THE UNIVERSITY OF YAOUNDE I

FACULTY OF SCIENCE

POST GRADUATE SCHOOL FOR LIFE SCIENCES, HEALTH AND ENVIRONMENT

DOCTORAL RESEARCH UNIT FOR LIFE SCIENCES

UNIVERSITE DE YAOUNDE I

FACULTE DES SCIENCES

CENTRE DE RECHERCHE ET DE FORMATION DOCTORALE EN SCIENCES DE LA VIE, SANTE 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
UNITE DES AGENTS ANTIMICROBIENS ET DE BIOCONTROLE

Antibacterial activity of metabolites from endophytic fungi inhabiting Cameroonian Annona muricata against the causative agents of Urinary Tract Infections

Thesis presented in partial fulfilment of the requirements for the award of the Degree of Doctorate/Ph.D in Biochemistry

by

YIMGANG Victorine Lorette
Registration N° 12R0999
M.Sc in Biochemistry

Supervised by:

FEKAM BOYOM Fabrice
Professor
University of Yaoundé I

Academic year : 2024-2025

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DEPARTMENT OF BIOCHEMISTRY

CERTIFICATE OF CORRECTION OF THE Ph.D THESIS

ATTESTATION DE CORRECTION DE LA THESE DE DOCTORAT Ph.D

The undersigned members of jury involved in the Ph.D thesis in Biochemistry entitled : **« Antibacterial activity of metabolites from endophytic fungi inhabiting Cameroonian *Annona muricata* against the causative agents of urinary tract infections»** defended on Monday, 02nd of December 2024 at 8 : 00 am in the S01/S02 Room of the Nouveau Block Pédagogique, Faculty of Science, University of Yaoundé I by **Mrs. YIMGANG Victorine Lorette (registration number 12R0999)**, are hereby certifying that the candidate has effected the corrections of the above mentioned thesis as requested by the examiners.

Therefore, they are satisfied with the corrections made and are recommending the doctorate/Ph.D degree to be awarded to the candidate.

Yaoundé, the 30th January 2025

Supervisor	Examiners	President of jury	Head of Department
FEKAM BOYOM Fabrice	KORO KORO Francioli	PENLAP Veronique	MOUNDIPA FEWOU Paul

NNANGA NGA Emmanuel

ASSAM ASSAM Jean Paul
Jean Paul Assam Assam (Ph.D)
Associate Professor of Microbiology

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ACADEMIC YEAR 2023/2024

(by Department and by Grade)

LAST UPDATE 04 June 2024

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49	ZEMO GAMO Franklin	Assistant	On duty

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27.	LIBALAH Moses BAKONCK	Senior Lecturer	On duty
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14.	DJIADEU NGAHA Michel	Senior Lecturer	On duty
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16.	KIKI Maxime Armand	Senior Lecturer	On duty

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31.	MANN MANYOMBE Martin Luther	Assistant	On duty
32.	MEFENZA NOUNTU Thiery	Assistant	On duty
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17.	WOAFO Paul	Professor	On duty
18.	ZEKENG Serge Sylvain	Professor	On duty

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25.	MVOGO ALAIN	Associate Professor	On duty
26.	NDOP Joseph	Associate Professor	On duty
27.	SIEWE SIEWE Martin	Associate Professor	On duty
28.	VONDOU Derbetini Appolinaire	Associate Professor	On duty
29.	WAKATA née BEYA Annie Sylvie	Associate Professor	***Director/HTTC/UYI***
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33.	CHAMANI Roméo	Senior Lecturer	On duty
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4.	NDAM NGOUPAYOU Jules-Remy	Professor	On duty
5.	NDJIGUI Paul-Désiré	Professor	***Head of Department***
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7.	NKOUMBOU Charles	Professor	On duty
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18.	METANG Victor	Associate Professor	On duty
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30.	ESSONO Jean	Senior Lecturer	On duty
31.	MAMDEM TAMTO Lionelle Estelle, épouse BITOM	Senior Lecturer	On duty
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33.	NGO BELNOUN Rose Noël	Senior Lecturer	On duty
34.	NGO'O ZE ARNAUD	Senior Lecturer	On duty
35.	NOMO NEGUE Emmanuel	Senior Lecturer	On duty
36.	NTSAMA ATANGANA Jacqueline	Senior Lecturer	On duty
37.	TCHAPTCHET TCHATO De P.	Senior Lecturer	On duty
38.	TEHNA Nathanaël	Senior Lecturer	On duty
39.	FEUMBA Roger	Senior Lecturer	On duty
40.	MBANGA NYOBE Jules	Senior Lecturer	On duty

41.	KOAH NA LEBOGO Serge Parfait	Assistant	On duty
42.	TENE DJOUKAM Joëlle Flore, épouse KOUANKAP NONO	Assistant	On duty

Distribution of permanent lecturers in the faculty of science according to departments

NUMBER OF LECTURERS					
Department	**Professors**	**Associate Professors**	**Senior Lecturers**	**Assist. Lecturers**	**Total**
BCH	8 (01)	20 (12)	9 (04)	6 (05)	**43 (22)**
BPA	11 (01)	19 (09)	16 (05)	3 (02)	**49 (17)**
BPV	6 (01)	10 (02)	14 (08)	2 (00)	**32 (11)**
CI	7 (01)	14 (04)	5 (01)	1 (00)	**27 (06)**
CO	7 (01)	15 (05)	11 (05)	1 (00)	**33 (11)**
RE	1(00)	/	/	/	**1(0)**
IN	2 (00)	2 (00)	14 (01)	4 (00)	**22 (01)**
MAT	1 (00)	8 (00)	19 (02)	5 (01)	**33 (03)**
MIB	3 (01)	7 (03)	9 (05)	5 (02)	**24 (11)**
PHY	18 (01)	12 (04)	11 (01)	1 (00)	**42 (06)**
ST	10 (00)	17 (03)	13 (03)	3 (01)	**43 (07)**
Total	**74 (07)**	**124 (42)**	**121 (35)**	**31 (11)**	**350 (95)**

A total of……**349 (95)**, with :

Professors……**73 (07)**

Associate Professors…….**124 (42)**

Senior Lecturers…….**121 (35)**

Assistant Lecturers……..**31 (11)**

( ) = Number of women…….**95**

DEDICATION

This piece of work is dedicated to my grand-mother **Kembou Justine**

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LIST OF ABBREVIATIONS

ATCC : American Type Culture Collection

AMb : *Annona muricata* bark

AMC : *Annona muricata* compound

AMF : *Annona muricata* fraction

AMf : *Annona muricata* fruit

AMl : *Annona muricata* leave

AMpe : *Annona muricata* peducle

AMr : *Annona muricata* root

AMrb : *Annona muricata* root bark

AMs : *Annona muricata* seed

AMsb : *Annona muricata* stem bark

AMSF : *Annona muricata* sub-fraction

AMtf : *Annona muricata* thorn of fruit

AMtw : *Annona muricata* twig

BLAST : Basic Local Alignment Search Tool

CAT : Catalase

CBS : Centraalbureau voor Schimmelcultures

CC_{50} : Cytotoxic Concentration 50

CFU : Colony Forming Units

CLSI : Clinical Laboratory Standard Institute

COSY : Correlated Spectroscopy

CPC : Centre Pasteur du Cameroun

DMEM : Dulbecco's Modified Eagle Medium

DMSO : Dimethylsulfoxide

DNA : Deoxyribonucleic Acid

DPPH : 1,1-diphenyl-2-picrylhydrazyle

EDTA : Ethylene Diamine Tetraacetic Acid

EtOAC : Ethyl acetate

FRAP : Ferric Reducing Antioxidant Power

HCA : Hierachical Cluster Analysis

Hex : Hexane

HMBC : Heteronuclear Multiple Bond Correlation

HNC : Herbier National du Cameroun

IC_{50} : Inhibitory Concentration 50

ITS : Internal Transcriber Spacer

$MBEC_{50}$: Minimum Biofilm Eradication Concentration 50

$MBIC_{50}$: Minimum Biofilm Inhibitory Concentration 50

MEGA : Molecular Evolutionary Genetics Analysis

MeOH : Methanol

MIC : Minimal Inhibitory Concentration

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

NA : Nutrient Agar

NB : Nutrient Broth

NCBI : National Center for Biotechnology Information

NMR : Nuclear Magnetic Resonance

OD : Optical Density

PBS : Phosphate buffered saline

PCA : Principal Component Analysis

PCR : Polymerase Chain Reaction

PDA : Potatoes Dextrose agar

PDB : Potatoes Dextrose Broth

RNA : Ribonucleic acid

RNS : Reactive Nitrogen Species

ROS : Reactive Oxygen Species

RSA_{50} : 50% Radical Scavenging Activity

SI : Selectivity Index

SOD : Superoxide dismutase

TLC : Thin Layer Chromatography

UTI : Urinary tract infection

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ABSTRACT

Urinary tract infections (UTIs), caused predominantly by bacteria, are a global health challenge exacerbated by rising multidrug resistance. This underlines the urgent need for new antibiotics with known or new mechanisms of action. Endophytic fungi, particularly those residing in medicinal plants used for UTIs treatment, could be a promising approach for novel antibiotic discovery. Therefore, this study investigates the potential of endophytic fungi from *Annona muricata* as a source of novel antibacterial compounds with activity against the causative agents of UTIs.

Crude ethyl acetate extracts from 41 different endophytic fungi were screened against three bacterial strains using the broth microdilution method and fungi producing active crude extracts were identified using ITS1-5.8S rRNA-ITS2 nucleotide sequences. Extracts derived from the seventeen identified fungi were tested for cytotoxicity on Vero cells and the DPPH and FRAP assays were used to investigate their antioxidant activity. The most promising extract underwent column chromatography separation, and the resulting fractions, pure sub-fractions and pure compounds were evaluated for antibacterial, antioxidant, and cytotoxic properties. The modes of action of the two most active compounds were further investigated on the most sensitive strains.

Out of the 41 extracts, 17 demonstrated antibacterial activity with Minimum Inhibitory Concentrations (MICs) ranging from 3.125 to 100 µg/mL, with no cytotoxicity observed at 100 µg/mL. The active fungi, identified as belonging to six genera including *Aspergillus*, *Curvularia*, *Fusarium*, *Meyerozyma*, *Penicillium*, and *Talaromyces* with varying levels of antioxidant activity of their derived extracts. The most promising extract, from *Fusarium sp.* AMtw3, was fractionated into nine fractions, yielding fifteen pure sub-fractions and two compounds, AMC6 and AMC20. AMC6, identified as 6-methyl-3-(prop-1-en-1-yl)-5,6-dihydro-2H-pyran-2-one, was the most active with an MIC of 0.39 µg/mL against *K. oxytoca* and *S. aureus* ATCC 43300. Regarding modes of action, Principal Component analysis (PCA) indicated that AMC20 known as friedelin, exerts a pronounced bactericidal effect through time kill kinetic assay and also favors membrane permeability on *K. oxytoca*.

This study highlights that endophytic fungi from *Annona muricata* can produce bioactive metabolites which can serve as a starting point for new antibiotics targeting UTI pathogens.

Keywords: Urinary tract infections, Endophytic fungi, *Annona muricata*, Antibacterial activity, Metabolites, Mode of action

RESUME

Les Infections du Tractus Urinaire (ITU), majoritairement causées par les bactéries, posent un défi mondial croissant en raison de la résistance accrue aux antibiotiques. Cela souligne l'urgente nécessité de découvrir de nouveaux antibiotiques avec des mécanismes d'action connus ou nouveaux. Les champignons endophytes présents dans les plantes médicinales pourraient constituer une solution prometteuse. Ainsi, cette étude visait à investiguer le potentiel des champignons endophytes d'*Annona muricata* en tant que source de nouveaux composés antibactériens contre les bactéries uropathogènes.

Les extraits bruts à l'acétate d'éthyle de 41 champignons endophytes ont été testés sur trois bactéries via la microdilution en milieu liquide. Les champignons produisant les extraits actifs ont été identifiés en utilisant les séquences ITS1-5.8S rRNA-ITS2. Lesdits extraits (17) ont été évalués pour leur cytotoxicité sur les cellules Vero et leur activité antioxydante à travers les tests DPPH et FRAP. L'extrait prometteur a été fractionné par chromatographie sur colonne, et les fractions, sous fractions pures et composés purs ont été testés pour les activités précédentes. Les modes d'action des deux composés les plus actifs ont été évalués sur les souches les plus sensibles.

Sur les 41 extraits, 17 ont démontré une activité antibactérienne avec des Concentrations Minimales Inhibitrices (CMI) allant de 3,125 à 100 µg/mL et aucune cytotoxicité n'a été observée à 100 µg/mL. Les champignons actifs, ont été identifiés comme appartenant à six genres dont *Aspergillus, Curvularia, Fusarium, Meyerozyma, Penicillium, et Talaromyces* avec des activités antioxydantes des extraits dérivés variables. Le fractionnement de l'extrait prometteur, provenant de *Fusarium sp*. AMtw3, a permis d'obtenir neuf fractions, quinze sous fractions pures et deux composés, AMC6 et AMC20. AMC6, identifié comme 6-méthyl-3-(prop-1-en-1-yl)-5,6-dihydro-2H-pyran-2-one, était le plus actif avec une CMI de 0,39 µg/mL sur *K. oxytoca* et *S. aureus* ATCC 43300. En ce qui concerne les modes d'action, l'Analyse en Composantes Principales (ACP) a montré que AMC20, connu sous le nom de friedéline, exerce un effet bactéricide prononcé et favorise également la perméabilité membranaire sur *K. oxytoca*.

Cette étude montre que les champignons endophytes d'*Annona muricata* peuvent produire des métabolites bioactifs qui peuvent servir de point de départ pour de nouveaux antibiotiques ciblant les bactéries responsables des Infections du Tractus Urinaire.

Mots-clés : Infections urinaires, champignons endophytes, *Annona muricata*, activité antibactérienne, métabolites, mode d'action.

Introduction

INTRODUCTION

Urinary Tract Infections (UTIs) are the second most frequent bacterial infections that affect humans after respiratory tract infections (Murray *et al*., 2021; Ngong *et al*., 2021). The incidence of UTIs results in 8 million visits to hospital emergency departments and 100,000 hospital admissions (May *et al*., 2019). The World Health Organization recognizes UTI as a prevalent infection and a significant global health challenge in terms of morbidity, healthcare costs, and antimicrobial resistance rates (Alhazmi *et al*., 2023). UTIs are among the most widespread infectious diseases, affecting millions worldwide annually with an estimated annual cost of $1.6 billion in the United States alone (Alhazmi *et al*., 2023; Simmering *et al*., 2017).High frequency of Enterobacteriaceae involved in urinary tract infections have been reported in several Africa countries with 89.17% in Nigeria, 39.13% in Uganda, 10.1% in Ghana and 21.2% among children in Gambia (Kebbeh *et al*., 2023). In Cameroon, a recent report has shown a prevalence of 31% among pregnant women attending antenatal care in some Integrated Health Centers in Buea Health District (Ngong *et al*., 2021).

Patients suffering from UTIs are commonly treated with several families of antibiotics such as quinolones, sufonamides, and beta-lactam. Thus, to have a suitable treatment, clinicians should prescribe an appropriate antibiotic according to the uropathogen's susceptibility identified via antimicrobial susceptibility testing (Alkhawaldeh *et al*., 2022). However, intense and inappropriate use of these antibiotics has led to the development of multidrug-resistant (MDR) strains of bacteria leading to complications in the management of UTIs (Biswas & Sinha, 2018). This situation is as serious as the total economic cost of antibiotic resistance was estimated to be over $20 billion in direct healthcare and $35 billion in lost productivity per year (Prestinaci *et al*., 2015). So, searching for novel compounds to overcome this growing problem of resistance is ultimately and absolutely necessary for the management of UTIs (Gadisa & Tadesse, 2021).

The use of microbial biotopes appears as a reproducible source and an exciting field of study in the search of new medicines when compared to other types of natural sources, such as plants and animals (Silva *et al*., 2022). Amongst them, one such unexplored and less studied are endophytic fungi, which are microorganisms that colonize healthy tissues of plants without causing apparent disease symptoms in their host (Patchett & Newman, 2021). They fully demonstrated their abilities to serve as a storehouse of a range of metabolites of different chemical classes, including alkaloids, flavonoids, steroids, terpenoids, and phenolic compounds. Some compounds

show pleiotropic and interesting pharmacological activities, including antimicrobial, antioxidant, anti-diabetic, anti-malarial, and antitumor properties (Caruso *et al*., 2022; Kaaniche *et al*., 2019; Manganyi & Ateba, 2020; Meshram *et al*., 2022; Toghueo *et al*., 2019). It is necessary to emphasize that one of the characteristics that makes them quite attractive. is their capability of producing identical or similar metabolites to those produced by the host plant. More so, the possibilities to manage the production of metabolites of interest in an optimized way during their fermentation process exist. Interestingly, they are renewable, readily available and eco-friendly sources of biologically active natural products (Silva *et al*., 2022). To date, the worldwide plant population is over 400 000 and many more are yet to be documented (Omomowo *et al*., 2023). Cameroon, particularly with its robust biodiversity (over 4,000 plant species per degree squared), needs to be harnessed because it can significantly contribute to this fight against antibiotic resistance and the development of new antimicrobials (Arsene *et al*., 2022; Onana, 2015). Among these species of plants, *Annona muricata,* a plant of the Annonaceae family, is a medicinal plant used in Cameroon and worldwide to treat various diseases including microbial infections (Roger *et al*., 2015). In addition, several previous studies carried out in our laboratory have demonstrated the ability of this plant's extracts to produce bioactive metabolites (Boyom *et al*., 2011; Yamthe *et al*., 2015). Therefore, we hypothesized that endophytic fungi residing inside tissues of *A. muricata* produce active metabolites against resistant bacterial pathogens causative agents of UTIs. This study aims to investigate the antibacterial and modes of action of metabolites isolated from *Annona muricata* endophytic fungi of Cameroon against the resistant causative agents of urinary tract infections.

Research questions:

1) What are the potent antimicrobial, non cytotoxic and antioxidant extracts from endophytic fungi of *Annona muricata*?
2) What is the composition of the most selective extract?
3) What are the potential modes of action of selected compounds and their structures?

Specific objectives:

1) Identify potent endophytic antimicrobial, non cytotoxic and antioxidant fungi from *Annona muricata*
2) Explore the most selective extract using a bioguided approach
3) Determine the potential modes of action of selected active compounds and their structures

Chapter I: Literature review

CHAPTER I: LITERATURE REVIEW

I.1 Generality on bacteria

I.1.1 Definition

Bacteria are microscopic single-celled organisms, typically 0.2 to 250 µm long, that lack a nuclear membrane (prokaryotic) but a circular chromosomal DNA located in the cytoplasm. Many bacteria contain another extra-chromosomal DNA structure called a plasmid. They are surrounded by a complex wall and often have flagella (Schulz & Jørgensen, 2001). There are thousands of different kinds of bacteria, and they live in various environments all over the world. Some bacteria live in the bodies of humans, animals, and plants and can be pathogenic or not to these organisms. Others however live in the soil, water, and air (Fredrickson *et al*., 2004). To reproduce, they generally use the asexual pathway, which may depend on biotic or abiotic stress. Thus, when environmental conditions are favorable (presence of nutrients), scissiparity or multiplication is favored. However, when the nutrient environment is depleted by desiccation, for example, the reproduction by spores is favored (Koch, 2002).

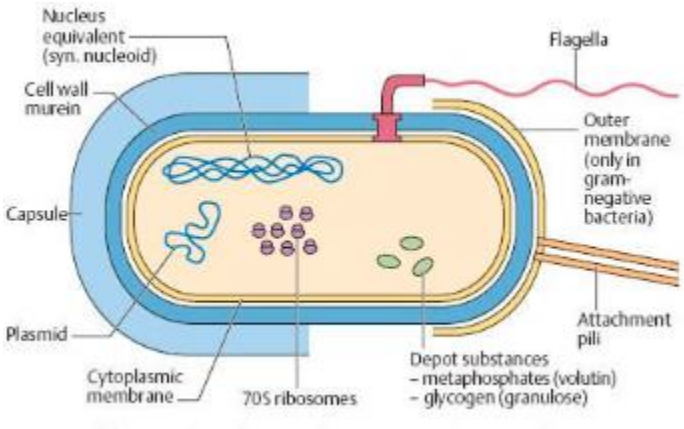


Figure 1: Bacteria cell structure

I.1.2 Classification of bacteria

Bacteria are classified and identified to distinguish one organism from another and to group similar organisms by criteria of interest. The grounds for the classification commonly used are morphology or shape and their reaction to the Gram stain.

I.1.2.1 Classification based on the morphology or shape

In the year 1872 scientist Cohn classified bacteria into 3 major types depending on their shapes as follows:

- **Cocci**

These types of bacteria are unicellular, spherical, or elliptical in shape. Either they may remain as a single cell or may aggregate together for various configurations (Pommerville & Pommerville, 2014). They are as follows:

- ✓ **Monococcus**: they are also called micrococcus and are represented by single, discrete round. Example: *Micrococcus flavus*.
- ✓ **Diplococcus**: the cell of the Diplococcus divides in a particular plane and after division, the cells remain attached to each other. Example: *Diplococcus pneumonia.*
- ✓ **Streptococcus:** here the cells divide repeatedly in one plane to form a chain of cells. Example: *Streptococcus pyogenes.*
- ✓ **Tetracoccus:** this consists of four round cells, which defied in two planes at a right angle to one another. Example: *Gaffkya tetragena.*
- ✓ **Staphylococcus**: here the cells are divided into three planes forming a structure like bunches of grapes giving an irregular configuration. Example: *Staphylococcus aureus.*
- ✓ **Sarcina**: in this case, the cells divide into three planes but they form a cube like configuration consisting of eight or sixteen cells but they have a regular shape. Example: *Sarcina lutea*.

- **Bacilli**

Based on the arrangement of rod-shaped cells, bacilli are classified into various types (Pommerville & Pommerville, 2014):

- ✓ **Bacillus:** single unattached cell, that looks like a rod. Example: *Bacillus cereus.*
- ✓ **Diplobacilli:** two rods are attached to each other and found in pairs after cell division. Example: *Moraxella bovis.*
- ✓ **Streptobacilli:** due to cell division in one plane, bacilli are arranged in a chain. Genus Streptobacillus contains gram-negative, aerobic or facultative anaerobic bacteria. Example: *Streptobacillus moniliformis.*
- ✓ **Coccobacilli:** these are short compared to other bacilli and oval in shape, they appear like a coccus. Example: *Chlamydia trachomatis.*

✓ **Palisades:** the bacilli after cell division bend and therefore are arranged in a palisade, fence-like structure. Example: *Corynebacterium diptheria*.

- **Spirals**

They can be further categorized depending in part on how much spiraling they show (Talaro & Chess, 2015).As such, we have:

✓ **Vibrio**: they are comma-shaped bacteria, appearing like curved rods. They typically live in aquatic environments. Example: *Vibrio cholerae*.

✓ **Spirilla**: it is another subgroup of bacteria with a more rigid, corkscrew-like spiral shape. Example: *Campylobacter jejuni*.

✓ **Spirochete**: they are long, thin and flexible corkscrew-shaped bacteria. Example: *Treponema pallidum*.

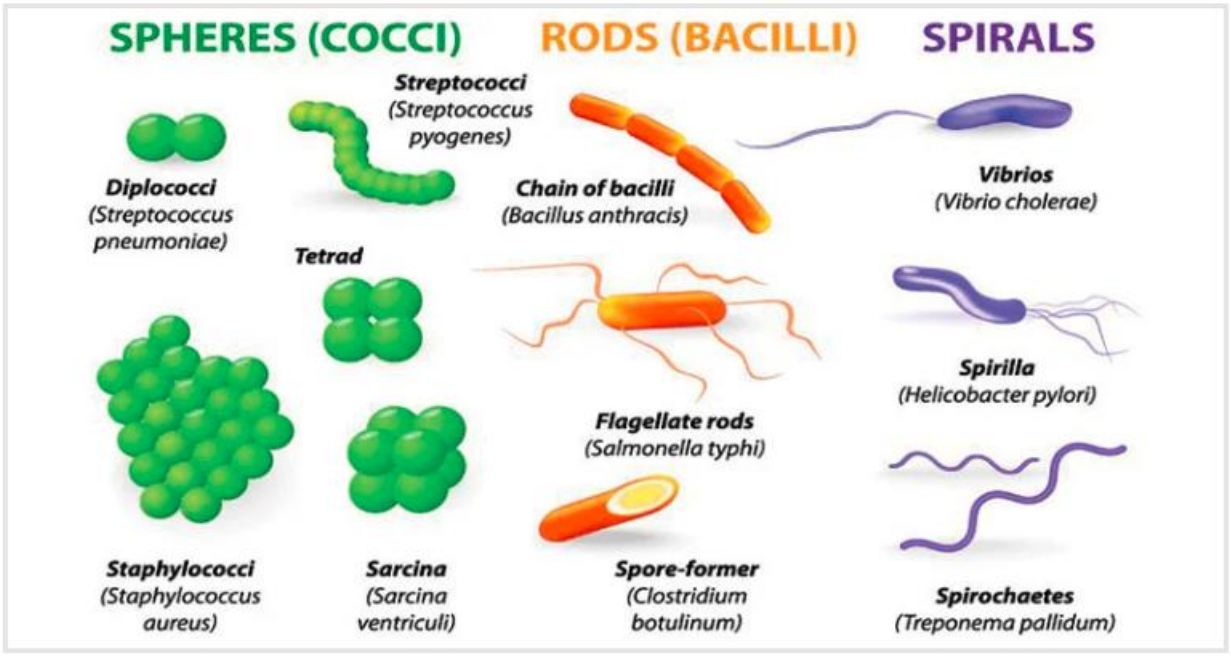


Figure 2: Representative cell morphology of bacteria

I.1.2.2 Classification based on reaction to the Gram stain

Hans Christian Gram developed the staining method in 1884 to distinguish the two types of bacteria (Gram-positive and Gram-negative bacteria). This staining method uses crystal violet dye and the classification is based on the color they turn in during the staining. This reaction gives gram-positive organisms a blue color when viewed under a microscope. Although gram-negative

organisms classically have an outer membrane, they have a thinner peptidoglycan layer, which does not hold the blue dye used in the initial dying process (Sizar *et al*., 2024).

The following table provides an overview of differences between Gram-positive and Gram-negative bacteria:

Table I: Difference between Gram-Positive and Gram-Negative Bacteria (Silhavy *et al*., 2010)

Element of comparison	**Gram-positive bacteria**	**Gram-negative bacteria**
Color after gram testing	Blue or purple	Pink or red
Membranes	No protective outer membrane.	Two membranes (one inner and one outer)
Cell wall	A single-layered, smooth cell wall	A double-layered, wavy cell-wall
Cell Wall thickness	Around 20 to 80 nanometres	Around 8 to 10 nanometres
Peptidoglycan Layer	It is a thick layer/ also can be multilayered	It is a thin layer/ often single-layered.
Lipopolysaccharide	Absent	Present
Toxin Produced	Exotoxins	Endotoxins or Exotoxins
Lipid and lipoprotein content	Low	High
Periplasmic space	Absent	Present
Teichoic acids	Present	Absent
Porins	Absent	Present

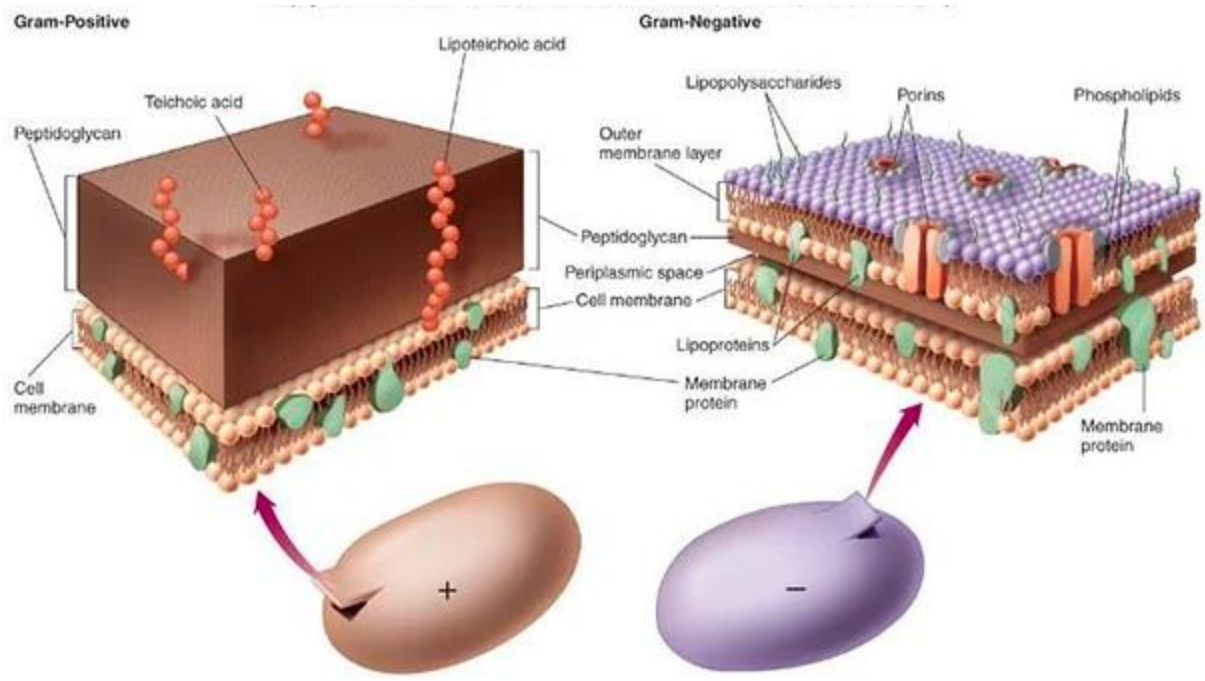


Figure 3: Difference between Gram-Positive and Gram-Negative Bacteria cell wall

I.2 Urinary Tract Infections (UTIs)

I.2.1 Definition

Urinary tract infections (UTIs) are infections caused by the presence, growth and spread of microorganisms in the urinary tract with or without the presence of specific symptoms (Daswani, 2019). They affect any part of the urinary system like the kidneys, ureters, bladder and urethra. When it affects the lower urinary tract, it is known as a simple cystitis (bladder infection) or urethra (urethritis infection) and when it affects the upper urinary tract it is known as pyelonephritis (kidney infection) (Ditkoff *et al*., 2018; Flores-Mireles *et al*., 2015). UTI is said to exist when a bacterial colony count of greater than or equal to 10^5 colony-forming units per ml of a typical urinary tract organism in properly collected mid-stream "clean catch" urine (Zumla, 2010).

I.2.2 Epidemiology

Urinary tract infections are the leading causes of morbidity in the general population, and are the second most common infectious disease after respiratory infections (Addis *et al*., 2021). According to a study, over 404.6 million people all around the globe had UTIs in 2019, and nearly 236,786 died as a result of UTIs the same year (Zeng *et al*., 2022). Except among infants and the

elderly, the infection occurs more commonly in women than in men with about 40–60% of women experiencing one episode in their lives and 30–44% experiencing recurrent UTI (rUTI). For women between 1 year and up to 50 years, UTI and recurrent UTI are predominantly diseases (Alhaj *et al*., 2025; Fazly Bazzaz *et al*., 2021).One of the reasons attributable to this fact is the presence of shorter urethra in women, which makes it easier for bacteria or other microbes to reach the bladder or urinary tract and cause infection (Alhaj *et al*., 2025).The Global Burden Disease study 2019 (Figure 4) analyzed data from 1990 to 2019 and estimated 404.61 million cases of UTIs, 236,790 deaths in 204 countries and territories in low, low-middle, middle, high-middle, and high regions (Yang *et al*., 2022).

In Sub-Saharan Africa, the prevalence of UTIs varies from country to country. Mwang'onde & Mchami (2022) did a review of UTI's data from sub-Saharan Africa countries published from 2000 to 2021 and reported an overall average prevalence of 32.12% with the highest prevalence recorded in South Africa (67.6%), followed by Nigeria (43.65%) and Zambia (38.25). In Cameroon, Djim-Adjim-Ngana *et al*., (2023) reported a prevalence of 26.3% among 144 UTI's patients attending health facilities in Garoua, the capital of the North Cameroon region. In addition, a recent investigation reported a prevalence of 31.62% among 215 patients surveyed at the Regional Hospital Bafoussam and Dschang Regional Annex Hospital (Bayaba et al., 2025).

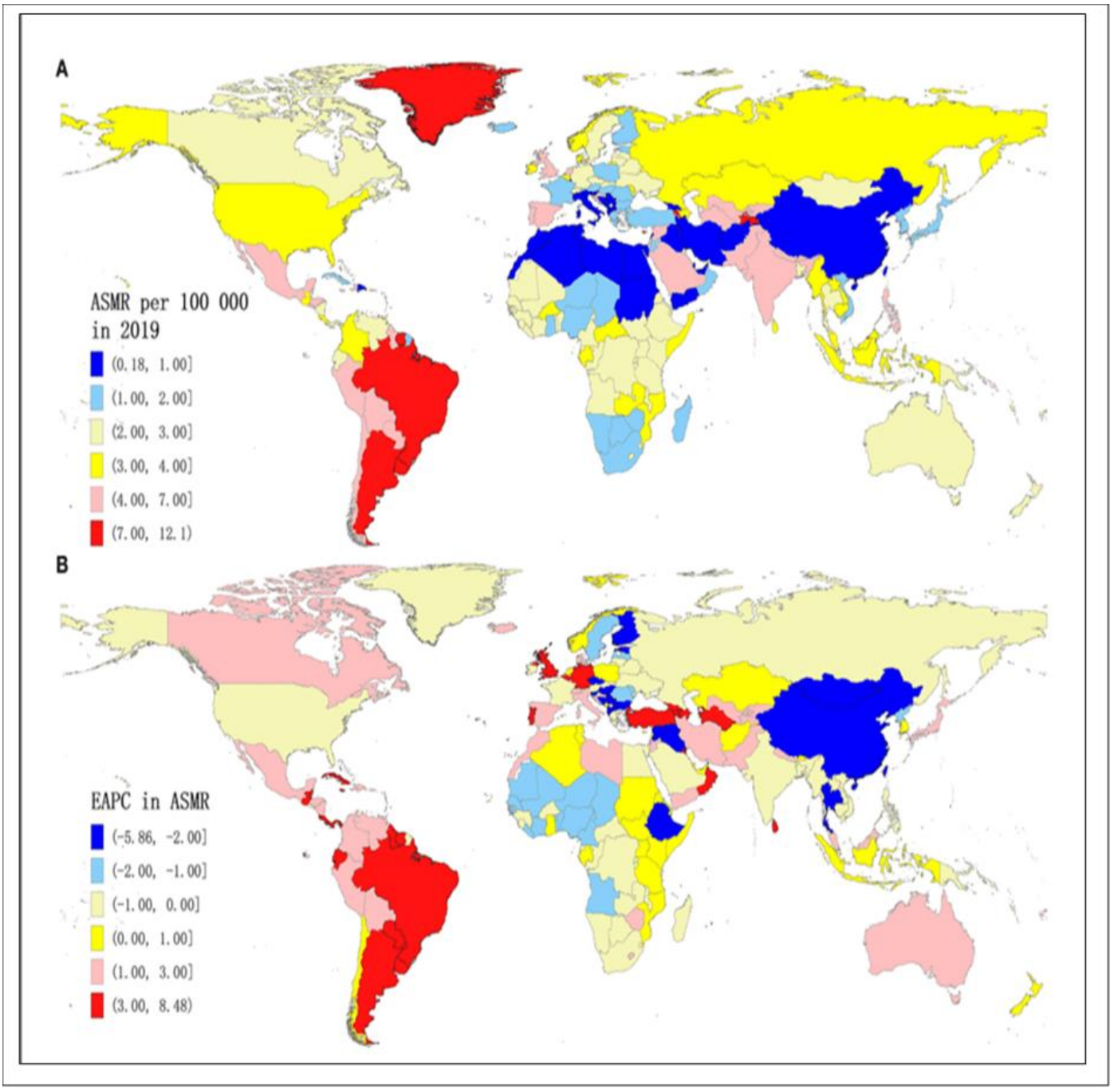


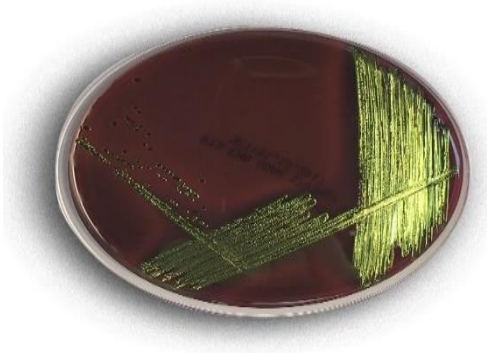
Figure 4: Global mortality of urinary tract infection for both sexes across 204 countries and territories. (a) Age Standardized Mortality Rate (AMSR) of urinary tract infection in 2019; (b) Estimated Annual Percentage Charge (EAPC) in the AMSR of urinary tract (Yang *et al*., 2022).

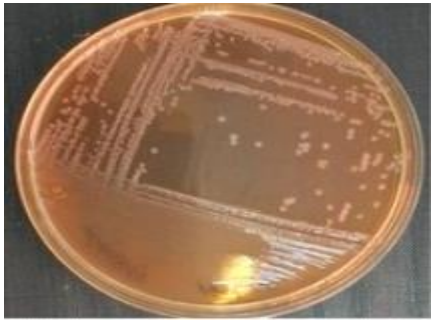
I.2.3 Causative agents and pathogenesis

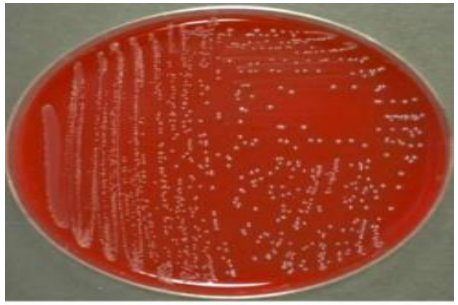
I.2.3.1 Causative agents

Urinary tract infections (UTIs) are mainly caused by bacteria, while the involvement of other microorganisms, such as fungi viruses and parasites, is quite rare (Mancuso *et al*., 2023). Bacteria alone are responsible for more than 95% of UTIs cases and are caused by a wide range of pathogens, including Gram-negative and Gram-positive bacteria (Farajnia *et al*., 2009). The most common causative agent is Uropathogenic *Escherichia coli* (UPECs) responsible for approximately 80% of urinary tract infections then followed by *Staphylococcus* species that contributes from 10% to 15%. In addition, *Klebsiella*, *Pseudomonas*, *Proteus* and *Enterococcus* species are also reported from UTIs patients (Lagha *et al*., 2019; Vasudevan, 2014).

Table II: Some bacteria responsible for UTIs and characteristics

Bacteria	Gram type	Respiratory type	Some biochemical parameters	Characteristics	Picture
Escherichia coli	Gram-negative	Facultative anaerobic	-Catalase positive -Indole positive - H_2S positive - Coagulase negative - Urease negative - Lipase negative - Beta Lactamase positive - Glucose Positive	Green metallic sheen colonies, Convex, smooth, mucoid, and circular	 Eosin methylene blue
Staphylococcus aureus	Gram positive	Aerobic and facultative anaerobic	-Catalase positive -Indole negative - H_2S negative - Coagulase positive - Urease positive - Lipase positive - Beta Lactamase positive - Glucose Positive	Ferments the mannitol which results in a yellow color of the culture medium	 Mannitol Salt Agar

Klebsiella oxytoca	Gram-negative	Aerobic and facultatively anaerobic	-Catalase positive -Indole positive - H_2S negative - Urease positive - Lipase negative - Beta Lactamase positive - Glucose Positive	Large, mucoid, pink to purple colonies with no metallic green sheen and circular	 Eosin methylene blue
Klebsiella Pneumoniae	Gram-negative	Aerobic and facultatively anaerobic	Catalase positive -Indole negative - H_2S negative - Urease positive -Lactose positive - Lipase negative - Beta Lactamase positive - Glucose Positive	Pink colored colonies, mucoid, convex and circular	 Mac conkey
Proteus mirablis	Gram-negative	Facultatively anaerobic	Catalase positive -Indole negative - H_2S positive - Urease positive - Lipase positive - Beta Lactamase positive - Glucose Positive	Yellowish, white or colourless and translucent colonies.	 Mac conkey

Enterococcus faecalis	Gram positive	Facultative anaerobe	-Catalase negative -Indole negative - H_2S negative - Urease positive - Beta Lactamase positive - Glucose Positive -Lactose positive	Small (pinpoint colonies), smooth, gray or grayish-white, non-hemolytic (γ-hemolytic) in appearance	 Blood agar

I.2.3.2 Pathogenesis of Bacterial Urinary tract infections

Generally, UTIs begin with colonization of the periurethral area by bacteria, followed by their migration into the bladder. In this organ, adhesion of uropathogens is mediated by bacterial fimbriae and adhesins resulting in invasion and colonization of the superficial umbrella cells and biofilm formation. Once sufficient bacterial colonization occurs, bacteria may ascend on the ureter towards the kidneys and then produce tissue-damaging toxins like proteases, hemolysins, urease, colony-necrotizing factors, siderophores, and polysaccharide coatings. These toxins cause tissue necrosis and facilitate invasion, giving rise to a typical cystitis. Thereafter, bacteria can spread and infect the renal parenchyma and cause an inflammatory response called pyelonephritis. If untreated, the inflammatory cascade continues leading to nephritis and causing acute kidney injury (Flores-Mireles *et al*., 2015; Lila *et al*., 2023).

