergillus* sp. N454, *Aspergillus* sp. N13, *Aspergillus* sp. N18 and *Curvularia* sp. N101 was the most active against all tested bacteria. It is reported that *Aspergillus* and *Curvularia* genera fungi produce antimicrobial compounds **(Han *et al*., 2014; Liu *et al*., 2017)**.

The mode of antibacterial action was investigated to understand the mechanism by which active extracts kill bacteria (*H. influenzae* and *E. coli*). The bacteriolysis assay revealed that the inhibitory effect exhibited by the crude extracts could be related to bacterial cell lysis. The inhibitory effect obtained with endophytic fungi extracts suggested the hypothesis that active metabolites content in each extract might have entered the bacterial cell through bacterial porins, after which they might have affected the bacterial enzymes, causing cell lysis and death **(Kotzekidou *et al*., 2008)**. Additionally, the cell lysis observed may be due to the weakening of the cell wall and the subsequent rupture of the cytoplasmic membrane due to osmotic pressure rather than a specific action on the cytoplasmic membrane **(Carson *et al*., 2002)**. Furthermore, the permeability of the outer membrane of *E. coli* ATCC 25922 and *H. influenzae* ATCC 49247 was significantly impaired by the addition of fungal extract into the culture, as indicated by the decrease of the relative absorbance in the bacterial solution. The outer membrane of Gram-negative bacteria is made by lipoproteins, phospholipids, and strong lipopolysaccharide molecules, which confer to her impermeability to various antibacterial agents **(Sharma *et al*., 2013)**. The control of the permeability of the cellular membrane is a crucial regulatory factor for various cellular functions, including cell metabolism maintenance, solute transport, and energy transduction processes **(Cox *et al*., 1998; Ayres *et al*., 1999)**. These potent extracts may contain hydrophobic compounds which enhance its accumulation inside periplasmic space, inducing the degradation of some necessary bacterial enzymes such as alkaline phosphatase leading to cellular lysis **(Carson *et al*., 2002; Hao *et al*., 2009; Moreira *et al*., 2005; Lambert *et al*., 2001)**. Further investigation on the action of extracts on bacteria membrane shows that each active extract tested may also have ability to alter the permeability of the bacterial cell by reducing its osmoregulatory ability to penetrate or exclude toxic materials resulting in their loss of salt tolerance **(Miksusanti *et al.*, 2008)**. This hypothesis is supported by the fact that the treatment of *E. coli* and *H. influenzae* with fungal extracts reduced the ability of the bacteria to form colonies on media containing KCl. Many investigators also reported the studies on salt tolerance of bacteria (**Carson *et al*., 2002; Bajpai *et al*., 2013;**

Patra and Baek, 2016). However, when *E. coli* ATCC 25922 and *H. influenzae* ATCC 49247 were treated with endophytic fungi extracts they did not significantly lose 260-nm-absorbing materials, suggesting that nucleic acids were not lost through a damaged on the cytoplasmic membrane. The inactivity of extracts on the inner membrane compared to the outer membrane can be explained by their difference in composition, which is essentially made of phospholipids (**Carson *et al*., 2002)**.

Several pathogens produced catalase to defend themselves against attacks by hydrogen peroxide, a weapon commonly used by macrophages, in addition to oxidative stress **(Iwase *et al*., 2013)**. Fungal extracts tested revealed a high inhibition of catalase activity of *E. coli* and *H. influenzae*, indicating that those extracts can be used to prevent DNA damage caused by hydroxyl radicals (OH-) issued from the decomposition of hydrogen peroxide by pathogenic bacteria **(Iwase *et al*., 2013)**. The potential of extracts to prevent oxidative stress was also investigated through the DPPH radical scavenging assay and FRAP reducing power. Antioxidants are known to prevent oxidative stress-mediated toxicity caused by oxygen-free radicals **(Hajhashemi *et al*., 2010)**. It is evident from the results that the fungal extracts contain radical scavenging compounds as previously reported **(Toghueo *et al*., 2016c; Patil *et al*., 2014; Tejesvi *et al*., 2008)**. Iron is essential for life because it is required for oxygen transport, respiration, and the activity of many enzymes. However, iron is highly reactive and catalyzes oxidative changes in lipids, proteins, and other cellular components. It causes lipid peroxidation through the reaction and decomposes the lipid hydroxide into peroxyl and alkoxyl radicals, perpetuating the chain reactions **(Govindappa *et al*., 2013)**. This study may be noted that only endophytic fungal extract from *Aspergillus* sp. N13 showed promising reducing potential indicating its antioxidant potential.

The results obtained are precise concerning the non-cytotoxicity of fungal extracts from *Aspergillus* sp. N454, *Aspergillus* sp. N18, and *Curvularia* sp. N101. However, *Aspergillus* sp. N13 exhibited high cytotoxic activity against Vero cells. This cytotoxicity could be attributed to the quality and quantity of compounds produced by this isolate. Endophytic fungi, particularly from the *Aspergillus* genus, are excellent producers of potent cytotoxic metabolites **(Wang *et al*., 2006)**. The difference observed in cytotoxicity found among active extracts from the *Aspergillus* genus could be due to culture conditions that could have favored or disfavored the production of toxic compounds **(Fischer and Dott, 2003; Jarvis and Miller, 2005)**. The previous investigation of endophytic fungal *Aspergillus* sp. 58 isolated from the bark of *T. catappa* showed that ethyl acetate extract obtained from a culture in potatoes dextrose broth was nontoxic, however, when the growth medium was supplemented with DMSO, the extract obtained was highly cytotoxic against HEK293T cell line **(Toghueo *et al*., 2018)**. **Watanabe *et al.*, (2004)** also reported that *Aspergillus fumigatus* cultured in high oxygen concentration produced toxic gliotoxins compounds. **Kamei *et al*., (2002)** also reported the production of cytotoxic molecules produced by *Aspergillus fumigatus* when cultured in the presence of macrophages.

The antibacterial-guided fractionation of ethyl acetate extract from *Aspergillus* sp. N454 led to ten fractions (F1 – F10) of which fraction F2 being the most potent with activity magnification by 2 folds against *H. influenzae* ATCC 49247, *P. aeruginosa* HM 601, and *K. pneumoniae* ATCC 13883. The subsequent fractionation of this active pool F2 led to 17 sub-fractions (KIMS1-KIMS17), out of which sub-fraction KIMS4 showed excellent activity with 10-40-fold improvement in potency regardless of the pathogenic strains tested. The antibacterial effect of fractions and sub-fractions is probably due to the presence of active constituents in the mixture. Indeed, the compositional analysis of active sub-fractions KIMS4, KIMS5, KIMS14, and KIMS17 led to the detection of 13, 7, 6, and 6 compounds respectively. Some of these compounds were already isolated in fungi culture and their antibacterial activity reported. Thus, N-Acetyl tryptamine, Usnic acid, Sinapic acid, Kaurane-17,18-dioic acid, 9,10-Epoxyoctadecenoic acid, 1-Hexadecanoylglycerol, Gibberellin A4 or A7, cyclo (Leu-Pro), cyclo (L-Leu-L-Pro), and Methyl 3,4,5-trimethoxycinnamate identified through UPLC-MS belonged to several classes, including alkaloids, steroids, glycosides and flavonoids. These classes of phytochemicals are known to be associated with antibacterial effects **(Strobel and daisy 2003)**. Flavonoids are known to affect the activity of sortase, which is an enzyme responsible of the adhesive property of bacterial cell wall, leading to interruption of biofilm development **(Santiago *et al*., 2015; Cushnie and Lamb, 2011; Saleem *et al*., 2010; Ozçelik *et al*., 2006)**. The steroids probably influenced the membrane integrity in all organisms **(Perumal *et al*.,2013)**. The antibacterial activity of sinapic acid has been demonstrated in various studies on human pathogens. **Engels *et al*., (2012)** demonstrated the antibacterial activity of sinapic acid against *Escherichia coli* (MIC: 0.7 g/L) and *Staphylococcus aureus* (MIC: 0.3 g/L). Usnic acid was reported to inhibit the growth of numerous Gram-positive bacteria by inhibiting RNA synthesis, with additional direct mechanisms, such as impairment of DNA replication **(Maciag-Dorszynska *et al*., 2014)**. **Narasimha *et al*., 2004** reported that Cinnamic acid derivative exhibited strong antibacterial activity against Gram-positive and Gram-negative bacteria. Isomers of ent-kaurane diterpenoids were founded to possess antibacterial activity against methicillin-resistant *Staphylococcus aureus* strain with a MIC range of 0.05–0.50 mg/ml **(Yang *et al*., 2016)**. Hierarchical clustering has been recently utilized for fingerprinting using HPLC –MS, to ascertain the interrelationships between the phytochemical profiles of plant extracts and their biological activities **(Kicel *et al*., 2016; Xu *et al*., 2017; Wang *et al*., 2018; Zeng *et al*., 2018; Gan *et al*., 2019)**. In the present study, HCA regrouped the promising sub-fractions in different clusters of two and two members based on their respective phytochemical contents. These results led us to conclude that the potent antibacterial activity exhibited by the sub-fractions could be due to specific compounds that are distributed in each of the active sub-fractions.

Endophytic fungi, mainly *Aspergillus* species, have emerged as an ideal source for producing a variety of natural metabolic compounds that can efficiently be exploited as antibacterial sources **(Nielsen *et al*., 2009)**. Modern molecular methods have demonstrated that the genetic potential of fungi from *Aspergillus* genus in terms of producing new secondary metabolites and the number of bioactive compounds has been underestimated **(Fisch *et al*., 2009; Scherlach and Hewerck, 2009)**. It was shown that many biosynthetic genes of these fungi remain silent under standard laboratory conditions **(Fisch *et al*., 2009)**. One strategy that has successfully been applied to solve the above-highlighted difficulties in drug discovery is the application of fermentation in the presence of small organic chemicals **(Guo *et al*., 2014)**. Therefore, the effect of small chemical elicitors on the production of antibacterial metabolites by the endophytic fungi *Aspergillus* sp. N454 in culture was studied. The results achieved in this study indicated that small chemicals have a significant impact on the antibacterial activity and qualitative and quantitative diversity of secondary metabolites produced by *Aspergillus* sp. N454. The results revealed that prominent small chemical used mostly potentialized the antibacterial activity against gram negative bacteria than gram positive. This result can be explained by the difference in the chemical composition of the outer membrane which confer different resistant mechanisms to antibacterial agents in each group of bacteria as described by **Sharma *et al*., (2013).**Results also revealed that nicotine has considerably increased the antibacterial potential (PI from 2-64) followed by ethanol (PI fold from 2 to

32.05), chloroform (PI fold 2 to 128.20), and acetone (PI fold from 2-4). Numerous small organic chemicals have been founded to underpin the production of cryptic specialized metabolites through different mechanisms. Nicotinamide was reported as histone deacetylases inhibitors of class three (HDAC of class III) **(Moore *et al*., 2012)**. Ethanol and dimethyl sulfoxide were reported to act on gene activation by causing mistranslation and inducing stress response **(Chen *et al*., 2000; Pettit, 2011)**. The culture of *Phomopsis* sp. N114 in the presence of 1-butanol (1% v/v) significantly improved the antiplasmodial potency of the afforded extract by 36.3 and 8.72-fold against Pf3D7 and PfINDO strains, respectively **(Toghueo *et al*., 2018)**. Other organic solvents such as acetone, 1-butanol, chloroform, acetonitrile, and methanol have also been reported to induce a shift in the metabolic profile of fungi **(Pettit, 2011; Toghueo *et al*., 2016a, 2018)**.