During this process of invasion, uropathogens used various virulence factors to survive, invade organs and overcome host immune surveillance such as:

- **Iron scavenging**: uropathogens like UroPathogenic *Esherichia coli* (UPEC) and *Klebsiella oxytoca* produces several siderophores such as aerobactin, yersiniabactin, enterochelin, etc. allowing the sequestration of iron from iron-binding proteins of a normal host thus promoting their proliferation and survival (Flores-Mireles *et al*., 2015; Tarkkanen *et al*., 1992).
- **Toxins production:** UPEC secretes cytotoxic necrotizing factor 1 (CNF1), affects actin remodelling in the host cell through three small RHO GTPases: RAC1, RHOA and cell division control 42 (CDC42). α-haemolysin (HlyA), another toxin produced by UPEC, is able to stimulate host cell lysis through pore formation and facilitate iron release and nutrient acquisition (Flores-Mireles *et al*., 2015).
- **Production of urease:** it is an enzyme present in Uropathogens of *Klebsiella* spp, *Staphylococcus* spp. and is the major bacterial virulence in this group of microorganisms. This enzyme catalyzes the hydrolysis of urea to carbon dioxide and ammonia resulting in high urine pH. Thus, the accumulation of ammonia becomes toxic for the uroepithelial cells, inducing direct tissue damage (Flores-Mireles *et al*., 2015; Konieczna *et al*., 2012).

- **Formation of biofilms:** they are communities of cells enclosed in the extracellular polymeric matrix composed of nucleic acids, proteins, and enzymes that bind to living and non-living surfaces. The most important adhesives structures involved in the formation process are pili (type1 pili, type 3 pili, P pili, S pili, etc.), flagella and fimbrae found in UPEC, *Staphylococcus* spp, and *Klebsiella* spp. Biofilms play an important role during UTIs by increasing the survival of bacteria in the urinary tract by protecting them against the cleaning effects of hydrodynamic forces, host defense mechanisms, phagocytosis, and antibiotics (Flores-Mireles *et al*., 2015; Ghasemian *et al*., 2019).

I.2.4 Treatment of UTIs: available antibiotics and their mechanisms of action

UTIs are usually treated empirically with antibiotics (Table III) as recommended by primary guidelines (Tan & Chlebicki, 2016). In Cameroon, most of them are prescribed in routine (Table IV). They are molecules capable of inhibiting growth (bacteriostatic antibiotics) or even killing bacteria (bactericidal antibiotics) by acting on one or more metabolic pathways essential to the life of the bacteria but without affecting the host cell (Nemeth *et al*., 2015). The chosen antimicrobial depends on extent of infection (complicated or uncomplicated), common local pathogens and resistance patterns (Tan & Chlebicki, 2016).

Table III: Families, mechanism of action and side effects of antibiotics used against UTIs (Kapoor *et al*., 2017; Overington *et al*., 2006; Pandey & Cascella, 2022)

Type of UTI	Antibiotic families	Molecule	Mode of action	Side effects
Uncomplicated UTIs	**Nitrofuran**	Macrodantin, Macrobid	Inhibition of the citric acid cycle as well as synthesis of DNA, RNA, and protein	Diarrhoea, dyspepsia, abdominal pain, constipation, vomiting, Loss of appetite
	Sulfonamide	Bactrim, Septra	Sulfamethoxazole inhibits the synthesis of dihydrofolic acid. Trimethoprim inhibits thymidine and DNA synthesis.	New or unusual joint pain, seizure, increased or decreased urination
	Phosphonomycins	Monurol	Inhibition of the production of the peptidoglycan precursor UDP N-acetylmuramic acid (UDP-MurNAc)	Nausea, upset stomach, mild diarrhea, sore throat, runny nose, etc
Complicated UTIs	**β-lactam**	Amoxicillin-clavulanate, Cefdinir,cephalexin, Cefaclor and cefpodoxime-proxetil , Ceftriaxon	Inhibition of the synthesis of the peptidoglycan layer	Diarrhea, rash, vomiting and nausea

Quinolone	Ciprofloxacin, Levofloxacin, lomefl oxacin (Maxaquin), norfloxacin, rufloxacin , ofloxacin, and finafloxacin	Inhibition of nucleic acid synthesis	Trouble sleeping, nausea, loss of appetite, vomiting

Table IV: Commonly prescribed antibiotics to treat UTIs in Cameroon (Akoachere *et al*., 2012; Arsene *et al*., 2022; Djim-Adjim-Ngana *et al*., 2023; Nzalie *et al*., 2016)

Type of UTI	Antibiotic families	Molecule
Uncomplicated UTIs	**Nitrofuran**	Nitrofurantoin
complicated UTIs	**Quinolone**	Ciprofloxacin, ofloxacin, levofloxacin, Nalidixic acid
	β-lactam	Ceftriaxon, Amoxicillin-clavulanate, ampicillin

Without antibiotics, the management of UTIs will be basically impossible. Therefore, the uncontrolled use of these molecules has led to the development of superbug pathogens which is a serious public health threat.

I.2.5 The rise of antibiotics resistance in UTIs

I.2.5.1 Generalities

Antimicrobial agents are the cornerstone of modern medical practice. They are effective in managing from easy to life-threatening diseases. However, infections such as UTIs are now becoming more and more difficult to treat due to the increasing development of resistance to available antimicrobial agents (Paul, 2018). Resistant pathogens are of two types; natural and acquired:

- **Natural or intrinsic resistance** is the ability of pathogens to resist in the presence of an antibiotic as a consequence of their natural structural or functional characteristics. (Martinez, 2014). Moreover, some genes naturally occurring in the bacteria are only expressed to resistance levels after exposure to an antibiotic, thus inducing this type of resistance (Jia *et al*., 2016).
- **Acquired resistance** corresponds to the ability of microbial pathogens to support a much higher concentration of antibiotics (Schwarz & Chaslus-Dancla, 2001). It can occur through a mutation that occurs in the DNA of the cell during the replication. The mutant strains are capable of transferring the mutation to the progeny via the vertical pathway. We can also have resistance acquired through DNA transfer via transformation, transposition and conjugation horizontal gene transfer (C Reygaert & Department of Biomedical Sciences, Oakland University William Beaumont School of Medicine, Rochester, MI, USA, 2018).

So far, several causative agents of UTIs have been reported as resistant to antibiotics. In fact, several strains of uropathogenic bacteria including *E. coli*, *S. aureus* and *Klebsiella* species have already been constantly reported to be resistant to antibiotics belonging to fluoroquinolones, beta-lactamase, Fosfomycin and nitrofurantoin (Lagha *et al*., 2019; Paul, 2018). These bacteria have exhibited resistance to antibiotic classes using various fundamental mechanisms including (1) enzymatic degradation of antibacterial drugs, (2) alteration of bacterial drug targets, (3) changes in membrane permeability to antibiotics and (4) active drug efflux pumps (Peterson & Kaur, 2018). Furthermore, these bacteria pathogens have the ability to form biofilms structures responsible for the long-lasting persistence of bacteria in the genitourinary tract. This is the major virulence factor associated with urinary tract infection pathogens (Lagha *et al*., 2019).

I.2.5.2 Biofilms as the main mechanism of antibiotic resistance during UTIs

Biofilm is defined as a microbiologically derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or each other and embedded in a matrix of extracellular polymeric substances (EPS) that they have produced. This matrix accounts for about 90% biomass, exhibiting an altered phenotype with respect to growth rate and gene transcription (Flemming & Wingender, 2010). Biofilm formation (Figure 2) is carried out in five steps:

(1) Reversible attachment of planktonic bacteria to surfaces: here, bacteria attach to the substratum (polystyrene catheters, prosthetic devices, stents or rough surfaces like renal stones) via the cell pole or the flagellum, followed by longitudinal attachment;

(2) Irreversible attachment to surfaces: it is branded by the reduction in flagella reversal rates, a reduction in flagella gene expression and the production of biofilm matrix components;

(3) Formation of a complex layer of biomolecules: cell clusters that are several cells thick appear and embed in the biofilm matrix;

(4) Acquisition of a three-dimensional structure: biofilm grows into 3D macro-colony and persisted cells become the dominant phenotype;

(5) Biofilm detachment: this step is characterized by the degradation of matrix components, with dispersed cells being motile and ready to spread and colonize new surfaces (Crouzet *et al*., 2014; Soto, 2014).

About 65–80% of total human infections are associated with biofilm formation including urinary tract infections (UTIs) (H. Pinto *et al*., 2021). During UTIs, biofilm development is considered as a determinant virulence factor making bacteria persistent in the genitourinary organs (Costerton *et al*., 1999). Bacteria adhere to urinary epithelium urinary directly and develop biofilms that invade the renal tissue. causing pyelonephritis or chronic bacterial prostatitis. In addition, biofilm can also form on catheters and many others prosthetic devices causing their blockage (Soto, 2014). In fact, the environmental conditions created on the catheter surface make it an ideal site for bacterial attachment and formation of biofilm structures (Ong *et al*., 2008). In this type of medical device, microorganisms producing urease, an enzyme that hydrolyzes urea to ammonium ions, can cause encrustation, the formation of infected bladder calculi, and urinary obstruction. The formation of ammonium ions increases the pH of the urine, finally causing the precipitation of magnesium and calcium phosphate crystals These crystals form a layer that protects bacteria from the antimicrobial effects of compounds used for coating or impregnating the catheters (Stickler *et al*., 1998). Therefore, bacteria living within the biofilm are less sensitive to antibiotics than their planktonic counterparts and therefore concentrations of antibiotics required to inhibit bacteria within a biofilm can be 10 to 1000 times higher than those used to inhibit the same bacteria in the planktonic state (Simões, 2011). Several mechanisms have been put forward to explain this strong reduction in antibiotic activity:

- **Antibiotic penetration failure:** the exopolysaccharide matrix acts as a barrier to the diffusion of the antibiotic. Indeed, they are negatively charged and work as an ion exchange resin that can sequester positively charged and hydrophobic antibiotics, such as aminoglycosides (Nadell *et al*., 2015).
- **Slow growth of bacteria:** the metabolism of the bacteria stabilizes at a low level, thus limiting the action of antibiotics requiring cell division and active metabolism. Due to the specific environment of the biofilm, where the deepest zones, rich in acidic residues, are poor in oxygen and nutrients, the bacteria have a slowing metabolism (Blanco *et al*., 2016).

- **Persistence of bacteria:** an increasing dose of antibiotic leads to an initial decrease in the number of living bacteria in a biofilm, up to a threshold beyond which increasing the doses no longer influence the number of bacteria killed, due to the persistence of bacteria persists regardless of the dose administered (Öner, 2013).

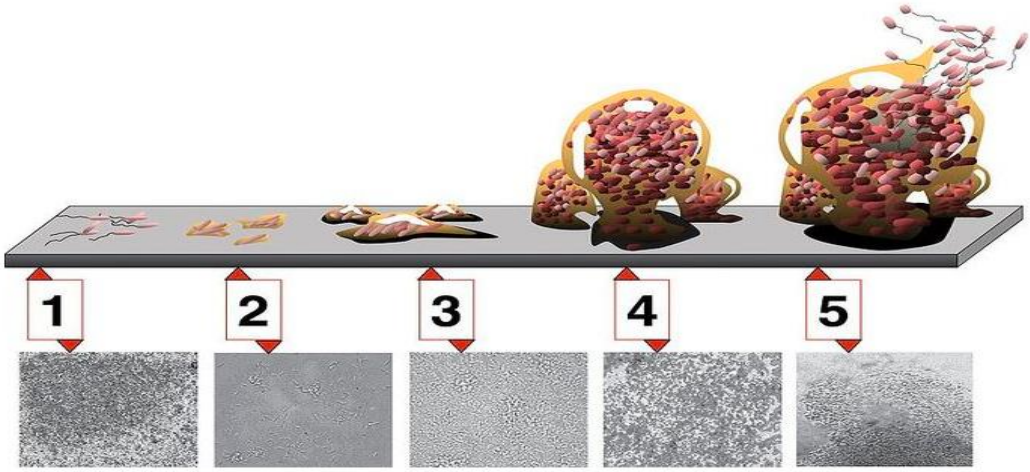


Figure 5: Biofilm formation steps. (1) Reversible attachment of planktonic bacteria to surfaces; (2) Irreversible attachment to surfaces; (3) Formation of the external matrix; (4) Biofilms acquire a three-dimensional structure; (5) Biofilm detachment

I.2.6 Oxidative stress and Urinary tract infections

Oxidative stress refers to an imbalance between oxidants and anti-oxidants in favor of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage (Sies, 2015). Reactive oxygen and nitrogen species (ROS/RNS) produced under oxidative stress are known to damage all cellular biomolecules (lipids, sugars, proteins and polynucleotides) (Murphy *et al*., 2011). To counteract the harmful effects taking place in the cell, the system has developed strategies like prevention of damage, repair mechanism to alleviate the oxidative damages, physical protection mechanism against damages, and the final most important is the antioxidant defense mechanisms. The latter include some antioxidants produced in the body (endogenous), and those obtained from the diet or taken externally (exogenous) (Ďuračková, 2010).

During UTI, tissue damage of many organs bladder is associated with an increased production of reactive oxygen species (ROS). Likewise, the urine of patients with acute UTI contained malondialdehyde (MDA), a product of oxidative stress and marker of lipid peroxidation (Kurutas *et al*., 2005). This possible damage by ROS can be prevented by the endogenous

antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) (Matés *et al*., 1999). Overall, UTI causes oxidative stress and increases lipid peroxidation level leading to insufficiency of antioxidant enzymes such as those mentioned above and therefore patients with UTI may benefit from antioxidants (Kurutas *et al*., 2005).

The increasing drug resistance among bacteria associated with side effects has made therapy of UTI difficult. This resistance problem renewed the efforts to accelerate the search for effective antibacterial agents against pathogenic microorganisms resistant to current antibiotics (Lagha *et al*., 2019). Natural products are the primary source of antibiotics, most of which are produced by microorganisms. Microorganisms, particularly fungi, represent a large and still resourceful pool for discovering novel compounds to combat antibiotic resistance in human pathogens (Toghueo, 2020).

I.3 Plant's Endophytic fungi as a source of new antibiotics

I.3.1 Definition

Endophytic fungi are microorganisms present in various plants and capable of colonizing healthy internal tissues extracellularly or intracellularly without causing disease symptoms (Strobel *et al*., 1999) and are present in all plant investigated so far (Huang *et al*., 2001; Verma *et al*., 2007).

I.3.2 Transmission of endophytic fungi

Transmission of endophytic fungi in plants can be horizontal or vertical.

- **Horizontal transmission** requires the production of external spores and their dispersion to colonize other plants. The spores are transported from one plant to another. This mode of transmission can be favored by the action of wind, phytophagous insects. These insects are responsible for disseminating the spores. In addition, the spores endophytic spores of Alternaria, Cladosporium, Aspergillus, Penicillium and other non-specific taxa are ubiquitous in the atmosphere, sporulating when the host tissue senesces and dies. This appears to be the predominant mechanism for the dispersal of endophytes (Deshmukh *et al.*, 2016).

- **Vertical transmission** occurs when the seed or seedling is infected by the endophyte and then transmitted to the plant. These seeds then give rise to asymptomatic infected. The life of endophytes within host plants involves interaction with them (Deshmukh *et al*., 2016).

I.3.3 Interaction between endophytic fungi and host plant

The interaction between endophytic fungi and their host is variable from fungus to fungus and from host to host. These interactions depend on many factors such as species, ecological habitat of the plant, phylogeny and soil nutrients which qualitatively and quantitatively influence the production of secondary metabolites in plants (Jia *et al*., 2016). Elements such as temperature, moisture, and soil nutrition levels within the ecological conditions component are key elements in determining the types and amount of secondary metabolites of host plants, which would indirectly affect the diversity of the endophytic population. For example, under conditions of low annual sunshine and high annual humidity, medicinal host plants produce more nutrients suitable for colonization, reproduction, and dissemination of endophytic fungi (Wu *et al*., 2013). In contrast, under cold climatic conditions and inappropriate oxygen levels, only certain types of host species can grow properly. Therefore, only a limited number of particular endophytic fungi could colonize the corresponding host plants, which leads to a certain degree of regional specificity on the population structure of endophytes (Jia *et al*., 2016). Similarly, the outcome (positive, negative, or neutral) of the relation between endophytic fungi and their host can be controlled by the nature of the latter's genomes and consequently has an impact on the type of secondary metabolites produced (Moricca & Ragazzi, 2008).

These fungi enhance the growth of the plants and providing resistance to biotic and abiotic stress pathogens through the production of antimicrobial agents and growth regulators (Gera Hol *et al*., 2007). In addition, they boost the production and accumulation of secondary metabolites, including bioactive compounds used as drugs, produced originally by the medicinal plants. In return, they receive from their host, organic nutrients, protection and the opportunity for propagation to the next generation (Clay & Schardl, 2002). Symbiotic endophytic fungi, as an abundant resource for metabolites have the special ability to produce the same or similar bioactive compounds originating from their host plants as well as other bioactive constituents.

On the other hand, plant availability is viewed as the limiting factor in the research success of some natural products. Overall, a large quantity of plants is required to produce sufficient amounts of the bioactive compounds for clinical use (Strömstedt *et al*., 2014). Sometimes, compounds are isolated from endangered or highly endemic plants. This raises major concerns regarding biodiversity conservation. In this regard, microorganisms present the opportunity to

discover a plethora of compounds using a large-scale production and offer a renewable source (Alvin *et al*., 2014).

I.3.4 Antimicrobial metabolites from endophytic fungi

Endophytes, the most important integral part of plant tissues, are a progressively significant area of research in many fields because of their chemical miscellany and their ability to produce many unique secondary metabolites that can be utilized for fuel, medicine, restoration (bioremediation) and agriculture (biocontrol or plant growth promotion) (Ferdous *et al*., 2019). Several endophytic fungi produce important therapeutic with drugs numerous biological properties, including antibacterial, antifungal, immunosuppressants, antiviral, antiparasitic, antioxidant, anti-inflammatory, and anticancer properties (Ferdous *et al*., 2019; Manganyi & Ateba, 2020; Toghueo *et al*., 2019; Toghueo, 2020). These bioactive metabolites might be structurally classified into alkaloids benzopyranones, chinones, peptides, phenols, quinones, flavonoids, steroids, terpenoids, tetralones, xanthones, and others (Fadiji & Babalola, 2020). Over the past few decades, several of them isolated from endophytic fungi are reported to be broad-spectrum compounds with action on a range of bacteria (Caruso *et al*., 2022; Digra & Nonzom, 2023; Ibeabuchi Jude Ali *et al*., 2023; Kouipou Toghueo & Boyom, 2019; Omomowo *et al*., 2023; Sundaramoorthy *et al*., 2015; Toghueo, 2020). They eventually constituted a valuable source of novel leads for the discovery of new antibiotics. Endophytic fungi in a medicinal plant produce similar bioactive compounds as the host plant, according to researchs (Strobel *et al*., 1999; Zhao *et al*., 2019). These findings reduced plant overexploitation due to the biotechnological use of endophytes in bioactive compound production. In addition, studies on endophytic fungi have contributed primary taxonomic data on the distribution, diversity, and novel species description. Exploring the diversity of endophytic fungi in medicinal plants is therefore critical to discovering new sources of bioactive compounds and protecting endangered medicinal plants (Hussein *et al*., 2024).

I.3.5 Method used to obtain endophytic fungi

In general, the common method employed to obtain endophytic fungi is relatively simple, but involve steps that must be strictly followed to avoid misinterpretation. After the plant material collection, the first step is the superficial disinfection of the sampled tissue to eliminate the epiphytic microbiota followed by (Dos Reis *et al*., 2022) fragmentation and distribution onto

culture medium and incubation. After the fungal growth, the third step consists of the purification of isolates (Dos Reis *et al*., 2022).

I.3.5.1 Collection and sampling of plant material

The first step to studying endophytic microorganisms is the collection of plant material. However, there is no universal consensus on the adequate number of individuals and/ or samples per individual that should be considered. It is strongly recommended a representative sampling according to the objectives proposed by the study. After collecting the plant material, the samples should be placed in a sterile plastic container or bag, transferred to a cool box, and preferably processed within 24 h after sampling under aseptic conditions (Dos Reis *et al*., 2022).

I.3.5.2 Surface disinfection of plant material

Several methodological obstacles appear when studying endophytes, including the complete removal of microorganisms that make up the epiphytic microbiota. The samples should be washed in running tap water for some time to remove debris, dust, other particles, and main epiphytes (e.g. epiphytic bacteria, yeasts and filamentous fungi) prior to the superficial disinfection of the material to enhance the process and fragmented into small pieces (5 mm × 5 mm are commonly used). Effective methods of surface disinfection to remove the epiphytic microbiota are mandatory first steps for studying endophytic microorganisms, which includes chemical or physical procedures. The chemical sterilization of the sample surface is the most used method for removing microorganisms from the rhizoplane. The most widely used consist of three basic steps: (1) submersion of the tissue in ethanol; (2) immersion of the tissue in the main sterilizing agent, and (3) successive washing with distilled water previously autoclaved. The main sterilizing agents used here are sodium hypochlorite and mercury chloride ($HgCl_2$). Other sterilizing agents can also be used such as hydrogen peroxide, paraquat and peroxyacetic acid in ethanol (Dos Reis *et al*., 2022). The other method consisting of physical surface disinfection can be mediated by sonication or ultraviolet (UV) light. In the case of sonication, the plant tissue previously washed in tap water should be placed in a sonicator or sonication bath containing buffer solution where the sound waves will be propagated, promoting the removal and lysis of microbial cells from the sample surface (Saldierna Guzmán *et al*., 2020). Overall, the choice of the disinfection protocol is a critical step to studying endophytic microorganisms and the selected methodology should be able to completely remove the epiphytic microbiota without interfering with the endophytic microbiota.

Therefore, the chosen method should consider the characteristics of the analyzed tissue/plant species (Burgdorf *et al.*, 2014).

I.3.5.3 Cultivation conditions, isolation and culture purification

There are many culture media available for seeding of tissue fragments and purification of isolates with Potato Dextrose Agar (PDA) as the most frequently used. Other growth media such as Malt Extract Agar (MEA), Tryptone Soybean Agar (TSA), Tryptone Bovine Extract Agar (TBEA), etc can be also used. Depending on the purpose of the study and the characteristics of the habitat (i.e. host plant species), the medium commonly used can be nutritionally supplemented and/or replaced by a specifc medium to better achieve the objectives of the study (Dos Reis *et al*., 2022; Hamzah *et al*., 2018; Pietro-Souza *et al.*, 2017). The different plant parts are placed on Petri dishes containing the chosen culture medium. Isolated mycelial fragments from the cultured petri dishes are subcultured on a new medium until the obtention of pure culture (Dos Reis *et al.*, 2022).

I.3.6 Fermentation techniques and crude extract production

The endophytic microorganisms have enormous potential to synthesize various biologically active metabolites. Therefore, it is necessary to design an appropriate cultivation system for their commercial exploitation. The endophytic fungi can be cultivated using liquid submerged (SmF) or solid state fermentation (SSF) (Patil *et al*., 2016).

I.3.6.1 Submerged fermentation (SmF)

Submerged fermentation system also called liquid fermentation system is a technique of cultivation of microorganisms in liquid broth medium that breaks down the supplied nutrients to compound (Costa *et al*., 2017). It is the most commonly method used in industrial fermentation (Kapoor *et al*., 2016). There are four types of submerged fermentation processes: batch culture, fed batch culture, continuous culture and perfusion batch culture; (a) Batch cultivation is the simplest mode of operation, in which the microorganisms are inoculated in fixed volume of fermentation medium for a defined time, after which the broth is collected for downstream processing; (b) Fed batch process mode: concentrated components of the nutrients are gradually added to the batch culture; (c) In continuous reactors, fresh medium is continuously added and broth from the bioreactor is continuously withdrawn. This allows the fermentation process to be operated for long periods; (d) In the perfusion batch cultivation mode, there is an equal volume of

addition of new medium and withdrawal of cell free broth (Ouedraogo & Tsang, 2021). SmF processes can be easily scaled up with ease of automation and do not suffer from the heat mass transfer limitations. However, the major drawbacks associated with this method are the low productivity, high production cost, and complexity of the medium (Dhillon & Surindara Kaura, 2016)

I.3.6.2 Solid State Fermentation (SSF)

Solid-state fermentation (SSF) is defined as the microbial growth on solid substrates in the absence of water or low content. However, solid material should have enough moisture content to enable growth (Gupta & Pandey, 2019). Among the microorganisms that most adapt to this type of fermentation are fungi, which are more favorable to the high production of enzymes . Among the enzymes that can be produced in SSF are amylases, proteases, xylanases, cellulases, and pectinases . These are produced by fungi directly on substrates, such as cereals or cereal derivatives. The substrates for SSF are generally residues or by-products of agroindustry. Among these are rice, wheat, barley, corn, soybeans, sugarcane, corn cob, wheat bran, and rice straw and others are also considered viable materials for biotransformation (Confortin *et al*., 2019; Gupta & Pandey, 2019). Today, two types of SSF systems can be distinguished, depending on the nature of the solid phase used: (a) SSF on natural solid substrates, and (b) SSF on impregnated inert supports .The first and most commonly used (and most often described) system involves cultivation on a natural material (cassava, potato, etc) this system is referred to as 'cultivation on natural substrates'. The second system, which is not as frequently used, involves cultivation on an inert support (vermiculite, polyurethane foam, etc) impregnated with a liquid medium (Ooijkaas *et al*., 2000). Although SSF has various advantages, the most considerable one is the use of agricultural and agro-industrial residues as a substrate that leads to an economical process with low initial costs of the process. Besides, the extraction yield is high than during submerged fermentation. The most challenging aspect of the SSF process is the difficulties in heat and mass transfer due to the lack of homogeneity in the solid particles (Khosravi & Razavi, 2021)

I.4 *Annona muricata*

A. muricata Lin known as soursop (English), Ebom beti (Cameroon) and graviola (Portuguese), is a plant species of the genus Annona, of the Annonaceae family, order Magnoliales

and division Magnoliophyta. The genus Annona comprises over 70 species among which *A. muricata* is the most widely grown. Its synonyms are *Annona bonplandiana* Kunth; *Annona cearensis* Barb. Rodr; *Annona macrocarpa* Werckle´; *Annona muricata* var. borinquensis Morales and *Guanabanus muricatus* M. Go´mez (Boyom *et al*., 2011; A. C. de Q. Pinto *et al*., 2005).

I.4.1 Ecology and distribution

A. muricata is native to the warmest tropical areas in South and North America and is now widely distributed throughout tropical and subtropical parts of the world, including India, Malaysia West and Central Africa. *A. muricata* survives in the humid tropical and subtropical lowlands. It is common on the coast and is found on slopes. Planted for its fruit, it has become wild or naturalized in thickets, pastures and along roads. The species is commonly cultivated in home gardens and is found in rural garden areas on volcanic and raised limestone islands, where it is occasionally naturalized. Trees are not found on atolls. They withstand very little frost (Coria-Téllez *et al*., 2018; A. C. de Q. Pinto *et al*., 2005).

I.4.2 Traditional uses

Possessing a wide range of uses due to its presence in many countries, the various organs of *A. muricata* are used in traditional medicine for the treatment of many diseases especially cancer and infectious diseases. The most widely used preparation in traditional medicine is the decoction of bark, root, seed or leaf with varying applications. The fruit is used as natural medicine for arthritic pain, neuralgia, arthritis, diarrhea, cancer dysentery, fever, malaria, parasites, rheumatism, skin rushes and worms, and it is also eaten to elevate a mother's milk after childbirth. The leaves are employed to treat cystitis, diabetes, headaches and insomnia. Moreover, internal administration of the leaf's decoction is believed to exhibit anti-rheumatic and neuralgic effects, whereas the cooked leaves are topically used to treat abscesses and rheumatism (Adewole & Caxton-Martins, 2009; De Sousa *et al*., 2010; Yajid *et al*., 2018). In Indonesia, the Caribbean islands and South Pacific countries, the leaves are used in baths to treat skin ailments (Boulogne *et al*., 2011). The ingestion of leaves decoction is used to treat discomfort associated with colds, flu and asthma. Natives of Malaysia use *A. muricata* leaves to treat cutaneous (external) and internal parasites (Coria-Téllez *et al*., 2018). In Cameroon the decoction of leaves of this plant is used against malaria, convulsion, Helminthiasis and microbial infections (Boyom *et al*., 2011; Roger *et al*., 2015; Tsabang *et al*., 2012).

I.4.3 Previous studies on endophytic fungi from *A. muricata*

Endophytic fungi isolated from *Annona muricata* encompass a diverse range of genera with notable biological activities. Common genera include *Aspergillus, Penicillium Fusarium* and *Trichoderma* which are known for their production of bioactive compounds. Other genera such as *Phomopsis, Pseudoficoccum, Periconia, Neocosmospora* etc also contribute to the diverse fungal community within soursop (Abdel-Rahman *et al*., 2019; Department of Pharmaceutical and Medicinal Chemistry, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Nigeria *et al*., 2018; Hasan *et al*., 2022; Silva *et al.*, 2024; Toghueo *et al*., 2019).

In the same way, some studies have demonstrated the different biological properties of extracts of endophytic fungi from *Annona muricata*. These studies substantially sought at identifying potent extracts that could be progressed for chemical analysis in order to characterize potential active compounds that can serve as starting points for new medicines against infectious diseases.

In this regard, the chemical and biological active guided investigation of crude extract from the endophytic fungus *Periconia* sp. obtained from leaves led to the characterization of two compounds Pericoannosin A and F both with anti-HIV activity with IC_{50} of 69.6 and 29.2 μM (D. Zhang *et al*., 2015). In a subsequent investigation, the anti-HIV compound, Pericoannosin B (IC_{50} value of 18.0 μM) was also isolated from the same extract by Liu *et al*., (2017). The antiplasmodial potential of endophytic fungi isolated from different organs of *A. muricata* was investigated against sensitive (Pf3D7) and resistant (PfINDO) strains of *Plasmodium falciparum*. According to the authors, 17.76% (n = 27) of fungi tested were found to completely inhibit the growth of Plasmodium strains at 10 μg/mL. Overall, the extracts from *Penicillium citrinum* AMrb11 (IC_{50}: 0.84–0.93 μg/mL) and *Neocosmospora rubicola* AMb22 (IC_{50}: 0.39–1.92 μg/mL) showed the highest promising activity against all three plasmodial strains with selectivity indexes ranging from 34.71 to 180.97 (Toghueo *et al*., 2019). Abdel-Rahman *et al*., (2019) reported the antimicrobial activity of extract from *Aspergillus niger* isolated from *A. muricata* seeds against *S. aureus* (IZD 12 mm ± 0.5), *P. aeruginosa* (IZD 15 mm ± 0.0), and *E. coli* (IZD 14 mm ± 0.0).

It is obvious that endophytic fungi from *Annona muricata* can produced secondary metabolites with potency against a broad spectrum of infectious pathogens, including bacteria. Therefore, support the aim of this study which is to search for potential active compounds against uropathogens causing UTIs.

Chapter II: Material and Methods

CHAPTER II: MATERIAL AND METHODS

II.1. Presentation of the research framework and materials

II.1.1 Research Framework

This research was mainly carried out in three areas namely:

- University of Yaoundé I (Departments of Biochemistry and Organic Chemistry) for performing biological assays and compound isolation;
- Institute of Natural Resources and Agrobiology of Salamanca (IRNASA) (Plant-Microorganism Interaction Research Group), Spain for fungal identification;
- University of South Florida (Department of Chemistry), USA for NMR analyses of compounds;

II.1.2 Material

II.1.2.1 Microbiological material

- Forty-one endophytic fungi previously isolated from various healthy and mature plant organs, including roots, root bark, fruit, fruit thorns, seeds, twigs, leaves, stem bark, bark, and peduncles;
- Three bacterial species were used in this investigation:
  - ✓ *Staphylococcus aureus* ATCC 43300, gram positive, cocci-shaped, aerobic and facultative anaerobic bacterium is a Methicillin and Oxacillin resistant strain obtained from the American Type Culture Collection (ATCC);
  - ✓ *Escherichia coli* ATCC 25922, gram negative, rod-shaped, facultative anaerobic bacterium is a quality control strain obtained from the American Type Culture Collection (ATCC);
  - ✓ *Klebsiella oxytoca*, gram negative, rod-shaped, aerobic and facultative anaerobic obtained from the "Centre Pasteur du Cameroun" (CPC) of Yaoundé-Cameroon is a clinical isolated from isolates urine of a patient suffering of urinary tract infection;
- Vero cell obtained from Centre Pasteur du Cameroun was used to perform cytotoxicity;

II.1.2.2 Culture media

- Nutrient Agar medium (NA; HiMedia, India) and Nutrient Broth (NB; HiMedia, India) medium were used for all antibacterial assays;
- Dulbecco's Modified Eagle Medium (DMEM) was used for cytoxicity assay;

II.1.2.3 Reagents, solvents and drugs

- **Reagents:** MTT, Resazurin, trypsin-EDTA, 2,2-diphenyl-1-picryl-hydrazyl (DPPH), Fe^{3+} ortho-phenanthroline, hydrogen peroxide, peptone, glucose, foetal bovin serum (FBS);
- **Solvents**: ethyl acetate, methanol, hexan, dicloromethane, Dimethysulfoxide (DMSO);
- **Drugs:** Ciprofloxacin, Penicillin/Streptomycin, L-ascorbic acid, Hydroxylamine, Podophyllotoxin;

II.1.2.4 Equipments, consumables, and glassware

Among those used, the most important were: UV lamp, TLC plate, column chromatography, NMR instrument, microplate reader, rotavapor, agarose (1% agarose) gel electrophoresis, primers, PCR kit, Microplates, petri dishes, Microtubes, centrifuges, laminar flow hood, CO_2 incubator, Simple incubator without CO_2, Autoclave, decanter bulb, culture bottle, flask, elernmeyer, beaker, bec bensen spout, funnel, scale, spatula, filter paper, sieve, bain marie.

II.1.2.5 Others

Phosphate-buffered saline, physiological water, distilled water, silica, NaCl;

II.2 Methods

II.2.1 Quality control of the medium used

Quality control (Basu *et al*., 2005; Curtis, 1985) of the medium used was carried out using several parameters as:

-Physical parameters such as color, clarity, cracked medium or crystallization;

- **pH Testing:** the pH of the media was measured to ensure it is within the specified range;

- **Sterility Testing** consisted of performing tests to confirm that the media is free from contaminants. It has been done by incubating the media under conditions that would promote the growth of contaminants and checking for any microbial growth;

-Growth Promotion Testing consisted of inoculating the media with known microorganisms to verify that it supports the growth of these organisms. This helps ensure that the media has the proper nutrient composition and is effective for its intended purpose.

II.2.2 Screening and characterization of potent endophytic fungi from *Annona muricata*

II.2.2.1 Source of endophytic fungi and transplanting procedure

The forty-one endophytic fungi used in this study were isolated in 2016 from healthy and mature organs (root, root bark, fruit, the thorn of fruit, seed, twig, leaf, stem bark, bark, and peduncle) of *Annona muricata* (3289/HNC) collected in Yaoundé (Figure 6), Cameroon, on January 10, 2016 (Toghueo *et al*., 2019). All endophytes were kept at -80°C in a 50% glycerol solution at the Antimicrobial & Biocontrol Agents Unit (AmBcAU), Laboratory for Phytobiochemistry and Medicinal Plants Studies, Department of Biochemistry, Faculty of Science, University of Yaoundé I, Cameroon (Toghueo *et al*., 2019). The number of isolates per organ are presented in Appendix 2. At the beginning of this study, explants from the preservation tubes were transferred to Potato Dextrose Agar (PDA) medium in petri dishes and kept under dark at room temperature. After 72 hours, the mycelium emerging from the explants was checked to ensure purity. Those contaminated were transferred on a new PDA until the pure isolates were obtained.

Figure 6: *Annona muricata* (A) Tree; (B) Flowers; (C) Fruits; (D) Leaves, (Yimgang, 2024)

II.2.2.2 Culture and extraction of metabolites from endophytic fungi

Each fungal strain was cultured on potato dextrose agar (PDA; HiMEDIA, India) at 25°C for seven days. Subsequently, three pieces (1 × 1-cm) of mycelium from these cultures were used to inoculate 100 mL of potato dextrose broth medium (PDB; HiMedia, India) in 250 mL Erlenmeyer flasks. Liquid cultures were grown for 7 days under static conditions in an incubator (Gallenkamp, UK) with a temperature set at 25 ± 2°C before extraction. After the incubation period, 100 mL of ethyl acetate (EtOAc) solvent was added to each culture, gently shaken, and kept overnight at room temperature. The organic phase was then decanted, and the aqueous phase was extracted three times with 100 mL of EtOAc. The organic phases were pooled (total = 300 mL) and evaporated to dryness at 40°C using a rotary evaporation system (Heidolph, Germany) to obtain crude fungal extracts

(Figure 7). The dry residue was diluted with 100% DMSO (Loba Chemie, India) to a stock concentration of 25 mg/mL, and stored in sterile conical flasks at 4°C until further use. Extraction yields of endophytic fungi (Table VI) were expressed in mg per 100 mL of PDB medium.

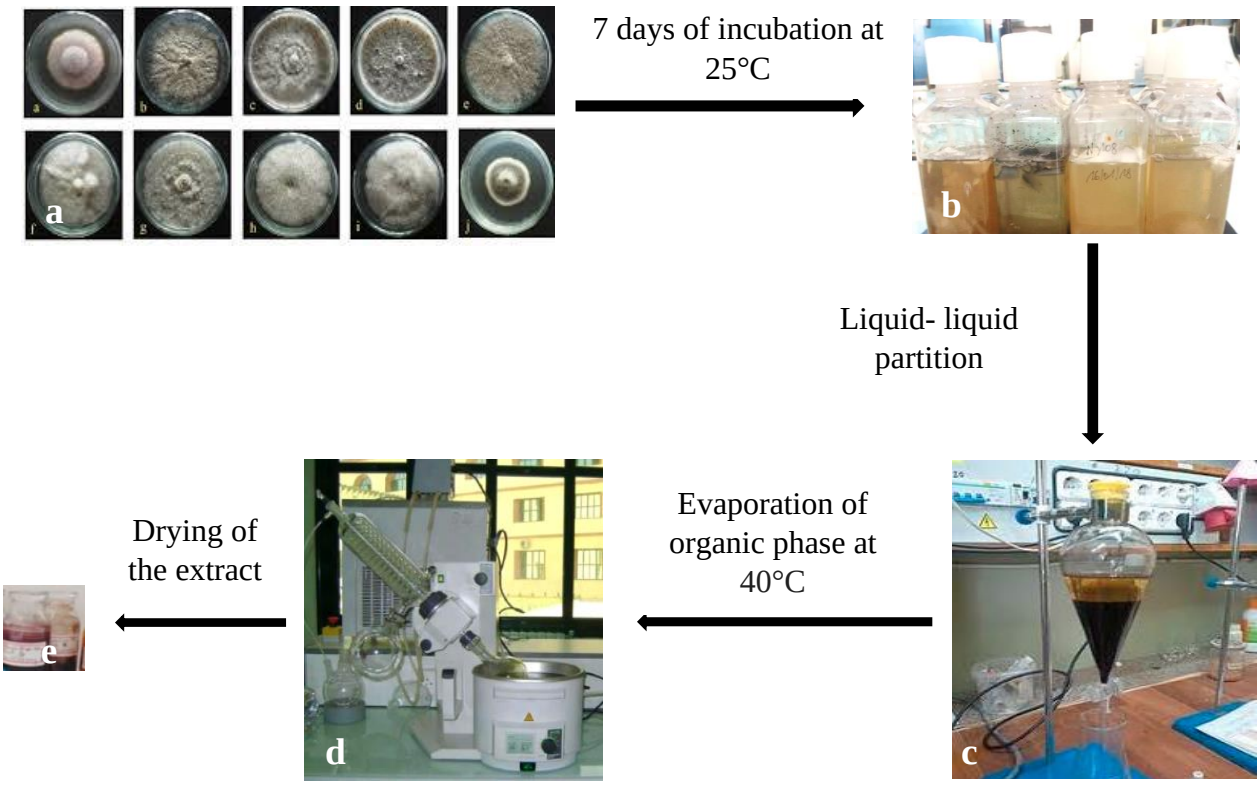


Figure 7: Culture of fungi for secondary metabolites production; (a) seven days old fungi cultures on PDA; (b) liquid medium with mycelium culture after seven days of fermentation at room temperature; (c) liquid liquid partition; (d) evaporation of the organic phase to obtain crude extract; (e) dry crude extract conserved in glass bottle.

II.2.2.3 Antibacterial screening

II.2.2.3.1 Microbial species subculture and culture media

Nutrient agar medium (NA; HiMedia, India) was used to revive the bacterial strains 24 h before each experiment, while nutrient broth (NB; HiMedia, India) medium was used for all antibacterial assays.

II.2.2.3.2 Preparation of bacterial suspensions

The different bacterial suspensions were prepared at $1.5x10^8$ CFU/mL using the 0.5 Mc Farland standard by comparison of the turbidity. Indeed, using a loop, a colony from the 24 h cultures on NA was aseptically removed and introduced into 10 mL of sterile physiological water (0.9% NaCl). After dispersion, the turbidity of this tube was compared to the 0.5 Mc Farland standard and then adjusted by dilution to the concentration of 10^7 CFU/mL

II.2.2.3.3 Preparation of stock solutions of the different extracts and the reference antibiotic

Stock solutions of extracts were prepared at 25 mg/mL by dissolving 25 mg of extract in 1 mL of 100% DMSO. Ciprofloxacin (Sigma Aldrich, USA) the reference drug was prepared in sterile distilled water at 1 mg/mL.

The intermediary solutions of extracts were prepared in 96-well microplates. Indeed, 180 µL of nutrient broth (NB) medium were introduced in the well of the plate; then, 20 µL of a stock solution (25 mg/ mL) were taken and introduced in the wells in order to reach a final concentration of 2500 µg/mL.

The final concentration of DMSO (Sigma–Aldrich, Germany) was at most 0.4% in wells during different assays. Prior to all assays, an experiment was ran consisting of testing the effect of DMSO at the concentration above (0.4%) on each bacterium used in this study. For this purpose, bacteria were treated with DMSO 0.4% followed by an incubation of 24 h at 37°C. At the end of this incubation period, no inhibition of bacterial growth was observed in comparison to negative control (bacteria alone).