Regarding the chemical composition, the HPLC profiling of extracts from *Aspergillus* treated with ethanol, nicotine, chloroform, and acetone showed in addition to the peaks found in control (untreated *Aspergillus* sp. N454), four, eight, three and two new peaks, respectively. Similarly, **Toghueo *et al*., (2016b)** reported induction of one new metabolite at 38.05 minutes by the fungus *Aspergillus niger* from *Terminalia catappa*. **Guo *et al*., (2014)** also reported that acetone was able to stimulate the production of new metabolites by *Eupenicillium* sp. Ethanol was also found by **Cueto *et al*., (2001)** to elicit the synthesis of a new chlorinated benzophenone antibiotic, pestalone, by the marine fungus *Pestalotia*. The HPLC profile of the extract from ethanol treatment inhibited the production of 3 compounds (compound 15 (RT≈10.524 min), 19 (RT≈11.777 min) and 20 (RT≈12.012 min)). This result suggests a possible inhibition of their synthesis in the presence of the chemical used. Likewise, **Toghueo *et al*., (2016b)** showed that the DMSO treatment inhibits the production of several compounds by *Aspergillus niger*. Comparison of the intensities of different peaks detected with HPLC indicated that the production of some compounds is elicitors-specific. The production of compounds 1 and 2 have increased more than double in the presence of nicotine and ethanol, respectively, while compound 6 production decreased when supplemented with nicotine and chloroform. Similar compositional variation trends were also observed with cyclohexane carboxaldehyde, 3,3-dimethyl-5-oxo- that was highly produced in the presence of ethanol (64.38%) in comparison to acetone (58.21%) **(Toghueo *et al*., 2016b)**. The results of hierarchical clustering analysis showed that the samples from various elicited cultures could be divided into three groups. This indicates that small chemical compounds significantly influence the content of constituents and therefore certainly lead to variation of pharmacological effects **(Kicel *et al*, 2016)**.

Conclusion and Perspectives

CHAPTER IV: CONCLUSION AND PERSPECTIVES

IV.1 Conclusion

This study reports the antibacterial and modes of action of some endophytic fungi's secondary metabolites against pneumonia related bacteria; the following conclusions were drawn.

Out of the 56 crude extracts tested, about 13% was considered very active, 66% partially active, and 21% non-active against all tested bacterial strains with MIC values ranging from 0.32µg/mL to 25µg/mL. The four more potent extracts (MIC<5µg/mL) (from *Aspergillus* sp. N454, *Aspergillus* sp. N13, *Curvularia* sp. N101, and *Aspergillus* sp. N18) significantly lysed the bacteria cells, increased outer membrane permeability, reduced salt tolerance, and inhibited bacterial catalase activity. They exhibited a DPPH free radical scavenging activity with IC_{50} ranging from 150.71 to 936.08 µg/mL. Three of the four potent extracts were non-cytotoxic against the Vero cells line (CC_{50}>100µg/mL).

Antibacterial guided fractionation of *Aspergillus* sp. N454 ethyl acetate crude extract lead to 10 fractions and 17 sub-fractions with fraction F2 (MIC: 0.39 - 12.5 µg/mL) and sub-fraction KIMS4 being the most active (MIC ranged 0.078 - 5 µg/mL). The fractionation of the crude extract to the subfractions have improved the antibacterial potential from 1.24 to 20 times. The chemical composition of most active sub-fractions showed the presence of 10 compounds, N-Acetyltryptamine, Usnic acid, Sinapic acid, Kaurane-1,18-dioic acid, 9,10-Epoxyoctadecenoic acid, 1 Hexadecanoylglycerol, Gibberellin A4, cyclo (Leu-Pro), cyclo (L-Leu-L-Pro), and Methyl 3,4,5-trimethoxycinnamate.

The presence of small chemicals elicitors in the cultured medium of *Aspergillus* sp. N454 increased the metabolites production up to 2.54 times with nicotine being the lead elicitor. They also increased proportion and the diversity of metabolites produced by *Aspergillus* sp. N454. The antibacterial activity in presence of elicitors was improved from 2 to 128 folds (MIC from 0.039 to 5 µg/mL) compared to the untreated culture. Extracts from cultures with ethanol, chloroform, acetone and nicotine mostly underpinned the antibacterial potential.

IV.2 Perspectives

This work is a starting point for discovering antibacterial compounds from endophytic fungi of Cameroonian medicinal plants. The followed-up study consists of:

- Perform large scale cultures of fungus *Aspergillus* sp. N454 in presence of elicitors for the isolation and identification of produced metabolites in presence of small chemical compounds;
- Realize the pharmacokinetic and pharmacodynamic studies of identified compounds.
- To study the in vivo effect of prominent compounds isolated

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CHAPITRE V: REFERENCES

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Appendices

APPENDICES

Appendices 1: Composition of Culture Media

Composition of Potatoes Dextrose Agar (PDA)

Potato peptone……………………………………………………………………4.0 g/L

Glucose…………………………………………………………………………20.0 g/L

Agar……………………………………………………………………………..15.0 g/L

pH……………………………………………………………………………..5.6

Composition of Potatoes Dextrose Broth (PDB)

Potato peptone……………………………………………………………………4.0 g/L

Glucose…………………………………………………………………………20.0 g/L

Final pH 5.6±0.2 at 25°C

Composition of Nutrient Agar (NA)

Beef extract ………………………………………………………………….1.0 g/L

Yeast extract ………………………………………………………………….2.0 g/L

Peptone ………………………………………………………………………….5.0 g/L

Sodium Chloride ……………………………………………………….….5.0 g/L

Agar ……….………………………………………………………………….15.0 g/L

Final pH 6.8 ± 0.2 at 25°C

Composition of Nutrient Broth (NB)

Beef extract ………………………………………………………………….1.0 g/L

Yeast extract ………………………………………………………………….2.0 g/L

Peptone ………………………………………………………………………….5.0 g/L

Sodium Chloride …………………………………………………….….5.0 g/L

Final pH 6.8 ±0.2 at 25°C

Appendices 2: Preparation of solutions and reagents

- **Preparation of MTT solution.**

12 mM MTT of stock solution was prepared by adding 1 mL of sterile PBS to one 5 mg vial of MTT (Component A). Mix using a vortex or sonication until dissolved and was stored for four weeks at 4°C protected from light. After that, 10 mL of 0.01 M HCl was added to one tube containing 1g of SDS. The solution was gently mixed by inversion or sonication until the SDS dissolves. For the cell viability assay, 1mL of MTT dye solution was diluted with 10 mL of complete DMEM. After the incubation time, 100 µL of DMSO100% was added to each well and mix thoroughly with the pipette, incubate at 37°C for 10 minutes.

- **10 X Phosphate Buffered Saline (PBS)**

80 g NaCl

2.0 g KCl (Merck)

14.4 g Na_2HPO_4 (Merck)

2.4 g KH_2PO_4 (Merck)

The ingredients were dissolved in ~800mL distilled H_2O, adjusted pH to 7.4. Then the final volume was adjusted to 1L with d'H_2O. The buffer was sterilized by autoclaving and stored at RT.

Appendices 3: Tables

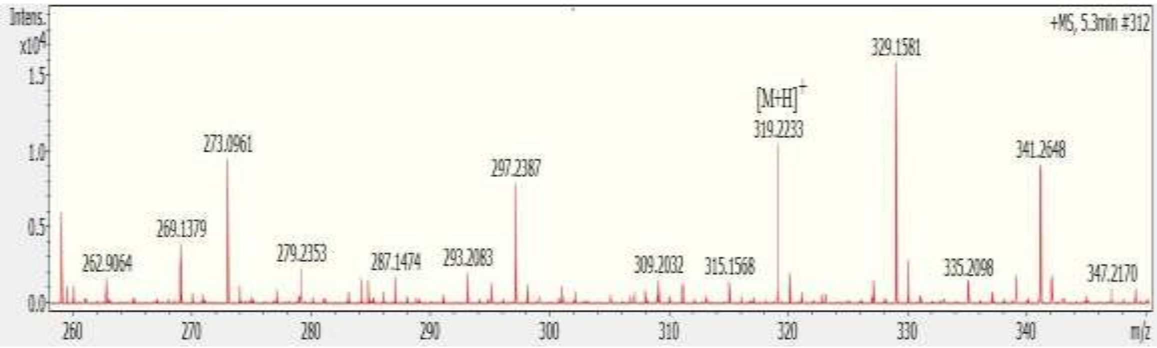
Table 1: Characteristics of bacterial strains used for antibacterial activity.

Bacterial strains	Acronym	References No	Characteristic
Streptococcus pneumoniae	*S. pneumoniae*	ATCC 49619	Clindamycin resistant strain
Klebsiella pneumoniae	*K. pneumoniae*	ATCC 13883	Sensitive strain
Pseudomonas aeruginosa	*P. aeruginosa*	HM 601	Sensitive strain
Haemophilus influenzae	*H. influenzae*	ATCC 49619	Sensitive strain
Escherichia coli	*E. coli*	ATCC 25922	Sensitive strain
Staphylococcus aureus	*S. aureus*	ATCC 43300	Methicillin and oxacyclin resistant strain
Staphylococcus aureus	*S. aureus*	BAA-977	Sensitive strain

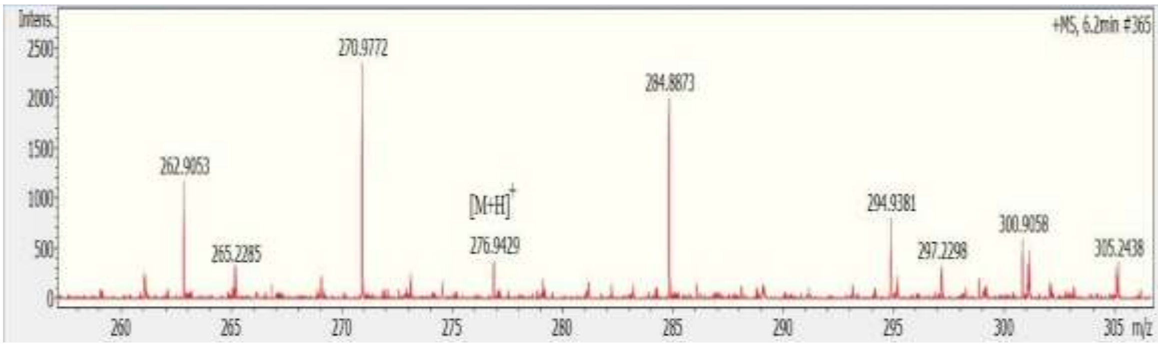
Abbreviations: ATCC, American Type Culture Collection;

Appendice4: Mass spectra of compounds identified in potent subfractions from the fractionation of Aspergillus sp. N454 extract

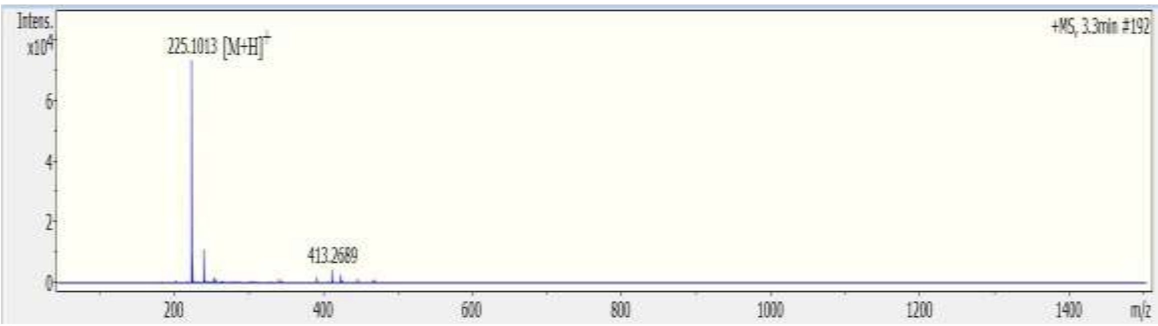
Appendices 4.1: HRESIMS spectrum of 9,10-Epoxyoctadecenoic acid



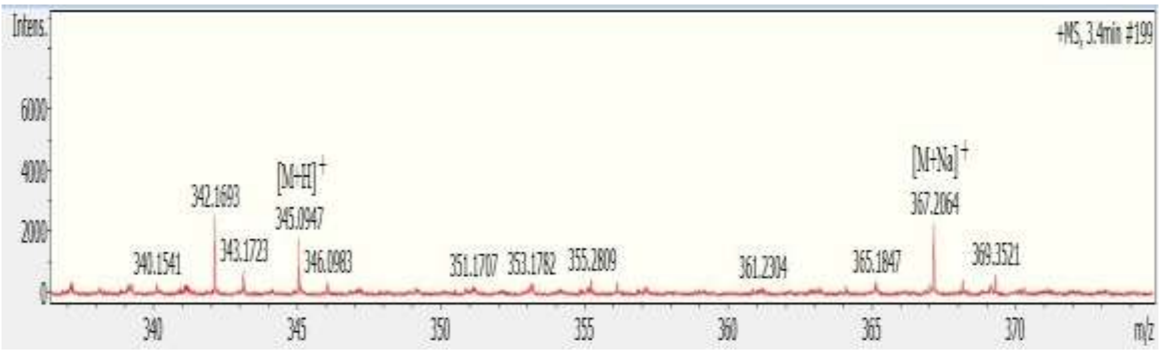
Appendices 4.2: HRESIMS spectrum of Methyl 3,4,5-trimethoxycinnamate



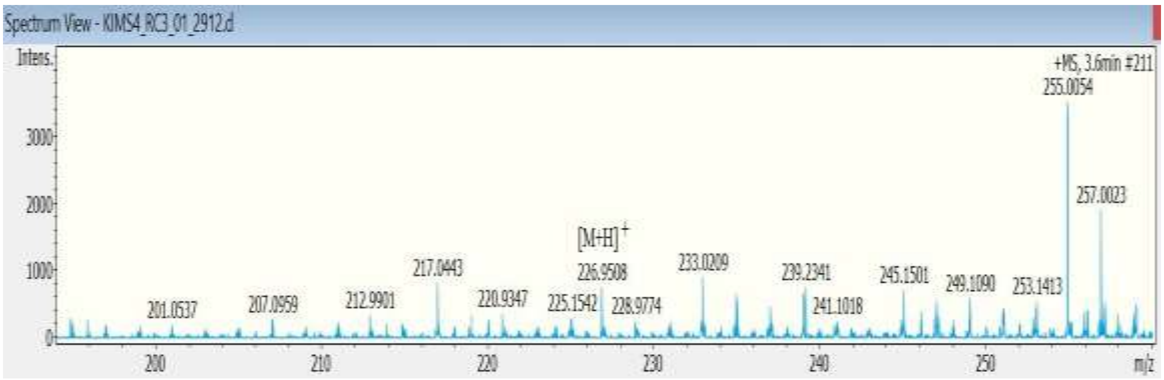
Appendices 4.3: HRESIMS spectrum ofN-Acetyl tryptamine



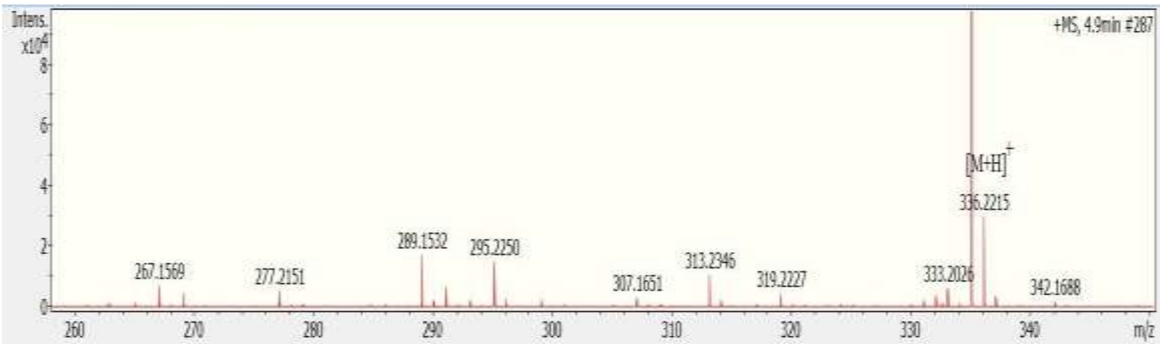
Appendices 4.4: HRESIMS spectrum of Usnic acid



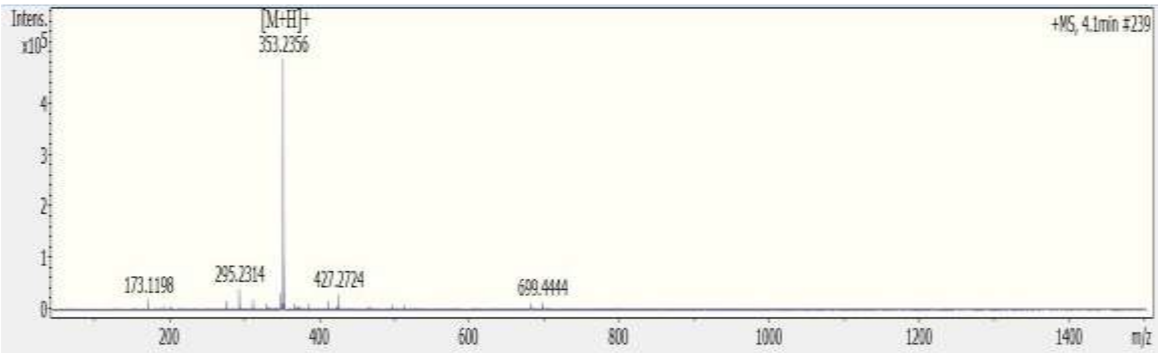
Appendices 4.5: HRESIMS spectrum of Sinapic acid



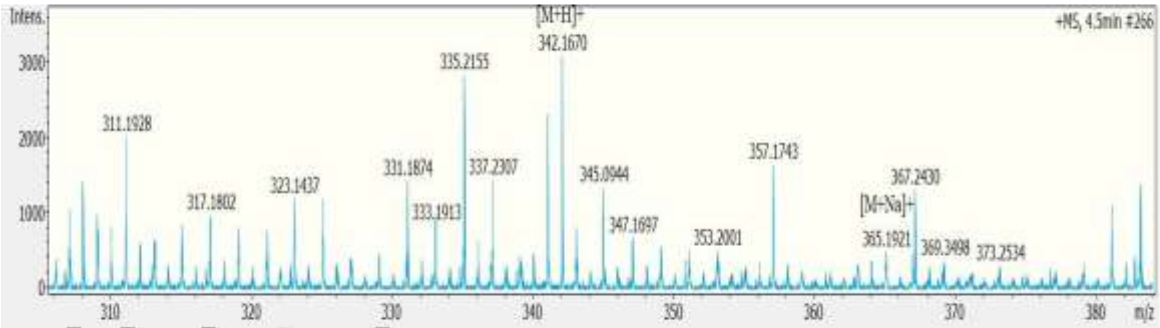
Appendices 4.6: HRESIMS spectrum of Kaurane-17,18-dioic acid



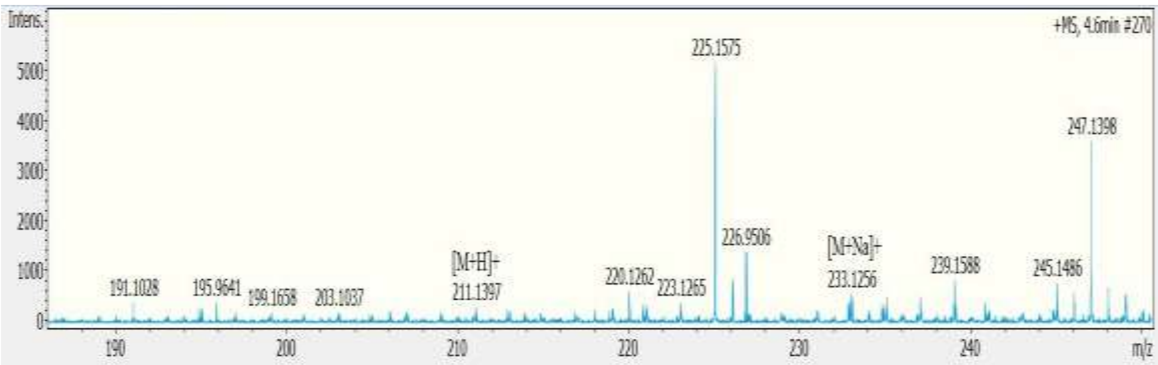
Appendices 4.7: HRESIMS spectrum of 1 Hexadecanoyl glycerol