II.2.2.3.4 Preliminary screening of endophytic fungi extracts

Each of the 41 crude ethyl acetate (EtOAc) extracts was screened at 100 µg/mL for antibacterial activity against three bacterial species following the M07-A9 protocol of the Clinical Laboratory Standards Institute (CLSI) (Clinical and Laboratory Standards Institute, 2015) with resazurin based method.

Principle

This method is based on the measurement of the fluorescence of resorufin at 570 nm and 590 nm as the emission and excitation wavelengths respectively formed following the reduction of resazurin by the deshydrogenases of viable cells.

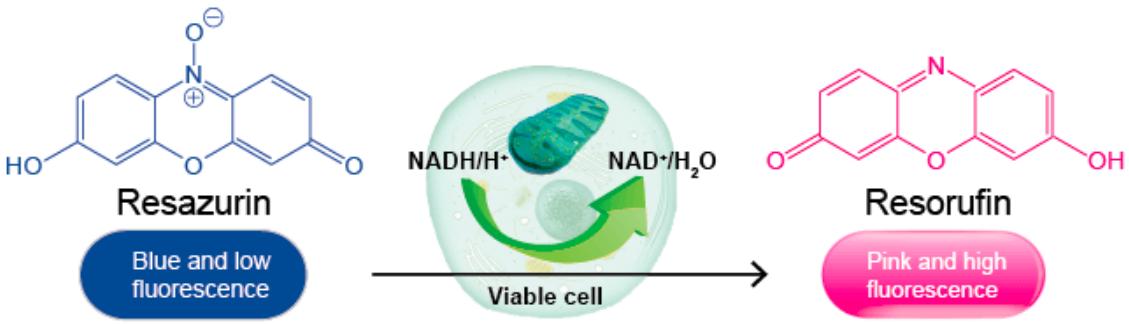


Figure 8: Enzymatic reduction of resazurin to resorufin

Procedure

Briefly, 92 µL of NB medium (HiMedia, India) were aseptically introduced into the wells of a 96-well microplate. Eight microliters (8 µL) of each extract (2500 µg/mL), were added to the wells followed by the addition of 100 µL of standardized bacterial suspension (10^7 CFU/mL). The tests were performed simultaneously for the negative control (NB + bacteria) and sterility control (NB alone). Ciprofloxacin (Sigma–Aldrich, Germany) at 64 µg/mL was used as the positive control. The plates were incubated at 37°C for 24 hours and after this period, 20 µL of a freshly prepared resazurin solution (0.15 mg/ml) was added to all the wells and the plates were incubated under the same conditions again for 30 minutes. Thereafter, wells in which no change in color from blue to pink was considered active. The test was performed in triplicate and repeated twice. Extracts that were consistently active on at least one of the bacteria tested were selected for molecular identification and the dose response study.

II.2.2.4 Molecular identification of potent antibacterial endophytes

II.2.2.4.1 DNA extraction

DNA was extracted from small mycelial fragments scraped from the surface of culture plates using a commercial kit (RedExtract-N-Amp Plant PCR Kits, Sigma Aldrich, USA). Briefly, the fungus was grown on a PDA dish. Fungal mycelium of 0.5 to 0.7 cm was cut and put into a 2 mL collection tube using a standard one-hole paper punch and 100µL of the

extraction solution were added to the collection tube. The sterile paper punch and forceps used prior to and between the handling of different samples. The close tubes were briefly vortexed and incubated at 95°C for 10min. A dilution solution (100 µL) was added and the mixture vortexed for 1 min. The diluted DNA extract was stored at 4°C (Sánchez Márquez *et al*., 2012).

II.2.2.4.2 PCR amplification

DNA amplification by PCR was then performed using the Extract-N-Amp PCR ReadyMix (this is a 2 × PCR reaction mix containing buffer, salts, dNTPs, Taq polymerase and JumpStart Taq antibody) and the primer pairs ITS4 and ITS5 from Invitrogen with a size of 500 bp (White *et al*., 1990) in a Gene Amp PCR system 9700 (Thermo Fisher Scientific-USA).

Primer sequences

ITS5: 5´- GGAAGTAAAAGTCGTAACAAGG- 3’

ITS4: 5´- TCCTCCGCTTATTGATATGC- 3´

The ITS1-5.8S rRNA-ITS2 region was amplified in a PCR and the amplification conditions were:

1) Initial denaturation 95°C for 3 min

2) Denaturation 95°C for 0.5-1 min.

3) Annealing 45-68°C for 0.5-1 min.

4) Extension 72°C 1-2 min.

5) Final extension 72°C 10 min.

6) Hold 4°C indefinitely

Steps 2, 3 and 4 were repeated 35 times. Each sample consisted of 10µL Taq polymerase master mix, 3µL primer mix (5µmoL/µL each), 2µL templates DNA and 2µL double distilled water.

II.2.2.4.3 DNA electrophoresis

PCR products and DNA ladders were loaded into the wells of an agarose gel after addition of loading buffer. Electrophoresis (Electrophoresis Power Supply-EPS 600) was conducted at 70 volts for 80 min. The gel consisted of 1% agarose (Sigma) in 100mL 50X TAE buffer solution. 2µL of Midori Green Direct DNA Stain (Invitrogen) had been added before casting. After electrophoresis, the gel was transferred into the UV-transluminator

(Fluor-S Multimager) to confirm that the PCR was successfully carried out. The DNA appears as yellow bands under UV light (figure 9) (Toghueo *et al*., 2017).

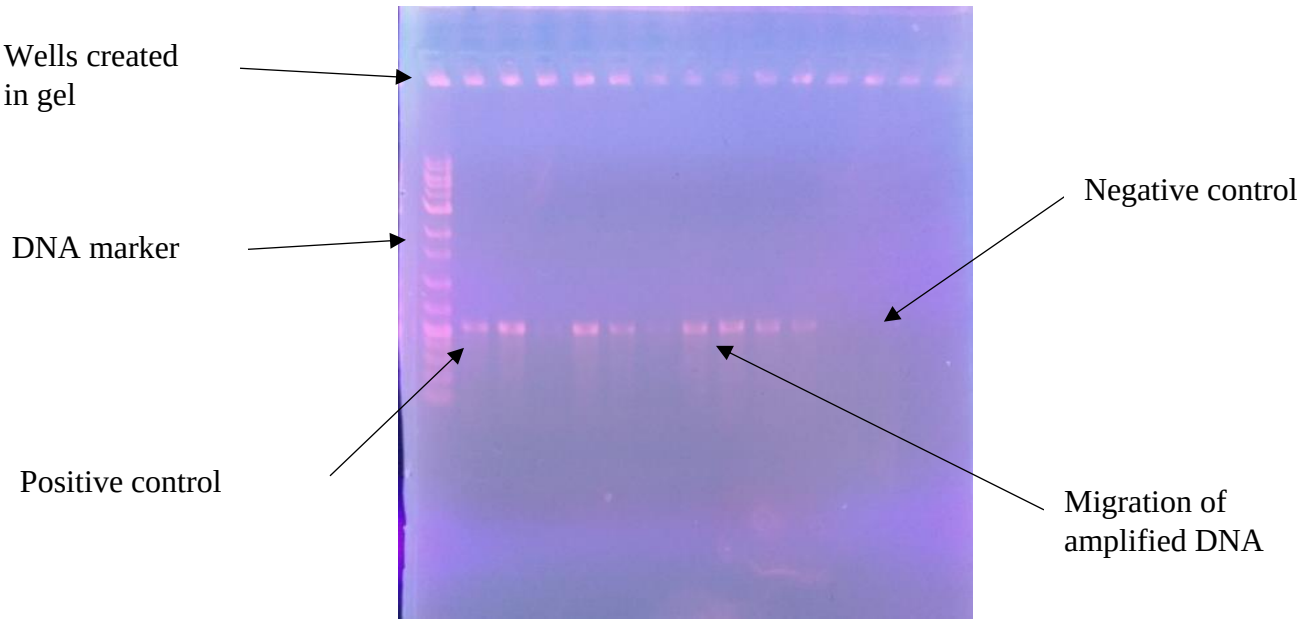


Figure 9: Agarose gel after migration of PCR product; Negative control: all reagents without amplified DNA, Positive control: DNA fragments of known sizes (DNA ladders).

II.2.2.4.4 DNA sequencing

II.2.2.4.4.1 Purification of PCR products

Two hundred microliters (200µL) of binding buffer were added to the 20µL of PCR product. The mixture was properly swirled by vortexing and then transferred onto the spin filter for centrifugation at 11.000 rpm for 3 min. The filtrate was removed and a second centrifugation was carried out for 2 min. The spin filter was placed into a new 1.5mL receiver tube and 20µL of elution buffer (or ddH2O) were directly added into the center of the spin filter and incubated for 1 min at room temperature. The mixture was centrifuged at 11.000 rpm for 1 min. The purified PCR products were stored at -20°C before sequencing (Toghueo *et al.*, 2017).

II.2.2.4.4.2 Quantification of DNA and preparation of reaction mixture for sequencing

After elution of PCR products, DNA was quantified using a Nanodrop spectrophotometer (Thermo Scientific, Germany).

- Total volume: 8µL (DNA + Primer + ddH2O)
- DNA concentration in the mixture = 60ng
- Concentration of Primer (ITS4) = 1µM

Example: For isolate 1 with DNA concentration at 19.30ng/µL, the reaction mixture was prepared as follow: 4µL of DNA + 3µL of ITS4 + 1µL of ddH2O.

The purified PCR amplicons were sequenced in a 3100 Genetic Analyzer (Applied Biosciences). Only one strand of the PCR amplicon was sequenced. The sequencing reaction was started at the 5' end of the ITS1-5.8S rRNA-ITS2 region, using primer ITS4.

II.2.2.4.5 Sequences and Phylogenetic analyses

The quality of the sequences obtained was analyzed by means of the sequencing reaction chromatograms, visualized with Chromas 2.4 software (Technelysium, Australia). Only sequences whose chromatograms showed discrete peaks and no ambiguous sections were used. The sequences were manually edited and compared with available data from GenBank databases.

The BLAST algorithm was used to find sequences similar to those obtained from fungal isolates. The criteria for identifying isolates were based on the similarity of their sequences to those of reliable reference isolates included in open access nucleotide databases. A dendrogram was made with the nucleotide sequences of the isolates and those of reference strains deposited in Centraalbureau Voor Schimmelcultures (CBS) and American Type Culture Collection (ATCC) collections. Sequences were aligned using the following parameters: pairwise alignment parameters (gap opening = 10 and gap extension = 0.1) and multiple alignment parameters (gap opening = 10, gap extension = 0.2, transition weight = 0.5, and delay divergent sequences = 25%) and optimized manually in MEGA 7.0. For the phylogenetic analyses based on maximum likelihood (ML), the best-fit models of nucleotide substitution for each data partition were determined using MEGA 7.0 software and incorporated into the analyses. Alignment gaps were treated as partial missing information, and one thousand replications estimated the robustness of the classifications. The

initial trees for the heuristic ML search were obtained by applying the neighbor-joining method to a matrix of pairwise distances estimated using the maximum composite likelihood approach, allowing some sites to be evolutionarily invariable. Groups of sequences at proximity within the same branch of the dendrogram were individually aligned to determine their similarity percentage. Sequences with close similarity with reference sequences used for phylogenetic analysis were considered to belong to the same species as the reference sequence (Toghueo *et al*., 2017).

II.2.2.5 Determination of Minimal Inhibitory Concentrations (MICs)

The active extracts were selected for the dose–response study for MIC determination on the three previous bacteria using the M07-A10 protocol (Clinical and Laboratory Standards Institute, 2015) with some modifications.

Principle

The micro-dilution method is based on the ability of a microorganism to grow and survive in a medium supplemented with antimicrobial substances**.** The lowest concentrations inhibiting the visible growth of bacteria were recorded as the MICs and were revealed by observation of no turbidity (no growth) and resazurin dye as described previously.

Procedure

To determine the MIC of the selected extracts, serial twofold dilutions of each extract were performed with final concentrations ranging from 1.5625 to 100 µg/mL for the extracts and from 0.117 to 7.5 µg/mL for the positive control. All test plates were incubated at 37°C for 24 hours. The turbidity was observed as an indication of growth, and the MIC was considered as the lowest concentration with no visible growth of microorganisms (no turbidity).In addition to this, the resazurin assay was also performed as described previously. Wells containing NB and bacteria constituted the negative control, while the sterility control contained NB alone. The test was performed in duplicate and repeated twice.

The criteria for activity of extracts against bacteria were defined as follows: very active (MIC < 25 µg/mL), partially active (MIC 25-100 µg/mL) and non-active (MIC >100 µg/mL).

II.2.2.6 Cytotoxicity assay of promising extracts

The cytotoxic effect of antibacterial extracts was assessed using the MTT assay as described by Mosmann, (1983), targeting normal monkey kidney Vero cells ATCC CRL1586 cultured in complete DMEM medium.

Principle

This method is based on the measurement of the absorbance at 570 nm of the formazan (Purple) formed following the reduction of MTT (yellow) by the dehydrogenases of viable cells (Babacan *et al*., 2021).

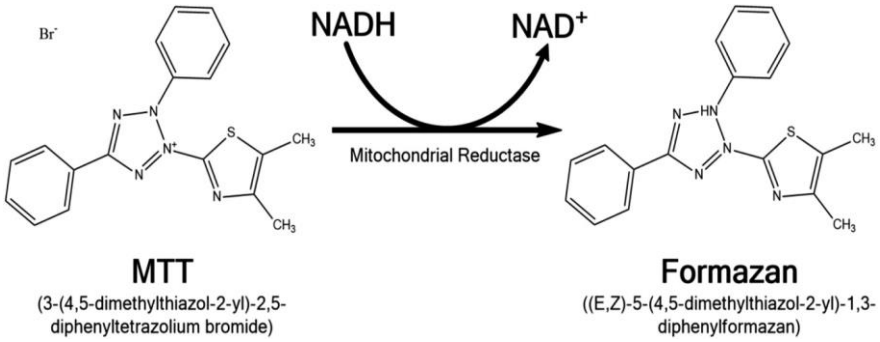


Figure 10: Enzymatic reduction of MTT to formazan

II.2.2.6.1 Preparation of Dulbecco's Modified Eagle Medium (DMEM)

Complete DMEM was prepared by supplementing 890 mL of incomplete DMEM with 10 mL (1%) of the antibiotic Penicillin/Streptomycin and 100 mL (10%) of inactivated FBS (in a water bath at 56°C for 30 minutes) to a final volume of one liter. The mixture was homogenized, filtered through a 0.22 µm millipore filter and stored at 4°C for further use.

II.2.2.6.2 *In vitro* culture of the Vero cell line

The Vero cell line was maintained in 75 cm^2 culture flasks (Tflask 75) containing complete DMEM medium. The medium was changed every 3 days after culture. For cell load preparation, cells were detached using trypsin-EDTA after incubation at 37°C for 3min. The culture medium was used to inactivate the trypsin and the detached cells were centrifuged at 1800 rpm for 5 min. The resulting pellet was suspended in 1 mL of medium and then the viability of the cells was determined. Cells were counted using trypan blue and was calibrated using a Neubauer chamber.

II.2.2.6.3 Determination of median cytotoxic concentrations (CC_{50})

Essentially, Vero cells at 5×10^3 cells/200 µL/well were seeded into 96-well flat-bottomed tissue culture plates (Corning, USA) in complete medium. Fifty microliters of serially diluted extract solutions (≤ 100 µg/mL) were added after 24 h of seeding, and the cells plus 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 a negative control (100% growth). Podophyllotoxin was used as positive control with concentrations ranging from 10 to 0.016 µM. 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, Männedorf, Switzerland) at 570 nm. From the obtained optical densities, the percentages of viable cells were calculated as follows:

Viability percentage = OD test/OD negative control × 100

The viability percentages were used to determine the median cytotoxic concentrations (CC_{50}) by analyzing the dose–response curves. Selectivity indexes (SI) were then calculated from the ratio of the median cytotoxic concentration (CC_{50}) to the minimum inhibitory concentration (MIC). The following criteria defined by Camacho *et al*., (2003) were used:

SI<1indicate a selective effect against the cell line while SI >1indicate a selective action against the tested bacteria

II.2.2.7 Antioxidant activity of fungal extracts

All active endophytic fungal extracts were investigated for their potential antioxidant activity using DPPH radical scavenging and ferric ion reducing antioxidant power (FRAP) assays.

II.2.2.7.1 DPPH radical scavenging assay

The potential free radical scavenging activity of fungal extracts was evaluated using the 2,2-diphenyl-1-picryl-hydrazyl (DPPH) assay previously described by Djague *et al*., (2020). Briefly, the extracts were first diluted to obtain extract concentrations of 62.5, 125, 250, 500, 1000, 2000, and 4000 µg/mL in a 96-well microplate. After that, twenty-five microliters (25 µL) of each dilution were introduced into a new microplate, and 75 µL of 0.02% DPPH were added to obtain final

concentrations of 15.625, 31.25, 62.5, 125, 250, 500, and 1000 µg/mL. The reaction mixtures were kept in the dark at room temperature for 30 min, after which the absorbance was measured at 517 nm against the blank (DPPH in methanol) using the microplate reader Infinite M200 (TECAN, Mannedorf, Switzerland). L-ascorbic acid was used as a positive control and was treated under the same conditions as the extracts with final concentrations of 0.391, 0.781, 1.563, 3.125, 6.25, 12.5, and 25 µg/mL. The percentage (%) radical scavenging activities of the extracts were calculated using the following formula, from which other parameters, such as the radical scavenging activity 50 (RSA_{50}), the effective concentration 50 (EC_{50}), and the antiradical power (ARP), were deduced. The assay was performed in triplicate and repeated twice.

Percentage of RSA = [(Abs control – Abs sample)/Abs control] × 100.

where RSA is the radical scavenging activity, Abs control is the absorbance of the blank (DPPH + methanol), and Abs sample is the absorbance of DPPH radical + fungal extract

Prior to the determination of radical scavenging parameters, a preliminary screening was carried out under the same conditions and at the concentration of 100 ug /mL only at the end of which those with Percentages of RSA ≥ 50 were selected for dose response study.

II.2.2.7.2 Ferric ion reducing antioxidant power (FRAP) assay

The assay was performed according to the method described by Djague *et al*., (2020). Briefly, the extracts were first dissolved for the DPPH assay. Twenty-five microliters from each dilution were added to 25 µL of 1.2 mg/mL Fe^{3+} solution in a new microplate. The plates were preincubated for 15 min at room temperature. Fifty microliters (50 µL) of 0.2% ortho-phenanthroline solution were added to obtain final extract concentrations of 15.625, 31.25, 62.5, 125, 250, 500, and 1000 µg/mL. The reaction mixtures were further incubated for 20 min at room temperature, after which the absorbance was measured at 505 nm using a 96-well microplate reader Infinite M200 (TECAN, Mannedorf, Switzerland) against the blank (25 µL methanol + 25 µL Fe^{3+} + 50 µL ortho-phenanthroline). Hydroxylamine was used as a positive control and was treated in the same way as the extracts with final concentrations of 0.103, 0.206, 0.413, 0.825, 1.65, 3.30, and 6.60 µg/mL. The assay was performed in triplicate. From a concentration-activity curve of NH_2OH used as a standard, the optical densities of the test wells were projected, and the results were expressed quantitatively as µg equivalent NH_2OH/g of extracts.

Based on its antibacterial activity and non-cytotoxicity against normal cell line, extract from *Fusarium* sp AMtw3, was selected for the chromatography separation to characterize potential active ingredients

II.2.3 Fractionation of *Fusarium sp.* AMEtw3 extract

II.2.3.1 Large scale cultivation of *Fusarium sp*. AMEtw3

The larger scale culture of *Fusarium sp* AMEtw3 was performed in 120 glass containers of 1 L glass filled each with 500 mL of PDB medium. Briefly, 1 cm^2 agar pieces from each seven days old culture on PDA medium were inoculated in liquid medium (PDB) and incubated at room temperature for 7 days under static conditions. After this incubation period, 300 mL of ethyl acetate was added in each container, the mixture macerated for 24 hours and then extracted by liquid-liquid partition. This last step was repeated thrice. The ethyl acetate phase was concentrated in a rotary evaporator at 70º C to obtain crude extract. The crude residue obtained was weighed and subjected to column chromatography.

II.2.3.2 Silica gel chromatography of large scale extract

The ethyl acetate extract of endophytic fungi *Fusarium* sp AMEtw 3 (25 g) was subjected to silica gel column chromatography. For this purpose, the extract was weighed and mixed with an equivalent volume of silica. The mixture was evaporated and allowed to dry. The column was packed with silica and saturated with *n*-hexane. The mixture of extract with silica was added to the column and eluted with different solvents system of increasing polarities (*n*-Hex 100%, *n*-Hex-EtOAc 90:10, *n*-Hex-EtOAc 70:30, *n*-Hex-EtOAc 50:50, *n*-Hex-EtOAc 20:80, EtOAc 100%, EtOAc-MeOH 80:10, EtOAc-MeOH 65:35, MeOH 100%). Eight hundred and fourteen (814) fractions of 100 mL each were collected and subsequently pooled into nine fractions (AMF1-AMF9) based on their thin layer chromatography (TLC) profile. Each spot on the TLC plate was visualized by UV lamp (254 and 365 nm) and by heating at about 110 °C after spraying with 20% H_2SO_4 (v/v).). Each fraction was then dried, weighed and tested for antibacterial, cytotoxicity and antioxidant activities as described previously.

Following this step, six fractions (except fraction AMF1 (*n*-Hex 100%), AMF4 (*n*-Hex/EtOAc 50 %) and AMF9 (MeOH 100 %) were submitted to a second chromatography for purification.

II.2.3.3 Rechromatography of fractions

- Fraction AMF2 (*n*-Hex/EtOAc 10 %): 3.730 g were dissolved in hexane and then fixed on 10 g of silica. Subsequently the elution was done with the *n*-Hex/EtOAc mixture of increasing polarity (*n*-Hex-EtOAc 95:5, *n*-Hex-EtOAc 90:10, *n*-Hex-EtOAc 85:15, *n*-Hex-EtOAc 80:20, *n*-Hex-EtOAc 75:25, *n*-Hex-EtOAc 65:35, *n*-Hex-EtOAc 50:50, *n*-Hex-EtOAc 40:60). Two hundred and forty-seven (247) tubes of 100 mL each were collected and pooled into twenty-two (22) based on their TLC profile. Overall, six pure sub-fractions (AMSF1, AMSF5, AMSF10, AMSF12, AMSF15, AMSF19) were obtained from collections 1-27, 28-47, 48-89, 90-117, 151-180.

- Fraction AMF3 (*n*-Hex/EtOAc 30 %): 4.960 g were dissolved in hexane and then fixed on 12 g of silica. Thereafter a mixture of solvent *n*-Hex/AcOEt (*n*-Hex-EtOAc 80:20, *n*-Hex-EtOAc 75:25, *n*-Hex-EtOAc 70:30, *n*-Hex-EtOAc 65:35, n-Hex-EtOAc 60:40, *n*-Hex-EtOAc 50:50, n-Hex-EtOAc 30:70, *n*-Hex-EtOAc 40:60, *n*-Hex-EtOAc 20:80) was used to elute the column and lead to three hundred and twenty-four tubes collected (324) and regrouped into 25. Four pure sub-fractions (AMSF3, AMSF11, AMSF13, AMSF14) and two compounds (AMC6, AMC20) were precipitated from 1-29, 30-61, 62-94, 95-118, 119-150, 196-217.

- Fractions AMF5 (*n*-Hex/EtOAc 80 %) and AMF6 (*n*-Hex/EtOAc 100 %): 1.426 g and 1.276 g respectively for each fraction were submitted separately to column chromatography by using solvent systems *n*-Hex-EtOAc 60:40, *n*-Hex-EtOAc 50:50, *n*-Hex-EtOAc 50:50, *n*-Hex-EtOAc 40:60, *n*-Hex-EtOAc 20:80, EtOAc 100. One hundred and forty-five (145) and one hundred and sixty (160) collections were respectively obtained from AMF5 and AMF6 regrouped in 15 and 12 groups. Pure sub-fractions AMSF8 and AMSF1 were purified respectively from 8-24 (*n*-Hex/EtOAc 80 %) and 45-89 (*n*-Hex/EtOAc 100 %) groups.

- Fractions AMF7 (EtOAc/MeoH 10%) and AMF8 (EtOAc/MeoH 35 %): these two fractions were combined based on their similar profile on TLC plate. So, the mixture obtained were dissolved in EtOAc/MeoH 10% and fixed on 15 g of silica. Then, elution was carried out with EtOAc-MeoH 10:80, EtOAc-MeoH 5:5, MeoH 100, $CDCl_3$ 100, $CDCl_3$-MeoH 95:5, $CDCl_3$-MeoH 90:10, $CDCl_3$-MeoH 85:15, $CDCl_3$-MeoH 80:20, $CDCl_3$-MeoH 75:25, $CDCl_3$-MeoH 70:30 resulting in two hundred and twenty-three tubes (223). These tubes were pooled into fifteen (15) based on their TLC profile. At the end of the purification three pure sub-fractions named AMSF7, AMSF9 and AMSF17 were obtained respectively from 17-40, 91-124, 178- 210 groups.

At the end of this second chromatography of the six fractions, 15 pure sub-fractions and two compounds were purified (Figure 11), dried, weighed and tested for antibacterial activity. Active ones were tested for their cytotoxicity and antioxidant activities as described previously.

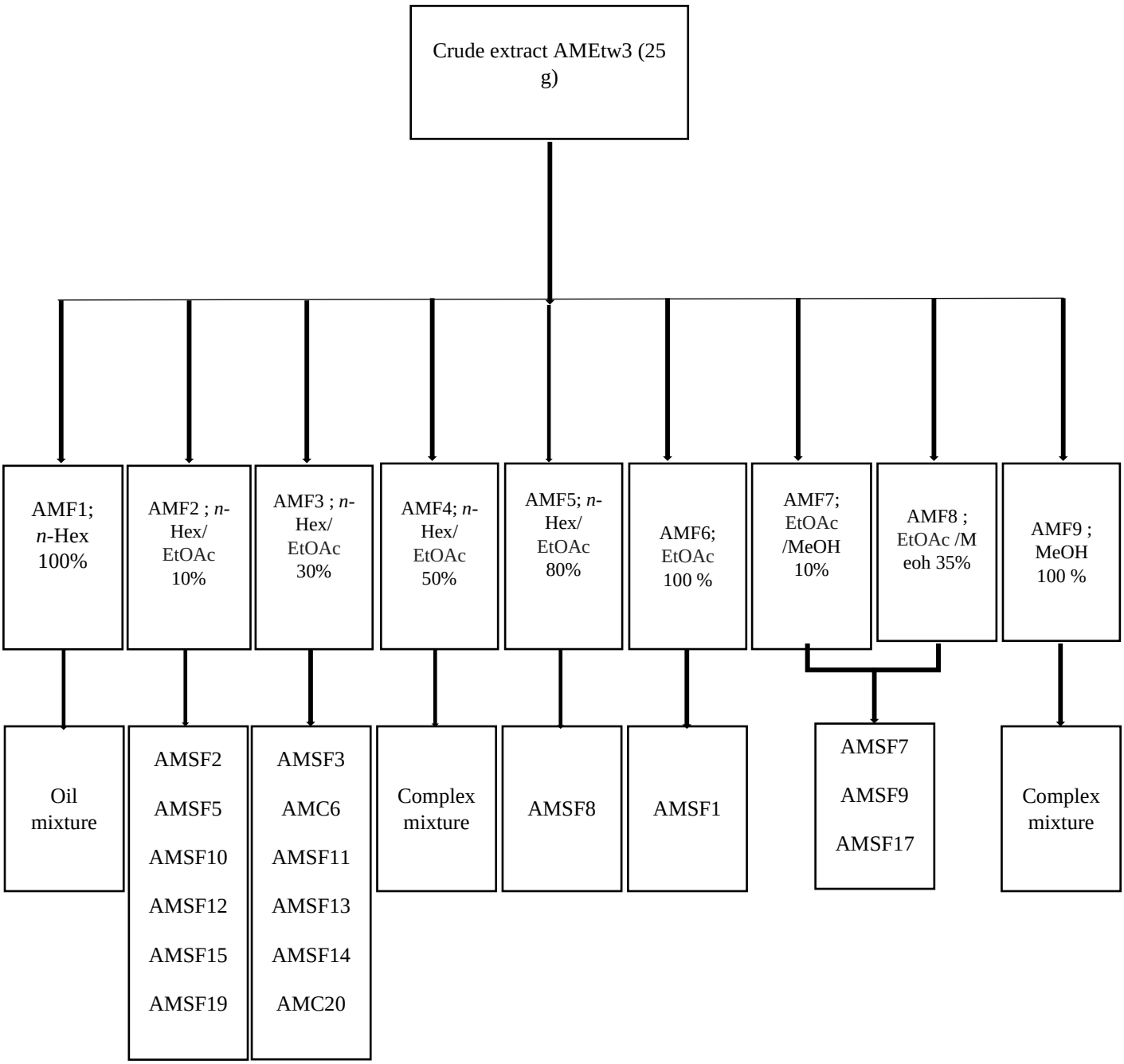


Figure 11: Protocol for the isolation of fractions, pure sub-fractions and compounds from the extract of *Fusarium* sp. AMEtw3

The two compounds (AMC6 and AMC20) exhibiting the best inhibitory activity against the three bacterial pathogens were selected for the mode of action study. The two most sensitive bacterial strains (*S. aureus* ATCC 43300 and *K. oxytoca*) were used for this part of work.

II.2.4 Possible modes of action of the two most potent compounds and their structure elucidation

II.2.4.1 Possible modes of action of the two most potent compounds

II.2.4.1.1 Inhibition of catalase activity

The catalase inhibitory activity of compounds was carried out as described by Mbekou *et al*., (2021). Compounds at the MIC concentration were added to a test tube containing 400 µL of hydrogen peroxide (H_2O_2) (40 mM) and 400 µL of phosphate buffer saline (PBS) (Sigma–Aldrich, Germany). The mixture was then transferred to another tube containing 200 µL of a bacterial suspension (1.5×10^8 UFC/mL) prepared in 0.9 % NaCl. 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 using the microplate reader Infinite M200 (TECAN, Mannedorf, Switzerland). The phosphate buffer constituted the blank, while bacterial strains in the phosphate buffer without any inhibitory substance were used as a negative control. The mixture of ciprofloxacin at 0.468 and 0.234 µg/ mL, respectively, for *S. aureus* ATCC 43300 and *K. oxytoca*, phosphate buffer, and bacterial strain constituted a positive control. The percentage of remaining H_2O_2 was determined according to the following formula:

% of remaining H_2O_2= Abs sample –Abs negative control ×100/ Abs negative control

*A*bs negative control is the absorbance of H_2O_2 without the compound, and Abs sample is the absorbance of H_2O_2 with the compound.

II.2.4.1.2 Bacteriolysis activity of compounds

The potential bacteriolytic ability of the antibacterial compounds was determined according to the method described by Mbekou *et al*., (2021) Briefly, the bacterial suspension ($5x10^7$ CFU/mL) was treated with compounds at MIC, 2 MIC, and 4 MIC and incubated at 37˚C. The optical density (OD) at 620 nm was measured at four different incubation periods, including 0 h, 1 h, 2 h, and 4 h, using an Infinite M200 microplate reader (TECAN, Mannedorf, Switzerland). A decrease in OD at 620 nm indicated bacterial cell lysis. Corresponding dilutions of the compounds were used as

blanks. Ciprofloxacin was used as a positive control at 0.468 and 0.234 µg/mL for *S. aureus* ATCC 43300 and *K. oxytoca*, respectively. 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.2.4.1.3 Outer membrane permeability assay

The potential effect of compounds on the outer membranes (OM) permeability of *S. aureus* ATCC 43300 and *K. oxytoca* was determined according to the method described by Mbekou *et al*., (2021) An overnight culture ($5x10^7$ CFU/mL) was inoculated into nutrient broth containing the compounds at 1/16 MIC, 1/8 MIC, 1/4 MIC, 1/2, MIC, MIC, 2 MIC, and 4 MIC. The media was then poured into sterilized 96-well microplates (100µL) and incubated at 37˚C for 24 h. After the incubation time, the growth of *S. aureus* ATCC 43300 and *K. oxytoca* was measured at 450 nm using an Infinite M200 microplate reader (TECAN, Mannedorf, Switzerland). The bacterial growth curve (OD/450 nm) as a function of extract concentration (µg/mL) was plotted. Ciprofloxacin (concentration ranged from 0.01 to 0.93 µg/mL and 0.06 to 1.84 µg/mL, respectively, for *K. oxytoca* and *S. aureus* ATCC 43300) was used as the positive control, and each test was conducted in triplicate.

II.2.4.1.4 Integrity of the Cell Membrane

The integrity of the cell membrane of *K. oxytoca* and *S. aureus* ATCC 43300 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 compounds at 4 MIC for different periods (0, 30, 60, 90, 120 and 240 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 0.468 and 0.234 µg/ mL, respectively, for *S. aureus* ATCC 43300 and *K. oxytoca* was used as 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.2.4.1.5 Antibacterial time-kill kinetic assay

The time-kill kinetic of active compounds was determined according to the method described by Babii *et al*., (2016). Compounds concentrations at MIC, 2 MIC and 4 MIC were

prepared by serial twofold dilution in a 96-well microplate. One hundred microliters (100 µL) of bacterial suspension (10^7 CFU/mL) were added, and the plate was incubated at 37˚C for different time intervals (0, 1, 2, 4, 6, 8, 10, 12, and 24 h). Following each incubation period, the cell suspensions were appropriately diluted (in NaCl 0.9%), and the ODs of the resulting solution were measured at 620 nm using an Infinite M200 microplate reader (TECAN, Mannedorf, Switzerland). Ciprofloxacin at 0.468 and 0.234 µg/ mL, respectively, for *S. aureus* ATCC 43300 and *K. oxytoca* was used as a positive control, while the bacteria incubated with NB were used as growth controls. The test was performed in triplicate, and the results are presented as the mean ± SD.

II.2.4.1.6 Determination of antibiofilm properties

II.2.4.1.6.1 Biofilm quantification

Biofilm production by *S. aureus* ATCC 43300 and the *K. oxytoca* isolate was performed as described by Cruz *et al*., (2018). Briefly, a single colony was taken from the overnight bacterial culture, inoculated into 0.9% (w/v) saline solution and vortexed to ensure that the bacterial suspension was homogeneous. Bacterial suspensions were adjusted to 1×10^7 colony forming units (CFU/mL) by diluting with appropriate nutrient broth supplemented with 2% glucose. An aliquot of 200 µL of bacterial suspension per well was dispensed into a 96-well flat-bottom microplate. The plate was then incubated at 37˚C for 24 hours. After this incubation period, planktonic cells were carefully removed, and adhered/biofilm cells were washed twice with 0.9% NaCl. Next, 100 µL of resazurin solution at 0.15 mg/mL prepared in sterile phosphate buffered saline (PBS; Sigma–Aldrich, Germany) was added to each well-containing biofilm. Microplates were incubated in the dark at 37˚C for 1 hour, after which the microplate reader Infinite M200 (TECAN, Mannedorf, Switzerland) was used to measure the relative fluorescence units (RFU) (λEx 530 nm and λEm 590 nm). The relative fluorescence units obtained were used to plot a histogram to examine the quantity of biofilms formed by each microorganism.

II.2.4.1.6.2 Biofilm Inhibition assay

The inhibitory potential of active compounds against biofilm formation by *S. aureus* ATCC 43300 and *K. oxytoca* was investigated using the method previously described by Cruz *et al*., (2018) with slight modifications. Briefly, 100 µL of each bacterial strain suspension ($1x10^7$ CFU/mL) was incubated with compounds at MIC, 2 MIC, and 4 MIC for 48 h at 37˚C. After incubation, planktonic cells were removed by washing the wells very delicately with 0.9% NaCl. Next, 100 µL of diluted

resazurin solution was added to each well-containing biofilm. Microplates were placed in the dark and incubated at 37˚C for 1 hour. A microplate reader Infinite M200 (TECAN, Mannedorf, Switzerland) was then used to measure the relative fluorescence units (RFU) (λEx 530 nm and λEm 590 nm) after incubation. Ciprofloxacin was used as a positive control and tested also at MIC, 2 MIC, and 4 MIC. Wells containing only bacteria-free medium constituted the negative control, and the assay was performed in triplicate and repeated twice. The percentages of inhibition were estimated (Biofilm inhibition% = RFU control−RFU sample/RFU control×100), and the Minimum Biofilm Inhibitory Concentrations 50 ($MBIC_{50}$) were determined using the inhibition percentages associated with various concentrations of compounds.

II.2.4.1.6.3 Biofilm eradication assay

The assay was performed using *S.aureus* ATCC 43300 and *K.oxytoca* as defined by Saising *et al*., (2011) and under the same conditions as in biofilm inhibition assay. The only difference here is that biofilms were allowed to grow prior in nutrient broth supplemented with 2% glucose during 24 h as in biofilm quantification assay before treatment with the compounds. At the end of the experiment, relative fluorescence units (RFU) (λEx 530 nm and λEm 590 nm) were recorded with an Infinite M200 microplate reader (TECAN, Mannedorf, Switzerland). The assay was performed in triplicate and repeated twice. The percentages of eradication were estimated (Biofilm eradication% = RFU control−RFU sample/RFU control×100), and the Minimum Biofilm Eradication Concentrations 50 ($MBEC_{50}$) were calculated based on the eradication percentages with the different concentrations of compounds.

II.2.4.2 Spectroscopic data treatment and structure elucidation

Further, the two compounds used for modes of action were submitted to 1D and 2D NMR for structure elucidation. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Inova instrument operating at 500 MHz for ^{1}H, 125 MHz for ^{13}C. 2D HMBC and COSY were acquired at 600 and 150 MHz. The 1D and 2D NMR spectra were processed with MestReNova software 9.0, and the structures were drawn with the Chemdraw Ultra 12.02 software. The structures were determined on the basis of their spectral data and by comparison of their spectroscopic data with those in the literature.

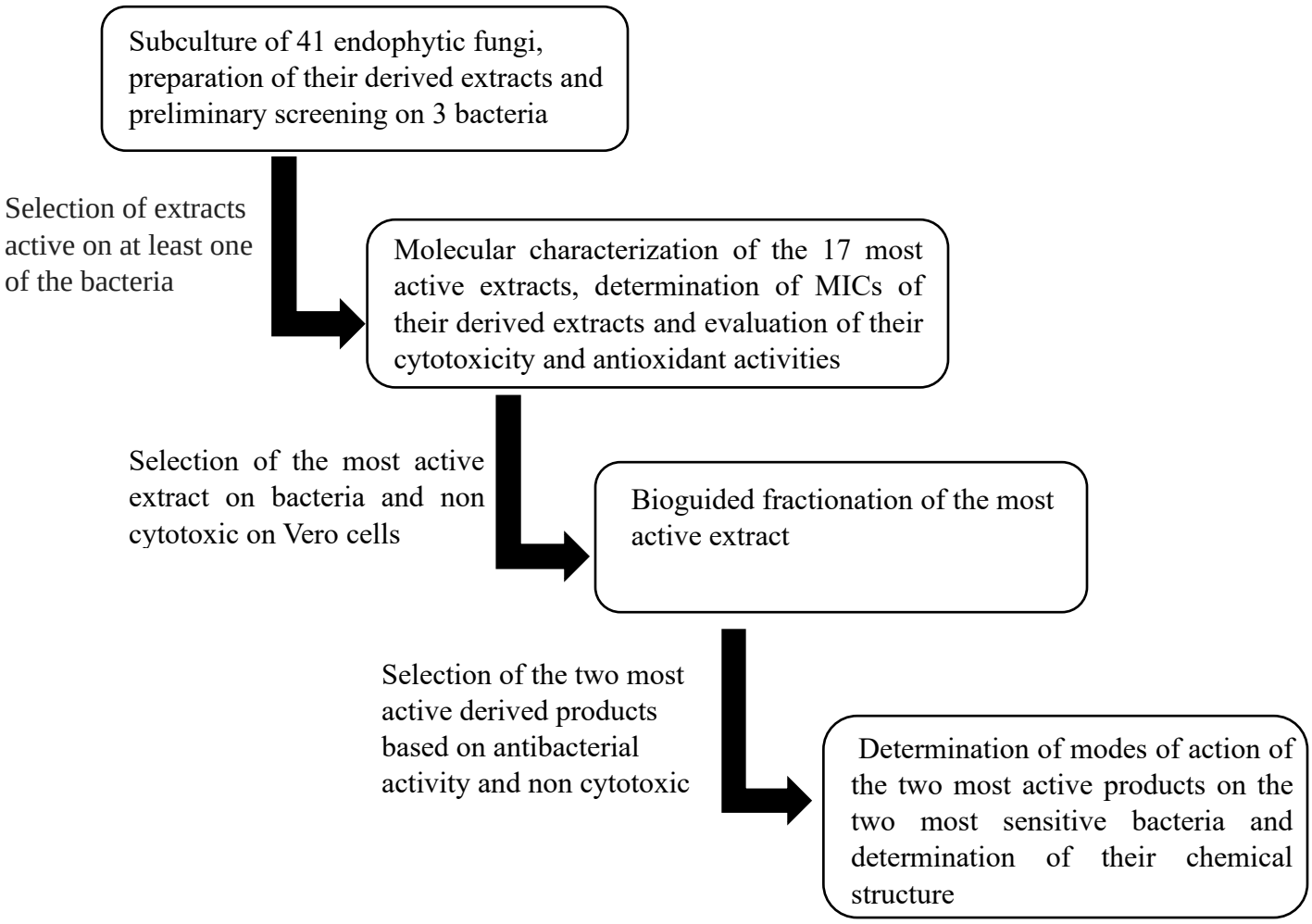


Figure 12: Summary of activities carried out

II.3 Statistical and phylogenetic analyses

- ✓ **Biological assays:** Raw data collected from at least two independent experiments were used to calculate the different percentages of inhibition and eradication with Microsoft Excel Software 2016. Statistical Analyses were carried out using one-way ANOVA with GraphPad Prism 8.0. Data are expressed as the mean ± SD of experiments performed in triplicate. Error bars represent the SD, and significant differences for multiple comparisons were determined by the Turkey test at $p < 0.05$. Graphs and histograms were also plotted with GraphPad Prism 8.0. The correlation between the different modes of action of compounds was analyzed using Principal Component Analysis (PCA) and the Hierarchical Cluster Analysis (HCA) with XLSTAT 2022 software.

- ✓ **Molecular characterization:**

  The BLAST algorithm was employed to identify sequences similar to those retrieved from fungal isolates in GenBank and MEGA 7.0 software was used to build the phylogenetic tree.

Chapter III: Results and Discussion

CHAPTER III: RESULTS AND DISCUSSION

III.1 Results

III.1.1 Screening and characterization of potent endophytic fungal strains

III.1.1.1 Antibacterial screening

In the present study, forty-one endophytic fungi isolated from ten organs of the medicinal plant *A. muricata* growing in Cameroon were subjected to antibacterial screening against three pathogenic bacteria causative agents of UTIs. Fungi were fermented in potato dextrose broth medium and extracted with ethyl acetate which yielded crude extracts with masses ranging from 12 to 402 mg depending on the cultured fungus. AMEs1 (402 mg) followed by AMEs3 (264 mg) and AMErb9 (258 mg) isolated from seeds (s) and root bark (rb) produced the highest amount of crude metabolites (Table VI).

The 41 crude extracts were screened at a single concentration of 100 µg/mL against three bacterial causative agents of UTIs (*E. coli* ATCC 25922, *K. oxytoca*, and *S. aureus* ATCC 43300). The results (Appendix 2) showed that 17 (41.46%) were active, among which 16 exhibited broad-spectrum activity against the three pathogens, while one (AMEr10) was only active against *S. aureus* ATCC 43300. Crude metabolites exhibiting activity were produced by endophytic fungi isolated from root (2), root bark (3), fruits (4), seeds (2), the thorn of fruits (2), twigs (1), bark (1), and stem bark (2). No endophytes from leaves or peduncles showed any inhibitory activity against the tested pathogens. Based on their antibacterial activity at 100 µg/mL, seventeen fungi were selected for molecular identification and further studies.

III.1.1.2 Molecular identification of endophytic fungi

The ITS rDNA region of all the 17 selected fungi was sequenced, and the identification was performed by comparison with published sequences in GenBank. The results from the BLAST search revealed that 16 endophyte sequences showed 98.32–100% similarity with sequences from previously identified fungi in the NCBI database. However, eleven of the endophytes' sequences investigated showed higher similarity with more than two different fungal species. AMr9 showed similarity with six different species of *Aspergillus*, and AMtf15, AMr10, AMrb9, AMs9, and AMb7 were similar to 3 different *Aspergillus* spp. AMrb1 and AMrb11 were similar to three *Penicillium* species, AMf4 was similar to two *Curvularia* species, and AMf3 was similar to more

than two *Meyerozyma* species. The sequence of AMtf5 showed only 96.88% similarity with the strain *Talaromyces clemensii* NR_168822.1. New sequences generated in this study were deposited in the NCBI GenBank nucleotide database (www.ncbi.nlm.nih.gov; Table V).

In order to infer the evolutionary history of endophytic fungi, the Neighbor-Joining method was used (Figure 13). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as the evolutionary distances used to infer the phylogenetic tree. With the bootstrap support of each clade over 80, the ML phylogenetic analysis showed that the seventeen endophytic fungi belonged to six genera from the phylum Ascomycota including *Aspergillus*, *Curvularia*, *Fusarium*, *Penicillium* and *Talaromyces*. Confirming the results from the BLAST search, nine endophytes (AMr10, AMCb7, AMs9, AMrb9, AMf4, AMf3, AMtf5, AMrb1, and AMr9) were classified at the genus level. In comparison, only eight endophytes could be identified at the species level, including AMf6, AMsb1, AMs3, AMrb11 and AMf1 as *Penicillium citrinum*, AMsb23 as *Talaromyces annesophieae*, AMtf15 as *Aspergillus austwickii* and AMtw3 as *Fusarium waltergamsii*. Overall, fungal species from the *Aspergillus* genus were identified in five organs: root (2), bark, seed, root bark, and thorn of fruits. *Penicillium* species were identified among fungal isolated from fruits (2), root bark (2), stem bark, and seeds. The *Talaromyces* species were identified in thorn of fruits and stem bark, while *Myerozyma*, *Fusarium*, and *Curvularia* were identified only in fruits, twigs, and fruits. Overall, the 17 selected fungi were mainly dominated by *Penicillium* and *Aspergillus*, accounting for 35.29% of the total.

Table V: Identity of the seventeen endophytic fungi with activity against bacteria causative agents of UTIs

Organs	Fungus code	Fungal identity	GenBank Accession No.	Blast Results		
				Closest Related Species	Query coverage (%)	Percent identity (%)
Root	AMr9	*Aspergillus sp.*	OM959526	*A. foetidus* NR_163668.1	100	100
				A. welwitschiae NR_137513.1	100	100
				A. awamori NR_077143.1	100	100
				A. tubingensis NR_131293.1	100	99.60
				A. costaricensis NR_103604.1	100	99.60
				A. niger NR_111348.1	98	100
	AMr10	*Aspergillus sp.*	OM959517	*A. austwickii* NR_171607.1	100	99.60
				A. aflatoxiformans NR_171606.1	100	99.60
				A. oryzae NR_135395.1	100	99.40
	AMrb1		OM959527	*P. herquei* NR_103659.1	100	99.26

		Penicillium sp.		*P. verrucisporum* NR_153275.1	100	99.26
				P. sanshaense NR_153276.1	100	98.76
Root bark	AMrb9	*Aspergillus sp.*	OM959523	*A. clavatonanicus* NR_135410.1	100	100
				A. longivesica NR_135411.1	100	99.80
				A. giganteus NR_135403.1	100	99.80
	AMrb11	*Penicillium citrinum*	OM959528	*P. citrinum* NR_121224.1	100	99.40
Fruit	AMf6	*Penicillium citrinum*	OM980761	*P. citrinum* NR_121224.1	100	99.53
	AMf3	*Meyerozyma sp.*	OM959515	*M. caribbica* NR_149348.1	99	99.57
				M. carpophila NR_152984.1	100	100
	AMf4	*Curvularia sp.*	OM959516	*C. kenpeggii* NR_158447.1	100	98.32
	AMf1	*Penicillium citrinum*	OM959525	*P.citrinum* NR_121224.1	100	99.56
Seed	AMs3	*Penicillium citrinum*	OM959520	*P. citrinum* NR_121224.1	100	98.68
	AMs9	*Aspergillus sp.*	OM959518	*A. clavatonanicus* NR_135410.1	100	100
				A. longivesica NR_135411.1	100	99.79
				A. giganteus NR_135403.1	100	99.79
	AMtf15	*Aspergillus austwickii*	OM959513	*A. austwickii* NR_171607.1	100	99.57%

Thorn of fruit	AMtf5	*Talaromyces sp.*	OM959521	*T. clemensii* NR_168822.1	100	96.88
Twig	AMtw3	*Fusarium waltergamsii*	OM959524	*F. waltergamsii* NR_159548.1	93	99.77
Stem bark	AMsb23	*Talaromyces annesophieae*	OM959519	*T. annesophieae* NR_170732.1	100	99.17
	AMsb1	*Penicillium citrinum*	OM959514	*P. citrinum* NR_121224.1	100	99.53
Bark	AMb7	*Aspergillus sp.*	OM959522	*A. austwickii* NR_171607.1	100	99.60
				A. flatoxiformans NR_171606.1	100	99.60
				A. oryzae NR_135395.1	100	99.40

AMr: *A. muricata* root; AMrb: *A. muricata* root bark; AMf: *A. muricata* fruit; AMs: *A. muricata* seed; AMtf: *A. muricata* thorn of fruit; AMtw: *A. muricata* twigs; AMl: *A. muricata* leaves; AMb: *A. muricata* bark; AMsb: *A. muricata* stem bark; AMpe: *A. muricata* peducle

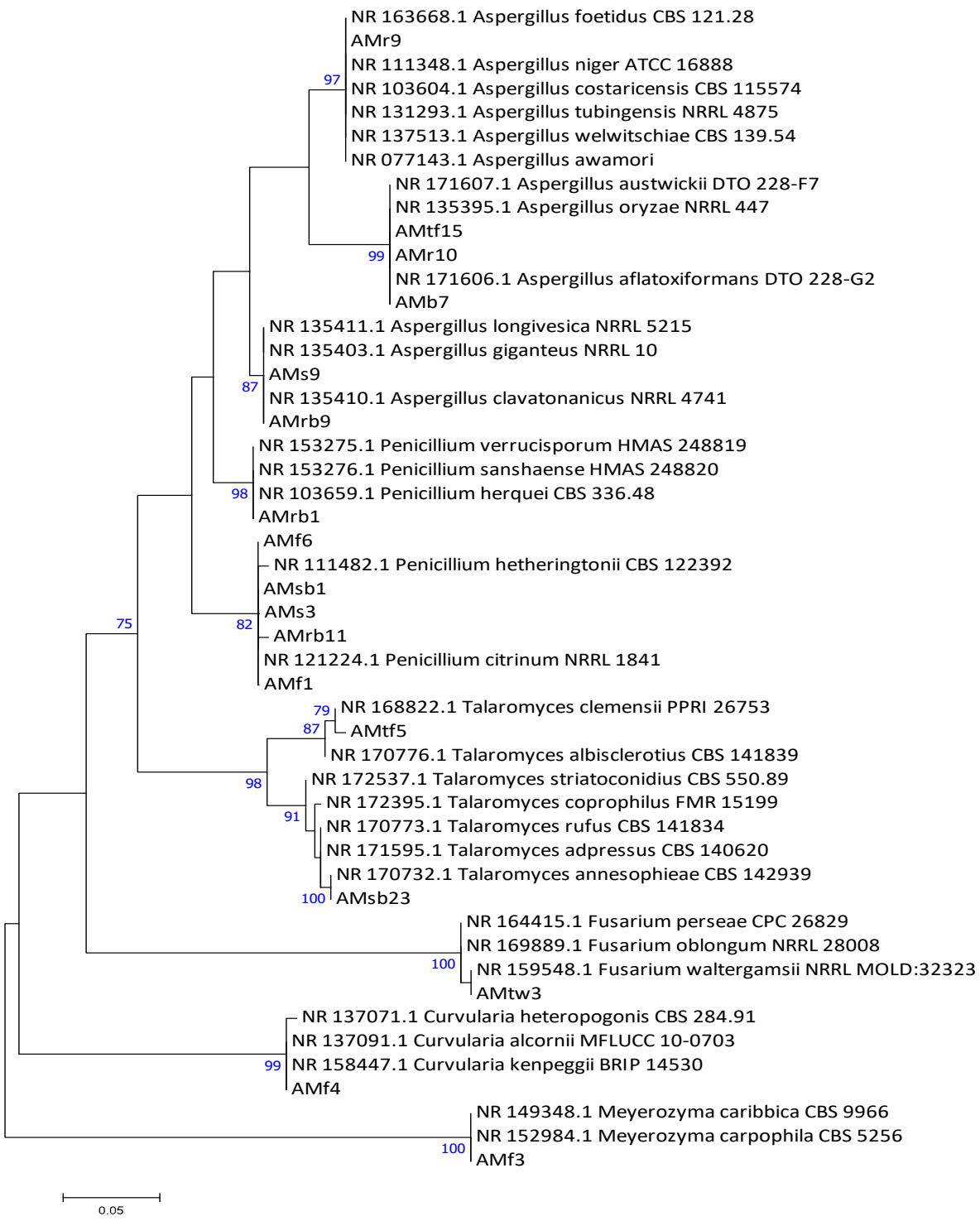


Figure 13: Molecular phylogenetic tree generated by Maximum Likelihood analysis based on ITS sequence alignments of endophytic fungal isolates; ATTC: American Type Culture Collection; CBS: Centraalbureau Voor Schimmelculture; NRRL: Agricultural Research Service Culture Collection ; DTO: working collection of the Applied and Industrial Mycology department housed

at the CBS-KNAW Fungal Biodiversity Centre, Utrecht, the Netherlands; HMAS: Human Microbiome Association Studies; FMR: Facultad de Medicina, Reus, Tarragona, Spain fungus collection ; CPC: personal collection of Pedro W. Crous housed at Westerdijk Fungal Biodiversity Institute ; MFLUCC: Mae Fah Luang University Culture Collection; BRIP: Plant Pathology Herbarium;

ML bootstrap support values (ML ≥ 70 %) are shown at the nodes. Isolates from *Annona muricata* are coded. The scale bar indicates 0.05 expected changes per site. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model. The tree with the highest log likelihood (-1028.13) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying the Maximum Parsimony method. A discrete Gamma distribution was used to model evolutionary rate differences among sites (2 categories (+*G*, parameter = 0.4638)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 49 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 221 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

III.1.1.3 Minimum inhibition concentrations (MICs), cytotoxic concentrations 50 (CC_{50}) and Selectivity Indexes (SI) of selected extracts

The 17 selected crude extracts were submitted to a dose-response study for MIC determination, and the results are summarized in Table VI. All extracts exhibited activity against at least one of the tested bacteria with the MIC values ranging from 3.125 to 100 µg/mL depending on the extracts and microorganisms. Extract from *F. waltergamsii* AMEtw3 (MIC: 3.125 µg/mL) was the most active against *S. aureus* ATCC 43300, followed by *Aspergillus* sp. AMEtf15, *P. citrinum* AMEf6 and *P. citrinum* AMEs3 (MIC: 9.375 µg/mL). When tested against *K. oxytoca*, extracts *F. waltergamsii* AMEtw3 (MIC: 3.125 µg/mL) and *P. citrinum* AMEsb1 (MIC: 3.125 µg/mL) were the most active, followed by *P. citrinum* AMEf6 (MIC: 4.687 µg/mL). Against *E. coli* ATCC 25922, *F. waltergamsii* AMEtw3 (MIC: 6.25 µg/mL) exhibited the best activity, followed by *P. citrinum* AMEf6 and *P. citrinum* AMEsb1 (MIC: 9.375 µg/mL).

The results from the dose-response study also revealed that extracts from endophytes belonging to the same genus isolated from the same or different organs displayed very different potency and activity profiles. For instance, *Aspergillus sp*. AMEr10 was inactive (MIC > 100 µg/mL) against *K. oxytoca* and *E. coli* ATCC 25922 and displayed weak activity against *S. aureus* ATCC 43300 (MIC: 100 µg/mL) while *Aspergillus sp*. AMEr9 isolated from the same organ (root) was very active against the three bacteria (MIC: 12.5 µg/mL). The extract from *P. citrinum* AMEsb1 from stem bark was four times more potent against *K. oxytoca* (MIC: 3.125 µg/mL) than extract produced by *P. citrinum* AMEs3 isolated from seeds (MIC: 12.5 µg/mL). *Aspergillus spp* AMEr9, AMErb9, and AMEs9 isolated from roots, root bark, and seeds displayed a similar activity profile against the three pathogens (Table VI). In addition to their antibacterial activity, all selected extracts displayed non cytotoxicity against Vero cells ATCC CRL1586 with the median cytotoxic concentrations (CC_{50}) greater than 100 µg/mL. By assessing their respective selectivity indexes (Table VII), it was observed that all the obtained values were greater than 1 thus. testifying to the non-cytotoxicity of the extracts.

Table VI: Yields (mg), Minimum Inhibitory Concentrations (MICs) and Cytotoxic Concentrations 50 (CC_{50}) of selected extracts

Plant organs	**Fungal Extracts**	**Yield (mg)**	**MIC (µg/mL ± SD)**			**CC_{50} (µg/mL ± SD)**
			***S. aureus* ATCC 43300**	***K. oxytoca* Isolate**	***E. coli* ATCC 25922**	**Vero cells ATCC CRL1586**
Roots	*Aspergillus sp.* AMEr9	195	12.5±0.00	12.5±0.00	12.5±0.00	> 100
	Aspergillus sp. AMEr10	12	100±0.00	> 100	> 100	> 100
Root bark	*Penicillium sp*. AMErb1	95	18.75±8.83	12.5±0.00	12.5±0.00	> 100
	Aspergillus sp. AMErb9	258	12.5±0.00	12.5±0.00	12.5±0.00	> 100
	Penicillium sp. AMErb11	61	12.5±0.00	12.5±0.00	12.5±0.00	> 100
Fruits	*Penicillium citrinum* AMEf6	22	9.375±4.19	4.687±2.20	9.375±4.41	> 100
	Meyerozyma sp. AMEf3	67	50±0.00	25±0.00	37.5±17.67	> 100
	Curvularia sp. AMEf4	216	12.5±0.00	9.375±4.41	12.5±0.00	> 100
	Penicillium citrinum AMEf1	126	12.5±0.00	9.375±4.41	12.5±0.00	> 100
	Penicillium citrinum AMEs3	264	9.375±4.19	12.5±0.00	25±0.00	> 100

Seeds	*Aspergillus sp*. AMEs9	139	12.5±0.00	12.5±0.00	18.75±8.83	> 100
Thorns of fruit	*Aspergillus sp*. AMEtf15	56	9.375±4.41	6.25±0.00	12.5±0.00	> 100
	Talaromyces sp. AMEtf5	95	18.75±8.83	6.25±0.00	12.5±0.00	> 100
Twigs	*Fusarium waltergamsii* AMEtw3	48	3.125±0.00	3.125±0.00	6.25±0.00	> 100
Stem bark	*Talaromyces annesophieae* AMEsb23	25	12.5±0.00	6.25±0.00	18.75±8.83	> 100
	Penicillium citrinum AMEsb1	194	12.5±0.00	3.125±0.00	9.375±4.41	> 100
Barks	*Aspergillus sp*. AMEb7	24	12.5±0.00	6.25±0.00	18.75±8.83	> 100
Ciprofloxacin		NA	0.468±0.00	0.234±0.00	0.234±0.00	NA
Podophyllotoxin		NA	NA	NA	NA	0.177±0.05

AMEr: *A. muricata* root; AMErb: *A. muricata* root bark; AMEf: *A. muricata* fruit; AMEs: *A. muricata* seed; AMEtf: *A. muricata* thorn of fruit; AMEtw: *A. muricata* twigs; AMEb: *A. muricata* bark; AMEsb: *A. muricata* stem bark; CC_{50}: Cytotoxic Concentrations 50; MIC: Minimum Inhibitory Concentration; NA: Not Applicable.

Table VII: Selectivity Indexes (SI) of selected extracts

Plant organs	Fungal Extracts	CC_{50} Vero cells/MIC *S. aureus* ATCC 43300	CC_{50} Vero cells/MIC *K. Oxytoca* Isolate	CC_{50} Vero cells/MIC *E. coli* ATCC 25922
Roots	*Aspergillus sp*. AMEr9	>8	>8	>8
	Aspergillus sp. AMEr10	>1	>1	> 1
Root bark	*Penicillium sp*. AMErb1	>5.33	>8	>8
	Aspergillus sp. AMErb9	>8	>8	>8
	Penicillium sp. AMErb11	>8	>8	>8
Fruits	*Penicillium citrinum* AMf6	>10.66	>21.36	>10.66
	Meyerozyma sp. AMEf3	>2	>4	>2.66
	Curvularia sp. AMEf4	>8	>10.66	>8
	Penicillium citrinum AMEf1	>8	>10.66	>8
Seeds	*Penicillium citrinum* AMEs3	>9.38	>8	>4
	Aspergillus sp. AMEs9	>8	>8	>5.33
Thorns of fruit	*Aspergillus sp*. AMEtf15	>10.66	>16	>8
	Talaromyces sp. AMEtf5	>5.33	>16	>8
Twigs	*Fusarium waltergamsii* AMEtw3	>32	>32	>16

Stem bark	*Talaromyces annesophieae* AMEsb23	>8	>16	>5.33
	Penicillium citrinum AMEsb1	>8	>32	>10.66
Barks	*Aspergillus sp*. AMEb7	>8	>16	>5.33

AMEr: *A. muricata* root; AMErb: *A. muricata* root bark; AMEf: *A. muricata* fruit; AMEs: *A. muricata* seed; AMEtf: *A. muricata* thorn of fruit; AMEtw: *A. muricata* twigs; AMEb: *A. muricata* bark; AMEsb: *A. muricata* stem bark; CC_{50}: Cytotoxic Concentrations 50; MIC: Minimum Inhibitory Concentration; NA: Not Applicable.

III.1.1.4 Antioxidant activity of fungal extracts

III.1.1.4.1 DPPH radical scavenging activity

The ability of the 17 antibacterial extracts to scavenge the free radical DPPH was measured at an absorbance of 517 nm after a preliminary screening at 1000 µg/mL. Globally, among the 17 extracts tested, only 7 showed a percentage of RSA ≥ 50 and were used for the dose response study. From the results, the radical scavenging activity of the seven tested extracts ranged from 146.05 to 799.75 µg/mL (Table VIII). Although their activity was significantly lower ($p \leq 0.05$) than that of ascorbic acid (RSA_{50} 8.92 µg/mL), the extract from *T. clemensii* AMEtf5 exhibited the highest scavenging activity (RSA_{50} 146.05 µg/mL) followed by an extract from *Aspergillus* sp. AMEr9 (RSA_{50} 176.9 µg/mL) obtained respectively from the thorn of fruits and roots of *A. muricata*. Interestingly, although *Aspergillus* spp from roots displayed good potency, another *Aspergillus* isolate from the thorn of fruits was weakly active (RSA_{50} 663.35 µg/mL). Similarly, while the extract from *P. citrinum* AMEsb1 from stem bark was potent (RSA_{50} 211.30 µg/mL), the *P. citrinum* AMEf6 from fruits was the less active (RSA_{50} 799.75 µg/mL) of the tested extracts. The same difference in activity could be observed with the *Talaromyces* species from the thorn of fruits (RSA_{50} 146.05 µg/mL) and stem bark (RSA_{50} 536.15 µg/mL). These observations could suggest that the organ of isolation of endophytic isolates could substantially influence the potency of each microbial species.

Table VIII: DPPH radical scavenging parameters of the seven active endophytic fungi extracts

Extracts	RSA_{50} (µg/mL± SD)	CE_{50}	ARP
***Aspergillus sp*. AMEtf15**	663.35 ± 4.73^{a}	$3.31 \times 10^{4\mathbf{a}}$	3.02×10^{-5a}
***T. clemensii* AMEtf5**	146.05 ± 4.31^{b}	0.73×10^{4b}	13.69×10^{-5b}
***P. citrinum* AMEf6**	799.75 ± 11.66^{c}	3.99×10^{4c}	2.50×10^{-5c}
***F. waltergamsii* AMEtw3**	282.30 ± 0.98^{d}	1.41×10^{4d}	7.09×10^{-5d}
***Aspergillus sp* AMEr9**	176.90 ± 0.84^{e}	0.88×10^{4e}	11.36×10^{-5e}
***T. annesophieae* AMEsb23**	536.15 ± 38.39^{f}	2.68×10^{4f}	3.73×10^{-5e}
***P.citrinum* AMEsb1**	211.30 ± 1.13^{g}	1.05×10^{4g}	9.52×10^{-5f}
Vitamin C	8.92 ± 1.065^{h}	0.0446×10^{4h}	224.21×10^{-5h}

RSA_{50}: radical scavenging activity 50; EC_{50}: Efficient Concentration 50; ARP: Antiradical Power ; AMEr: *A. muricata* root; AMEf: *A. muricata* fruit; AMEtf: *A. muricata* thorn of fruit; AMEtw: *A. muricata* twigs; AMEsb: *A. muricata* stem bark. Along the column, values carrying the same letter superscripts are not significantly different ($p > 0.05$), and values carrying different letters are significantly different ($P < 0.05$).

III.1.1.4.2 Reduction of Fe^{3+} ions by ortho-phenanthroline

The seventeen extracts were also screened for the ability to reduce the Fe^{3+} to Fe^{2+}. The results from table IX showed a significant correlation between the concentration of the extracts and their reducing power. By projecting the optical densities of the extracts on a concentration-activity curve of NH_2OH used as standard, the results showed that only extracts from five endophytic fungi including AMEtf15, AMEf6, AMEs3, AMEf4 and AMEf1 exhibited activity (12.03, 12.28, 11.97, 12.35 and 12.37 µg equivalent NH_2OH/g respectively) at 1000 µg/mL. Moreover, at concentrations 1000, 500, 250 and 125 µg/mL, the ferric ion reducing capacities of all these five extracts were not significantly different ($P > 0.05$).

Overall, out of the seventeen extracts screened for ferric ion reducing capacities, extract AMS9 displayed the weakest activity at all concentrations.

Table IX: Quantitative evaluation of Fe^{3+} reducing power by endophytic extracts of *A. muricata*

Code of extracts	**µg equivalent NH_2OH/g of extract± SD**						
Concentrations (µg/mL)	**1000**	**500**	**250**	**125**	**62.5**	**31.25**	**15.625**
***Aspergillus sp.* AMEtf15**	12.03±0.59 a	11.46±0.16 b	11.45±0.40 c	11.36±0.34	7.96±0.20	7.65±0.07	7.28±0.20
***Penicillium sp* AMErb1**	2.98±0.10	1.99±0.06	1.45±0.01	1.06±0.02	0.91±00	0.79±0.2	0.68±0.11
***P. citrinum* AMEf6**	12.28±0.36 a	11.88±0.12 b	11.77±0.43 c	11.72±0.38	8.25±0.50	7.88±0.09	7.70±0.07
***P.citrinum* AMEs3**	11.97±0.36 a	11.70±0.16 b	11.57±0.16 c	11.48±0.06	7.84±0.09	4.12±0.35	3.76±0.43
***Meyerozyma sp.*AMEf3**	1.06±0.00	0.95±0.08	0.84±0.03	0.83±0.06	0.82±0.06	0.79±0.10	0.67±0.01 f
***Penicillium sp.* AMErb11**	1.06±0.02	0.87±0.06	0.82±0.19	0.79±0.11 c	0.74±0.21	0.74±0.16	0.65±0.03
***Curvularia sp.*AMEf4**	12.35±0.42 a	11.74±0.25 b	11.53±1.06 c	11.30±0.74 d	11.15±0.95 e	10.28±0.00 f	8.47±0.43
***Talaromyces sp.*AMEtf5**	3.92±0.18	2.67±0.08	1.76±0.08	1.26±0.03	0.95±0.07	0.84±0.02	0.81±0.13

***Aspergillus sp.*AMEb7**	1.10±0.00[a]	1.08±0.01[ab]	0.87±0.02[abc]	0.80±0.02[abcd]	0.76±0.00[bcde]	0.73±0.06[cdef]	0.69±0.20[cdef]
***Aspergillus sp* AMEsb23**	4.47±0.26[f]	2.86±0.05[e]	1.71±0.07[a]	1.32±0.28[ab]	1.01±0.15[abc]	0.95±0.20[bcd]	0.81±0.14[bcd]
***F. waltergamsii* AMEtw3**	2.26±0.00[e]	1.61±0.01[d]	1.41±0.02[a]	1.29±0.06[a]	1.08±0.06[b]	1.01±0.01[b]	0.69±0.02[c]
***P.citrinum*AMEf 1**	12.37±0.02[a]	11.83±0.16[a b]	11.72±0.25[ab c]	11.70±0.22[abc]	8.11±0.37[d]	7.95±0.17[d]	4.35±0.32[e]
***Aspergillus sp* AMEr9**	3.79±0.19[f]	2.68±0.12[e]	1.86±0.00[d]	1.36±0.09[a]	1.15±0.05[ab]	0.89±0.03[abc]	0.89±0.19[abc]
***Aspergillus sp.*AMErb9**	1.09±0.01[e]	0.94±0.00[d]	0.84±0.03[a]	0.81±0.00[a]	0.74±0.01[b]	0.68±0.00[bc]	0.67±0.00[c]
***Aspergillus sp* AMEsb1**	4.90±0.26[e]	3.27±0.13[a]	2.20±0.09[a]	1.57±0.03[ab]	1.2±0.07[abc]	1.06±0.22[abcd]	0.79±0.00[acd]
***Aspergillus sp.*AMEr10**	1.43±0.03[f]	1.20±0.00[a]	1.16±0.02[ab]	1.05±0.11[abc]	0.95±0.02[bcd]	0.91±0.05[cde]	0.72±0.02[e]
***Aspergillus sp.* AMEs9**	0,96±0.06 [e]	0.74±0.04[d]	0.62±0.00[a]	0.54±0.0.2[cd]	0.32±0.08[f]	0.30±0.02[bcd]	0.16±0.01[f]

AMEr: *A. muricata* root; AMErb: *A. muricata* root bark; AMEf: *A. muricata* fruit; AMs: *A. muricata* seed; AMEtf: *A. muricata* thorn of fruit; AMEtw: *A. muricata*; AMEb: *A. muricata* bark; AMEsb: *A. muricata* stem bark. Along the line, values carrying the same letter are not significantly different (P>0.05), and values carrying different letters are significantly different (P<0.05)

III.1.2 Fractionation results

Twenty-five grams of extract from the large scale culture of *Fusarium* sp AMEtw3 were subjected to partition column chromatography using silica gel 60 (Macherey-Nagel, Sigma-Aldrich, St. Louis, MO). Eight hundred and fourteen fractions were collected and grouped on the basis of their thin layer chromatography (TLC) profiles into nine major fractions labelled AMF1; AMF2; AMF3; AMF4; AMF5; AMF6; AMF7; AMF8; AMF9 with masses ranging from 0.371 to 10.501 g for a total mass of 24.112 g (Table X). This mass represented 96.44 % of the mass of the originated crude extract indicating that only 3.56 % were lost during the elution process. Six (6) fractions were subjected to chromatography again to obtain 15 pure sub-fractions and two compounds. All were tested for antibacterial, cytotoxicity and antioxidant activities.

III.1.2.1 MICs (µg/mL), CC_{50} (µg/mL) and SI of the fractions obtained after column chromatography

Obtained fractions were tested for their antibacterial activity against the three pathogenic bacteria and the cytotoxicity against the Vero cell line. Results obtained are summarized in the tables X and XI below:

Table X: Minimum Inhibitory Concentrations (MICs) and Cytotoxic Concentrations 50 (CC_{50}) of fractions

Fractions code	Mass (g)	MIC (µg/mL ± SD)			CC_{50} (µg/mL ± SD)
		***S. aureus* ATCC 43300**	***K. oxytoca* Isolate**	***E. coli* ATCC 25922**	**Vero cells ATCC CRL1586**
AMF1	5.240	3.125±0.000	3.125±0.000	6.125±0.000	> 100
AMF2	3.730	1.56±0.000	0.78±0.000	3.125±0.000	> 100
AMF3	4.960	3.125±0.000	1.56±0.000	3.125±0.000	> 100
AMF4	2.259	> 100	50±0.000	> 100	> 100

AMF5	1.426	6.25±0.000	3.25±0.000	12.5±0.000	> 100
AMF6	1.276	6.25±0.000	3.125±0.000	25±0.000	> 100
AMF7	1.726	6.25±0.000	1.56±0.000	25±0.000	> 100
AMF8	1.870	25±0.000	12.5±0.000	> 100	> 100
AMF9	1.625	100±0.000	50±0.000	> 100	> 100
Ciprofloxacin	NA	0.468±0.000	0.234±0.000	0.234±0.000	NA
Podophyllotoxin	NA	NA	NA	NA	0.177±0.05

AMF: *Annona muricata* fraction; CC_{50}: Cytotoxic Concentrations 50; MIC: Minimum Inhibitory Concentration NA: not applicable

Table XI: Selectivity Indexes (SI) of fractions

Fractions code	**CC_{50} Vero cells/MIC *S. aureus* ATCC 43300**	**CC_{50} Vero cells/MIC *K. Oxytoca* Isolate**	**CC_{50} Vero cells/MIC *E. coli* ATCC 25922**
AMF1	>32	>32	>16
AMF2	>64.10	>128.20	>32
AMF3	>32	>64.10	>32
AMF4	>1	>2	> 1
AMF5	>16	>32	>8
AMF6	>16	>32	>4
AMF7	>16	>64.10	>4
AMF8	>4	>8	> 1
AMF9	>1	>2	> 1

AMF: *Annona muricata* fraction; CC_{50}: Cytotoxic Concentrations 50; MIC: Minimum Inhibitory Concentration NA: not applicable

From the results obtained in table X, the different fractions showed varying MIC values ranging from 0.78 to > 100 µg/mL depending on the fractions and microorganisms. The fraction AMF2 showed the best activities on all the tested microorganisms with MIC ranging from 0.78 to 3.125 µg/mL. Compared to the originated crude extract, this fraction has improved the activity by 2 folds on *S. aureus* ATCC 43300 and *E. coli* ATCC 25922 and 4 folds on *K. oxytoca.* The fraction AMF9 was the least active with MICs >100, 100 and 50 µg/mL respectively against *E. coli* ATCC 25922, *S. aureus* ATCC 43300 and *K. oxytoca* . Fractions AMF8 and AMF9 were inactive against *E. coli* ATCC 25922 at 100 µg/mL.

Overall, compared to the crude extract, fractions AMF5 AMF6, AMF7, AMF8 and AMF9 although having shown activity, did not improve the antibacterial activity particularly on *S. aureus* ATCC 43300 and *E. coli* ATCC 25922. Conversely, the activity decreased by 2 folds for fractions AMF5, AMF6, AMF7, 8 folds for fraction AMF8 and 32 folds for AMF9 on *S. aureus* ATCC 43300. On *E. coli* ATCC 25922, this decrease of activity was observed with fractions AMF5, AMF6 and AMF7 with respective folds of 2, 4 and 4. Among them, AMF7 improved the activity (2 folds) on *K. oxytoca* isolate with an MIC of 1.56 µg/mL. Moreover, the fractions tested showed no cytotoxicity against Vero cells ATCC CRL1586 with the median cytotoxic concentrations (CC_{50}) greater than 100 µg/mL. All these fractions however presented good selective action against bacteria since their respective SI were greater than 1

III.1.2.2 MICs (µg/ml), CC_{50} (µg/mL) and SI of products obtained after chromatography of fractions

The purification of each fraction made it possible to obtain seventeen products (fifteen pure sub-fractions and two compounds). Pure sub-fractions AMSF3, AMSF11 and AMSF15 were not tested due to their small quantity. The remaining 14 products were tested for activity against the three bacteria uropathogens. The tested compounds exhibited activity with MIC values ranging from 0.195 to >100 µg/mL (Table XII). The results of the cytotoxicity activity of the ten most active samples are also reported in the tables below (XII and XIII).

Table XII: Minimum Inhibitory Concentrations (MICs) and Cytotoxic Concentrations 50 (CC_{50}) of the pure sub-fractions and compounds

Fraction of origin	**Pure sub-fractions/Compounds code**	**MIC (µg/mL ± SD)**			**CC_{50} (µg/mL ± SD)**
		***S. aureus* ATCC 43300**	***K. oxytoca* Isolate**	***E. coli* ATCC 25922**	**Vero cells ATCC CRL1586**
AMF2	AMSF2	1.56±0.000	1.56±0.000	> 100	> 100
	AMSF5	> 100	> 100	> 100	/
	AMSF10	3.25±0.000	3.25±0.000	6.25±0.000	> 100
	AMSF12	> 100	> 100	> 100	/
	AMSF15	/	/	/	/
	AMSF19	> 100	> 100	> 100	/
AMF3	AMSF3	/	/	/	/
	AMC6	0.39±0.000	0.39±0.000	0.78	> 100
	AMSF11	/	/	/	/
	AMSF13	25±0.000	25±0.000	> 100	> 100
	AMSF14	> 100	> 100	> 100	/
	AMC20	0.39±0.000	0.39±0.000	12.5±0.000	> 100
AMF5	AMSF8	1.56±0.000	1.56±0.000	1.56±0.000	> 100
AMF6	AMSF1	1.56±0.000	0.78±0.000	1.56±0.000	> 100
	AMSF7	> 100	50±0.000	> 100	> 100
	AMSF9	100±0.000	100±0.000	> 100	> 100

AMF7 and AMF8	AMSF17	0.195±0.000	0.78±0.000	3.25±0.000	> 100
	Ciprofloxacin	0.234±0.000	0.468±0.000	0.234±0.000	> 100
	Podophyllotoxin	NA	NA	NA	0.177±0.05

AMF: *Annona muricata* fraction ; AMC: *Annona muricata* compound ; AMSF: *Annona muricata* sub-fraction ; CC_{50}: Cytotoxic Concentrations 50; MIC: Minimum Inhibitory Concentration; /: not tested

Table XIII: Selectivity Indexes (SI) of pure sub-fractions and compounds

Fraction of origin	**Pure sub-fractions/Compounds code**	**CC_{50} Vero cells/MIC *S. aureus* ATCC 43300**	**CC_{50} Vero cells/MIC *K. Oxytoca* Isolate**	**CC_{50} Vero cells/MIC *E. coli* ATCC 25922**
AMF2	AMSF2	>64.10	>64.10	> 1
	AMSF10	>30.76	>30.76	>16
AMF3	AMC6	>256.41	>256.41	>128.20
	AMC20	>256.41	>256.41	>8
AMF5	AMSF8	>64.10	>64.10	>64.10
AMF6	AMSF1	>64.10	>128.20	>64.10
AMF7 and AMF8	AMSF7	> 1	>2	> 1
	AMSF9	> 1	> 1	> 1
	AMSF17	> 512.82	>128.20	>30.76

AMF: *Annona muricata* fraction ; AMC: *Annona muricata* compound ; AMSF: *Annona muricata* sub-fraction ; CC_{50}: Cytotoxic Concentrations 50; MIC: Minimum Inhibitory Concentration.

The results demonstrate pure sub-fractions AMSF5, AMSF14 and AMSF19 were completely inactive against all three bacteria pathogens tested. Compound AMC6 was the most active on uropathogens with MIC values of 0.78. 0.39 and 0.39 µg/mL, followed by AMC20 (MICs:12.5, 0.39 and 0.39 µg/mL) respectively against *E. coli* ATCC 25922, *S. aureus* ATCC

43300 and *K. oxytoca*. The activity observed against tested bacteria was better than that of the parent extract and the original fraction AMF3. In fact, the antibacterial activity was improved with AMC6 compound by 8 folds on *S. aureus* ATCC 43300 and 4 folds on *K. oxytoca* and *E. coli* ATCC 25922 compared to fraction AMF3. Similarly, this activity was also improved by 8 folds against all the three bacteria used when compared to the crude extract (Figure 14). AMSF10, AMSF2, AMSF13 and AMSF7 tested on *S. aureus* ATCC 43300 decreased the antibacterial activity by 2 to 8 folds when compared to their parent fraction. When tested on *E. coli* ATCC 25922, no activity was observed with AMSF2 and AMSF13. In contrast, AMSF10 and AMSF7 exhibited decreased antibacterial activity by 2 and 32 folds respectively. Indeed, AMSF20, AMSF8, AMSF1 and AMSF17 showed an improvement of the activity compared to fractions by 4 to 8 folds, 2-128 folds respectively on *S. aureus* ATCC 43300 and *K. oxytoca.* Except for AMC20, these samples also showed improvement of antibacterial potency potential *on E. coli* ATCC 25922 by 4 -16 folds. Globally, with about 71.428 % (10/14) of products active against *K. oxytoca*, this strain was the most sensitive while *E. coli* ATCC 25922 was the least sensitive of the three strains. Concerning the cytotoxicity, all the products tested had a selectivity index greater than 1 and were therefore non-cytotoxic on the Vero cell line.

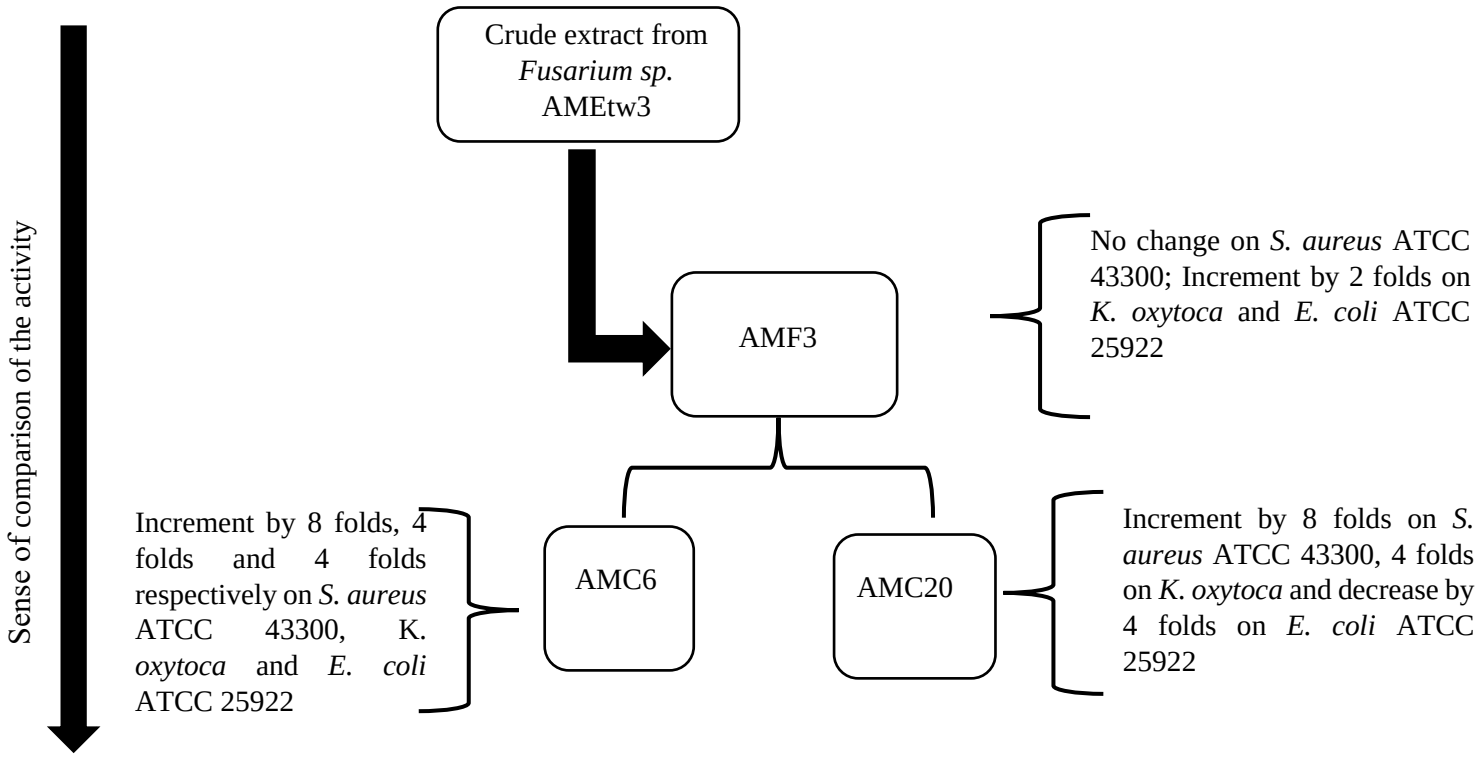


Figure 14: Evolution of the antibacterial activity from the selective crude extract to the two active isolated compounds (AMC6 and AMC20)

III.1.2.3 Antioxidant activity of active fractions, pure sub-fractions, and compounds

III.1.2.3.1 DPPH radical scavenging activity

Active obtained fractions and their active derived products were evaluated for their capacity to reduce the stable, purple-coloured radical DPPH into yellow-coloured DPPH-H.