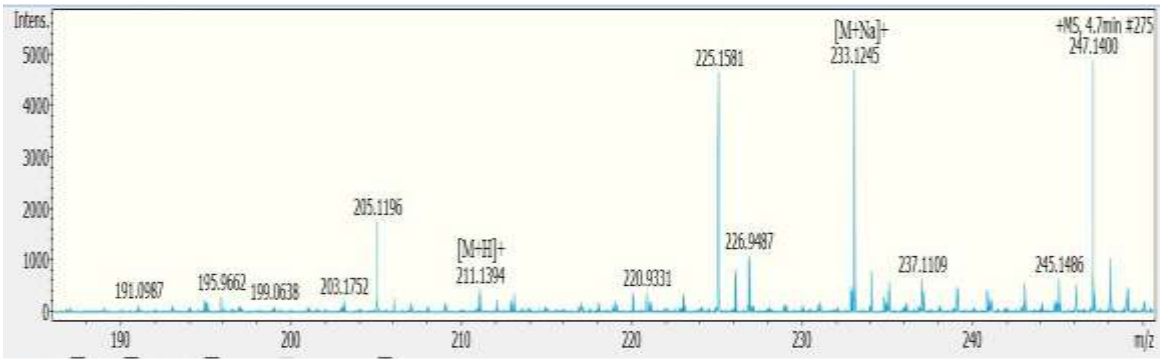
Appendices 4.8: HRESIMS spectrum ofGibberellin A4



Appendices 4.9: HRESIMS spectrum of Cyclo (Leu-Pro)



Appendices 4.10: HRESIMS spectrum of Cyclo(L-Leu-L-Pro)


Intens.
+MS, 4.7min #275
247.1400
[M+Na]+
233.1245
225.1581
205.1196
[M+H]+
211.1394
226.9487
191.0987
195.9662
199.0638
203.1752
220.9331
237.1109
245.1486
m/z

f

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Research Article

Antibacterial and Mode of Action of Extracts from Endophytic Fungi Derived from *Terminalia mantaly*, *Terminalia catappa*, and *Cananga odorata*

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Emerging drug-resistant bacteria creates an urgent need to search for antibiotics drugs with novel mechanisms of action. Endophytes have established a reputation as a source of structurally novel secondary metabolites with a wide range of biological activities. In the present study, we explore the antibacterial potential of endophytic fungi isolated from different tissues of *Terminalia mantaly*, *Terminalia catappa*, and *Cananga odorata*. The crude ethyl acetate extracts of 56 different endophytic fungi were screened against seven bacterial strains using the broth microdilution method. The antibacterial modes of action of the most active extracts (04) were evaluated using *E. coli* ATCC 25922 and *H. influenzae* ATCC 49247 strains. Both the DPPH and FRAP assays were used to investigate their antioxidant activity, and their cytotoxicity against the Vero cell line was evaluated using the MTT assay. Out of the 56 crude extracts tested, about 13% were considered very active, 66% partially active, and 21% nonactive against all tested bacterial strains with MIC values ranging from 0.32 μg/mL to 25 μg/mL. The four more potent extracts (MIC <5 μg/mL) (from *Aspergillus* sp. N454, *Aspergillus* sp. N13, *Curvularia* sp. N101, and *Aspergillus* sp. N18) significantly lysed the bacteria cells, increased outer membrane permeability, reduced salt tolerance, and inhibited bacterial catalase activity. They exhibited a DPPH free radical scavenging activity with IC_{50} ranging from 150.71 to 936.08 μg/mL. Three of the four potent extracts were noncytotoxic against the Vero cells line ($CC_{50} > 100\ \mu$g/mL). Results from this investigation demonstrated that endophytes from Cameroonian medicinal plants might content potent antibacterial metabolites. The bioguided fractionation of these potent extracts is ongoing to isolate and characterise potential active ingredients.

1. Introduction

Pneumonia, a type of acute respiratory tract infection that affects the lungs, is considered one of the significant public health concerns. This disease is caused by several infectious agents, including bacteria, viruses, fungi, and parasites with bacteria responsible for most cases of mortality and morbidity. In fact, in 2017, pneumonia caused by bacteria was responsible for 808 694 children's death worldwide, with most of the cases reported in developing countries [1, 2]. For instance, pneumonia was responsible for 33.5% of morbidity among children under-five at the Wondo Genet district in Ethiopia [3] and 11.5% of deaths of children under-five in Cameroon in 2018 [4]. For the management of pneumonia, vaccines for the prevention (23-valent pneumococcal polysaccharide vaccine (PPV23), pneumococcal conjugate vaccine (PCV 13)) are available despites their limited protection [5]. On the other hand, antibiotics recommended and usually prescribed for the treatment are costly, inaccessible by the indigenous population, have several undesirables side effects, and more importantly, there is an increasing loss of effectiveness due to the emergency multidrug-resistant bacteria strains [1, 6]. Therefore, it is crucial and urgent to search for novel, more effective, less toxic, and safe antibiotic drugs with multiple modes of action to fight pathogens resistance [7].

Over the past two decades, endophytic fungi have been reported as excellent sources of structurally novel and bioactive secondary metabolites which can constitute perfect starting points for developing new potential antibiotic drugs. Indeed, metabolites produced by endophytic fungi belong to several classes, including alkaloids, lignans, terpenoids, flavonoids, tannins, and steroids, and exhibit multiple biological activities including antibacterial [8–12]. Moreover, our previous study showed that extracts from some endophytic fungi from *Terminalia catappa*, *Terminalia mantaly*, and *Cananga odorata*, three Cameroonian medicinal plants exhibit potent inhibition against various pathogenic bacteria and parasites strains [13]. Therefore, we hypothesized that extracts from these endophytic fungi may produce active metabolites against bacteria pathogens causative agents of human pneumonia. The present study was undertaken to investigate the antibacterial and the modes of action of endophytic fungi extracts isolated from the plants mentioned above.

2. Materials and Methods

2.1. Endophytic Fungi Isolates and Extracts Preparation. A total of fifty-six isolates (Supplementary material 1) of endophytic fungi from *Terminalia catappa* (51244/HNC), *Terminalia mantaly* (64212/HNC), and *Cananga odorata* (42250/HNC) were used in this study [14]. For extract preparation, each fungal isolate was cultivated by placing agar blocks of actively growing pure culture (3 mm diameter) in a 200 mL Erlenmeyer flask containing 100 mL of Potatoes Dextrose Broth (PDB) medium (Sigma Aldrich, USA). Flask was incubated at 25°C for 7 days. After the incubation period, 100 mL of ethyl acetate was added to each culture, shaken, and kept overnight at room temperature. The mixture was then transferred to a separatory funnel, the organic phase was collected, and the solvent was then removed at 70°C using a rotary vacuum evaporator (Heidolph, Germany). The dry solid residue was prepared at 25 mg/mL in DMSO 100% (Loba Chemie, India) and kept at 4°C before the antibacterial screening. The positive controls Amoxicillin and Ciprofloxacin were prepared at 2 mg/mL in sterile distilled water.

2.2. Bacteria and Culture Conditions. The bacteria strains used in this investigation were obtained from the American Type Culture Collection (ATCC) including *Streptococcus pneumoniae* ATCC 49619, *Klebsiella pneumoniae* ATCC 13883, *Staphylococcus aureus* BAA-977, *Staphylococcus aureus* ATCC 43300, *Haemophilus influenzae* ATCC 49247, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* HM 601. Twenty-four hours before each experiment, bacteria were subcultured on nutrient agar tubes at 37°C.

2.3. Determination of Minimum Inhibitory Concentration (MIC) of Extracts. The minimum inhibitory concentration (MIC) of extracts was determined according to the M07-A9 Clinical Laboratory Standards Institute microdilution method using 96-wells microtitre plates [15]. Briefly, 4 μL of extracts and reference drugs (Amoxicillin and Ciprofloxacin) from stock solutions were introduced in the well followed by the addition of 96 μL of bacteria inoculum standardised at 10^6 CFU/mL. A blank column was included for sterility control, while bacterial strains in the culture medium without any inhibiting substance were negative control. The concentrations of extracts ranged from 0.195 μg/mL to 25 μg/mL, and that of Ciprofloxacin and amoxicillin ranged from 0.562 μg/mL to 128 μg/mL. After 24 hours of incubation at 37°C, the turbidity was observed as an indication of growth. MIC was defined as the lowest concentration inhibiting the visible growth of bacteria. All tests were performed in triplicate.

2.4. Determination of Possible Modes of Action of Promising Extracts

2.4.1. Measurement of the Lytic Activity of Extracts. The determination of extracts' lytic activity was performed as described by Limsuwan and Voravuthikunchai [16]. An overnight bacterial culture was used to prepare bacterial suspension at 0.5 Mc Farland in NaCl 0.9%. The bacterial suspension was treated with extracts at MIC, 2 MIC, and 4 MIC and incubated at 37°C. The optical density (OD) at 620 nm was measured at four different periods, including 0 h, 1 h, 2 h, and 4 h using the microplate reader Infinite M200 (TECAN). A decrease in OD 620 nm indicated bacterial cell lysis. Corresponding dilutions of the extract were used as blanks. Ciprofloxacin at 2 μg/mL was used as a positive control. The results were expressed as a ratio of the OD at each time interval versus the OD at 0 min (in %). All assays were carried out in triplicate.

2.4.2. Integrity of the Cell Membrane. The integrity of the cell membrane of *Haemophilus influenzae* ATCC 49247 and *Escherichia coli* ATCC 25922 was carried out as previously described by Carson et al. [17] with slight modifications. Briefly, the test bacteria in the exponential growth phase were washed and suspended in sterile peptone water (0.1 g/100 mL). The bacterial strains (5.10^7 CFU/mL) were incubated with extracts at 4 MIC for different periods (0, 30, 60, 90, and 120 min). The mixtures were then centrifuged at 5000 rpm for 10 minutes, after which the UV absorbance of the supernatant was measured at 260 nm using the microplate reader Infinite M200 (TECAN). The untreated bacterial cultures in sterile peptone water served as the negative control. Ciprofloxacin at 2 μg/mL was a positive control, and each test was performed in triplicate. Results were expressed in terms of the optical density of 260 nm absorbing materials in each interval for the ultimate time.

2.4.3. Outer Membrane Permeability Assay. Outer membrane (OM) permeability of *H. influenzae* ATCC 49247 and *E. coli* ATCC 25922 was determined according to the method described by Oliviera et al. [18] with small modifications.

An overnight culture (5.10^7 CFU/mL) was inoculated into nutrient broth containing the extracts at MIC, 2MIC, and 4 MIC. The media was then poured into sterilised 96-well microplates (100 μL) and incubated at 37°C for 24 h. After the incubation time, the growth of *H. influenzae* ATCC 49247 and *E. coli* ATCC 25922 was measured at 450 nm using the Microplate Reader Infinite M200 (TECAN). The graph of bacterial growth parameter (OD/450 nm) in the function of extract concentration (μg/mL) was plotted. Ciprofloxacin (concentration ranged from 1 to 4 μg/mL) was used as positive control, and each test was conducted in triplicate.

2.4.4. Salt Tolerance Assay. The salt tolerance effect of *H. influenzae* ATCC 49247 and *E. coli* ATCC 25922 treated with the MIC concentration of the extracts was evaluated on nutrient agar plates supplemented with different potassium concentrations chloride (KCl) [19]. 24 h before the experiment, bacteria strains were cultured on a nutrient agar medium and incubated at 37°C. The overnight culture was treated with extracts at 4 MIC and further incubated for 60 min at 37°C. The samples were then serially diluted (Dilution Factor = 100) and inoculated on nutrient agar plates supplemented with different concentrations of KCl (0%, 2.5%, 5.0%, and 10.0%). Each plate was incubated for 24 hours at 37°C. Ciprofloxacin at 4 μg/mL was used as a positive control. After the incubation period, both the controls and treated plates were compared, the colonies were counted, and the results were expressed in terms of Log10 CFU/mL. The experiment was performed in triplicate.

2.4.5. Inhibition of Catalase Activity. The catalase inhibitory activity of the extracts was evaluated using the protocol described by Weydert and Cullen [20] with slight modifications. Extracts at the MIC concentration were added in a test tube containing 400 μL of hydrogen peroxide (40 mM) and 400 μL of PBS. The mixture then transferred in another tube containing 200 μL of a bacterial suspension (1.5×10^8 UFC/mL). The samples were incubated at 37°C for 30 min; after which, they were centrifuged at 1200 rpm for 10 min. The supernatants were collected, and their optical density (OD) read at 232 nm. The phosphate buffer constituted the blank, while bacterial strains in phosphate buffer without any inhibiting substance was used as a negative control. The mixture of Ciprofloxacin, phosphate buffer, and bacterial strain constituted positive control. The percentage of remaining hydrogen peroxide was determined according to the following formula (1).

$$\%\text{of remaining H2O2} = \frac{\left(A_{\text{sample}} - A_{\text{negative control}}\right) \times 100}{A_{\text{negative control}}}. \quad (1)$$

$A_{\text{negative control}}$ is the absorbance of H_2O_2 without the extract, and A_{sample} is the absorbance of H_2O_2 with the extract.

2.5. Evaluation of the In Vitro Antioxidant Potential and Cytotoxicity of Promising Extracts

2.5.1. DPPH Radical Reduction Assay. DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical scavenging assay was performed according to the method described by Scherer and Godoy [21]. Briefly, 25 μL of extracts dissolved in methanol were added to wells of a microtiter plate in triplicate followed by 75 μL of DPPH solution (0.01%) to yield extract solution concentration ranging from 1000 to 1.95325 μg/mL. The content was mixed and incubated for 30 min in the dark at 25 ± 2°C after which the absorbance was taken at 517 nm using a Microplate Reader Infinite M200 (TECAN). Ascorbic acid was used as a standard antioxidant with final concentrations ranging from 25 to 0.195 μg/mL. The results were expressed through the calculation of the DPPH• inhibition percentage according to the following formula (2).

$$\text{Inhibition of DPPH (\%)} = \frac{\left(A_{\text{control}} - A_{\text{sample}}\right) \times 100}{A_{\text{control}}}, \quad (2)$$

where A_{control} is the DPPH• radical absorbance without the extract and A_{sample} is the DPPH• absorbance with the extract.

The concentration of extract proportional to a 50% inhibition of DPPH• radical (IC_{50}) was obtained by analysing the extract solution concentration versus inhibition percentage graphic. Thus, lower extract concentrations (μg/mL) mean greater antioxidant capacity provided by the analysed extract.

2.5.2. Ferric Ion Reducing Antioxidant Power (FRAP) Assay. The ferric reducing antioxidant power of potent extracts was determined using the method described by Benzie et al. [22]. 25 μL of extracts at different concentrations (7.8125-4000 μg/mL) were introduced into a microtiter plate, and 25 μL of a solution of Fe^{3+} at 1.2 mg/mL were added. The plates were preincubated for 15 min at room temperature, and 50 μL of 0.2% ortho-phenanthrolin were then added to obtain a final extract concentration ranging from 1000 to 1.95325 μg/mL. The reaction mixtures were further incubated for 15 min at room temperature, and the absorbance was measured at 505 nm using a microplate reader Infinite M200 (TECAN) against the blank (made of 25 μL methanol +25 μL Fe3++50 μL ortho-phenanthrolin). Ascorbic acid was used as positive control and tested at concentrations ranging from 0.103 to 6.60 μg/mL. The assay was performed in triplicate.

2.5.3. Evaluation of the Cytotoxicity of Promising Extracts. The cytotoxic effect of antibacterial extracts was assessed using the MTT assay [23], targeting normal monkey kidney Vero cells ATCC CRL1586 cultured in complete medium containing 13.5 g/L DMEM (Gibco, Waltham, MA USA), 10% fetal bovine serum (Gibco, Waltham, MA, USA), 0.21% sodium bicarbonate (Sigma-Aldrich, New Delhi, India), and 50 μg/mL gentamicin (Gibco, Waltham, MA, USA). Essentially, Vero cells at 5×10^3 cells/200 μL/well were seeded into 96-well flat-bottomed tissue culture plates

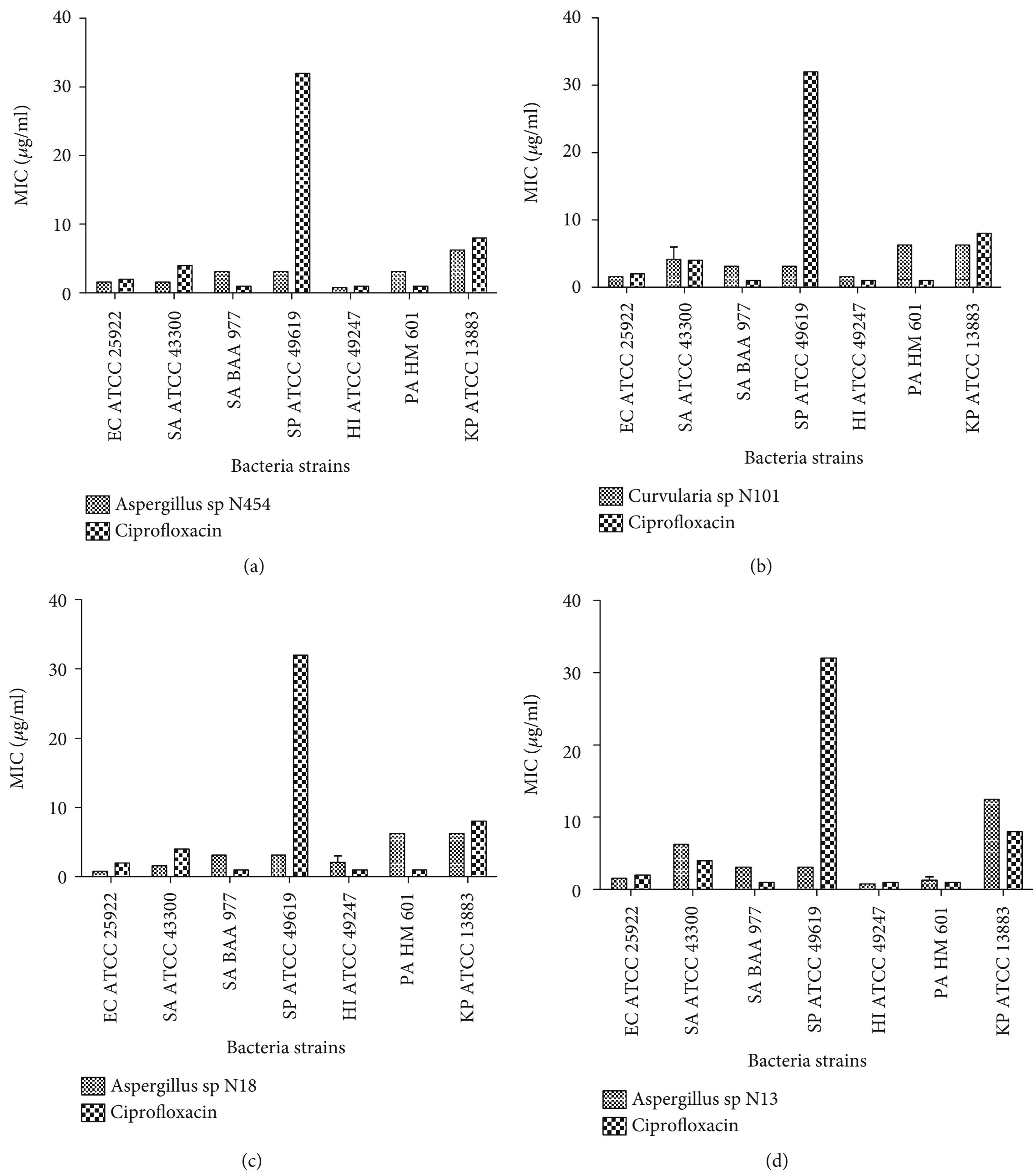


Figure 1: Minimal inhibitory concentration (MIC) of the most potent extracts on bacteria strains. The errors bars represent the standard deviation of measurement of a sample in three separate runs. (a) *Aspergillus* sp. N454. (b) *Curvularia* sp. N101. (c) *Aspergillus* sp. N18. (d) *Aspergillus* sp. N13. SA: *Staphylococcus aureus*; EC: *Escherichia coli*; SP: *Streptococcus pneumoniae*; HI: *Haemophilus influenzae*; PA: *Pseudomonas aeruginosa*; KP: *Klebsiella pneumoniae*.

(Corning, USA) in complete medium. Fifty microliters of serially diluted extract solutions ($\leq$200 μg/mL) were added after 24 h of seeding and cells plus test substance incubated for 48 h in a humidified atmosphere at 37°C and 5% CO_2. DMSO (0.4% (v/v) was added as negative control (100% growth). Twenty microliters of a stock solution of MTT (5 mg/mL in 1x phosphate-buffered saline) were added to each well, gently mixed, and incubated for an additional 4 h. After spinning the plate at 1500 rpm for 5 min, the supernatant was carefully removed and 100 μL of 100% DMSO (v/v) was added to dissolve the formazan. The plate was read on a microtiter plate reader (Infinite M200 (TECAN)) at 570 nm. The 50% cytotoxic concentrations (CC_{50}) of extracts were determined by analysis of the dose-response curves.

2.6. Statistical Analysis. Data collected from at least three independent experiments were analysed using One-Way ANOVA using Graph Pad Prism 5. Data are expressed as mean ± SD of experiments performed in triplicate. Error bars represent the SD, and a, b, and c represent $p < 0.05$, $^{**}p < 0.001$, $^{***}p < 0.0001$, respectively, significant difference compared to untreated sample.