Five fractions were found to scavenge DPPH with a percentage of RSA ≥ to 50 during the preliminary screening. The table XIV below shows that scavenging activity 50 (RSA_{50}) of the five selected fractions ranged from 186.23 to 875.54 µg/mL with AMF3 ($RSA_{50:}$ 186.23 µg/mL) being the most active followed by AMF2 ($RSA_{50:}$ 224.67 µg/mL). These activities show a little improvement as compared to that of their parent extract ($RSA_{50:}$282.30 µg/mL) but are still lower than that of vitamin C (RSA50: 8.92 µg/mL). Fractions AMF4 ($RSA_{50:}$ 662.78 µg/mL), AMF7 ($RSA_{50:}$ 445.23 µg/mL), and AMF8 ($RSA_{50:}$ 875.54 µg/mL) displayed weak ability to scavenge the free radical DPPH and therefore did not improve the scavenging effect of the originated extract.

Table XIV: DPPH radical scavenging parameters of the active fractions

Fractions	RSA_{50} (µg/mL± SD)	CE_{50}	ARP
AMF2	224.67 ± 2.67^{a}	1.12×10^{4a}	8.90×10^{-5a}
AMF3	186.23 ± 1.23^{a}	0.93×10^{4a}	10.73×10^{-5a}
AMF4	662.78 ± 3.90^{d}	3.31×10^{4d}	3.01×10^{-5d}
AMF7	445.23 ± 0.73^{b}	2.22×10^{4b}	4.49×10^{-5b}
AMF8	875.54 ± 2.90^{f}	4.37×10^{4f}	2.28×10^{-5f}
Vitamin C	8.92 ± 1.065^{g}	0.0446×10^{4h}	224.21×10^{-5h}

RSA_{50}: radical scavenging activity 50; EC_{50}: Efficient Concentration 50; ARP: Antiradical Power; AMF: *Annona muricata* fraction. Along the column, values carrying the same letter superscripts are not significantly different ($p > 0.05$), and values carrying different letters are significantly different ($P < 0.05$).

The initial screening of the fourteen products (twelve pure sub-fractions and two compounds) at 1000 µg/mL revealed six active (five pure sub-fractions and one compound) at the threshold (percentage of RSA ≥ to 50). Results summarized in table XV clearly show the results from the dose response study leading to a radical scavenging activity 50 (RSA_{50}) value. Thus, AMSF17 ($RSA_{50:}$ 123.45 µg/mL), AMSF9 ($RSA_{50:}$ 234.23 µg/mL) and AMSF8 ($RSA_{50:}$ 245.56 µg/mL) displayed the best activities among all compounds and pure sub-fractions tested. Compared to their different original fractions AMF5, AMF7, AMF8 and the crude extract the activity has been significantly improved. Similarly, the purification of the AMF6 ($RSA_{50:} > 1000$ µg/mL) fraction leading to the pure sub-fraction AMSF1 ($RSA_{50:}$ 789.34 µg/mL) has enhanced his scavenging effect. On the other hand, AMSF2 ($RSA_{50:}$ 368.12 µg/mL) and AMC6 ($RSA_{50:}$ 556.34 µg/mL) respectively purified from AMF3 ($RSA_{50:}$ 186.23 µg/mL) and AMF2 ($RSA_{50:}$ 224.67 µg/mL) have shown a decrease in activity when compared to the fraction of origin.

Table XV: DPPH radical scavenging parameters of the active pure sub-fractions and compounds

Pure sub-fractions/Compound	RSA_{50} (µg/mL± SD)	CE_{50}	ARP
AMSF1	789.34 ± 5.76^{e}	3.94×10^{4e}	2.53×10^{-5e}
AMSF2	368.12 ± 0.34^{b}	1.84×10^{4b}	5.43×10^{-5b}
AMC6	556.34 ± 0.56^{c}	2.78×10^{4c}	3.59×10^{-5c}
AMSF8	245.56 ± 7.89^{a}	1.22×10^{4a}	8.14×10^{-5a}
AMSF9	234.23 ± 9.34^{a}	1.17×10^{4a}	8.53×10^{-5a}
AMSF17	123.45 ± 2.56^{a}	0.61×10^{4a}	16.20×10^{-5a}
Vitamin C	8.92 ± 1.065^{g}	0.0446×10^{4h}	224.21×10^{-5h}

RSA_{50}: radical scavenging activity 50; EC_{50}: Efficient Concentration 50; ARP: Antiradical Power; AMC: *Annona muricata* compound; AMSF: *Annona muricata* sub-fraction. Along the column, values carrying the same letter superscripts are not significantly different ($p > 0.05$), and values carrying different letters are significantly different ($P < 0.05$).

III.1.2.3.2 Reduction of Fe^{3+} ions by ortho-phenanthroline

The result showed that, of all the fractions and at corresponding concentrations, AMF2, AMF8 and AMF3 exhibited moderate activity (14.89, 11.76 and 10.82 µg equivalent NH_2OH/g respectively) at 1000 µg/mL (Table XVI) which is higher than that of the parent extract (2.26 µg equivalent NH_2OH/g). Globally apart from fraction AMF7(1.89 µg equivalent NH_2OH/g), all the other fractions displayed ferric ion reducing capacities greater than that of extract from *Fusarium* sp. AMtw3 which still remains very weak.

Table XVI: Quantitative evaluation of Fe^{3+} reducing power by fractions

Code of fractions	**µg equivalent NH_2OH/g of fraction ± SD**						
Concentrations (µg/mL)	**1000**	**500**	**250**	**125**	**62.5**	**31.25**	**15.625**
AMF1	5.78 ± 0.23^{a}	4.12±0.4^{a}	4.067±0.34^{a}	3.98±1.23^{a}	1.89±0.76^{bc}	1.09±0.09^{bc}	0.98±0.67^{c}
AMF2	14.89±3.02^{a}	14.04±0.56^{a}	11.56±0.43^{b}	8.64±1.56^{c}	5.12±1.23^{d}	5.04±0.69^{de}	4.04±0.23^{e}
AMF3	10.82±0.34^{a}	10.34±0.34^{a}	10.01±0.21^{a}	8.24±0.23b^{c}	6.23±1.09^{cd}	4.23±1.09^{d}	4.10±0.03^{d}
AMF4	9.56±0.34^{a}	9.23±0.23^{a}	7.23±1.34^{b}	6.23±1.45^{b}	3.13±0.89^{c}	2.23±0.04^{c}	1.78±0.12^{d}
AMF5	4.34±0.34^{a}	4.23±0.92^{a}	2.34±0.45^{b}	2.20±0.10^{b}	2.01±0.23^{bc}	1.89±0.87^{cd}	1.45±0.56^{d}
AMF6	3.56±0.89^{e}	2.89±0.45^{a}	2.56±0.45^{a}	1.89±0.45^{ab}	1.02±0.45^{b}	0.65±0.05^{bc}	0.32±0.021^{bcd}
AMF7	1.89±0.33^{f}	1.76±0.23^{e}	1.56±0.34^{a}	1.34±0.008^{ab}	0.98±0.098^{abc}	0.43±0.023^{bcd}	0.32±0.032^{bcd}
AMF8	11.76±0.23^{a}	11.23±0.65^{ab}	9.23±0.23^{abc}	8.34±.0.34^{bc}	4.34±1.09^{bcd}	2.23±0.32^{d}	1.09±0.06^{e}
AMF9	8.98±0.23^{a}	8.54±0.23^{a}	6.28±0.23^{b}	5.23±0.23^{b}	3.24±0.34^{de}	2.98±0.45^{fe}	1.03±0.03^{f}

AMF: *Annona muricata* fraction. Along the column, values carrying the same letter superscripts are not significantly different (p >0.05), and values carrying different letters are significantly different (P < 0.05)

Out of the ten compounds/pure sub-fractions tested, AMSF17 (20.23 µg equivalent NH_2OH/g) was the most potent at 1000 µg/mL and also better than its source fractions AMF7 (1.89 µg equivalent NH_2OH/g) and AMF8 (11.76 µg equivalent NH_2OH/g). Moreover, the table XVII also indicates that samples AMSF2, AMSF8, AMSF10, AMSF13 and AMC20 (8.90, 3.09, 6.56 and 10.67 µg equivalent NH_2OH/g, respectively) at the highest concentration tested show no improvement of the activity when compared to their parent fractions. AMC6, AMSF7, AMC1 and AMSF9 show improved this activity. Overall, the fractions obtained did not significantly improve ferric ion reducing power.

Table XVII: Quantitative evaluation of Fe^{3+} reducing power by pure sub-fractions and compounds

Pure sub-fractions/ compounds code	**µg equivalent NH2OH/g of pure sub-fraction/compound± SD**						
Concentrations (µg/mL)	**1000**	**500**	**250**	**125**	**62.5**	**31.25**	**15.625**
AMSF1	6.56±0.87^{a}	4.89±0.36^{b}	4.82±1.45^{b}	3.78±0.93^{cd}	3.02±0.23^{cd}	2.34±0.34^{de}	2.23±0.34^{e}
AMSF2	8.90±0.34^{a}	8.67±0.98^{a}	8.45±1.09^{a}	6.76±0.23^{b}	6.23±0.25^{b}	6.10±0.27^{bc}	5.90±1.34^{c}
AMC6	11.67±23^{a}	11.56±0.23^{a}	11.02±0.34^{a}	9.45±0.76^{bc}	9.21±0.99^{bc}	8.23±0.98^{c}	8.10±0.02^{c}
AMSF7	3.09±0.02^{a}	3.06±0.98^{a}	2.67±0.45^{b}	2.02±0.34^{cd}	1.78±0.034^{d}	1.67±0.34^{d}	0.89±0.04^{e}
AMSF8	4.89±0.34^{a}	4.67±0.90^{a}	3.98±0.90^{b}	3.23±0.32^{b}	3.12±0.23^{bc}	2.34±19.4^{c}	1.45±0.34^{d}
AMSF9	4.56±076^{a}	4.23±0.34^{a}	4.10±0.34^{a}	3.12±0.08^{bc}	3.00±0.00^{bc}	2.12±0.03^{bc}	2.01±0.01^{d}
AMSF10	6.56±0.98^{a}	6.23±0.34^{a}	5.87±0.23^{b}	5.34±0.23^{bc}	4.98±0.67^{c}	4.76±0.23^{c}	2.56±2.90^{d}

AMSF13	1.098±0.09[a]	1.06±0.098[a]	0.98±0.23[a]	0.45±0.04[b]	0.32±0.045[b]	0.26.0.067[c]	0.15±0.078[d]
AMSF17	20.23±0.23a	20.12±1.231[a]	18.56±0.02[b]	18.23±0.09[b]	18.02±1.45[bc]	17.64±0.34[c]	17.56±0.34[c]
AMC20	10.67±0.34[a]	9.23±0.45[b]	9.21±0.23[bc]	8.90±1.09[bc]	8.67±0.23[bc]	8.34±0.23[c]	7.32±1.23[cd]

AMC: *Annona muricata* compound; AMSF: *Annona muricata* sub-fraction. Along the column, values carrying the same letter superscripts are not significantly different ($p > 0.05$), and values carrying different letters are significantly different ($P < 0.05$)

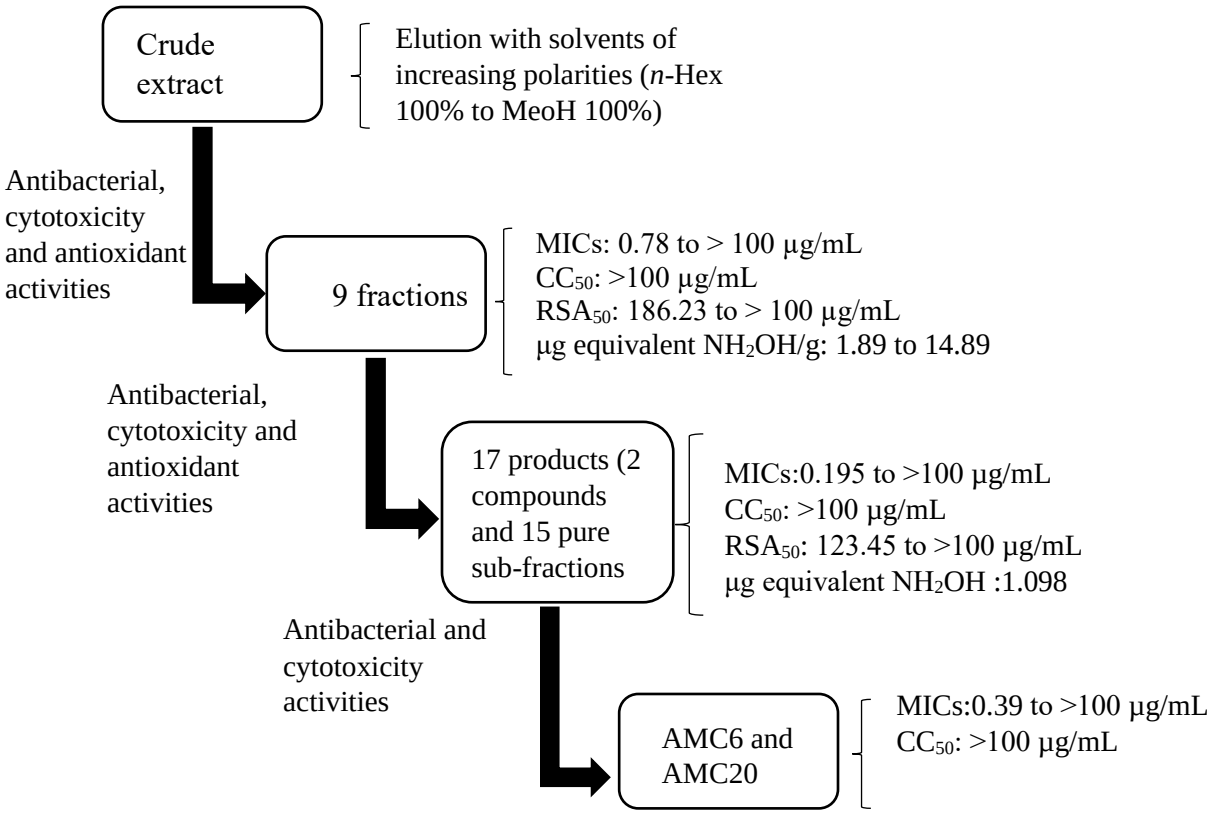


Figure 15: Summary of the fractionation of AMEtw3 extract

III.1.3 Modes of action of promising compounds and their structure elucidation

III.1.3.1 Modes of action of promising compounds

III.1.3.1.1 Effect of compounds on the catalase activity

The inhibition of the catalase activity of *S. aureus* ATCC 43300 and *K. oxytoca* was evaluated by comparing the amount of H_2O_2 remaining in the medium after the addition of compounds to the control (Table XVIII). The percentages of remaining H_2O_2 in bacteria culture treated with compounds were ranged from 55.93-56.82% and 65.32-67.79%, respectively, for *S. aureus* ATCC 43300 and *K. oxytoca.* This finding highlights the ability of tested compounds to exert a certain degree of inhibition against the activity of the bacterial catalase enzyme. The catalase inhibition activity exhibited by the two compounds on *S. aureus* ATCC 43300 was comparable to that of the positive control, Ciprofloxacin, since no statistically significant difference was observed ($p > 0.05$). On the contrary, the catalase inhibition capacities of these two last compounds were significantly higher ($P < 0.05$) than that of the positive control on *K. oxytoca*.

Table XVIII: Percentage of remaining H_2O_2 after evaluation of the effect of compounds on catalase activity of *S. aureus* ATCC 43300 and *K. oxytoca* at the MIC concentration

	Remaining H_2O_2 (%) ± SD	
Compounds	***K. oxytoca***	***S. aureus* ATCC 43300**
AMC6	67.79 ± 0.41^{b}	56.82 ± 1.97^{a}
AMC20	65.32 ± 0.62^{b}	55.93 ± 1.77^{a}
Ciprofloxacin	54.12 ± 2.89^{ab}	53.59 ± 2.70^{a}

Data are expressed as the mean±SD. Along the column, values carrying the same letter superscripts are not significantly different ($p > 0.05$), and values carrying different letters are significantly different ($P < 0.05$). AMC6: *A. muricata* compound 6, AMC20: *A. muricata* compound 20.

III.1.3.1.2 Lytic effect of compounds

The bacteriolysis assay was performed to investigate if active compounds inhibit *S. aureus* ATCC 43300 and *K. oxytoca* through cell lysis. Because viable bacteria absorb light at 620 nm, any decrease in optical density at this wavelength could be used as an indication of bacteriolysis. The cell lysis activity exhibited by compounds is summarized in Figure 16. Globally, the treatment of the bacterial cells with compounds (AMC6 and AMC20) caused significant cell lysis at all tested concentrations after 4h of incubation. Compound AMC6 (76.456%) exhibited the highest bacteriolytic activity at 4 MIC against *K. oxytoca* with no significant difference ($p > 0.05$) than that of the Ciprofloxacin (74.984%), which served as positive control. Likewise, no statistically significant difference in the lytic activity was observed ($p > 0.05$) with compound AMC20 at 2MIC and MIC on the two bacteria tested and this activity was lower in comparison to the positive control. The reduction of the bacterial population was more pronounced against *K. oxytoca*, with relative absorbance percentages varying from 23.544-100%.

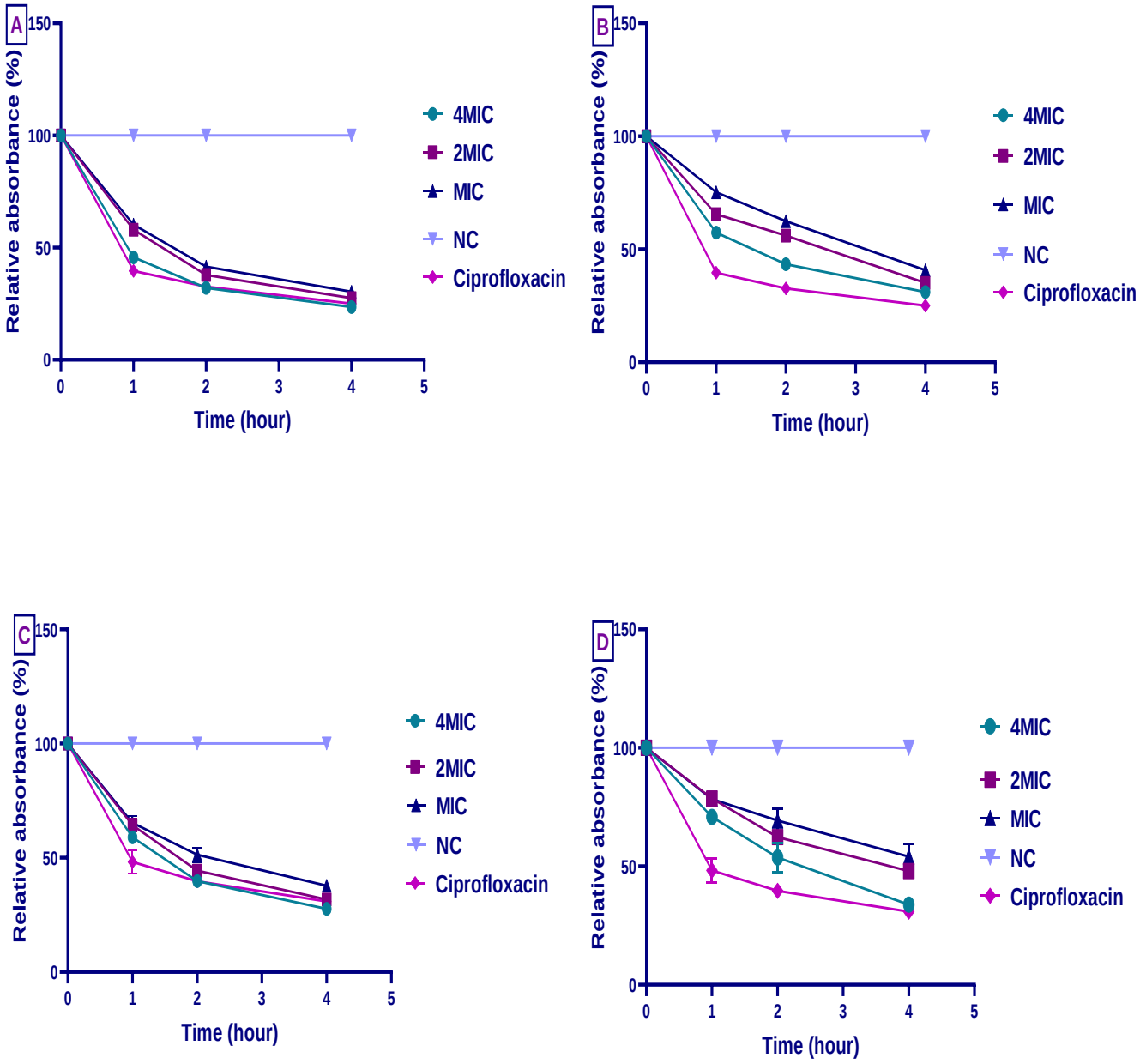


Figure 16: The bacteriolytic activity of compounds on *S. aureus* ATCC 43300 and *K. oxytoca* . (A) AMC6 on *K. oxytoca*; (B) AMC20 on *K. oxytoca*; (C) AMC6 on *S. aureus*; (D) AMC20 on *S. aureus*; AMC6: *A. muricata* compound 6; AMC20: *A. muricata* compound 20. MIC: Minimum Inhibitory Concentration, NC: Negative Control. Data are expressed as the mean ± SD

III.1.3.1.3 Effect of compounds on the permeability of outer cell membrane

The capacity of compounds to permeabilize the outer cell membrane was determined throughout 24 h at seven different concentrations. The figure 17 shows that the permeabilization activity was compound and concentration-dependent. Overall, the two compounds resulted in a decrease in the optical density with the increasing concentration of compounds. This trend could indicate the loss of intracellular ingredients by the chelation of divalent cations. It can also be noted that the destabilization power of the outer membrane of both bacteria by the different compounds was generally not significantly different ($p > 0.05$) than those of ciprofloxacin at their corresponding concentrations

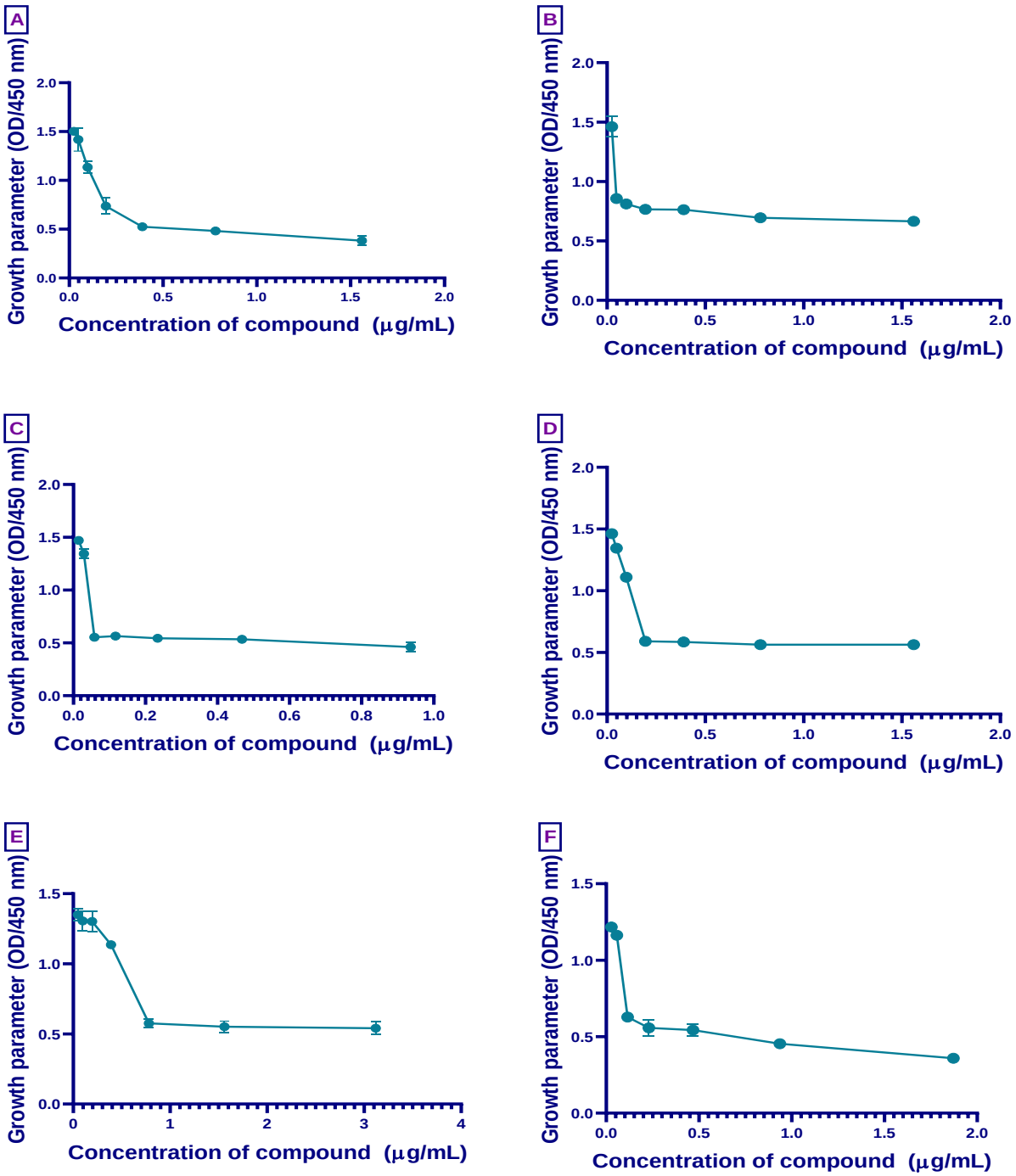


Figure 17: Effect of compounds on the outer membrane permeability of *S. aureus* ATCC 43300 and *K. oxytoca*; (A) AMC6 on *K. oxytoca*; (B) AMC20 on *K. oxytoca*; (C) Ciprofloxacin on *K. oxytoca*; (D) AMC6 on *S. aureus*; (E) AMC20 on *S. aureus*; (F) Ciprofloxacin; AMC6: *A.*

muricata compound 6; AMC20: *A. muricata* compound 20. Data are expressed as the mean ± SD.

III.1.3.1.4 The effect of compounds on the integrity of the cell membrane

The leakage of intracellular components from *S. aureus* ATCC 43300 and *K. oxytoca* cytoplasm after exposure at MIC of different compounds was carried out by measuring the absorbance of the supernatant at 260 nm. For this purpose, the presence of cytoplasmic elements in the culture broth is an indicator of damage to the bacterial membrane. The results presented in figure 18 show a moderate concentration-dependent increase in optical density compared to the control after 120 min and 60 min of incubation respectively on *K. oxytoca* and *S. aureus* ATCC 43300. Overall, the maximum effect was observed with AMC6 on *K. oxytoca* at 240 min (OD= 0.946), but this activity is significantly lower ($p < 0.05$) than that of the ciprofloxacin (OD= 1.173).

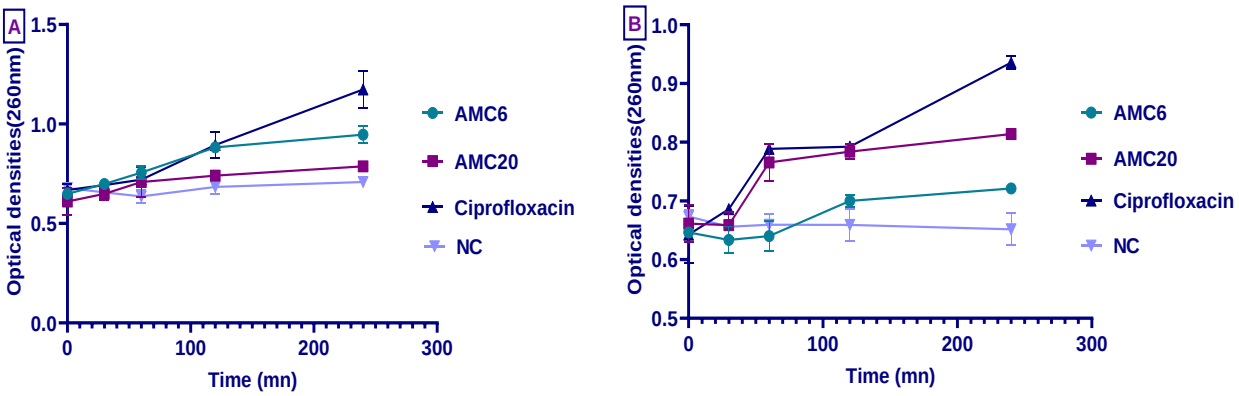


Figure 18: Effect of compounds on nucleic acids release leakage from (A) AMC6 and AMC20 on *K. oxytoca*; (B) AMC6 and AMC20 on *S. aureus* ATCC 43300 at their 4MIC concentration; AMC6: *A. muricata* compound 6, AMC20: *A. muricata* compound 20, NC: Negative Control Data are expressed as the mean ± SD.

III.1.3.1.5 Bacterial growth curves

The growth kinetics of bacteria strains exposed to three different concentrations (MIC, 2MIC and 4MIC values) of compounds for 24 h were compared to bacteria growth without

antibiotic treatment (Figure 19). As revealed by the reading of optical densities at 620 nm, the two compounds at all tested concentrations significantly inhibited the growth of the two bacteria pathogens compared to the negative control. Globally, although there was an initial increase in the number of viable cells during the first 2-4 hours of incubation, a gradual decrease in the bacterial population was noted from the 5^{th} to the 24^{th} hour, depending on the compound and the microorganism. The maximum bactericidal effect was noted after 24 h of incubation with the decrease in bacterial population by 5.033-6.812 times for *S. aureus* ATCC 43300 and 3.098-10.053 times for *K. oxytoca*. Compound AMC6 was the most efficient killing on *K. oxytoca* at all tested concentrations, with 5.658, 5.906, and 10.053 folds reduction of bacteria population at MIC, 2MIC and 4MIC, respectively. The killing power of this compound at MIC concentration was significantly greater ($P < 0.05$) than the activity of ciprofloxacin used as positive control at the same concentration known to exert a bactericidal effect. Against *S. aureus* ATCC 43300, there was no significant difference ($p > 0.05$) between the two compounds but their activity was significantly lower than that of the positive control.

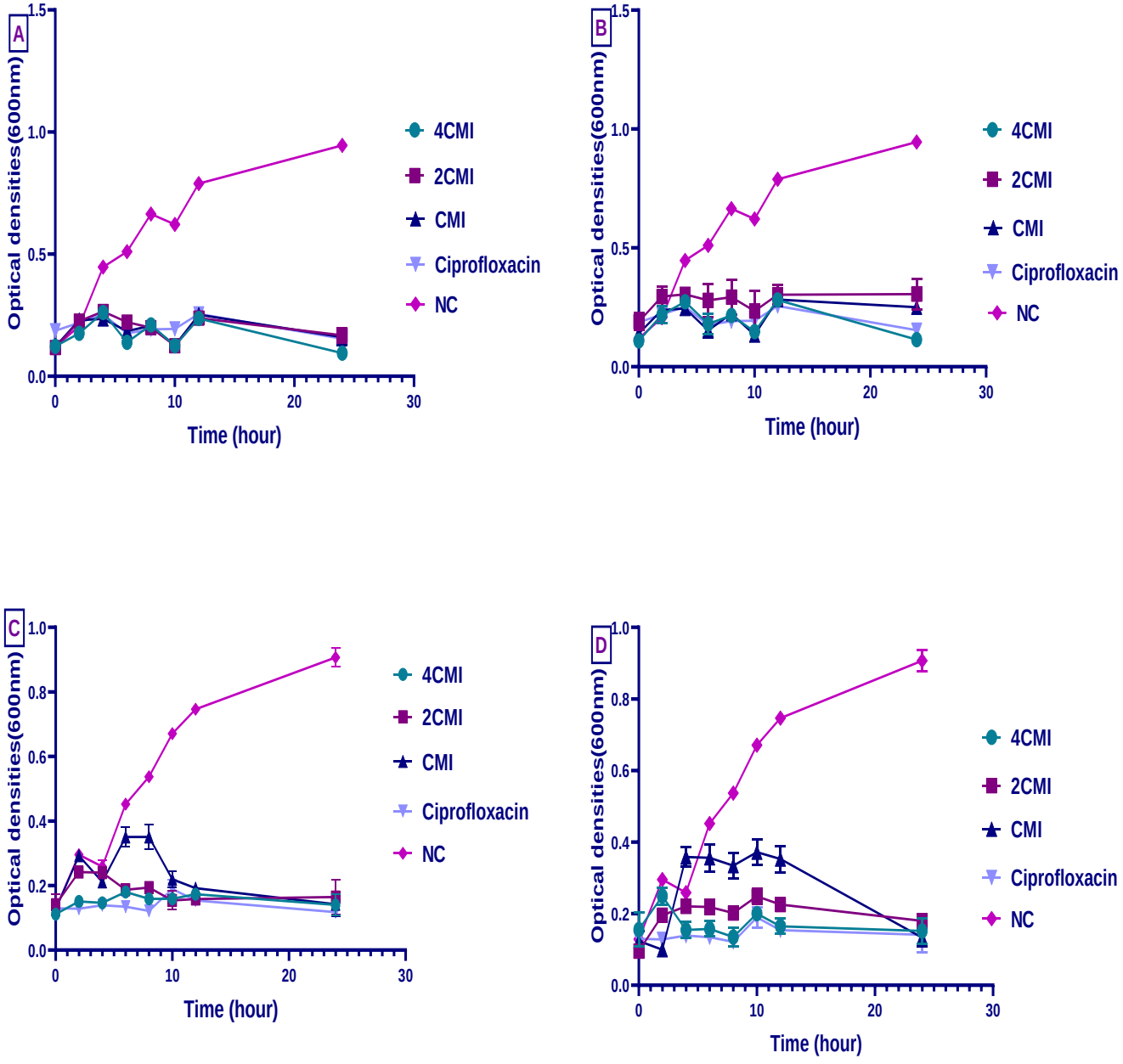


Figure 19: Activity kinetics of compounds on *S. aureus* ATCC 43300 and *K. oxytoca*; *(*A) AMC6 on *K. oxytoca*; (B) AMC20 on *K. oxytoca*; (C) AMC6 on *S. aureus*; (D) AMC20 on *S. aureus* ATCC, MIC: Minimal Inhibitory Concentration; NC: Negative Control. Data are expressed as the mean ± SD.

III.1.3.1.6 Effect of compounds on biofilm

III.1.3.1.6.1 Biofilm quantification

Biofilm production by *K. oxytoca* and *S. aureus* ATCC 43300 in NB medium supplemented with 2% glucose after 24 hours of incubation was expressed in terms of the mean of fluorescence values from independent assays performed in triplicate. Figure 20 showed that after 24 hours of incubation, both *K. oxytoca* and *S. aureus* ATCC 43300 could form a significant amount of biofilm materialized by the mean relative fluorescence units (RFU) (λ_{Ex} 530 nm and λ_{Em} 590 nm) from two independent assays over 10000. There was no significant difference ($P > 0.05$) between the amounts of biofilm formed by the two bacteria.

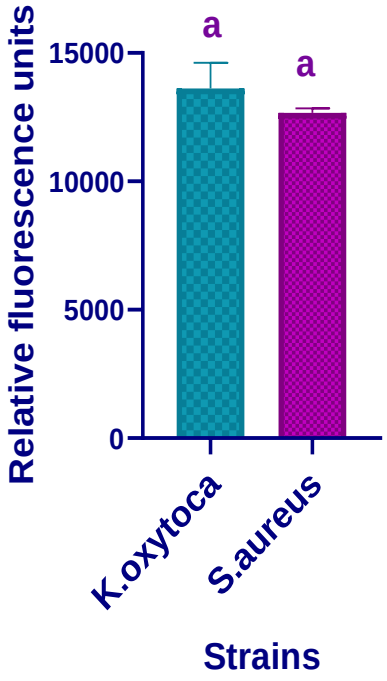


Figure 20: Biomass of biofilms produced by *K. oxytoca* and *S. aureus* ATCC 43300 after 48 hours in Nutrient broth (NB) medium supplemented with 2% glucose. The experiment was performed twice in triplicate, and data are expressed as the mean ± SD. Values with the same letter express no significant difference at $p > 0.05$.

III.1.3.1.6.2 Effect of compounds on formation and eradication of biofilm

Bacterial biofilms play a significantly important role in urinary tract infections (UTIs), responsible for persistent infections causing relapses and acute prostatitis (Delcaru *et al*., 2016). Bacterial forming biofilms are difficult to eradicate due to several factors, including persistent

cells showing reduced metabolism that leads to higher levels of antimicrobial resistance and the natural resistance conferred to bacteria by the biofilms structure (Delcaru *et al*., 2016). Therefore, the search for new therapeutic agents is necessary. In this respect, the selected compounds were also evaluated for their potential to inhibit the biofilms formation by *S. aureus* ATCC 43300 and *K. oxytoca*. The determination of the Minimum Biofilm Inhibitory Concentration 50 ($MBIC_{50}$), defined as the concentration of compound required to reduce by half the biofilm formation, revealed different degrees of inhibition of compounds against both bacteria pathogens (Table XIX). The $MBIC_{50}$ values ranged from 0.07-0.09 µg/mL against *K. oxytoca* and 0.08-0.19 µg/mL on *S. aureus* ATCC 43300. *K. oxytoca* was more sensitive than *S. aureus*. However, there was no significant difference ($p > 0.05$) between biofilm inhibition capacity of the two compounds and the positive control.

Table XIX: Minimum Biofilm Inhibitory Concentration 50 ($MBIC_{50}$) and Eradication Concentration Biofilm 50 ($MBEC_{50}$) values of compounds against *K. oxytoca* and *S. aureus* biofilm formation and eradication.

Compounds	***K. oxytoca***		***S. aureus* ATCC 43300**	
Parameters	**$MBIC_{50}$ (µg/mL± SD)**	**$MBEC_{50}$ (µg/mL± SD)**	**$MBIC_{50}$ (µg/mL± SD)**	**$MBEC_{50}$ (µg/mL± SD)**
AMC6	0.07 ± 0.00^{a}	0.20 ± 0.01^{a}	0.08 ± 0.00^{a}	0.182 ± 0.00^{a}
AMC20	0.09 ± 0.00^{a}	0.19 ± 0.00^{a}	0.19 ± 0.02^{b}	0.29 ± 0.01^{b}
Ciprofloxacin	0.03 ± 0.00^{a}	$0.075\pm0.0.07^{b}$	0.07 ± 0.00^{a}	0.21 ± 0.00^{b}

Data are expressed as the mean ± SD. Along the column values carrying the same letter superscripts are not significantly different ($p > 0.05$), and values carrying different letters are significantly different ($P<0.05$). AMC6: *A. muricata* compound 6, AMC20: *A. muricata* compound 20.

The ability of compounds to prevent the further formation of biofilm was also investigated on the two bacteria. The table above indicated that Minimum Biofilm Eradication Concentration 50 ranged from 0.182 to 0.29 µg/mL. The best activity was exhibited by compound AMC6 ($MBEC_{50}$: 0.182 µg/mL) compound on *S. aureus* ATCC 43300 and is significantly greater than that of the positive control ($MBEC_{50}$: 0.21 µg/mL). Compounds AMC6 ($MBEC_{50}$: 0.20 µg/mL) and AMC20 ($MBEC_{50}$: 0.19 µg/mL) showed the same activity on *K. oxytoca* which is significantly lower ($p < 0.05$) than activity of the ciprofloxacin ($MBEC_{50}$: 0.075

µg/mL). Overall, the two compounds have high eradicating capacities of biofilm against *K. oxytoca* and *S. aureus.*

III.1.3.1.7 Comparison of modes of action by Means of Principal Components Analysis (PCA) and Hierarchical Clustering analysis (HCA)

The aim of this analysis was to compare the different modes of action of the two compounds on each of the bacteria used and, even more importantly, to find out whether any correlation exists among the five modes of action investigated. Statistical analysis was performed using principal components analysis and hierarchical clustering (with Euclidian distance as a similarity measure and Ward's linkage).