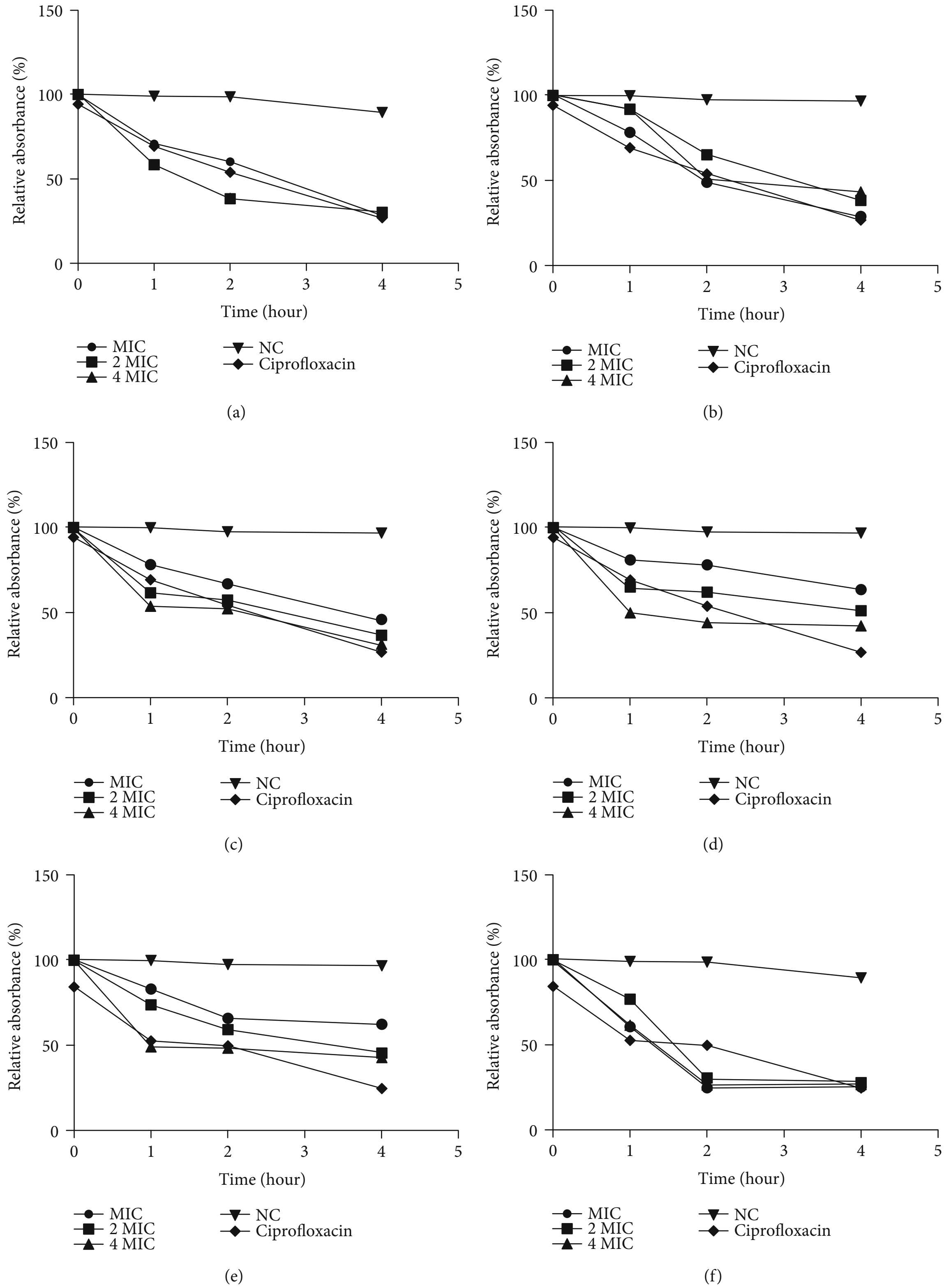


Figure 2: Continued.

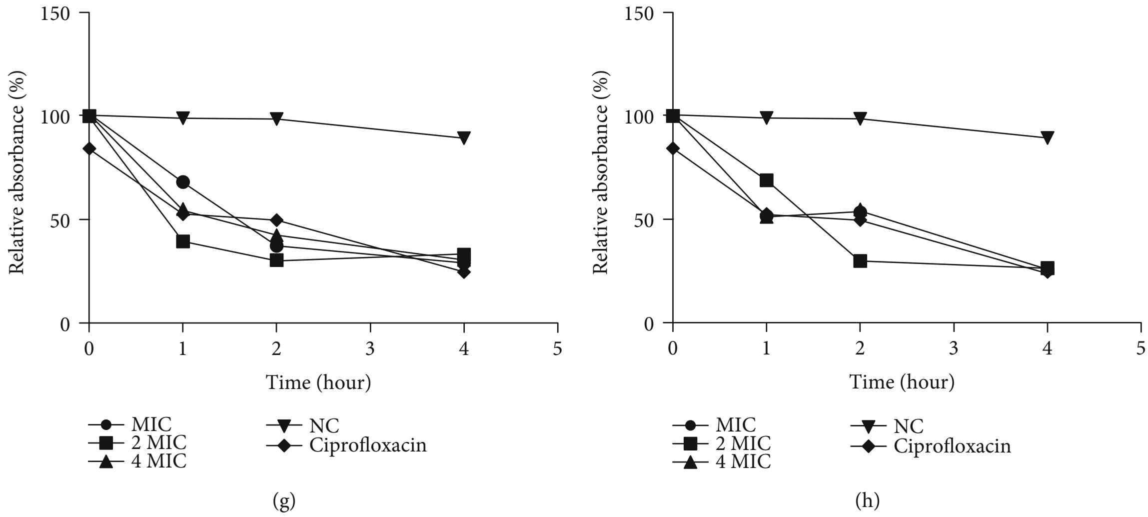


Figure 2: Bacteriolytic activity of endophytic fungi extracts on *E. coli* ATCC 25922 and *H. influenzae* ATCC 49247. (a) *Aspergillus* sp. N454 on *E. coli*. (b) *Aspergillus* sp. N18 on *E. coli*. (c) *Aspergillus* sp. N13 on *E. coli*. (d) *Curvularia* sp. N101 on *E. coli*. (e) *Aspergillus* sp. N454 on *H. influenzae*. (f) *Aspergillus* sp. N18 on *H. influenzae*. (g) *Aspergillus* sp. N13 on *H. influenzae*. (h) *Curvularia* sp. N101 on *H. influenzae*. Data are expressed as the mean ± SD. MIC: minimal inhibitory concentration; NC: negative control.

3. Results

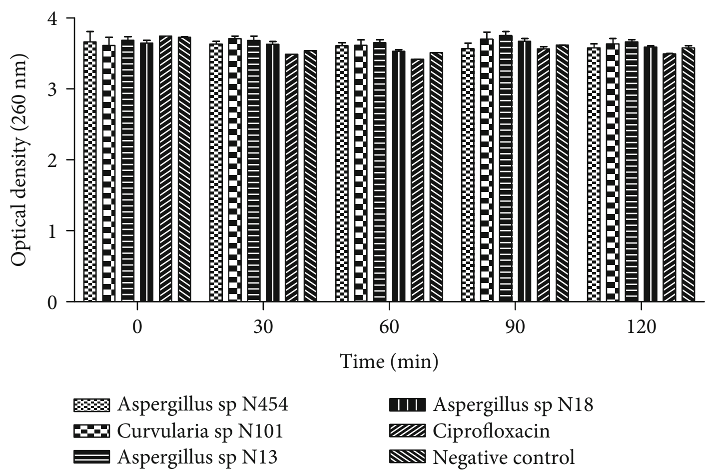
3.1. Extraction Yield and Antibacterial Potency of Endophytic Extracts. The extraction yield of endophytic fungi ranged from 52 to 200 mg for 200 mL of culture medium, with the *unidentified fungal* N445 from *C. odorata* root producing the higher amount of metabolites (Supplementary material 2). Each of the fifty-six extracts was screened for antibacterial activity against seven pathogenic bacteria. The MIC ranged from 0.39 to >25 μg/mL depending on the fungal extract and the tested pathogen (Supplementary material 2). Our criteria for the activity of extracts against bacteria were defined as follows: very active (MIC <5 μg/mL), partially active (MIC 5-15 μg/mL), and nonactive (MIC >15 μg/mL). Therefore, out of the 56 extracts, eight were very active (13%) with the more potent (MIC <5 μg/mL) being extracts from *Aspergillus* sp. N454, *Aspergillus* sp. N18, *Curvularia* sp. N101, and *Aspergillus* sp. N13 isolated, respectively, from *C. odorata* and *T. catappa* (Figure 1). Thirty-seven extracts (66%) showed moderate potency with MIC between 5 and 15 μg/mL, while 12 extracts (21%) with MIC >15 μg/mL were considered as inactive. Overall, endophytic *Aspergillus* spp. exhibited the best activity (MIC ranged from 0.78 to 6.25 μg/mL), while *E. coli* ATCC 25922 and *H. influenzae* ATCC 49247 were the more sensitive pathogenic strains. To continue our investigation, the four most prominent crude extracts, including *Aspergillus* sp. N454, *Aspergillus* sp. N18, *Curvularia* sp. N101, and *Aspergillus* sp. N13 (Figure 1), were selected for the mode of action and antioxidant studies.

3.2. The Mode of Action of Promising Extracts

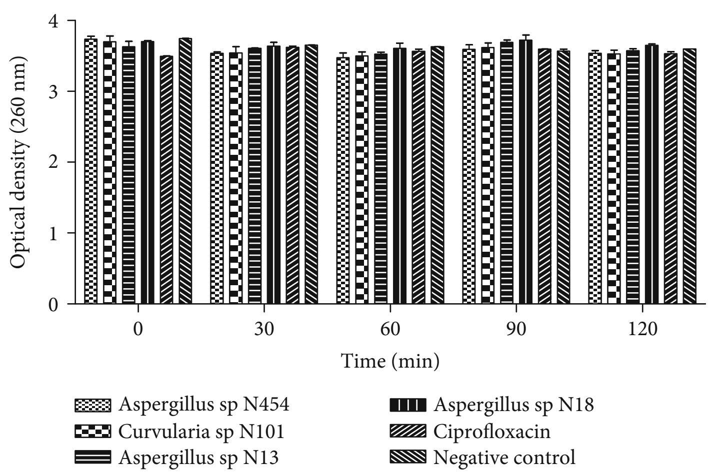
3.2.1. Bacteriolytic Effect of Selected Extracts. The bacteriolysis assay was performed to investigate if active extracts inhibit *H. influenzae* ATCC 49247 and *E. coli* ATCC 25922 through cell lysis. A viable bacteria absorb light at 620 nm; therefore, bacteriolysis occurs if the optical density of the medium at 620 nm decreased with time [17]. The cell lysis activity exhibited by fungi extracts is summarised in Figure 2. Globally, the bacterial cells' treatment with fungal extracts caused gram-negative bacteria cell lysis at all tested concentrations. This cell lysis was more significant than 50% and 55% for *E. coli* and *H. influenzae*, respectively, after 4 hours of incubation at 2 MIC and 4 MIC depending on the tested extract. The reduction of the bacterial population was more drastic in the case of *H. influenzae* than *E. coli*. Overall, the bacteriolysis was higher with an extract from *Aspergillus* sp. N18 (71%) on *E. coli* at 4 MIC and *Curvularia* sp. N101 (75%) on *H. influenzae* at 2 MIC, respectively.

3.2.2. The Effect of Extracts on the Integrity of the Cell Membrane. The cytoplasmic membrane's integrity was analysed by determining the release of cellular materials including nucleic acids, proteins, metabolites, and ions, which were absorbed at 260 nm into the bacterial suspensions. Treatment of *H. influenzae* ATCC 49247 and *E. coli* ATCC 25922 with potent fungal extracts at the MIC concentrations indicated no significant cell leakage of 260 nm absorbing material in a time-dependent manner. This absence of nucleotide leakage was observed until the 120th minute of incubation (Figures 3(a) and 3(b)). The same result was obtained in the control group treated with Ciprofloxacin, revealing its disability to damage the tested bacteria strains' cytoplasmic membrane.

3.2.3. The Effect of Extracts on the Permeability of Outer Cell Membrane. Bacterial cell membrane permeability was determined in terms of optical density at 450 nm (Figures 4(a) and 4(b)). Measurement of the optical density of bacterial cells treated with endophytes extracts demonstrated that extracts from *Aspergillus* sp. N454, *Aspergillus* sp. N18, *Aspergillus*



(a)



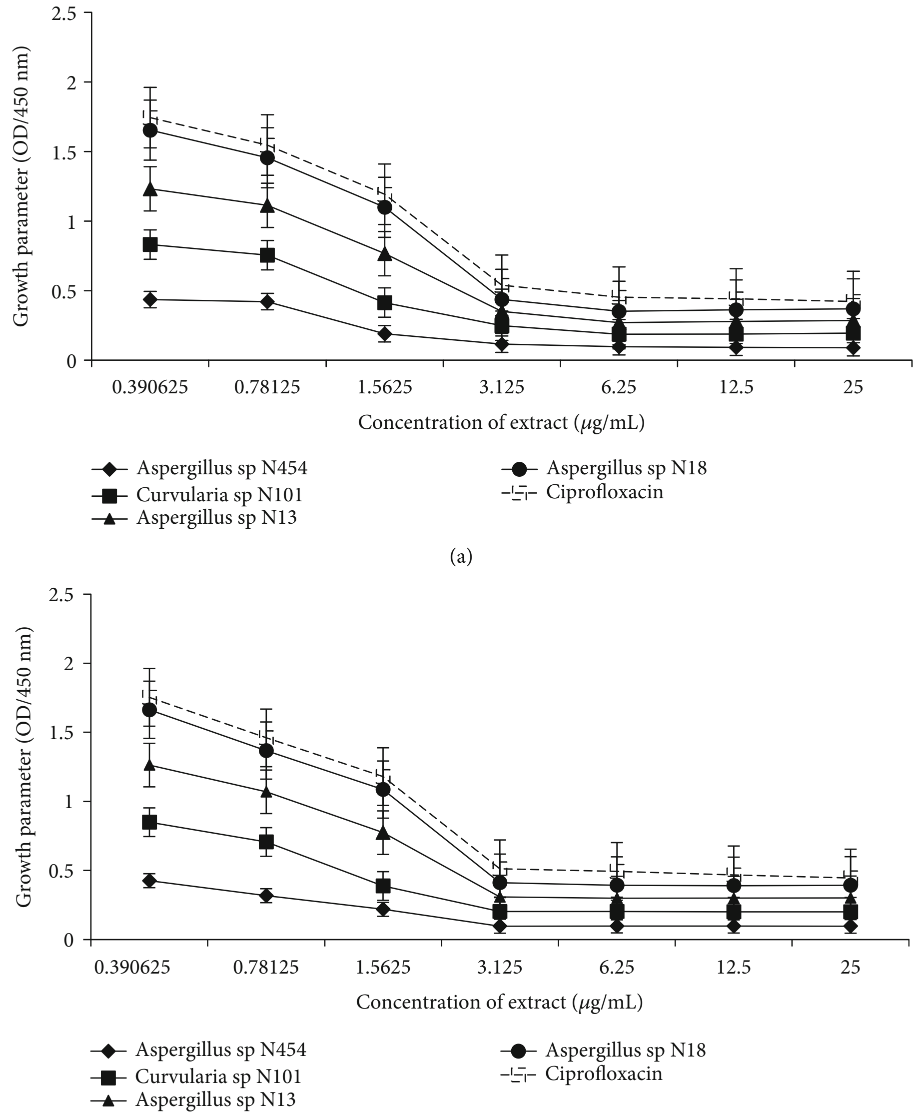
(b)

Figure 3: Total nucleotide leakage from (a) *Escherichia coli* ATCC 25922 and (b) *Haemophilus influenzae* ATCC 49247 treated with different endophytic fungi extracts at their MIC concentration. Data are expressed as the mean ± SD.

sp. N13, and *Curvularia* sp. N101 affect the permeability of *E. coli* ATCC 25922 and *H. Influenzae* ATCC 49247. All extracts resulted in a decrease of the optical density at 450 nm, which indicated leakage of intracellular components, including electrolytes from the cells. Furthermore, in both treated bacteria, when the concentration of extracts increases from 0.39 to 3.125 μg/mL, there was a sharp decrease in the optical density which corresponds to the loss of viability of tested bacteria (Figures 4(a) and 4(b)). The inhibition of the growth of *H. influenzae* and *E. coli* was more intense when treated with an extract from *Aspergillus* sp. N454 as compared to antibiotic Ciprofloxacin at almost all the concentrations tested.

3.2.4. The Effect of Extracts on the Loss of Salt Tolerance Capacity. The effect extracts on salt tolerance of bacteria are summarised in Figures 5(a) and 5(b). The addition of KCl to nutrient agar (NA) medium significantly reduced the colony-forming units of treated *E. coli* ATCC 25922 and *H. influenzae* ATCC 49247. *H. influenzae* treated with KCl and fungal extract revealed that the number of bacteria able to form colonies on NA-KCl was not significantly reduced at 2.5% and 5% of KCl concentration compared to the control (0% KCl), while, at 10% of KCl, a minimal bacterial growth was recorded. However, regarding *E. coli*, the proportion of bacteria able to form colonies on KCl nutrient agar was significantly reduced when KCl was used at 5% and 10% g/L. In general, the reduction of colonies formation while treated with extracts was proportional to the medium's KCl concentration.

3.2.5. The Effect of Extracts on the Catalase Activity. The inhibition of the catalase activity of *E. coli* and *H. influenzae* was evaluated by comparing the amount of H_2O_2 remaining in the medium after the addition of fungal extracts to the control. The results obtained were summarised in Figure 6 below. The percentages of remaining H_2O_2 in bacteria culture treated with extracts were ranged from 28.48 to 47.63% for *E. coli* and from 55.28 to 76.67% for *H. influenzae* highlighting the ability of tested extracts to exert a certain degree of inhibition against the activity of the bacterial catalase enzyme. The crude extract from *Aspergillus* sp. N18



(b)

Figure 4: Effect of endophytic fungi extracts on the membrane permeability of (a) *H. influenzae* ATCC 49247 and (b) *E. coli* ATCC 25922. Data are expressed as the mean ± SD.

exerted the highest degree of inhibition against *H. influenzae* (%of remaining $H_2O_2 = 47.63$) and *E. coli* (%of remaining $H_2O_2 = 76.67$). The catalase inhibition activity exhibited by *Aspergillus* sp. N18 is comparable to that of the positive control, Ciprofloxacin showing a percentage of remaining H_2O_2 of 45.09 and 81.57% on *H. influenzae* and *E. coli*, respectively.

3.3. Antioxidant Potential and Cytotoxicity of Selected Extracts. To understand the effect of active extracts to fight against oxidative stress, the DPPH and the Fe^{3+} reducing ability of extracts were evaluated (Table 1). The results show that the inhibition concentration 50 (IC_{50}) of DPPH ranged from 150.71 to 936.08 μg/mL depending on tested extracts. Extract of *Aspergillus* sp. N454 showed a high scavenging activity with IC_{50} of 150.71 μg/mL, whereas the extract from *Curvularia* sp. N101 showed the least antioxidant activity ($IC_{50} > 1000$ μg/mL). Concerning the Fe^{3+}-reducing power assay, only the ethyl acetate extract from *Aspergillus* sp. N13 exhibited activity, although very weak with RC_{50} of 760.96 μg/mL (Table 1).

The cytotoxicity of the extracts was tested against Vero cells ATCC CRL1586. As shown in Table 1, extract from *Aspergillus* sp. N13 was cytotoxic against Vero cells ATCC CRL1586 (CC_{50} of 14.285 μg/mL), while no cytotoxic activity was observed with other tested extracts ($CC_{50} > 100$ μg/mL). These values were within the cutoff point of the National Cancer Institute criteria for noncytotoxicity crude extract ($IC_{50} < 20$ μg/mL).

4. Discussion

The antibacterial activity of the crude ethyl acetate extracts of different endophytic fungi isolated from *Cananga odorata*, *Terminalia mantaly*, and *Terminalia catappa* was evaluated

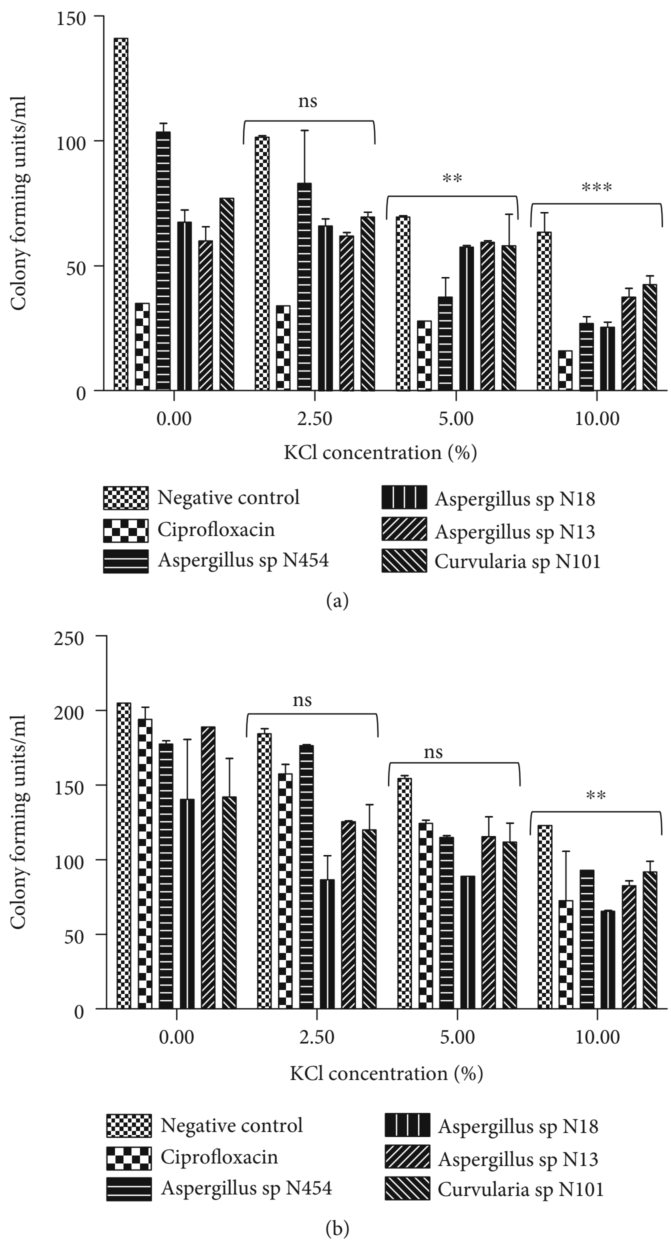


Figure 5: Effect of potent extracts on reducing salt tolerance of (a) *E. coli* ATCC 25922 and (b) *H. influenzae* ATCC 49247 at the MIC concentration. The error bars represent the standard deviation of measurement of a sample in three separate sample runs. ns: nonsignificant; ***: significantly different compared to the untreated samples ($p < 0.001$); **: significantly different compared to the untreated samples ($p < 0.01$).