III.1.3.1.7.1 Principal Component Analysis on *K. oxytoca*

PCA generates plots of the distribution of the different compounds following the different modes of action. Globally, the most interesting points are usually those that are close enough to one of the axes, and far enough from the origin. Moreover, points close to each other are very similar. In this study, the biplot obtained shows that the compound AMC6 acts preferentially on *K. oxytoca* by inhibiting biofilm formation and catalase activity (cluster II). Furthermore, bacteriolysis, outer membrane permeability and time kill kinetics are the main modes of action by which the AMC20 compound acts on the same bacteria (cluster I). Ciprofloxacin, the standard drug, prefers integrity of the cell membrane and biofilm eradication (cluster III) (Figure 21).

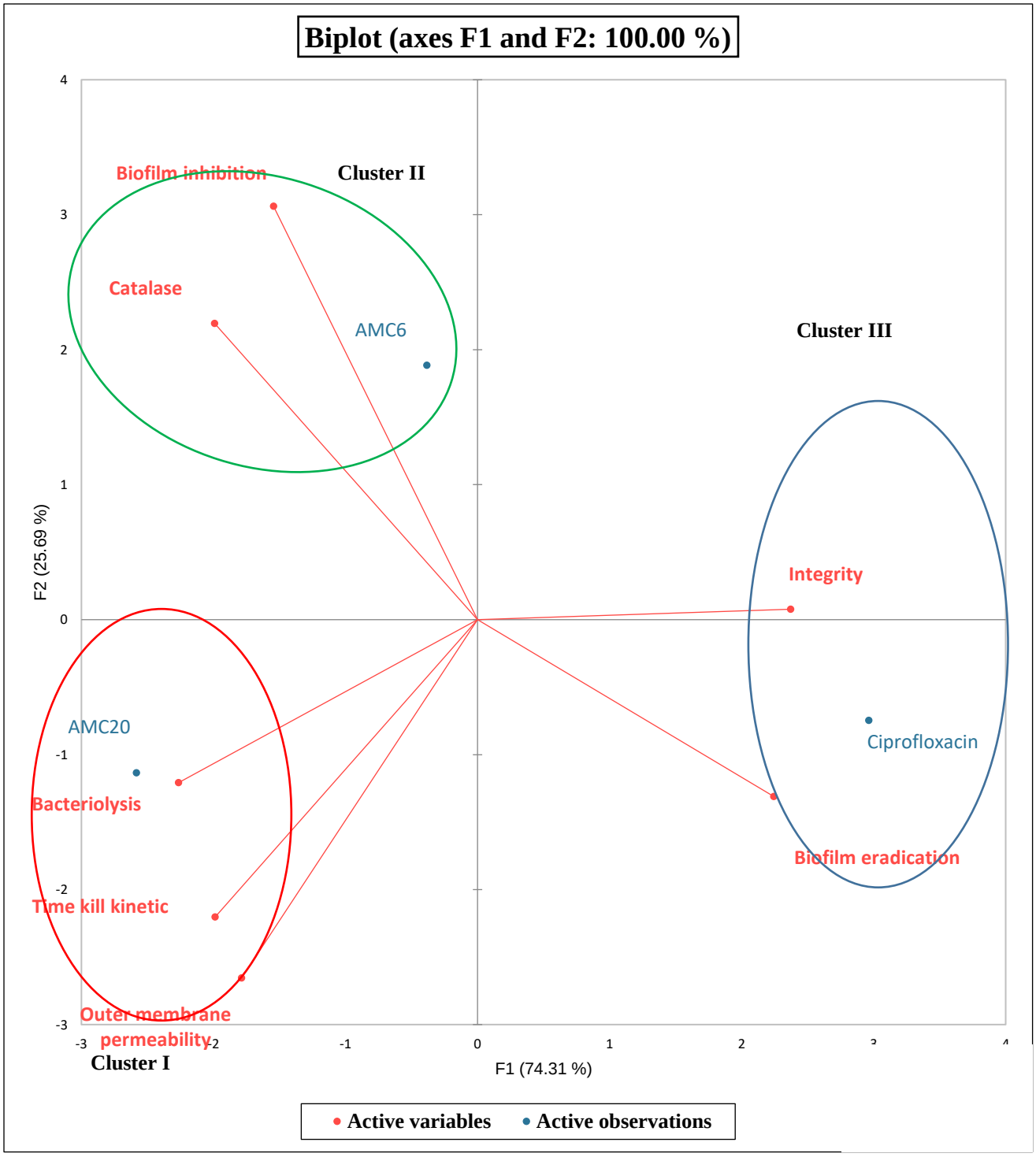


Figure 21: Principal Components analysis (PCA) of modes of action of compounds on *K. oxytoca*

III.1.3.1.7.2 Hierarchical Clustering analysis on *K. oxytoca*

The findings of HCA are illustrated in a dendrogram, a plot that displays the arrangement of samples and their relationships in a tree shape. From the figure 22 below, the sample population can be classified into two main clusters at a distance of 14 units with ciprofloxacin belonging to the cluster I. Cluster II includes compounds AMC6 and AMC20. Samples connected by a shorter distance are more similar in terms of modes of action than those connected by a longer distance. It clearly appears that AMC6 and AMC20 exhibited close modes of action on *K. oxytoca.*

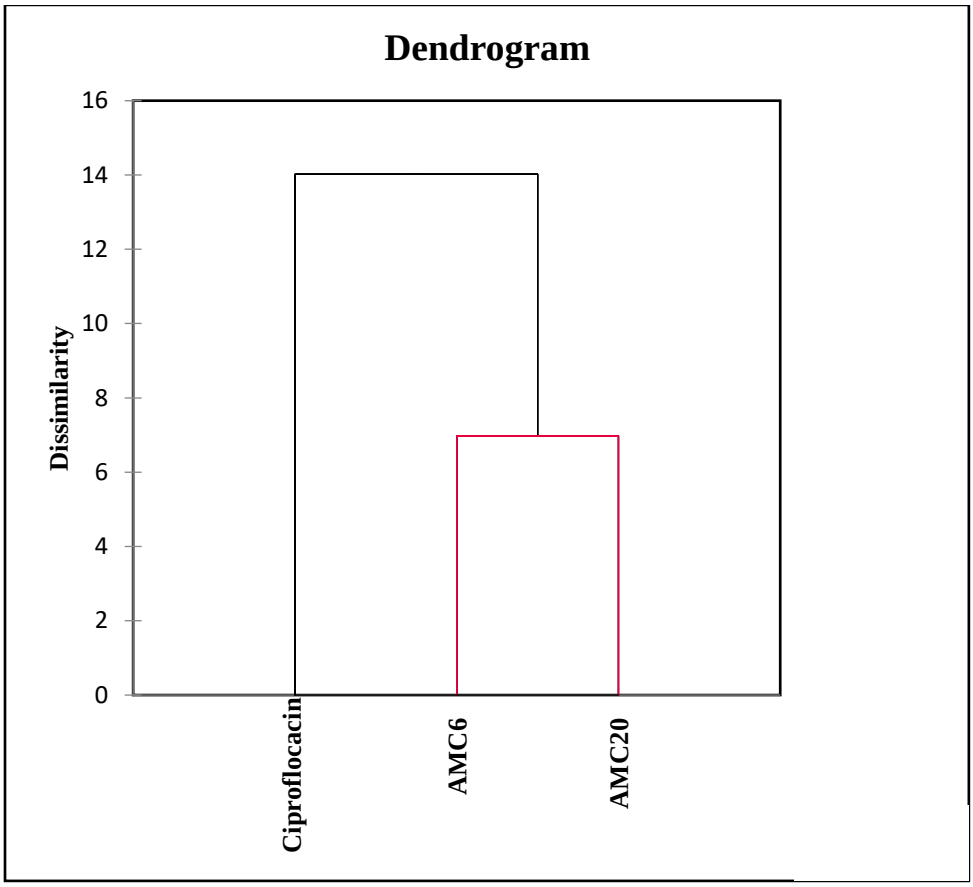


Figure 22: Hierarchical cluster analysis (HCA) of modes of action of compounds on *K. oxytoca*

III.1.3.1.7.3 Principal Components Analysis on *S. aureus* ATCC 43300

Biplot represented on Figure 23 showed that compound AMC20 acts on *S. aureus* selectively by bacterial lysis (cluster I). Similarly, AMC6 privileged catalase inhibition and the

reduction of bacterial population within 24 h (cluster III). Ciprofloxacin prefers to eradicate biofilm and destroys outer membrane permeability (cluster II).

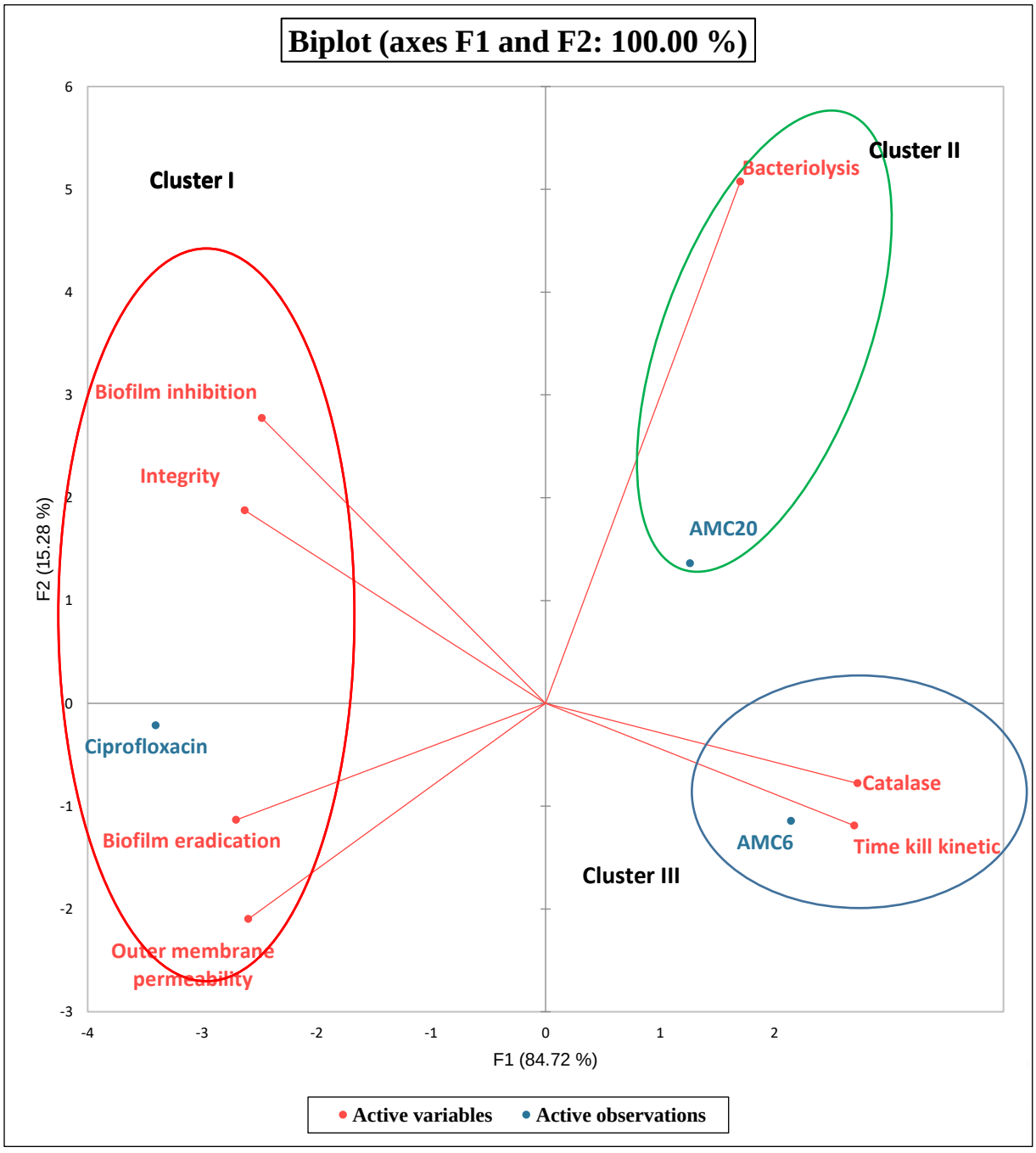


Figure 23: Principal Components analysis (PCA) of modes of action of compounds on *S. aureus* ATCC 43300

III.1.3.1.7.4 Hierarchical Clustering analysis on *S. aureus* ATCC 43300

The dendrogram of HCA of modes of action of compounds on *S. aureus* ATCC 43300 are shown on Figure 24. These samples are grouped into two major clusters at a distance of 250 units. Cluster I consist of AMC20 and AMC6. They are closer in terms of choice of preferred mode(s) of action Cluster II is represented by ciprofloxacin.

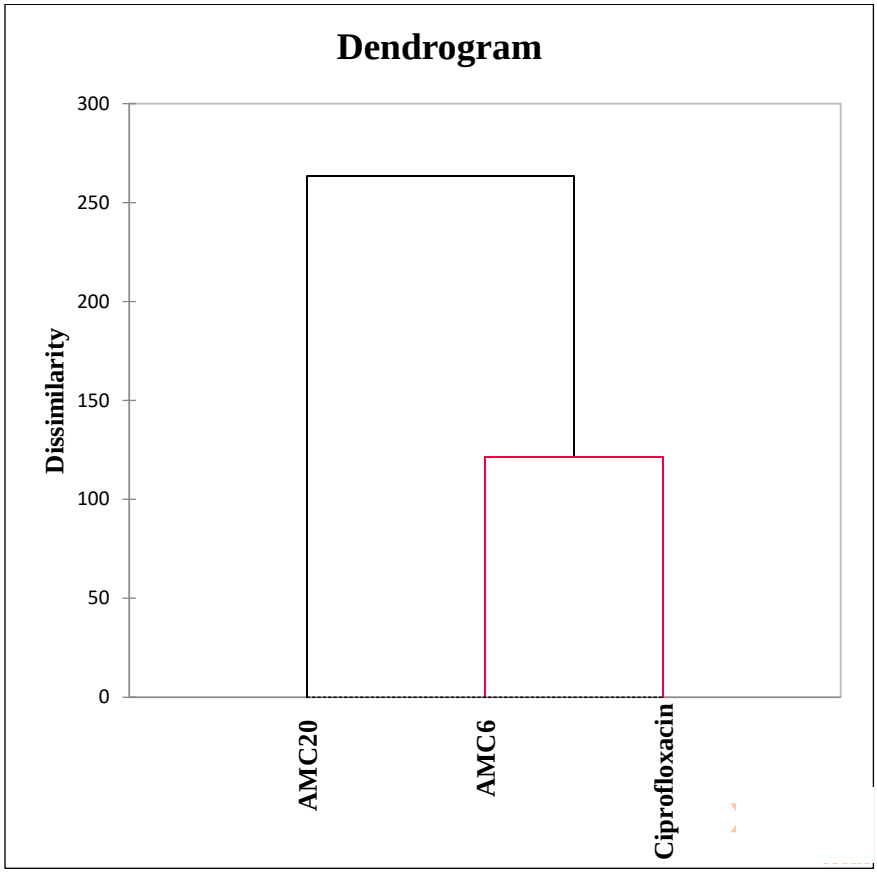


Figure 24: Hierarchical cluster analysis (HCA) of modes of action of compounds on *S. aureus* ATCC 43300

III.1.3.2 Structure elucidation of the two active compounds

III.1.3.2.1 Compound AMC6

The compound AMC6 was obtained as a white powder and soluble in DMSO. ^{13}C NMR (150 MHz, DMSO- d_6) δ_C 174.5, 143.9, 131.8, 126.4, 104.9, 72.0, 49.1, 34.3, 19.2, 18.6. The fully decoupled ^{13}C spectrum presents us with 10 signals corresponding to 10 carbon atoms (Figure 25). We observe:

- A signal at δ_C 174.5 corresponding to the carbonyl of lactone,

- Four aromatic signals at δ_C 143.9, 131.8, 126.4 and 104.9,
- A signal at δ_C 72.0 corresponds to an oxymethine,
- Two signals at δ_C 19.2 and 18.6 corresponding to the methyl groups.

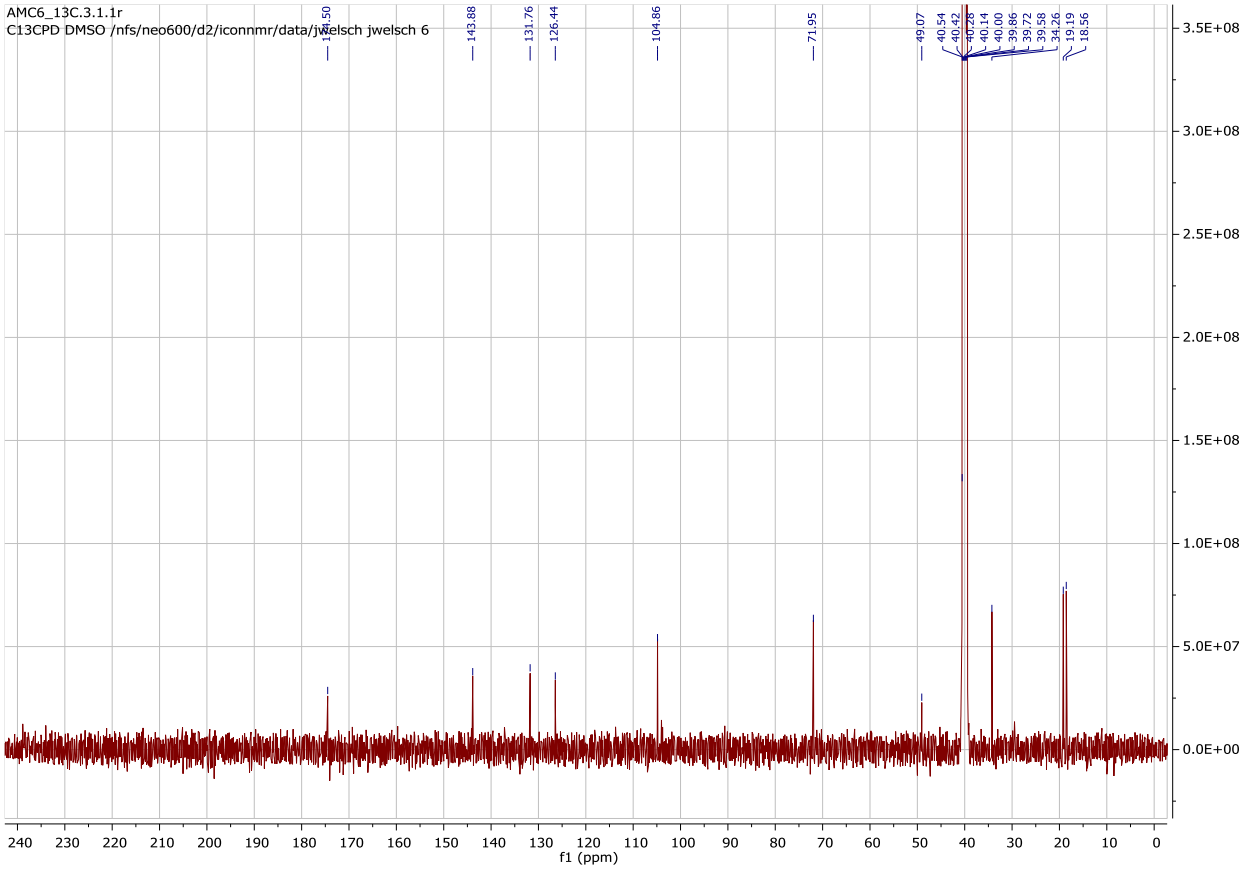


Figure 25: 13C NMR spectrum (DMSO-d6, 150 MHZ) of AMC6 compound

On the COSY spectrum (Figure 27), we count at least 12 families of protons among which :

^{1}H NMR (600 MHz, DMSO-d_6**)** δ_H 12.20, 6.29, 5.17, 4.25, 1.50, 1.48, 4.25, 0.80, 2.28, 2.19, 0.86, 2.02, 1.91.

We observe on this COSY spectrum scalar correlations of protons between (Figure 26):

- The signal at δ_H 12.2 corresponds to hydroxylate proton or nitrogenous proton with the signal δ_H 6.29,

- The signal at δ_H 4.25 with the signal at δ_H 1.91,

- The signal at δ_H 0.86 with the signal at δ_H 1.91,

- The signal at δ_H 4.25 with the signal at δ_H 5.17,

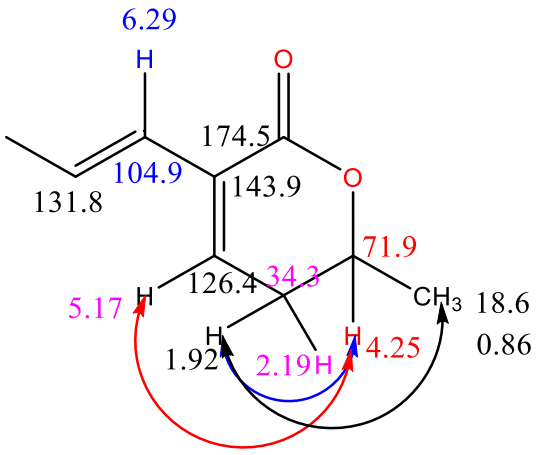


Figure 26: Correlated Spectroscopy (COSY) correlation between protons of AMC6 compound

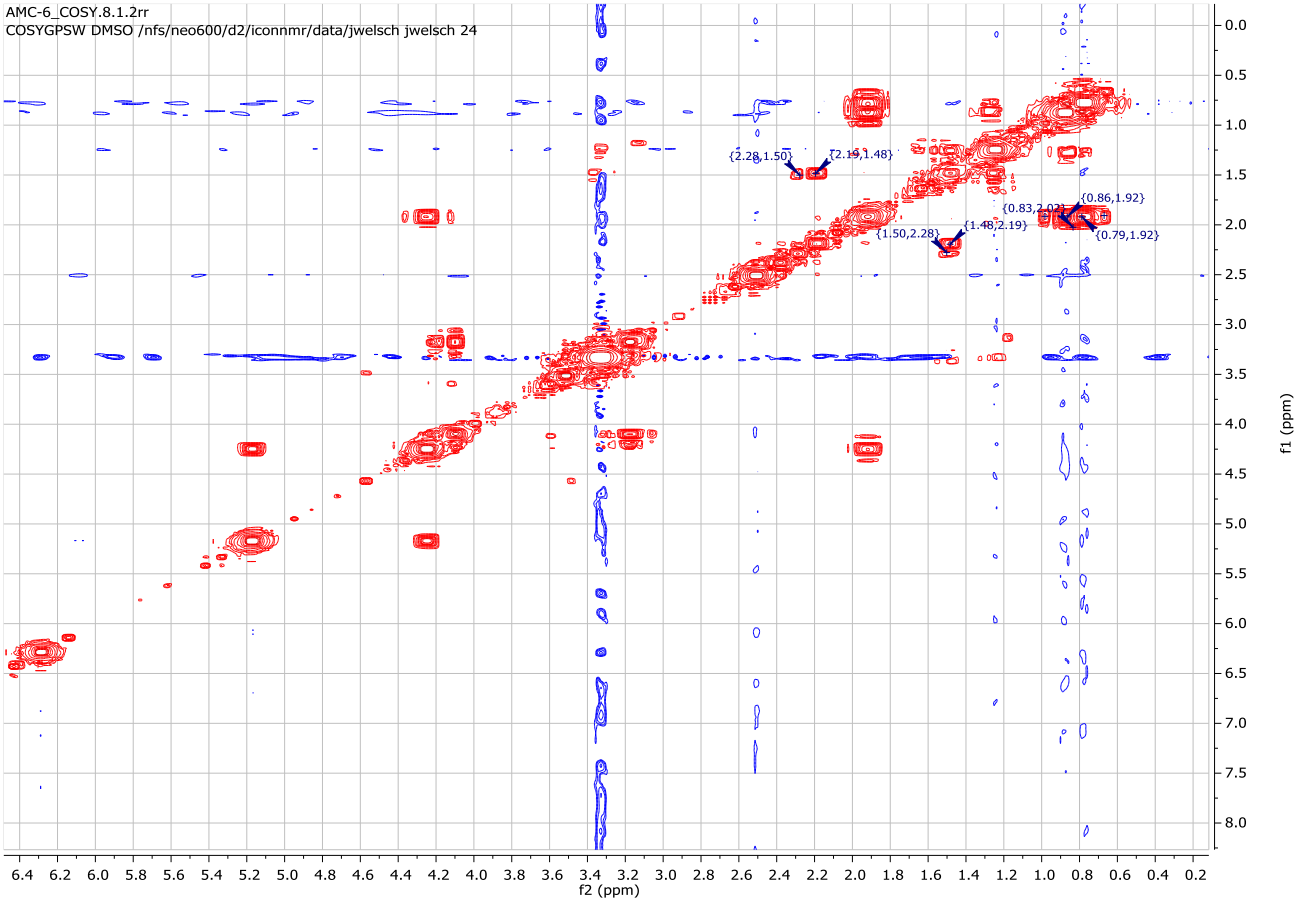


Figure 27: COSY spectrum of AMC6 compound

On its HMBC spectrum, we observe the correlations of protons to carbons ranging from $^{2}J_{C\text{-}H}$ to $^{4}J_{C\text{-}H.}$ (figure 29). We also observe the correlations of olefinic proton at δ_H 6.29 (carried

by the carbon at δ_C 104.9 which is justified by an element of symmetry of two spots) to the carbonyl of lactone at δ_C 174.5 and aromatic carbons at δ_C 143.9, 131.8, 126.4 and 104.9 respectively (figure 28).

- The correlation of proton at δ_H 2.19 to the carbonyl of lactone at δ_C 174.5

- The correlation of proton at δ_H 5.17 to the carbons at δ_C 34.3 and 71.9

- The Correlations of methyl group protons at δ_H 0.86 to oxymethine carbon at δ_C 71.9 and carbon at 34.3.

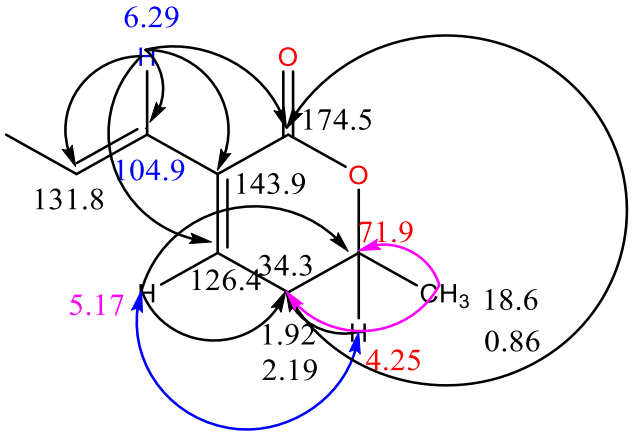


Figure 28: Heteronuclear Multiple Bond Correlation (HMBC) correlation between carbons and protons of AMC6 compound

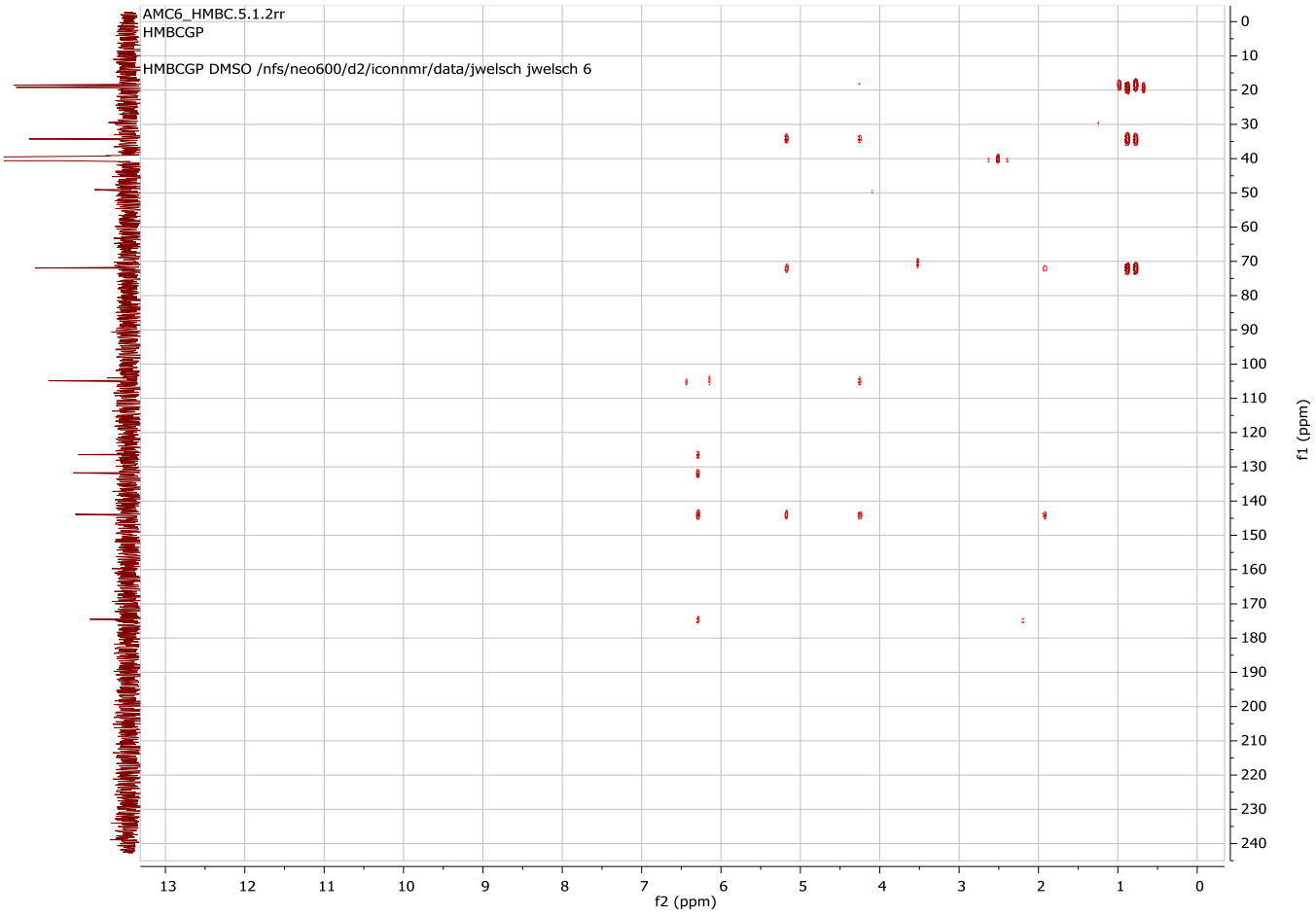
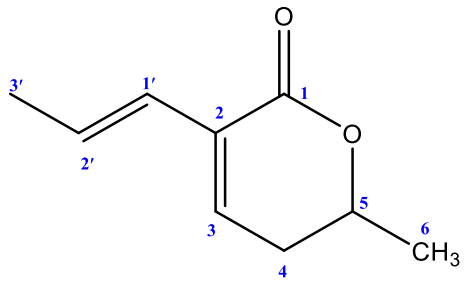


Figure 29: HMBC spectrum of AMC6 compound

Based on the above results, the following structure was deduced (Figure 30):



(*E*)-6-methyl-3-(prop-1-en-1-yl)-5,6-dihydro-2*H*-pyran-2-one

Figure 30: Structure of AMC6 compound

III.1.3.2.2 Compound AMC20

The compound AMC20 was obtained as a white powder and soluble in chloroform. Indeed, its carbon ^{13}NMR spectrum (Figure 31) shows the general appearance of a triterpenoid spectrum with 30 signals attributable to the 30 carbons of the molecule. Among these signals, the one at δ_C 6.8 is characteristic of triterpenoids of the friedelin series and attributable to the angular methyl carbon at position 23 (Mahato & Sen, 1997).

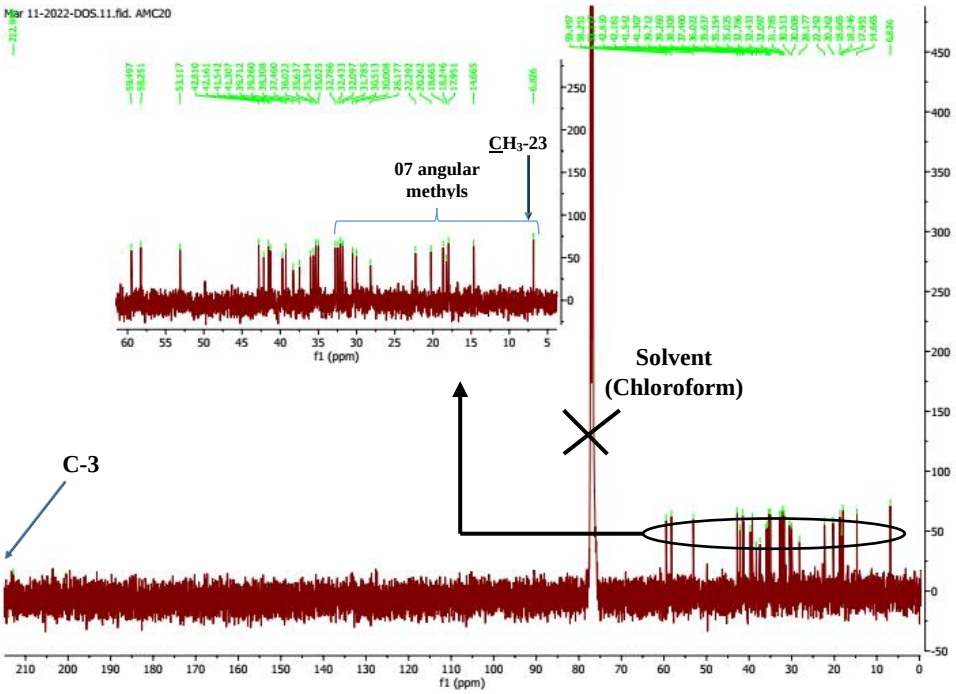


Figure 31: 13C NMR ($CDCl_3$, 150 MHz) of AMC20 compound

For its ^{1}H NMR spectrum (Figure 32), it presents a set of signals attributable to methyls (CH3-), methylenes (-CH2-), methines (CH) and quaternary carbons (C). Interpretation of all its spectroscopic data followed by comparison with literature (Table XX) allowed us to identify it to friedelin (Sousa *et al.*, 2012).

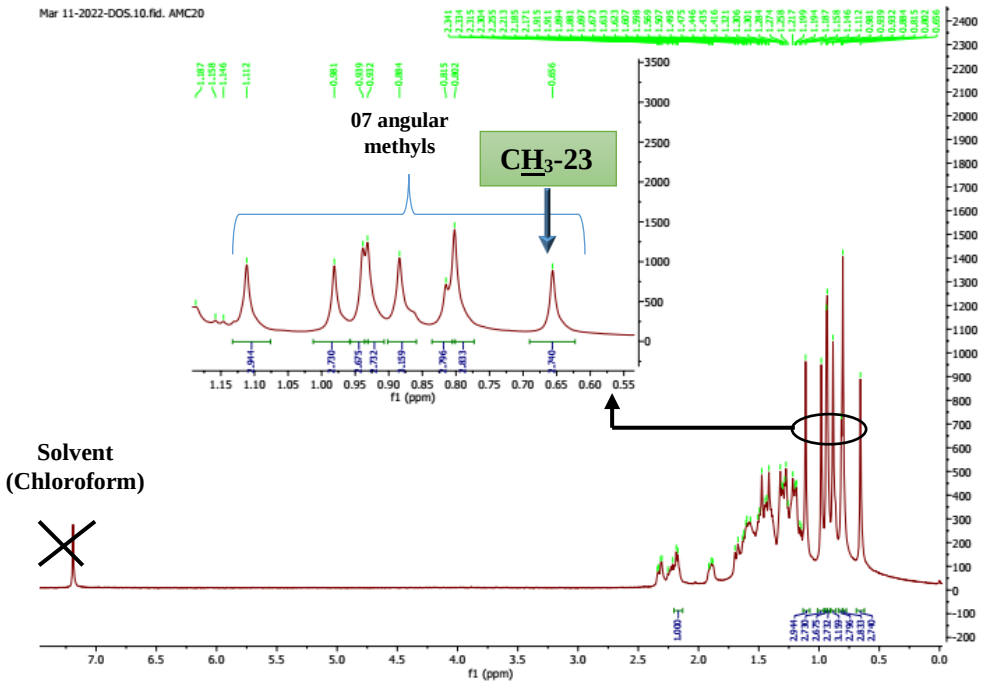


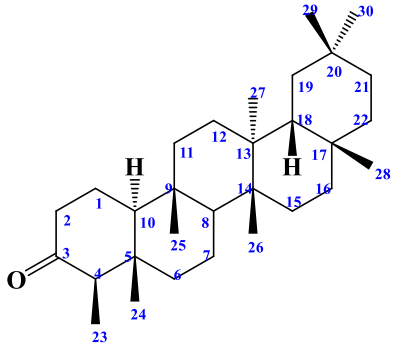
Figure 32: 1H NMR (CDCl3, 500 MHz) of AMC20 compound

Table XX: 13C NMR, 1H NMR data ($CDCl_3$, 125 MHz and 500 MHz) of AMC20 compared to Friedelin ($CDCl_3$, 100 MHz and 400 MHz)

	AMC20			**Friedelin (Sousa *et al.*, 2012)**		
N°	**δ ^{13}C**	**(*mt*)**	**δ ^{1}H**	**δ ^{13}C**	**(*mt*)**	**δ ^{1}H**
1	22,3	(CH_2)	1,62 (*m*)	22,3	(CH_2)	1,95 ; 1,71 (*ddd*)
2	41,5	(CH_2)	2,33 ; 2,23 (*m*)	41,5	(CH_2)	2,37 ; 2,27 (*ddd*)
3	*Nd*	(C)	-	213,2	(C)	-
4	58,3	(CH)	2,18 (*m*)	58,2	(CH)	2,25 (*q*)
5	42,2	(C)	-	42,1	(C)	-
6	41,3	(CH_2)	1,68 ; 1,22 (*m*)	41,3	(CH_2)	1,74 ; 1,28 (*d*)
7	18,2	(CH_2)	1,42 ; 1,32 (*m*)	18,2	(CH_2)	1,49 ; 1,36 (*m*)
8	53,1	(CH)	1,33 (*m*)	53,1	(CH)	1,38 (*dd*)

9	37,5	(C)	-	37,4	(C)	-
10	59,5	(CH)	1,46 (*m*)	59,5	(CH)	1,53 (*m*)
11	35,6	(CH_2)	1,39 ; 1,30 (*m*)	35,7	(CH_2)	1,45 ; 1,26 (*m*)
12	30,5	(CH_2)	1,30 (*m*)	30,5	(CH_2)	1,33 ; 1,32 (*m*)
13	39,7	(C)	-	39,7	(C)	-
14	38,3	(C)	-	38,3	(C)	-
15	32,8	(CH_2)	1,23 (*m*)	32,4	(CH_2)	1,47 ; 1,27 (*m*)
16	36,0	(CH_2)	1,50 (*m*)	36,0	(CH_2)	1,58 ; 1,35 (*m*)
17	30,0	(C)	-	30,0	(C)	-
18	42,8	(CH)	1,49 (*m*)	42,8	(CH)	1,56 (*m*)
19	35,4	(CH_2)	1,14 (*m*)	35,3	(CH_2)	1,37 ; 1,22 (*m*)
20	28,2	(C)	-	28,2	(C)	-
21	32,4	(CH_2)	1,45 (*m*)	32,8	(CH_2)	1,50 ; 1,31 (*m*)
22	39,3	(CH_2)	1,43 ; 0,87 (*m*)	39,2	(CH_2)	1,51 ; 0,95 (*m*)
23	6,8	(CH_3)	0,81 (*sl*)	7,0	(CH_3)	0,88 (*d*)
24	14,7	(CH_3)	0,66 (*s*)	14,6	(CH_3)	0,73 (*s*)
25	17,9	(CH_3)	0,80 (*s*)	17,9	(CH_3)	0,87 (*s*)
26	20,3	(CH_3)	0,94 (*s*)	20,2	(CH_3)	1,01 (s)
27	18,7	(CH_3)	0,98 (*s*)	18,6	(CH_3)	1,05 (*s*)
28	32,1	(CH_3)	1,11 (*s*)	32,1	(CH_3)	1,18 (*s*)
29	35,0	(CH_3)	0,90 (*s*)	35,0	(CH_3)	1,00 (*s*)
30	31,8	(CH_3)	0,93 (*s*)	31,8	(CH_3)	0,94 (*s*)

The interpretation of ^{1}H and ^{13}C NMR spectrum in comparison to the literature data allowed us to give the structure on the figure 33 below commonly known as Friedelin.



(4*R*,4a*S*,6b*R*,8a*R*,12a*R*,12b*S*,14a*S*,14b*S*)-4,4a,6b,8a,11,11,12b,14a-octamethylicosahydropicen-3(2*H*)-one

Figure 33: structure of AMC20 compound (friedelin)

III.2 DISCUSSION

The current study was designed to investigate the antibacterial and modes of action metabolites from endophytic fungi associated with *Annona muricata*, a plant traditionally used in Cameroon to treat microbial infections.

Crude ethyl acetate extracts from forty-one (41) endophytic fungi isolated from distinct organs of *A.muricata* were screened for their ability to inhibit *Escherichia coli* ATCC 25922, *Klebsiella oxytoca*, and *Staphylococcus aureus* ATCC 43300. Of the 41 extracts tested, 17 (41.46%) exhibited activity against at least one bacterium. Our previous screening of 56 extracts from endophytic fungi isolated from the three medicinal plants from Cameroon, *Terminalia mantaly, Terminalia catappa*, and *Cananga odorata*, against seven bacterial strains revealed that approximately 13% were very active against all tested bacterial strains (Mbekou *et al*., 2021). Another previous screening of 152 extracts from endophytic fungi from *A.muricata* against Plasmodium parasites revealed that over 17% of isolates exhibited activity (Toghueo *et al*., 2019). These results indicate that many endophytes inhabiting Cameroonian plants could produce active metabolites to inhibit various human pathogenic microbes.