with the aims of identifying potential active extract(s) that can be further investigated for the discovery of active principle useful in the treatment of lower respiratory tract infection as pneumonia. Endophytic fungi have enormous potential to produce an extensive range of bioactive secondary metabolites with therapeutic activity such as antibacterial, antifungal, and antioxidant [10–12]. In the present study, a total of 8 (13%) strains showed excellent antibacterial activity with a broad spectrum among which extracts from endophytic fungi *Aspergillus* sp. N454, *Aspergillus* sp. N13, *Aspergillus* sp. N18, and *Curvularia* sp. N101 was more active against all tested bacteria. Literature reveals that the fungi from *Aspergillus* and *Curvularia* genera can produce antimicrobial compounds [24, 25]. In the present work, the antibacterial mode was done to understand the mechanism of inhibition of active extracts against bacteria (*H. influenzae* and *E. coli*). The bacteriolysis assay results showed that the crude extracts' inhibitory effect could be related to bacterial cell lysis. Positive results obtained with endophytic fungi extracts suggested that each extract's active metabolites content might have entered into the bacterial cell through bacterial porins. They might have affected the bacterial enzymes, causing cell lysis and death [26]. Additionally, the cell lysis observed may also have been due to the weakening of the cell wall and the

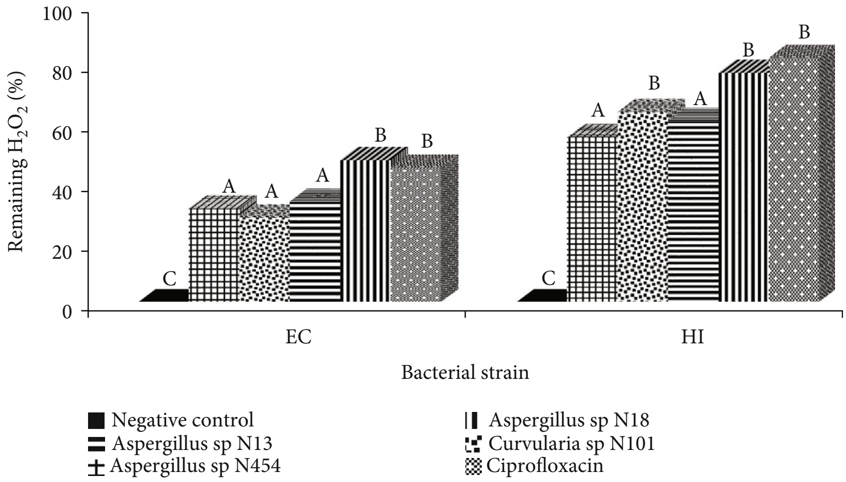


Figure 6: Effect of endophytic fungi extracts on catalase activity of *Escherichia coli* and *Haemophilus influenzae* at the MIC concentration. Data are expressed as the mean ± SD. Values with different letters express a significant difference at $p < 0.05$. EC: *Escherichia coli*; HI: *Haemophilus influenzae.*

Table 1: DPPH radical (IC_{50}) and RC_{50} of fungi extract and cytotoxicity (CC_{50}). Means ± SD.

Fungi name	IC_{50} (μg/mL)	RC_{50} (μg/mL)	CC_{50} (μg/mL)
Aspergillus sp. N13	250 ± 0.350^{c}	760 ± 0.07^{b}	14.28 ± 5.86
Curvularia sp. N101	>1000	>1000	>100
Aspergillus sp. N454	150.71 ± 0.02^{b}	>1000	>100
Aspergillus sp. N18	936.08 ± 1.93^{d}	>1000	>100
Ascorbic acid	2.71 ± 0.08^{a}	13.94 ± 0.27^{a}	—

Values carrying the same letter superscripts are not significantly different ($p > 0.05$). IC_{50}: inhibitory concentration 50 of DPPH radical; RC_{50}: Fe^{3+}-reducing concentration 50; CC_{50}: cytotoxic concentration 50; SD: standard deviation. Data are presented as mean values ± standard deviation of triplicate experiments.

subsequent rupture of the cytoplasmic membrane due to osmotic pressure rather than a specific action on the cytoplasmic membrane [17].

Further, the action of extracts on the permeability of the bacterial outer membrane was studied. The permeability of the outer membrane of *E. coli* ATCC 25922 and *H. influenzae* ATCC 49247 was significantly impaired by the addition of fungal extracts into the culture as indicated by the decrease of the relative absorbance in the bacterial solution. Gram-negative bacteria's outer membrane is made by lipoproteins, phospholipids, and vital lipopolysaccharide molecules, which confer to her impermeability to various antibacterial agents [27]. The control of the cellular membrane's permeability is a crucial regulatory factor for various cellular functions, including cell metabolism maintenance, solute transport, and energy transduction processes [28, 29]. These potent extracts may content hydrophobic compounds which enhanced their accumulation inside periplasmic space inducing the degradation of some necessary bacterial enzymes such as alkaline phosphatase leading to cellular lysis [17, 30–32].

Additionally, extracts may have altered the bacterial cell's permeability by reducing its osmoregulatory ability to penetrate or exclude toxic materials, resulting in their loss of salt tolerance [19]. This hypothesis is supported by the fact that the treatment of *E. coli* and *H. influenzae* with fungal extracts reduced the bacteria's ability to form colonies on media containing KCl. The studies on salt tolerance of bacteria were also reported by many investigators [17, 33, 34]. The leakage of the 260 nm absorbing material was evaluated to see whether potent extracts were also acting on the bacterial inner membrane. Unfortunately, *E. coli* ATCC 25922 and *H. Influenzae* ATCC 49247 treated with endophytic fungi extracts did not significantly lose 260-nm-absorbing materials, suggesting nucleic acids were not lost through a damaged cytoplasmic membrane. The inactivity of extracts on the inner membrane compare to the outer membrane can be explained by their difference in composition which is essentially made of phospholipids. Previous reports demonstrated that antimicrobial compounds such as chlorhexidine, hexachlorophene, phenethyl alcohol, tetracyclines, and polymyxin that act on cytoplasmic membrane induced structural and functional abnormalities of the bacterial membrane phospholipids bilayer [17, 35].

Inhibition of catalase activity of an extract can indicate the reduction of pathogen resistance towards oxidative stress. Several pathogens produced catalase in order to defend themselves against attacks by hydrogen peroxide, a weapon commonly used by macrophages, in addition to oxidative stress [36]. Fungal extracts tested revealed a high inhibition of catalase activity of *E. coli* and *H. influenzae* indicating that those extracts can be used to prevent DNA damage caused by hydroxyl radicals (OH-) issued from the decomposition of hydrogen peroxide by pathogenic bacteria [36].

The potential of extracts to prevent oxidative stress in human was also investigated through the DPPH radical scavenging assay and FRAP reducing power. Antioxidants are known to prevent oxidative stress-mediated toxicity caused by oxygen-free radicals [37]. It was evident from the results that the fungal extracts contain radical scavenging compounds as previously reported [13, 38, 39]. Iron is essential for life because it is required for many enzymes' activity, oxygen transport, and respiration. However, iron is an extremely reactive metal and catalyses oxidative changes in lipids, proteins, and other cellular components. It causes lipid peroxidation through the reaction and decomposes the lipid hydroxide into peroxyl and alkoxyl radicals that can perpetuate the chain reactions [40]. From this study, it may be noted that only endophytic fungal extract from *Aspergillus* sp. N13 showed promising reducing potential indicating its antioxidant potential.

Results obtained are precise concerning the noncytotoxicity of fungal extracts from *Aspergillus* sp. N454, *Aspergillus* sp. N18, and *Curvularia* sp. N101. However, *Aspergillus* sp. N13 exhibited high cytotoxic activity against Vero cell. This cytotoxicity could be attributed to the quality and quantity of compounds produced by this isolate. Endophytic fungi, particularly from the *Aspergillus* genus, have been reported as excellent producers of strong cytotoxic metabolites [41]. The difference observed in cytotoxicity found among active extracts from the *Aspergillus* genus could be due to culture conditions that could have favored or unfavored toxic compounds' production [42, 43]. For instance, our previous investigation of endophytic fungal *Aspergillus* sp. 58 isolated from the bark of *T. catappa* showed that ethyl acetate extract obtained from the culture in PDB was nontoxic; however, when the growth medium was supplemented with DMSO, extract obtained was highly cytotoxic against HEK293T cell line [44]. Watanabe et al. [45] also reported that *A. fumigatus* cultured produced toxic gliotoxins compounds in the presence of high oxygen concentration. Kamei et al. [46] also reported the production of cytotoxic molecules produced by *A. fumigatus* when cultured in the presence of macrophages.

5. Conclusion

In this study, ethyl acetate extracts of endophytic fungi derived from *T. catappa*, *T. mantaly*, and *C. odorata* demonstrated strong antibacterial activity against pathogenic bacteria implicated in pneumonia. Results indicated that fungal isolates of *Aspergillus* sp. N454, *Aspergillus* sp. N18, and *Curvularia* sp. N101 may be useful as an alternative to producing antibacterial drugs. Therefore, further investigation to isolate and identify the bioactive compounds responsible for these fungi's specified biological activities is currently ongoing.

Data Availability

Data used to support the findings of this study are all included within the article.

Conflicts of Interest

The authors declare no conflicts of interest.

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Supplementary Materials

Supplementary material 1: endophytic fungi: their host plant, isolation site and names. Supplementary material 2: extraction yield (mg/200 mL), and mimimal inhibitory concentration (μg/mL (mean ± SD)) of ethyl acetate extracts from the 56 endophytic fungi used in the present investigation. *(Supplementary Materials)*

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