The seventeen active endophytes identified by sequencing their ITS1-5.8S rRNA-ITS2 region belonged to the *Aspergillus*, *Curvularia*, *Fusarium*, *Meyerozyma*, *Penicillium*, and *Talaromyces* genera. Among them, 9 (52.94%) were identified at the genus level, while only eight (47.05%) were identified at the species level. Given the inability of the ITS sequence to give precise identification of our endophytic isolates, further analysis, such as the sequencing of additional genes followed by multilocus phylogenetic analysis, will be conducted to achieve the precise identification of these isolates (Helaly *et al*., 2018; Toghueo, 2020; Yoo & Eom, 2012; Toghueo *et al*., 2023). Nevertheless, these results obtained are in agreement with those of several authors who have also isolated fungi of the genus *Aspergillus*, *Fusarium* and *Penicillium* (Abdel-Rahman *et al*., 2019; Hasan *et al*., 2022; Silva *et al*., 2024) from different parts of the same plant. However, in the best of our knowledge, there is no reports in the literature on the isolation of endophytic fungi of *Curvularia, Meyerozyma* and *Talaromyces* genus from *Annona muricata*. Thus, the great diversity of fungal genera identified from the present study indicates that *A. muricata* hosts a large group of microorganisms capable of producing antibacterial metabolites

The extracts from the seventeen identified isolates were submitted to a dose–response study for MIC determination. All extracts exhibited activity against all tested bacteria, with MIC

values ranging from 3.125 to 100 µg/mL depending on the extracts and microorganisms. The most active extracts against the three bacterial pathogens were consistently from the Fusarium and Penicillium genera, with *F.waltergamsii* AMtw3 being the most active, followed by *P.citrinum* AMf6. These findings are in line with previous studies reporting the potential of endophytic fungi belonging to these genera as sources of active antimicrobial metabolites (Toghueo, 2020; Toghueo & Boyom, 2020). Fortunately, these active endophyte extracts were non cytotoxic against Vero cells at concentrations as high as 100 µg/mL using the MTT colorimetric method. These results suggest that the antibacterial activity of extracts reported in the present study could not be related to the toxicity effect; therefore, antibacterial compounds produced by these endophytes could have good selectivity against mammalian cells. Likewise, several authors have also obtained good antibacterial activity with non-cytotoxic extracts from endopytic fungi on mammalian cells. Indeed, (Mbekou et al., 2021) reported that extracts from 3 endophytic fungi isolated from *Terminalia catappa a*nd *Cananga odorata* with MICs less than 10 µg/mL on seven bacteria causing pneumonia were non cytotoxic on Vero cells. Similarly (Ariffin et al., 2011) described that two extracts from endophytic fungi isolated from *Pandanus amaryllifolius* and seaweed species have demonstrated promising activity on six bacteria without cytotoxicity on normal fibroblast (D551) cell line. On the other hand, crude ethyl acetate extracts from endophytes belonging to these genera and inhabiting other Cameroonian medicinal plants were previously reported for their weak cytotoxicity against normal cell lines such as HEK239T mammalian cells (Ateba *et al*., 2018; Toghueo *et al*., 2018, 2019). However, this non cytotoxicity of crude metabolites produced by these endophytes could also be related to the culture conditions. We previously found that extracts from *Fusarium* sp. N240 and *Xylaria* sp. N120 cultured in potato dextrose broth were cytotoxic against HEK239T cells but were non toxic when the fungi were grown in Czapek Dox medium (Toghueo *et al*., 2018).

The potential of extracts to prevent oxidative stress resulting from excess non neutralized reactive species in the body was also investigated through DPPH radical scavenging and FRAP reducing power assays. Antioxidants are tremendously important substances that can protect the body from damage caused by free radicals. The scavenging activity of the fungal extracts was measured by discoloration to yellow following the formation of a non radical (2,2-diphenyl-1-hydrazine) molecule (Ibrahim *et al.*, 2021). Fungal extracts AMEtf15, AMEf6, AMEtw3, AMEr9, AMEsb1, AMEsb23 and AMEf4 exhibited good DPPH radical scavenging activity

(RSA_{50} 146.05 to 799.75 µg/mL). The metal chelating capacity may also indicate its potential antioxidant activity (Mandel *et al*., 2006). The iron-chelating activity of all fungal extracts was determined by reaction with orthophenanthroline. The results showed that five extracts at the highest concentration tested, including AMEtf15, AMEf6, AMEs3, AMEf4, and AMEf1, exhibited iron-chelating activity (12.03, 12.28, 11.97, 12.35 and 12.37 µg equivalent NH_2OH/g, respectively). Overall, two extracts from *Aspergillus* sp. AMEtf15 and *P. citrinum* AMf6 exhibited DPPH radical scavenging and ferric ion reducing capacities. The antioxidant activities of these fungal extracts observed in the present study could be linked to their phytochemical content. Our previous compositional analysis of ethyl acetate extracts from endophytic fungi from *Annona muricata*, *Aspergillus* sp. AMEb7 and *P. citrinum* AMErb23 revealed the presence of 44 and 38 different secondary metabolites, with isolongifolene (30.18%) and octadecyl-3,5-di-tert-butyl-4-hydroxydrocinnamate (19.62%) being the most abundant in AMEb7 and AMErb23, respectively (Toghueo *et al*., 2019). In fact, secondary metabolites, such as flavonoids, phenolic acids, terpenoids, tannins, saponins, and alkaloids, exhibit significant antioxidant properties that can play a crucial role in managing urinary tract infections (UTIs). Their antioxidant mechanisms involve several key actions. For instance, flavonoids scavenge reactive oxygen species (ROS) and reactive nitrogen species (RNS), preventing cellular damage and inflammation. (Panche *et al*., 2016). Phenolic compounds, mitigate oxidative stress by enhancing endogenous antioxidant defense systems and this helps to protect the urinary tract lining from oxidative damage (Kumar & Goel, 2019). Tannins, have the ability to chelate metal ions, reducing their availability for catalytic reactions that generate free radicals (Zhang *et al*., 2023). These antioxidant mechanisms collectively contribute to reducing oxidative damage in UTIs, potentially leading to improved management and outcomes of the infection (Allameh & Salamzadeh, 2016) Overall, endophytic fungi from *Annona muricata* can produce a significant number of metabolites with the potential to act as both antimicrobials and antioxidants.

These promising results prompted us to have a special interest in the search for compounds responsible for the activities observed in the most active and selective extract, *Fusarium* sp. AMtw3. Therefore, a silica gel chromatography separation of extract AMtw3 was performed to obtain 9 fractions (AMF1-AMF9) and the chromatography of the fractions led to 15 pure sub-fractions and 2 compounds. Globally, all fractions were active on at least two bacteria tested with MIC ranging from 0.78 to >100 µg/mL. Among them, fraction AMF2

showed the best inhibitory potential with an improvement of the activity by 2-4 folds when compared to the crude extract. Out of all fractions tested, antibacterial activity was improved by most of them. The difference observed in the antibacterial activities between the different fractions might be related to the type and quantity of bioactive secondary metabolites (Muhammad *et al*., 2021). Meanwhile, the MIC values of compounds and pure sub-fractions ranged from 0.195 to >100 µg/mL with an overall improvement of the activity by 2-128 folds against bacteria regardless of the pathogenic strains tested. Compound AMC6 was the most active (MIC: 0.39, 01.39, 0.78 µg/mL) followed by AMC20 (0.78, 0.39, 12.5 µg/mL) respectively on *S. aureus* ATCC 43300, *K. oxytoca and E. coli* ATCC 25922. Conversely, sub-fractions such as AMSF10, AMSF2, AMSF13 and AMSF7 did not improve activity on *S. aureus* ATCC 43300. Regarding the DPPH scavenging activity, fractions AMF2 and AMF7 significantly improved the activity of their parent extract, but AMF4, AMF7 and AMF8 did not ameliorate this capacity. AMSF17, AMSF8 and AMSF1 showed moderate scavenging activity thus displaying an amelioration of the activity. On the contrary, the fractionation did not significantly enhance the iron ferric reducing power of both fractions and compounds. These changes observed in different activities could be due to the absence of interaction such as synergism and antagonism that might exist between metabolites before the purification process (Vaou *et al*., 2021).

Based on the antibacterial profile, the most two potent compounds were analyzed and the structures elucidated. Thus, these two compounds named AMC6 and AMC20 were respectively determined to be 6-methyl-3-(prop-1-en-1-yl)-5,6-dhihydro-2H-pyran-2-one and friedelin and they belong to lactones and triterpenes groups respectively (Djoukeng *et al*., 2005). Compounds can exert their antimicrobial effects on bacteria via one or various mechanisms of action. Therefore, several model assays were used to investigate the mode of antibacterial action of the two identified compounds (AMC6 and AM20) against *S. aureus* ATCC43300 and *K. oxytoca*. The two compounds inhibited the production of catalase by both pathogens. The ability of bacteria to produce catalase contributes to their pathogenicity by detoxifying the oxygen-dependent microbicidal products of phagocytic cells. The large concentration of H_2O_2 may overwhelm an organism's defenses and may prove fatal to microorganisms (Dwyer *et al*., 2014). Therefore, the loss of the ability to produce catalase by *S. aureus* and *K. oxytoca* indicates their inability to deactivate antimicrobials by catalase production (Daniels *et al*., 2020). These two

compounds also demonstrated their ability to affect both the inner and outer membranes of bacteria. The results from the bacteriolysis assay indicate that compounds cause gross membrane damage and provoke whole-cell lysis. This property was previously reported by Mbekou *et al*., (2021) with endophytic fungal extracts. The outer membrane permeability assay results showed a gradual decrease in optical densities with the increasing compounds concentration, which indicates membrane destabilization. Lethal injury of microbial cell membranes may alter their permeability and affect the membrane's ability to osmoregulate the cell adequately or exclude toxic materials (Gilbert, 1984). This antibacterial action can result in membrane expansion, increased membrane fluidity and permeability, disturbance of membrane embedded proteins, inhibition of respiration, and alterations in the ionic homeostasis between intracellular and extracellular compartments of bacteria, eventually leading to cell death (Trombetta *et al*., 2005). In fact, terpenoidal compounds are used in the treatment of bacterial infections due to their lipophilic properties which allow them to be easily interacting with the bacterial wall, interfering with the biosynthesis of its components as well as they can penetrate the bacterial cell (Djoukeng *et al*., 2005). In addition, the carboxy group in friedelin attached to carbon atom 3 increased lipophilicity and polarity. These properties are associated with solubility, permeability through cell membrane and ability to interact with the hydrophobic region contributing to the inhibitory activity of the bacteria (Cunha *et al*., 2007; de León *et al*., 2010). More specifically, oxygenated terpene merges to the polar head groups that make up the lipid fraction of the bacterial plasma membrane. They act by inducing alterations of the cell membrane affecting its permeability and therefore resulting in alterations of intracellular material. (Miksusanti *et al*., 2008). Further investigation on the action of compounds on the integrity of the cell membrane of the bacteria by measuring the release of 260 nm absorbing material (nucleic acids and derivatives) was carried out. The results of this study revealed low levels of absorption at 260 nm compared to the control indicating that the membrane integrity is partially affected. In fact, marked leakage of cytoplasmic material is considered indicative of gross and irreversible damage to the cytoplasmic membrane (Chovanová *et al*., 2013). So, the leakage of nucleic acids and proteins could cause the disorder of function in the synthesis of proteins and DNA materials and the inhibition of bacterial growth (J. Zhang *et al*., 2017). Furthermore, a study reported that lactones have the capacity to cause cytoplasmic membrane damage leading to biochemical decomposition of genetic material and therefore modifying the bacterial DNA. These changes resulted in to cell

death eventually and lead to reduction in the number of viable cells (Kowalczyk *et al*., 2021). The triterpenes have been reported to exert antimicrobial activities against both the antibiotic-susceptible and antibiotic-resistant bacteria, mainly via their abilities to promote cell rupture inhibition of protein and DNA synthesis, disintegration of the outer membrane and this due to the presence of the oxygenated and ester functional groups (Álvarez-Martínez *et al*., 2021; Guimarães *et al*., 2019; Włoch *et al*., 2020).All compounds also exert a bactericidal effect on both pathogens as materialized by reducing the bacterial population, as shown by the graph of optical densities versus incubation time. Bactericidal agents are sought after to fight against resistant bacteria because they attack and kill bacteria outright, preventing these cells from causing further damage within the body (Eng *et al*., 1991). Among bacterial therapeutic targets, biofilms remain the main virulence factor contributing to the pathogenesis and resistance of microbial pathogens (Flores-Mireles *et al*., 2015). Therefore, finding antibiotics with the ability to inhibit both planktonic cells and bacterial biofilm structure is of paramount importance. Our investigation revealed that compounds AMC6 and AMC20 strongly inhibited *S. aureus* ATCC43300 and *K. oxytoca* biofilm formation ($MBIC_{50:}$ 0.07 to 0.19 µg/mL). The inhibition of preformed biofilm by compounds from natural products has also been reported by (Ampofo *et al*., 2020). In addition to direct biofilm inhibition activity, compounds that are active against biofilm-forming organisms would also be of considerable advantage in treatment of UTIs. Therefore, the two compounds tested demonstrated a high ability to prevent biofilm formation. Terpenoids were reported to be ideal anti-adhesive compounds involved in the detachment of planktonic cells from the biofilm (Perumal & Mahmud, 2013). Equally protein levels during initial biofilm development were found to decrease fivefold after treatment with carvacrol, a compound in the terpenoid group (Knowles *et al*., 2005). In addition, their free oxo group is believed to further allow their ease diffusion through the polar polysaccharide biofilm matrix, and as such the efficient killing of the enclosed bacteria (Kostoglou *et al*., 2020). Further, friedelin being a hydrophobic compound, believed to influence the fatty acid composition of the cell membrane, and thus cell hydrophobicity which led to the eradication of the biofilm (Mahizan *et al*., 2019). Besides, studies have shown that lactones affect biofilm formation at low concentrations (Sordi *et al*., 2018). Thus, the two compounds have significant effects on both biofilm formation and the survival of the organisms in the established biofilms. These results

support the use of these compounds as alternative agents against biofilm production during UTIs infections.

With the purpose of revealing the preferred mode of action of each compound, two chemometric techniques consisting of principal component analysis (PCA) and hierarchical cluster analysis (HCA) were carried out. PCA biplot clearly demonstrated that compound AMC6 is more effective to inhibit biofilm and catalase on *K. oxytoca* while on *S. aureus* ATCC 43300, catalase and the reduction of bacteria population through time kill kinetic are its preferred modes of action. It was also observed that compound AMC20 tended to have higher cells lytic activity, induce the outer membrane permeability and exert bactericidal effect on *K. oxytoca. While on S. aureus* ATCC 43300 its effect was mainly focus on bacteriolysis. HCA showed strong similarities in term of the modes of action between the two compounds on *k. oxytoca.* In fact, the illustration of the hierarchy enables the identification of similarities and differences within and across compounds (Annemer *et al*., 2022).

Conclusion, Perspectives and Recommendations

CONCLUSION, PERSPECTIVES AND RECOMMENDATIONS

Conclusion

This work investigated the antibacterial and modes of action of metabolites of endophytic fungi from *Annona muricata* from Cameroon against the causative agents of urinary tract infections, the following conclusions were drawn:

1) Out of the forty-one extracts screened against three selected bacteria, seventeen were able to inhibit at least one bacterium with MICs values ranging from 3.125 to 100 µg/mL. The molecular identification of the selected endophytes producing active extracts revealed that they belonged to six genera known as *Aspergillus*, *Curvularia*, *Fusarium*, *Meyerozyma*, *Penicillium*, and *Talaromyces.*

2) Fractionation of *Fusarium sp*. AMtw3 acetate crude extract was conducted to nine fractions (MIC: 0.78 to >100 µg/mL), fifteen pure sub-fractions and two compounds (MIC: 0.39 to >100 µg/mL) with AMC6 being the most active compound (0.78. 0.39 and 0.39 µg/mL) respectively against *E. coli* ATCC 25922, *S. aureus* ATCC 43300 and *K. oxytoca*. Fractionation of crude extract to fraction and fraction to its derived products has improved the antibacterial activity respectively by 2-4 folds and 2-128 folds.

3) Among the seventeen compounds isolated, only the two most potent based on their antibacterial activity could be analyzed with spectroscopic methods and identified respectively as 6-methyl-3-(prop-1-en-1-yl)-5,6-dhihydro-2H-pyran-2-one (AMC6) and friedelin (AMC20). PCA and HCA performed on modes of action reveal that on *K. oxytoca* bacterium, compound AMC6 focuses its action on biofilm and catalase inhibition and AMC20 for its part induces the outer membrane permeability, lyse cells and exert bactericidal on the same bacteria. Bacteriolysis appears as the main mode of action of AMC20 on *S. aureus* ATCC 43300 and catalase inhibition and time kill kinetic for AMC6 on the same bacterium. Overall, both compounds are most close in term of exert modes of action on *k. oxytoca*

In summary, the results obtained in this study suggest that friedelin and 6-methyl-3-(prop-1-en-1-yl)-5,6-dhihydro-2H-pyran-2-one could serve as starting point for drug development against UTIs.

Perspectives

The present study being only a prelude in the identification of new UTIs molecules, we plan as future prospects to:

- Formulate a traditionally improved medicine with the extract of interest Fusarium *sp.* AMEtw3

- Perform spectroscopic analyses of promising pure sub-fractions

- Dose catalase and urease, which are biochemical markers

- Isolate and characterize resistance genes and identify mutations. on *K. oxytoca* isolate

- Investigate Urinary Tract Infection-specific resistance strains

- Determine the pharmacokinetic properties *in vitro* of the two promising compounds

Recommendations

At the end of this work, the following recommendations can be made:

To the general population:

-Promote good personal hygiene practices to prevent urinary tract infections, such as drinking enough water, urinating regularly and maintaining good intimate hygiene.

- Encourage people with symptoms of urinary tract infection to consult healthcare professionals for appropriate diagnosis and treatment.

- Encourage the careful and informed use of medicinal plants, more specifically *Annona muricata*, in particular by avoiding unnecessary self-medication.

To the government through the Ministries of Public Health, Scientific Research and Higher Education

-Encourage public-private collaboration to promote the development of new treatments based on local biodiversity and discoveries.

-Implement training programs for local health practitioners on the safe use of traditional remedies and their integration into modern medical practices.

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Appendices

APPENDICES

Appendix 1: Composition of Culture Media

Potatoes Dextrose Agar (PDA)

Constituents	**Concentration en (g/L)**
Potato peptone	4.0
Glucose	20.0
Agar	15.0
Final pH 5.6 ± 0.2 at 25°C	

Potatoes Dextrose Broth (PDB)

Constituents	**Concentration en (g/L)**
Potato peptone	4.0
Glucose	20.0
Final pH 5.6 ± 0.2 at 25°C	

Nutrient Agar (NA)

Constituents	**Concentration en (g/L)**
Beef extract	1.0
Yeast extract	2.0
Peptone	5.0
Sodium Chloride	5.0
Agar	15.0
Final pH 6.8 ± 0.2 at 25°C	

Nutrient Broth (NB)

Constituents	Concentration en (g/L)
Beef extract	1.0
Yeast extract	2.0
Peptone	5.0
Sodium Chloride	5.0
Final pH 6.8 ±0.2 at 25°C	

Dulbecco's Modified Eagle Medium (DMEM)

Constituents	Concentration en (g/L)
Calcium chloride dihydrate	0.2650
Non-hydrated ferric nitrate	0.00001
Magnesium sulfate anhydrous	0.09772
Potassium chloride	0.40000
Sodium chloride	6.4000
Glycine	0.0300
L-Arginine hydrochloride	0.0840
L-cystine dihydrochloride	0.06257
L-Glutamine	0.58400
L-Histidine hydrochloride monohydrate	0.04200
L-Isoleucine	0.1050
L-leucine	0.1050

L-Lysine hydrochloride	0.1460
L-Méthionine	0.0300
L-phénylalanine	0.0660
L-Serine	0.04200
L-Thréonine	0.0950
L-Tyrosine disodium salt	0.10379
L-Valine	0.0940
Choline chloride	0.0040
D-Ca-pantothenate	0.0040
Folic acid	0.0040
Nicotinamide	0.0040
Pyridoxal hydrochloride	0.0040
Riboflavin	0.0004
Thiamine hydrochloride	0.0004
i-Inositol	0.0072
D-Glucose	4.5000
Sodium salt of phenol red	0.0159
L-Tryptophan	0.0160

Appendix 2: Screening results of extracts from endophytic fungi from various organs of *Annona muricata*

Plant organs	**Extract codes**	***Escherichia coli* ATCC 25922**	***Klebsiella oxytoca* isolate**	***Staphylococcus aureus* ATCC 43300**

Roots	AMr5	-	-	-
	AMr7	-	-	-
	AMr8	-	-	-
	AMr9	+	+	+
	AMr10	-	-	+
Root bark	AMrb1	+	+	+
	AMrb9	+	+	+
	$AMrb10_1$	-	-	-
	AMrb11	+	+	+
	AMrb13	-	-	-
	AMrb25	-	-	-
Fruits	AMf1	+	+	+
	AMf2	-	-	-
	AMf3	+	+	+
	AMf4	+	+	+
	AMf5	-	-	-
	AMf6	+	+	+
Seeds	AMs1	-	-	-
	AMs3	+	+	+
	AMs9	+	+	+
	AMs10	-	-	-
Thorns of fruit	AMtf5	+	+	+

	AMtf6	-	-	-
	AMtf9	-	-	-
	AMtf12	-	-	-
	AMtf15	+	+	+
	AMtf16	-		
Twigs	AMtw3	+	+	+
	AMtw5	-	-	-
	AMtw7	-	-	-
	AMtw8	-	-	-
	AMtw	-	-	-
Leaves	AML3	-	-	-
	AML10	-	-	-
Stem bark	AMsb1	+	+	+
	AMsb14	-	-	-
	AMsb16	-	-	-
	AMsb23	+	+	+
	AMsb25	-	-	-
Barks	AMb7	+	+	+
Peducles	AMpe9	-	-	-
	PDB	-	-	-

-: not active at 100 µg /mL; +: active at 100 µg /mL; AMr: *A. muricata* root. AMrb: *A. muricata* root bark. AMf: *A. muricata* fruit. AMs: *A. muricata* seed. AMtf: *A. muricata* thorn of fruit. AMtw: *A. muricata* twigs. AMl: *A. muricata* leaves. AMb: *A. muricata* bark. AMsb: *A. muricata* stem bark. AMpe: *A. muricata* peducle. PDB: Potato Dextrose Broth.

Appendix 3: Means of ratio of the OD at each time interval versus the OD at 0 min (in %) of compounds on *K. oxytoca* and *S. aureus* ATCC 43300 during Bacteriolysis Activity

Bacteria	**Codes of compound**	**Concentrations**	**Means of ratio of the OD at each time interval versus the OD at 0 min (in %)**			
K. oxytoca			0	1	2	4
	AMC6	4MIC	100±0.000^{a}	45.737±1.056^{B}	32.082±0.38^{b}	23.544±0.771^{C}
		2MIC	100±0.000^{a}	57.996±0.852^{a}	37.801±0.44^{b}	27.527±0.739^{b}
		MIC	100±0.000^{a}	60.267±0.454^{a}	41.518±0.03^{b}	30.343±0.320^{b}
	AMC20	4MIC	100±0.000^{a}	57.408±0.748^{a}	43.418±1.29^{b}	31.106±0.574^{b}
		2MIC	100±0.000^{a}	65.554±2.440^{a}	56.072±1.810^{a}	35.062±0.022^{a}
		MIC	100±0.000^{a}	75.191±0.348^{a}	62.498±0.735^{a}	40.785±1.423^{a}
	Ciprofloxacin	MIC	100±0.000^{a}	39.584±1.337^{B}	32.627±0.63^{b}	25.016±0.302^{C}
	NC		100±0.000^{a}	100±0.000^{c}	100±0.001^{c}	100±0.000^{c}
***S. aureus* ATCC 43300**	AMC6	4MIC	100±0.000^{a}	58.992±1.525^{b}	39.817±2.42^{b}	27.668±0.619^{b}
		2MIC	100±0.000^{a}	64.274±2.756^{b}	44.362±0.765^{b}	31.731±1.593^{b}
		MIC	100±0.000^{a}	65.333±3.092^{b}	51.338±3.12^{a}	37.760±0.677^{a}

AMC20	4MIC	100±0.000[a]	70.848±0.947[b]	53.687±6.314[a]	33.807±1.088[b]
	2MIC	100±0.000[a]	78.619±1.63[b]	62.277±1.471[c]	47.958±0.680[a]
	MIC	100±0.000[a]	78.361±1.823[b]	69.272±4.921[c]	54.102±5.193[a]
Ciprofloxacin	MIC	100±0.000[a]	48.217±4.989[a]	39.687±0.48[b]	30.875±2.702[b]
NC		100±0.000[a]	100±0.000[c]	100±0.000[d]	100±0.000[c]

NC: Negative control, OD: Optical Density, MIC: Minimum Inhibitory Concentrations. Along the column and for each microorganism, values carrying the same letter superscripts are not significantly different ($p > 0.05$) and values carrying different letters are significantly different ($P < 0.05$).

Appendix 4: Means of growth parameter (OD/450 nm) of compounds on *K. oxytoca* and *S. aureus* ATCC 43300 during Outer Membrane Permeability assay

Bacteria	**Codes of compound**	**Means of growth parameter (OD/450 nm)**						
K. oxytoca	**Concentrations**	**4MIC**	**2MIC**	**MIC**	**1/2 MIC**	**1/4 MIC**	**1/8 MIC**	**1/16 MIC**
	AMC6	0.382±0.048 a	0.481±0.036 a	0.524±0.014 a	0.736±0.082 a	1.134±0.064 a	1.417±0.018 a	1.502±0.037 a
	AMC20	0.665±0.017 b	0.695±0.028 b	0.762±0.008 b	0.765±0.018 a	0.810±0.005 b	0.857±0.029 b	1.461±0.085 a
	Ciprofloxacin	0.461±0.045 a	0.534±0.003 a	0.544±0.027 a	0.565±0.010 b	0.553±0.023 c	1.344±0.041 a	1.470±0.009 a
***S. aureus* ATCC 43300**	AMC6	0.562±0.008 a	0.562±0.033 a	0.585±0.039 a	0.590±0.004 a	1.109±0.015 a	1.344±0.036 a	1.462±0.039 a
	AMC20	0.540±0.045 a	0.551±0.040 a	0.575±0.028 a	1.136±0.013 b	1.301±0.069 a	1.305±0.069 a	1.350±0.043 a
	Ciprofloxacin	0.359±0.025 b	0.454±0.031 a	0.543±0.039 a	0.557±0.054 a	0.627±0.031 b	1.163±0.026 b	1.217±0.002 a

NC: Negative control, OD: Optical Density, MIC: Minimum Inhibitory Concentrations. Along the column and for each microorganism, values carrying the same letter superscripts are not significantly different ($p > 0.05$) and values carrying different letters are significantly different ($P < 0.05$).

Appendix 5: Means of OD at each time interval versus on *K. oxytoca* and *S. aureus* ATCC 43300 during integrity cell membrane measurement at 260 nm

		Incubation times (h)				
Bacteria	**Compounds codes**	**0**	**30**	**60**	**120**	**240**
K. oxytoca	**AMC6**	0.648±0.023[a]	0.697±0.027[a]	0.756±0.033[a]	0.881±0.014[a]	0.946±0.043[a]
	AMC20	0.610±0.065[a]	0.648±0.030[a]	0.708±0.076[a]	0.74±0.026[b]	0.786±0.008[b]
	Ciprofloxacin	0.667±0.667[a]	0.692±0.692[a]	0.72±0.720[a]	0.894±0.894[a]	1.173±0.023[e]
	NC	0.678±0.025[a]	0.656±0.027[a]	0.636±0.033[b]	0.683±0.036[c]	0.698±0.026[d]
***S. aureus* ATCC 43300**	**AMC6**	0.646±0.008[a]	0.633±0.022[a]	0.64±0.025[b]	0.700±0.009[b]	0.721±0.002[b]
	AMC20	0.661±0.031[a]	0.658±0.022[a]	0.765±0.032[a]	0.783±0.012[b]	0.813±0.008[b]
	Ciprofloxacin	0.642±0.048[a]	0.686±0.007[a]	0.788±0.007[a]	0.792±0.004[b]	0.935±0.010[a]
	NC	0.673±0.011[a]	0.655±0.002[a]	0.659±0.018[b]	0.659±0.027[c]	0.651±0.027[d]

Along the column and for each microorganism, values carrying the same letter superscripts are not significantly different ($p > 0.05$) and values carrying different letters are significantly different ($P < 0.05$).

Appendix 6: Means of optical densities (620nm) at each time interval of compounds on *K. oxytoca* and *S. aureus* ATCC 43300 during time kill kinetics assay

			Incubation time (h)							
Bacteria	**Codes of compound**	**Concentrations**	**0**	**2**	**4**	**6**	**8**	**10**	**12**	**24**
K. oxytoca	**AMC6**	**4MIC**	0.122±0.014[a]	0.176±0.002[a]	0.26±0.009[a]	0.139±0.017[a]	0.209±0.038[a]	0.124±0.002[a]	0.236±0.009[a]	0.094±0.006[a]
		2MIC	0.117±0.004[a]	0.226±0.004[b]	0.264±0.009[a]	0.221±0.008[b]	0.198±0.013[b]	0.124±0.003[a]	0.238±0.007[a]	0.167±0.002[b]
		MIC	0.121±0.007[a]	0.23±0.009[b]	0.235±0.003[a]	0.184±0.000[a]	0.207±0.019[b]	0.126±0.005[a]	0.252±0.008[a]	0.16±0.032[b]
	AMC20	**4MIC**	0.108±0.009[a]	0.219±0.035[b]	0.273±0.012[a]	0.18±0.041[a]	0.215±0.006[b]	0.147±0.006[a]	0.281±0.001[a]	0.114±0.001[b]
		2MIC	0.189±0.036[a]	0.295±0.040[b]	0.304±0.026[b]	0.279±0.069[b]	0.292±0.072[b]	0.234±0.086[b]	0.302±0.040[b]	0.305±0.064[c]
		MIC	0.139±0.010[a]	0.238±0.010[b]	0.244±0.016[a]	0.153±0.031[a]	0.223±0.013[b]	0.133±0.017[a]	0.282±0.019[a]	0.25±0.007[c]
	Ciproflox acin	**MIC**	0.186±0.007[a]	0.219±0.010[b]	0.252±0.021[a]	0.176±0.027[a]	0.192±0.004[a]	0.193±0.012[a]	0.255±0.003[a]	0.153±0.014[b]
	NC		0.118±0.019[a]	0.206±0.022[b]	0.447±0.014[c]	0.51±0.012[c]	0.664±0.006[c]	0.621±0.010[c]	0.788±0.006[c]	0.945±0.14[d]
***S. aureus* ATCC 43300**	**AMC6**	**4MIC**	0.111±0.011[a]	0.15±0.017[a]	0.146±0.010[a]	0.179±0.013[a]	0.159±0.012[a]	0.159±0.013[a]	0.173±0.007[a]	0.14±0.032[a]
		2MIC	0.14±0.033[a]	0.241±0.009[b]	0.24±0.005[b]	0.187±0.015[a]	0.193±0.005[a]	0.153±0.028[a]	0.158±0.008[a]	0.164±0.053[a]
		MIC	0.126±0.006[a]	0.292±0.017[b]	0.211±0.014[b]	0.35±0.030[b]	0.35±0.038[b]	0.219±0.024[b]	0.192±0.005[a]	0.141±0.036[a]

AMC20	**4MIC**	0.155±0.004^{a}	0.249±0.023^{b}	0.154±0.023^{a}	0.157±0.021^{a}	0.134±0.025^{a}	0.199±0.013^{a}	0.164±0.021^{a}	0.151±0.034^{a}
	2MIC	0.095±0.002^{a}	0.195±0.019^{b}	0.22±0.019^{b}	0.218±0.020^{b}	0.201±0.018^{b}	0.248±0.021^{b}	0.225±0.017^{b}	0.18±0.011^{a}
	MIC	0.123±0.027^{a}	0.099±0.005^{a}	0.358±0.027^{c}	0.356±0.037^{b}	0.334±0.036^{b}	0.372±0.036^{b}	0.352±0.037^{b}	0.133±0.025^{a}
Ciproflox acin	**MIC**	0.129±0.008^{a}	0.128±0.009^{a}	0.138±0.002^{a}	0.134±0.002^{a}	0.121±0.001^{a}	0.189±0.029^{a}	0.154±0.002^{a}	0.117±0.011^{a}
NC		0.128±0.013^{a}	0.295±0.013^{b}	0.258±0.019^{b}	0.452±0.018^{c}	0.536±0.007^{c}	0.67±0.016^{c}	0.746±0.006^{c}	0.906±0.029^{b}

MIC: Minimum Inhibitory Concentrations. Along the column, values carrying the same letter superscripts are not significantly different ($p > 0.05$) and values carrying different letters are significantly different ($P<0.05$)

Appendix 7: Reduction factors of *S. aureus* ATCC 43300 and *K. oxytoca* isolate population by compounds after 24 hours of incubation

	Reduction of bacteria population after 24 hours					
Bacteria	***K. oxytoca***			***S. aureus* ATCC 43300**		
Codes of compounds	**4MIC**	**2MIC**	**MIC**	**4MIC**	**2MIC**	**MIC**
AMC6	10.053[a]	5.906[a]	5.658[a]	6.471[a]	6.425[a]	5.524[a]
AMC20	8.289[b]	3.780[b]	3.098[b]	6.812[a]	6.000[a]	5.033[a]
Ciprofloxacin	NA	NA	4.670[C]	NA	NA	7.110[b]

MIC: Minimum Inhibitory Concentrations. Along the column, values carrying the same letter superscripts are not significantly different ($p > 0.05$) and values carrying different letters are significantly different ($P < 0.05$).

Appendix 8: Amount of biofilm materialized by the mean relative fluorescence units on *K. oxytoca* and *S. aureus* ATCC 43300 after 48 hours of incubation

Strains	**Means of relative fluorescence units**
K. oxytoca	13614.5 ±29.760[a]
S. aureus	12665.75 ±173.240[a]

Along the column. values carrying the same letter superscripts are not significantly different ($p > 0.05$).

Appendix 9: Mean of inhibition percentages of *K. oxytoca* and *S. aureus* ATCC 43300 biofilm by compounds

		Concentrations						
Bacteria	**Codes of compounds**	**4MIC**	**2MIC**	**MIC**	**1/2 MIC**	**1/4 MIC**	**1/8 MIC**	**1/16 MIC**
K. oxytoca	**AMC6**	93.45±0.116	84.434±0.252	81.705±0.215	77.504±0.353	69.454±1.072	47.378±0.617	46.247±2.378
	AMC20	82.797±0.194	82.914±0.271	79.931±0.215	71.345±0.380	51.218±1.234	44.064±1.873	42.124±0.363
	Ciprofloxacin	93.182±1.533	88.731±0.880	77.693±0.849	64.164±1.399	54.61±0.724	48.242±0.062	39.131±0.204
S. aureus **ATCC 43300**	**AMC6**	81.905±0.224	58.71±0.939	54.727±2.319	52.365±0.113	50.741±1.544	43.934±0.185	39.327±3.752
	AMC20	73.645±0.724	73.267±0.168	62.14±1.927	54.601±1.651	53.19±0.621	49.724±0.429	41.791±2.144
	Ciprofloxacin	77.805±1.670	72.994±1.269	67.98±0.060	64.928±1.563	58.182±1.518	44.367±0.115	34.43±0.626

MIC: Minimum Inhibitory Concentrations

m

Appendix 10: Mean of eradication percentages of *K. oxytoca* and *S. aureus* ATCC 43300 biofilm by compounds

		Concentrations						
Bacteria	**Codes of compounds**	**4MIC**	**2MIC**	**MIC**	**1/2 MIC**	**1/4 MIC**	**1/8 MIC**	**1/16 MIC**
K. oxytoca	**AMC6**	60.447±0.398	55.328±0.415	53.467±2.038	49.324±1.413	35.635±1.432	29.158±4.849	22.272±1.002
	AMC20	58.227±0.485	54.567±1.127	52.523±0.562	50.126±0.816	43.936±1.620	38.287±0.912	25.3±1.438
	Ciprofloxacin	88.442±2.185	76.177±2.716	64.009±0.117	52.447±0.763	49.009±1.288	35.098±1.414	23.543±0.628
S. aureus **ATCC 43300**	**AMC6**	61.485±1.003	58.185±0.676	52.329±0.209	51.035±0.906	49.635±1.097	37.662±3.133	25.219±1.519
	AMC20	56.761±2.513	53.101±0.173	52.228±1.247	50.847±0.434	45.623±3.813	34.997±3.077	20.81±0.406
	Ciprofloxacin	67.587±0.437	57.914±0.029	55.4±0.696	50.778±0.014	44.322±0.630	33.83±1.319	22.276±1.004

MIC: Minimum Inhibitory Concentrations

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Crude metabolites from endophytic fungi inhabiting Cameroonian *Annona muricata* inhibit the causative agents of urinary tract infections

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Abstract

Urinary tract infections (UTIs) are common bacterial infections. The global emergence of multidrug-resistant uropathogens in the last decade underlines the need to search for new antibiotics with novel mechanisms of action. In this regard, exploring endophytic fungi inhabiting medicinal plants used locally against urinary tract infections could be a promising strategy for novel drug discovery. This study investigates crude metabolites from endophytic fungi isolated from *Annona muricata* as potential sources of antibiotic drugs to fight against uropathogens and reduce related oxidative stress. Crude ethyl acetate extracts from 41 different endophytic fungi were screened against three bacterial strains using the broth microdilution method, and fungi producing active crude extracts were identified using ITS1-5.8S rRNA-ITS2 nucleotide sequences. The antibacterial modes of action of the five most active extracts were evaluated using *Staphylococcus aureus* ATCC 43300 and *Klebsiella oxytoca* strains. 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 41 crude extracts tested, 17 were active with minimum inhibitory concentrations (MICs) ranging from 3.125 μg/mL to 100 μg/mL and were not cytotoxic against Vero cell lines with a cytotoxic concentration 50 (CC_{50}) >100 μg/mL. The more potent extracts (from *Fusarium waltergamsii* AMtw3, *Aspergillus* sp. AMtf15, *Penicillium citrinum* AMf6, *Curvularia* sp. AMf4, and *Talaromyces annesophieae* AMsb23) significantly inhibited bacterial catalase activity, lysed bacterial cells, increased outer membrane permeability, and inhibited biofilm formation, and the time-kill kinetic assay revealed concentration-dependent bactericidal activity. All seventeen extracts showed weak ferric iron-reducing power (1.06 to 12.37 μg equivalent NH_2OH/g of extract). In comparison, seven extracts exhibited DPPH free radical scavenging activity, with RSA_{50} ranging from 146.05 to 799.75 μg/mL. The molecular identification of the seventeen active fungi revealed that they belong to six distinct genera, including *Aspergillus*, *Curvularia*, *Fusarium*, *Meyerozyma*, *Penicillium*, and *Talaromyces*. This investigation demonstrated that fungal endophytes from Cameroonian *Annona muricata*, a medicinal

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Abbreviations: AMr, *Annona muricata* root; AMrb, Annona muricata root bark; AMf, *Annona muricata* fruit; AMs, *Annona muricata* seed; AMtf, *Annona muricata* thorn of fruit; AMtw, *Annona muricata* twigs; AMl, *Annona muricata* leaves; AMb, *Annona muricata* bark; AMsb, *Annona muricata* stem bark; AMpe, *Annona muricata* peduncle; ATCC, American Type Culture Collection used as reference strains for respective bacteria; NB, Nutrient Both; CPC, Centre Pasteur du Cameroun; CLSI, Clinical Laboratory Standard Institute; PDA, Potato dextrose agar; PDB, Potato dextrose broth; MIC, Minimal Inhibitory Concentration; BLAST, Basic Local Alignment Search Tool; CBS, Centraalbureau voor Schimmelcultures; CC_{50}, 50% cell cytotoxic concentration; DMEM, Dulbecco's minimum essential medium; PCR, Polymerase Chain Reaction; ITS, Iternal Transcribed Spacer; MEGA, Molecular Evolutionary Genetics Analysis; PCR, Polymerase Chain Reaction; NA, Not applicable; SD, Standard deviation; HNC, Herbier National du Cameroun; NCBI, National Center for Biotechnology Information; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium; FRAP, Ferric Ion Reducing Antioxidant Power; DPPH, 2,2-diphenyl-1-picryl-hydrazyl; RSA_{50}, 50% Radical Scavenging Activity; EC_{50}, 50% efficient concentration; ARP, Antiradical Power; EDTA, Ethylene Diamine Tetraacetic Acid-Na2; DNA, Deoxyribonucleic Acid.

plant used locally to treat bacterial infections, might contain potent antibacterial metabolites with multiple modes of action. The antibacterial-guided fractionation of these active extracts is currently ongoing to purify and characterise potential antibacterial active ingredients.

Introduction

Infectious diseases emerging throughout history have included some of the most feared plagues of the past. Recent outbreaks include Ebola, Zika, dengue, Middle East respiratory syndrome (MERS), severe acute respiratory syndrome (SARS), influenza, fungal infection, and more recently, COVID-19 [1, 2]. Although new infections emerging today are global problems, many old diseases and the looming specter of rising antimicrobial resistance are still with us. They are continuously jeopardising human health and various forms of social and economic well-being [1]. In this respect, urinary tract infections (UTIs) are among the most common bacterial infections affecting individuals of all ages and sexes [3]. Currently, it represents a serious public health problem, causing approximately 150 million cases per annum [4, 5], with an estimated annual cost of $1.6 billion in the United States alone [6]. In Cameroon, a recent investigation revealed a prevalence of 12% among children from 1 to 3 years of age at the Buea District Hospital [7].

Testing and treatment strategies vary for the management of UTIs, with empirical therapy being the use of broad-spectrum antibiotics [4, 8]. Unfortunately, in recent years, we have seen the utility of many of these antibiotic agents eroded because of their widespread use and the subsequent development of resistance [9–11]. According to the World Health Organization (WHO), antibiotic resistance remains one of the greatest threats to human health and poses a severe financial burden on healthcare systems worldwide [12]. The increase in resistance to many of the currently available oral drugs makes the management of UTIs caused by resistant pathogens a significant challenge. Therefore, there is an urgency to develop new effective antibiotics with a novel mechanism of action to overcome antibiotic resistance.

Natural products are the primary source of antibiotics, most of which are produced by microorganisms. Microorganisms, particularly fungi, represent a large and still resourceful pool for discovering novel compounds to combat antibiotic resistance in human pathogens. Their ability to produce structurally diverse and potent compounds has been known for decades, particularly with the discovery of penicillin G, a β-lactam antibiotic from *Penicillium notatum* by Sir Alexander Fleming [13–15]. Over the past decades, endophytic fungi living in a symbiotic association with medicinal plants without causing harm have been recognized as excellent sources of structurally novel and bioactive antimicrobial agents [12, 15, 16]. The Cameroonian biodiversity is rich, with thousands of plant species often used by local populations to treat various infections. In fact, with over 4,000 plant species per degree squared, Cameroon's plant diversity is higher than that of all other West African countries combined [17]. Therefore, the long-neglected microbiome of these plants deserves serious attention. Their investigation could hold a key for future great discoveries in the fight against antibiotic-resistant microbes and urinary tract infections.

In our continuous bioprospecting of endophytic fungi residing inside Cameroonian medicinal plants as sources of potent antimicrobial agents, we screened endophytic fungi from *Annona muricata* for their potential antibacterial activity. Our previous investigation revealed that these endophytes could produce crude metabolites with antiplasmodial activity [18]. *Annona muricata* is a multipurpose plant traditionally used in Cameroon and elsewhere to

treat several infectious diseases, including UTIs [19]. We hypothesized that these endophytic fungi might produce active metabolites against bacterial pathogens that are causative agents of UTIs. The present study investigates the antibacterial and modes of action of endophytic fungal extracts isolated from the plant mentioned above.

Materials and methods

Source of endophytic fungi

The forty-one endophytic fungi used in this study were isolated from healthy and mature organs (root, root bark, fruit, the thorn of fruit, seed, twig, leaf, stem bark, bark, and peduncle) of *Annona muricata* (3289/HNC). All plant samples were collected in Yaoundé, Cameroon, on January 10, 2016. Small pieces of plant materials measuring approximately 2 mm were surface disinfected through a 5-min rinse with 70% ethanol, followed by treatment with 1% active chlorine solution for 15 min, 2 min in 70% ethanol, and a final rinse three times in sterile distilled water. Disinfected material pieces were plated on potato dextrose agar (PDA; HiMEDIA, India) containing chloramphenicol (200 mg/L) and kept in the dark at room temperature (22–26˚C). After the emergence of endophyte mycelium from plant tissues into the agar, mycelial fragments were transferred to fresh PDA plates and maintained under natural light at room temperature. All endophytes were kept at -80˚C in a 50% glycerol solution at the Antimicrobial & Biocontrol Agents Unit (AmBcAU), Laboratory for Phytobiochemistry and Medicinal Plants Studies, Department of Biochemistry, Faculty of Science, University of Yaoundé I, Cameroon[18].

Molecular identification of potent antibacterial endophytes

The endophytic fungal isolates showing antibacterial activity against UTI pathogens at 100 μg/mL were identified based on the ITS1-5.8S rRNA-ITS2 nucleotide sequence. Briefly, fungal genomic DNA was extracted from mycelium grown in potato dextrose broth (PDB, HIMEDIA) using a commercial kit (RedExtract-N-Amp Plant PCR, Sigma Aldrich, USA). The extracted DNA concentration and purity (A260/A280 ratio) were measured with a Thermo Scientific NanoDrop 1000 Spectrophotometer (Thermo Scientific, Germany) using 1 μL of each sample. The ITS1-5.8S rRNA-ITS2 region was amplified by PCR using primers ITS4 and ITS5 (Sigma–Aldrich, Germany) and the protocol described by White *et al.* [20]. The reagents included in the kit were also used for PCR amplification using the following conditions: 95˚C for 2 min, followed by 35 cycles of 94˚C for 1 min, 54˚C for 1 min, and 72˚C for 1 min; and a final step of 72˚C for 10 min. The PCR product was analyzed by agarose (1% agarose) gel electrophoresis using 1×Tris EDTA (TE) buffer containing 1 μg/mL ethidium bromide (EtBr) and a constant voltage of 100 V for 20 min. DNA bands were visualized, and images were acquired using a Gel Doc XR+ imaging system (Bio–Rad Laboratories Inc., Germany). Amplicons were purified by filtration (MSB Spin PCRapace, Invitek, Germany), and only one strand of the PCR amplicon was sequenced. The sequence reaction was started at the 5′ end of the ITS1-5,8S rRNA-ITS2 region using the primer ITS4.

The BLAST algorithm was used to find sequences similar to those obtained from fungal isolates. The criteria for identifying isolates were based on the similarity of their sequences to those of reliable reference isolates included in open access nucleotide databases. A dendrogram was made with the nucleotide sequences of the isolates and those of reference strains deposited in Centraalbureau Voor Schimmelcultures (CBS) and American Type Culture Collection (ATCC) collections. Sequences were aligned using the following parameters: pairwise alignment parameters (gap opening = 10 and gap extension = 0.1) and multiple alignment parameters (gap opening = 10, gap extension = 0.2, transition weight = 0.5, and delay divergent

sequences = 25%) and optimized manually in MEGA 7.0. For the phylogenetic analyses based on maximum likelihood (ML), the best-fit models of nucleotide substitution for each data partition were determined using MEGA 7.0 software and incorporated into the analyses. Alignment gaps were treated as partial missing information, and one thousand replications estimated the robustness of the classifications. The initial trees for the heuristic ML search were obtained by applying the neighbor-joining method to a matrix of pairwise distances estimated using the maximum composite likelihood approach, allowing some sites to be evolutionarily invariable. Groups of sequences at proximity within the same branch of the dendrogram were individually aligned to determine their similarity percentage. Sequences with close similarity with reference sequences used for phylogenetic analysis were considered to belong to the same species as the reference sequence [21].

Culture and extraction of endophytic fungi

Each fungal strain was first cultivated on potato dextrose agar (PDA; HiMEDIA, India) at 25˚C for seven days. Subsequently, three pieces (1 × 1-cm) of mycelium from these cultures were used to inoculate 100 mL of potato dextrose broth medium (potato infusion 200 g, dextrose 20 g, pH 5.1 ± 0.2) (PDB; HiMedia, India) in 250-mL Erlenmeyer flasks. Liquid cultures were grown for 7 days under static conditions in an incubator (Gallenkamp, UK) with a temperature set at 25 ± 2˚C before extraction. After the incubation period, 100 mL of ethyl acetate (EtOAc) was added to each culture, gently shaken, and kept overnight at room temperature. The organic phase was then decanted, and the aqueous phase was extracted three times with 100 mL EtOAc. The organic phases were pooled (total = 300 mL) and evaporated to dryness at 40˚C using a rotary evaporation system (Heidolph, Germany) to obtain crude fungal extracts. The dry residue was weighed (S1 Table in S1 File), diluted with 100% DMSO (Loba Chemie, India) to a stock concentration of 25 mg/mL, and stored in sterile conical flasks at 4˚C until further use.

Screening of fungal extracts for antibacterial activity

Microbial species and culture media. Three bacterial species were used in this investigation. Two strains, *Staphylococcus aureus* ATCC 43300 and *Escherichia coli* ATCC 25922, were obtained from the American Type Culture Collection (ATCC) and *Klebsiella oxytoca*, and a clinical isolate was obtained from the "Centre Pasteur du Cameroun" (CPC) of Yaoundé-Cameroon. Nutrient agar medium (NA; HiMedia, India) was used to revive the bacterial strains 24 h before each experiment, while nutrient broth (NB; HiMedia, India) medium was used for all antibacterial assays.

Antibacterial screening and minimum inhibitory concentrations of endophytic extracts. Individual ethyl acetate (EtOAc) extracts of endophytes were screened (at a single concentration) for antibacterial activity. Subsequently, the active compounds were selected for the dose–response study for MIC determination.

Extracts were screened against three bacterial species at a single concentration of 100 μg/mL according to the M07-A9 protocol of the Clinical Laboratory Standards Institute (CLSI) [22]. Briefly, 92 μL of nutrient broth (NB; HiMedia, India) was aseptically introduced into the wells of a 96-well microplate. Eight microliters (8 μL) of each extract, initially prepared in an intermediate plate at 2500 μg/mL, was added to the wells followed by 100 μL of standardized bacterial suspension (2 x10^6 CFU/mL). The tests were performed simultaneously for the negative control (NB + bacteria) and sterility control (NB alone). Ciprofloxacin (Sigma–Aldrich, Germany) at 64 μg/mL was used as the positive control. The test was performed in triplicate, and the plates were incubated at 37˚C for 24 hours. After this period, extracts that were active

on at least one of the bacteria tested were selected to determine the minimum inhibitory concentrations (MICs).

To determine the MIC of the selected extracts, serial twofold dilutions of each extract were performed with final concentrations ranging from 1.5625 to 100 μg/mL for the extracts and from 0.117 to 7.5 μg/mL for the positive control. All test plates were incubated at 37˚C for 24 hours. The turbidity was observed as an indication of growth, and the MIC was considered the lowest concentration with no visible growth of microorganisms (no turbidity). Wells containing NB and bacteria constituted the negative control, while the sterility control contained NB alone. The final concentration of DMSO (Sigma–Aldrich, Germany) was at most 1%, and the preliminary test did not inhibit bacterial growth. The test was performed in duplicate and repeated twice.

Evaluation of possible modes of action of the most potent extracts

The five extracts (AMtw3, AMtf15, AMf6, AMf4, and AMsb23) exhibiting the best inhibitory activity against the three bacterial pathogens were selected for the mode of action study. The two most sensitive bacterial strains (*S. aureus* ATCC 43300 and *K. oxytoca*) were used for this study.

Inhibition of Catalase Activity. The catalase inhibitory activity of extracts was carried out as described by Mbekou *et al.* [23]. Extracts at the MIC concentration were added to a test tube containing 400 μL of hydrogen peroxide (H_2O_2) (40 mM) and 400 μL of phosphate buffer saline (PBS) (Sigma–Aldrich, Germany). The mixture was then transferred to another tube containing 200 μL of a bacterial suspension (1.5×10^8 UFC/mL) prepared in 0.9% NaCl. 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 using the microplate reader Infinite M200 (TECAN, Männedorf, Switzerland). The phosphate buffer constituted the blank, while bacterial strains in phosphate buffer without any inhibiting substance were used as a negative control. The mixture of ciprofloxacin at 0.468 and 0.234 μg/mL, respectively, for *S. aureus* ATCC 43300 and *K. oxytoca*, phosphate buffer, and bacterial strain constituted a positive control. The percentage of remaining H_2O_2 was determined according to the following formula:

$$\%\ \text{of remaining}\ H_2O_2 = \text{Abs sample} - \text{Abs negative control} \times 100/\text{Abs negative control}$$

*A*bs negative control is the absorbance of H_2O_2 without the extract, and Abs sample is the absorbance of H_2O_2 with the extract.

Bacteriolysis activity of extracts. The potential bacteriolytic ability of the antibacterial extracts was determined according to the method described by Mbekou *et al.* [23]. Briefly, the bacterial suspension ($5x10^7$ CFU/mL) 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 an Infinite M200 microplate reader (TECAN, Männedorf, Switzerland). A decrease in OD at 620 nm indicated bacterial cell lysis. Corresponding dilutions of the extract were used as blanks. Ciprofloxacin was used as a positive control at 0.468 and 0.234 μg/mL for *S. aureus* ATCC 43300 and *K. oxytoca*, respectively. 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.

Outer membrane permeability assay. The potential effect of extracts on the outer membrane (OM) permeability of *S. aureus* ATCC 43300 and *K. oxytoca* was determined according to the method described by Mbekou *et al.* [23]. An overnight culture ($5x10^7$ CFU/mL) was inoculated into nutrient broth containing the extracts at 1/16 MIC, 1/8 MIC, 1/4 MIC, 1/2

MIC, MIC, 2 MIC, 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 *S. aureus* ATCC 43300 and *K. oxytoca* was measured at 450 nm using an Infinite M200 microplate reader (TECAN, Männedorf, Switzerland). The bacterial growth graph (OD/450 nm) as a function of extract concentration (μg/mL) was plotted. Ciprofloxacin (concentration ranged from 0.01 to 0.93 μg/mL and 0.06 to 1.84 μg/mL, respectively, for *K. oxytoca* and *S. aureus* ATCC 43300) was used as the positive control, and each test was conducted in triplicate.

Antibacterial time-kill kinetics assay. The time-kill kinetics of active endophyte extracts were determined according to the method described by Babii *et al.* [24]. Here, extract concentrations of MIC, 2 MIC and 4 MIC were prepared by serial twofold dilution in a 96-well microplate. One hundred microliters (100 μL) of bacterial suspension (2 x 10^6 CFU/mL) was added, and the plate was incubated at 37˚C for different time intervals (0, 1, 2, 4, 6, 8, 10, 12, and 24 h). Following each incubation period, the cell suspensions were appropriately diluted (in NaCl 0.9%), and the ODs of the resulting solution were measured at 620 nm using an Infinite M200 microplate reader (TECAN, Männedorf, Switzerland). Ciprofloxacin at 0.468 and 0.234 μg/mL, respectively, for *S. aureus* ATCC 43300 and *K. oxytoca* was used as a positive control, while the bacteria incubated with NB were used as growth controls. The test was performed in triplicate, and the results are presented as the mean±SD.

Biofilm inhibition assay. *Biofilm Quantification*. Biofilm production by *S. aureus* ATCC 43300 and the *K. oxytoca* isolate was performed as described by Cruz *et al.* [25]. Briefly, a single colony was taken from the NA overnight bacterial culture, inoculated into 0.9% (w/v) saline solution and vortexed to ensure that the bacterial suspension was homogeneous. Bacterial suspensions were adjusted to 1×10^7 colony forming units (CFU/mL) by diluting with appropriate nutrient broth supplemented with 2% glucose. An aliquot of 200 μL of bacterial suspension per well was dispensed into a 96-well flat-bottom microplate. The plate was then incubated at 37˚C for 48 hours. After this incubation period, planktonic cells were carefully removed, and adhered/biofilm cells were washed twice with 0.9% NaCl. Next, 100 μL of resazurin solution at 0.15 mg/mL prepared in sterile phosphate buffered saline (PBS; Sigma–Aldrich, Germany) was added to each well-containing biofilm. Microplates were incubated in the dark at 37˚C for 1 hour, after which the microplate reader Infinite M200 (TECAN, Männedorf, Switzerland) was used to measure the relative fluorescence units (RFU) (λ_{Ex} 530 nm and λ_{Em} 590 nm). The relative fluorescence units obtained were used to plot a histogram to examine the amount of biofilms formed by each microorganism.

Biofilm inhibition assay

The inhibitory potential of active extracts against biofilm formation by *S. aureus* ATCC 43300 and *K. oxytoca* was investigated using the method previously described by Cruz *et al.* [25] with slight modifications. Briefly, 100 μL of each bacterial strain suspension (1x10^7 CFU/mL) was incubated with crude extracts at MIC, 2 MIC, and 4 MIC for 48 h at 37˚C. After incubation, planktonic cells were removed by washing the wells very delicately with 0.9% NaCl. Next, 100 μL of diluted resazurin solution was added to each well-containing biofilm. Microplates were placed in the dark and incubated at 37˚C for 1 hour. A microplate reader Infinite M200 (TECAN, Männedorf, Switzerland) was then used to measure the relative fluorescence units (RFU) (λ_{Ex} 530 nm and λ_{Em} 590 nm) after incubation. Ciprofloxacin was used as a positive control at 0.468 and 0.234 μg/mL for *S. aureus* ATCC 43300 and *K. oxytoca*, respectively. Wells containing only bacteria-free medium constituted the negative control, and the assay was performed in triplicate and repeated twice. The percent inhibition was estimated (Biofilm inhibition% = RFU control − RFU sample/RFU control × 100), and the inhibitory

concentration 50 (IC_{50s}) was calculated based on the percent inhibition with the different concentrations of extracts.

In vitro antioxidant activity of fungal extracts

All active endophytic fungal extracts were investigated for their potential antioxidant activity using DPPH radical scavenging and ferric ion reducing antioxidant power (FRAP) assays.

DPPH radical scavenging assay. The potential free radical scavenging activity of fungal extracts was evaluated using the 2,2-diphenyl-1-picryl-hydrazyl (DPPH) assay previously described by Djague *et al.*[26]. Briefly, the extracts were first diluted to obtain extract concentrations of 62.5, 125, 250, 500, 1000, 2000, and 4000 μg/mL in a 96-well microplate. After that, twenty-five microliters (25 μL) of each dilution was introduced into a new microplate, and 75 μL of 0.02% DPPH was added to obtain final concentrations of 15.625, 31.25, 62.5, 125, 250, 500, and 1000 μg/mL. The reaction mixtures were kept in the dark at room temperature for 30 min, after which the absorbance was measured at 517 nm against the blank (DPPH in methanol) using the microplate reader Infinite M200 (TECAN, Männedorf, Switzerland). L-ascorbic acid was used as a positive control and was treated under the same conditions as the extracts with final concentrations of 0.391, 0.781, 1.563, 3.125, 6.25, 12.5, and 25.0 μg/mL. The assay was performed in triplicate. The percentage (%) radical scavenging activities of the plant extracts were calculated using the following formula, from which other parameters, such as the radical scavenging activity 50 (RSA_{50}), the effective concentration 50 (EC_{50}), and the antiradical power (ARP), were deduced.

$$\text{Percentage of RSA} = [(\text{Abs control} - \text{Abs sample})/\text{Abs control}] \times 100.$$

where RSA is the radical scavenging activity, Abs control is the absorbance of the blank (DPPH + methanol), and Abs sample is the absorbance of DPPH radical + fungal extract.

Ferric ion reducing antioxidant power (FRAP) assay. The assay was performed according to the method described by Djague *et al.* [26]. Briefly, the extracts were first dissolved for the DPPH assay. Twenty-five microliters from each dilution was added to 25 μL of 1.2 mg/mL Fe^{3+} solution in a new microplate. The plates were preincubated for 15 min at room temperature. Fifty microlitres (50 μL) of 0.2% ortho-phenanthroline solution was added to obtain final extract concentrations of 15.625, 31.25, 62.5, 125, 250, 500, and 1000 μg/mL. The reaction mixtures were further incubated for 20 min at room temperature, after which the absorbance was measured at 505 nm using a 96-well microplate reader Infinite M200 (TECAN, Männedorf, Switzerland) against the blank (25 μL methanol + 25 μL Fe^{3+} + 50 μL ortho-phenanthroline). Hydroxylamine was used as a positive control and was treated in the same way as the extracts with final concentrations of 0.103, 0.206, 0.413, 0.825, 1.65, 3.30, and 6.60 μg/mL. The assay was performed in triplicate. From a concentration-activity curve of NH_2OH used as a standard, the optical densities of the test wells were projected, and the results were expressed quantitatively as μg equivalent NH_2OH/g of extracts.

Cytotoxicity assay of promising extracts

The cytotoxic effect of antibacterial extracts was assessed using the MTT assay as described by Mosmann, [27], 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 (Corning, USA) in complete medium. Fifty microliters of serially diluted extract solutions ($\leq$100 μg/mL) were

added after 24 h of seeding, and the cells plus 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 a negative control (100% growth). Twenty microliters of a stock solution of MTT (5 mg/mL in 1x phosphate-buffered saline) was 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, Männedorf, Switzerland) at 570 nm. The 50% cytotoxic concentrations (CC_{50}) of extracts were determined by analyzing the dose–response curves.

Statistical analysis

Data collected from at least three independent experiments were analyzed using one-way ANOVA with GraphPad Prism 5. Data are expressed as the mean ± SD of experiments performed in triplicate. Error bars represent the SD, and significant differences for multiple comparisons were determined by the Turkey test at $p < 0.05$.

Results

Screening and characterization of potent endophytic fungal strains

Antibacterial screening. In the present study, forty-one endophytic fungi isolated from ten organs of the medicinal plant *A*. *muricata* growing in Cameroon were subjected to antibacterial screening against three pathogenic bacteria causing UTIs. Fungi fermented in potato dextrose broth medium and extracted with ethyl acetate yielded crude extracts with masses ranging from 12 to 402 mg depending on the cultured fungus. AMs1 (402 mg) followed by AMs3 (264 mg) and AMrb9 (258 mg) isolated from seeds (s) and root bark (rb) produced the highest amount of crude metabolites (S1 Table in S1 File).

The 41 crude extracts were screened at a single concentration of 100 μg/mL against three bacterial causative agents of UTIs (*E. coli* ATCC 25922, *K*. *oxytoca*, and *S*. *aureus* ATCC 43300). The results (S1 Table in S1 File) showed that 17 (41.46%) were active, among which 16 exhibited broad-spectrum activity against the three pathogens, while one (AMr10) was only active against *S*. *aureus* ATCC 43300. Crude metabolites exhibiting activity were produced by endophytic fungi isolated from root (2), root bark (3), fruits (4), seeds (2), the thorn of fruits (2), twigs (1), bark (1), and stem bark (2). No endophytes from leaves or peduncles showed any inhibitory activity against the tested pathogens. Based on their antibacterial activity at 100 μg/mL, the seventeen fungi were selected for molecular identification and further studies.

Molecular characterization of endophytic fungi. The ITS rDNA region of all 17 selected fungi was sequenced, and the identification was performed by comparison with published sequences in GenBank (S2 File in S1 File). The results from the BLAST search revealed that 16 endophyte sequences showed 98.32–100% similarity with sequences from previously identified fungi in the NCBI database. However, eleven of the endophytes sequences investigated showed higher similarity with more than two different fungal species. AMr9 showed similarity with six different species of *Aspergillus*, and AMtf15, AMr10, AMrb9, AMs9, and AMb7 were similar to 3 different *Aspergillus* spp. AMrb1 and AMrb11 were similar to three *Penicillium* species, AMf4 was similar to two *Curvularia* species, and AMf3 was similar to more than two *Meyerozyma* species. The sequence of AMtf5 showed only 96.88% similarity with the strain *Talaromyces clemensii* NR_168822.1. New sequences generated in this study were deposited in the NCBI GenBank nucleotide database (www.ncbi.nlm.nih.gov; Table 1).

To infer the evolutionary history of endophytic fungi, the maximum likelihood (ML) method based on the Kimura 2-parameter model was used (Fig 1). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are

Table 1. Identity of the seventeen active endophytic fungi, their GenBank accession number, and their closest related species.

Organs	Fungus code	Fungal identity	GenBank Accession No.	Blast Results		
				Closest Related Species	Query coverage (%)	Percent identity (%)
Root	AMr9	*Aspergillus* sp.	OM959526	*A. foetidus* NR_163668.1	100	100
				A. welwitschiae NR_137513.1	100	100
				A. awamori NR_077143.1	100	100
				A. tubingensis NR_131293.1	100	99.60
				A. costaricensis NR_103604.1	100	99.60
				A. niger NR_111348.1	98	100
	AMr10	*Aspergillus* sp.	OM959517	*A. austwickii* NR_171607.1	100	99.60
				A. aflatoxiformans NR_171606.1	100	99.60
				A. oryzae NR_135395.1	100	99.40
Root bark	AMrb1	*Penicillium* sp.	OM959527	*P. herquei* NR_103659.1	100	99.26
				P. verrucisporum NR_153275.1	100	99.26
				P. sanshaense NR_153276.1	100	98.76
	AMrb9	*Aspergillus* sp.	OM959523	*A. clavatonanicus* NR_135410.1	100	100
				A. longivesica NR_135411.1	100	99.80
				A. giganteus NR_135403.1	100	99.80
	AMrb11	*Penicillium* sp.	OM959528	*P. citrinum* NR_121224.1	100	99.40
Fruit	AMf6	*Penicillium citrinum*	OM980761	*P. citrinum* NR_121224.1	100	99.53
	AMf3	*Meyerozyma* sp.	OM959515	*M. caribbica* NR_149348.1	99	99.57
				M. carpophila NR_152984.1	100	100
	AMf4	*Curvularia* sp.	OM959516	*C. kenpeggii* NR_158447.1	100	98.32
	AMf1	*Penicillium citrinum*	OM959525	*P.citrinum* NR_121224.1	100	99.56
Seed	AMs3	*Penicillium citrinum*	OM959520	*P. citrinum* NR_121224.1	100	98.68
	AMs9	*Aspergillus* sp.	OM959518	*A. clavatonanicus* NR_135410.1	100	100
				A. longivesica NR_135411.1	100	99.79
				A. giganteus NR_135403.1	100	99.79
Thorn of fruit	AMtf15	*Aspergillus* sp.	OM959513	*A. austwickii* NR_171607.1	100	99.57%
	AMtf5	*Talaromyces* sp.	OM959521	*T. clemensii* NR_168822.1	100	96.88
Twig	AMtw3	*Fusarium waltergamsii*	OM959524	*F. waltergamsii* NR_159548.1	93	99.77
Stem bark	AMsb23	*Talaromyces annesophieae*	OM959519	*T. annesophieae* NR_170732.1	100	99.17
	AMsb1	*Penicillium citrinum*	OM959514	*P. citrinum* NR_121224.1	100	99.53
Bark	AMb7	*Aspergillus* sp.	OM959522	*A. austwickii* NR_171607.1	100	99.60
				A. flatoxiformans NR_171606.1	100	99.60
				A. oryzae NR_135395.1	100	99.40

AMr: *A. muricata* root, AMrb: *A. muricata* root bark, AMf: *A. muricata* fruit, AMs: *A. muricata* seed, AMtf: *A. muricata* thorn of fruit, AMtw: *A. muricata* twigs, AMl: *A. muricata* leaves, AMb: *A. muricata* bark, AMsb: *A. muricata* stem bark, AMpe: *A. muricata* peduncle.

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shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as the evolutionary distances used to infer the phylogenetic tree. With the bootstrap support of each clade over 80, the ML phylogenetic analysis showed that the seventeen endophytic fungi belonged to six genera from the phylum Ascomycota, including *Aspergillus*, *Curvularia*, *Fusarium*, *Meyerozyma*, *Penicillium*, and *Talaromyces*. Confirming the results from the BLAST search, eleven endophytes (AMf15, AMr10, AMb7, AMs9, AMrb9, AMrb11, AMf4, AMf3, AMtf5, AMrb1, and AMr9) were classified at the genus level. In comparison, only six endophytes could be identified at the species level, including AMf6, AMsb1, AMs3 and AMf1 as *Penicillium citrinum*, AMsb23 as *Talaromyces annesophieae* and AMtw3 as *Fusarium*

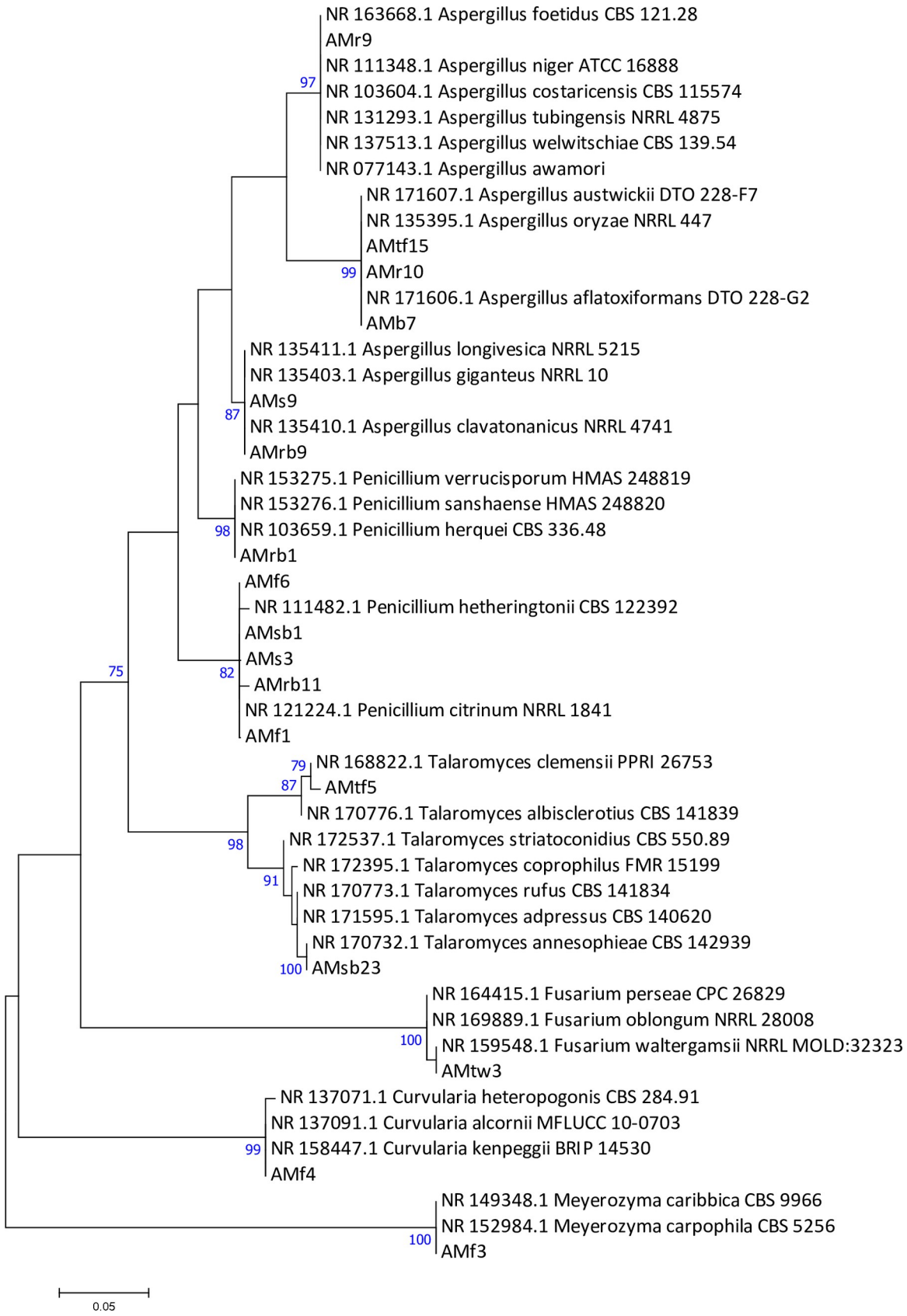
NR 163668.1 Aspergillus foetidus CBS 121.28
AMr9
NR 111348.1 Aspergillus niger ATCC 16888
97
NR 103604.1 Aspergillus costaricensis CBS 115574
NR 131293.1 Aspergillus tubingensis NRRL 4875
NR 137513.1 Aspergillus welwitschiae CBS 139.54
NR 077143.1 Aspergillus awamori
NR 171607.1 Aspergillus austwickii DTO 228-F7
NR 135395.1 Aspergillus oryzae NRRL 447
AMtf15
99
AMr10
NR 171606.1 Aspergillus aflatoxiformans DTO 228-G2
AMb7
NR 135411.1 Aspergillus longivesica NRRL 5215
NR 135403.1 Aspergillus giganteus NRRL 10
AMs9
87
NR 135410.1 Aspergillus clavatonanicus NRRL 4741
AMrb9
NR 153275.1 Penicillium verrucisporum HMAS 248819
NR 153276.1 Penicillium sanshaense HMAS 248820
98
NR 103659.1 Penicillium herquei CBS 336.48
AMrb1
AMf6
NR 111482.1 Penicillium hetheringtonii CBS 122392
AMsb1
AMs3
75
82
AMrb11
NR 121224.1 Penicillium citrinum NRRL 1841
AMf1
79
NR 168822.1 Talaromyces clemensii PPRI 26753
87
AMtf5
NR 170776.1 Talaromyces albisclerotius CBS 141839
98
NR 172537.1 Talaromyces striatoconidius CBS 550.89
NR 172395.1 Talaromyces coprophilus FMR 15199
91
NR 170773.1 Talaromyces rufus CBS 141834
NR 171595.1 Talaromyces adpressus CBS 140620
NR 170732.1 Talaromyces annesophieae CBS 142939
100
AMsb23
NR 164415.1 Fusarium perseae CPC 26829
NR 169889.1 Fusarium oblongum NRRL 28008
100
NR 159548.1 Fusarium waltergamsii NRRL MOLD:32323
AMtw3
NR 137071.1 Curvularia heteropogonis CBS 284.91
NR 137091.1 Curvularia alcornii MFLUCC 10-0703
99
NR 158447.1 Curvularia kenpeggii BRIP 14530
AMf4
NR 149348.1 Meyerozyma caribbica CBS 9966
NR 152984.1 Meyerozyma carpophila CBS 5256
100
AMf3
0.05

Fig 1. Molecular phylogenetic tree generated by Maximum Likelihood analysis based on ITS sequence alignments of endophytic fungal isolates. ML bootstrap support values (ML ≥ 70%) are shown at the nodes. Isolates from *Annona muricata* are coded. The scale bar indicates 0.05 expected changes per site. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model. The tree with the highest log likelihood (-1028.13) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying the Maximum Parsimony method. A discrete Gamma distribution was used to model evolutionary rate differences among sites (2 categories (+*G*, parameter = 0.4638)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 49 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 221 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

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waltergamsii. Overall, fungal species from the *Aspergillus* genus were identified in five organs: root (2), bark, seed, root bark, and thorn of fruits. *Penicillium* species were identified among fungi isolated from fruits (2), root bark (2), stem bark, and seeds. *Talaromyces* species were identified in the thorns of fruits and stembarks, while *Myerozyma*, *Fusarium*, and *Curvularia* were identified only in fruits, twigs, and fruits, respectively. Overall, the 17 selected fungi were mainly dominated by *Penicillium* and *Aspergillus*, accounting for 35.29% of the total.

Minimum inhibition concentrations (MICs) and cytotoxic concentrations 50 (CC_{50}) of selected extracts

The 17 selected crude extracts were submitted to a dose–response study for MIC determination, and the results are summarized in Table 2. All extracts exhibited activity against at least one of the tested bacteria, with MIC values ranging from 3.125 to 100 μg/mL depending on the extracts and microorganisms. Extract from *F. waltergamsii* AMtw3 (3.125 μg/mL) was the most active against *Staphylococcus aureus* ATCC 43300, followed by *Aspergillus* sp. AMtf15, *P. citrinum* AMf6 and *P. citrinum* AMs3 (MIC 9.375 μg/mL). When tested against *K. oxytoca*, extracts *F. waltergamsii* AMtw3 (MIC 3.125 μg/mL) and *P. citrinum* AMsb1 (MIC 3.125 μg/mL) were the most active, followed by *P. citrinum* AMf6 (MIC 4.687 μg/mL). Against *E. coli* ATCC 25922, *F. waltergamsii* AMtw3 (MIC 6.25 μg/mL) exhibited the best activity, followed by *P. citrinum* AMf6 and *P. citrinum* AMsb1 (MIC 9.375 μg/mL).

The results from the dose–response study also revealed that extracts produced by endophytes from the same genus and isolated from the same or different organs displayed very different potency and activity profiles. For instance, *Aspergillus* sp. AMr10 was inactive (MIC > 100 μg/mL) against *K. oxytoca* and *E. coli* ATCC 25922 and displayed weak activity against *S. aureus* ATCC 43300 (MIC 100 μg/mL), while *Aspergillus* sp. AMr9 isolated from the same organ (root) was very active against the three bacteria (MIC 12.5 μg/mL). The extract from *P. citrinum* AMsb1 from stem bark was four times more potent against *K. oxytoca* (MIC 3.125 μg/mL) than the extract produced by *P. citrinum* AMs3 isolated from seeds (MIC 12.5 μg/mL). Conversely, *Aspergillus* spp. AMr9, AMrb9, and AMs9 isolated from roots, root bark, and seeds displayed a similar activity profile against the three pathogens (Table 2). In addition to their antibacterial activity, all selected extracts displayed weak cytotoxicity against Vero cells ATCC CRL1586 with a median cytotoxic concentration (CC_{50}) greater than 100 μg/mL. Extracts from *F. waltergamsii* AMtw3, *P. citrinum* AMf6, *Aspergillus* sp. AMtf15, *Curvularia* sp. AMf4 and *T. annesophieae* AMsb23, produced by fungi from different genera, were selected to investigate their potential antibacterial mode of action.

Modes of action of promising extracts

Effect of extracts on catalase activity. The inhibition of the catalase activity of *S. aureus* ATCC 43300 and *K. oxytoca* was evaluated by comparing the amount of H_2O_2 remaining

Table 2. Yields (mg), minimum inhibitory concentrations (MICs) and cytotoxic concentrations 50 (CC_{50}) of selected extracts.

Plant organs	Fungal Extracts	Yield (mg)	MIC (μg/mL ± SD)			CC_{50} (μg/mL ± SD)
			***S. aureus* ATCC 43300**	***K. oxytoca***	***E. coli* ATCC 25922**	**Vero cells ATCC CRL1586**
Roots	*Aspergillus* sp. AMr9	195	12.5±0.00	12.5±0.00	12.5±0.00	> 100
	Aspergillus sp. AMr10	12	100±0.00	> 100	> 100	> 100
Root bark	*Penicillium* sp. AMrb1	95	18.75±8.83	12.5±0.00	12.5±0.00	> 100
	Aspergillus sp. AMrb9	258	12.5±0.00	12.5±0.00	12.5±0.00	> 100
	Penicillium sp. AMrb11	61	12.5±0.00	12.5±0.00	12.5±0.00	> 100
Fruits	*Penicillium citrinum* AMf6	22	9.375±4.19	4.687±2.20	9.375±4.41	> 100
	Meyerozyma sp. AMf3	67	50±0.00	25±0.00	37.5±17.67	> 100
	Curvularia sp. AMf4	216	12.5±0.00	9.375±4.41	12.5±0.00	> 100
	Penicillium citrinum AMf1	126	12.5±0.00	9.375±4.41	12.5±0.00	> 100
Seeds	*Penicillium citrinum* AMs3	264	9.375±4.19	12.5±0.00	25±0.00	> 100
	Aspergillus sp. AMs9	139	12.5±0.00	12.5±0.00	18.75±8.83	> 100
Thorns of fruit	*Aspergillus* sp. AMtf15	56	9.375±4.41	6.25±0.00	12.5±0.00	> 100
	Talaromyces sp. AMtf5	95	18.75±8.83	6.25±0.00	12.5±0.00	> 100
Twigs	*Fusarium waltergamsii* AMtw3	48	3.125±0.00	3.125±0.00	6.25±0.00	> 100
Stem bark	*Talaromyces annesophieae* AMsb23	25	12.5±0.00	6.25±0.00	18.75±8.83	> 100
	Penicillium citrinum AMsb1	194	12.5±0.00	3.125±0.00	9.375±4.41	> 100
Barks	*Aspergillus* sp. AMb7	24	12.5±0.00	6.25±0.00	18.75±8.83	> 100
Ciprofloxacin		NA	0.468±0.00	0.234±0.00	0.234±0.00	NA
Podophyllotoxin		NA	NA	NA	NA	0.177±0.05

AMr: *A. muricata* root; AMrb: *A. muricata* root bark; AMf: *A. muricata* fruit; AMs: *A. muricata* seed; AMtf: *A. muricata* thorn of fruit; AMtw: *A. muricata* twigs; AMb: *A. muricata* bark; AMsb: *A. muricata* stem bark; CC_{50}: Cytotoxic concentrations 50; MIC: Minimum inhibitory concentration; NA: Not applicable.

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in the medium after the addition of fungal extracts to the control (Table 3). The percentages of remaining H_2O_2 in bacterial cultures treated with extracts ranged from 49.17–54.58% and 46.99–55.70% for *S. aureus* ATCC 43300 and *K. oxytoca*, respectively, highlighting the ability of the tested extracts to exert a certain degree of inhibition against the activity of the bacterial catalase enzyme. The catalase inhibition activity exhibited by all fungal extracts was comparable to that of the positive control, ciprofloxacin, since no statistically significant difference was observed ($p>0.05$).

Table 3. Percentage of remaining H_2O_2 after evaluation of the effect of endophytic fungal extracts on catalase activity of *S. aureus* ATCC 43300 and *K. oxytoca* at the MIC concentration.

	Remaining H_2O_2 (%) ± SD	
Fungal extracts	***K. oxytoca***	***S. aureus* ATCC 43300**
***F.waltergamsii* AMtw3**	52.20±1.67^{ab}	51.66±1.50^{a}
Aspergillus sp. AMtf15	55.70±0.00^{a}	51.84±3.77^{a}
***P. citrinum* AMf6**	51.61±3.15^{ab}	54.58±2.36^{a}
***Curvularia* sp AMf4**	53.68±0.95^{ab}	50.23±4.31^{a}
***T.annesophieae* AMsb23**	46.99±1.78^{b}	49.17±3.53^{a}
Ciprofloxacin	54.12±2.89^{ab}	53.59±2.70^{a}

Data are expressed as the mean±SD. Along the column, values carrying the same letter superscripts are not significantly different ($p>0.05$), and values carrying different letters are significantly different ($P<0.05$). AMf: *A. muricata* fruit, AMtf: *A. muricata* thorn of fruit, AMtw: *A. muricata* twigs, AMsb: *A. muricata* stem bark.

https://doi.org/10.1371/journal.pone.0267246.t003

Lytic effect of extracts

The bacteriolysis assay was performed to investigate if active extracts inhibit *S. aureus* ATCC 43300 and *K. oxytoca* through cell lysis. Because viable bacteria absorb light at 620 nm, any decrease in optical density at this wavelength could be used as an indication of bacteriolysis. The cell lysis activity exhibited by fungal extracts is summarized in Fig 2. Globally, the treatment of the bacterial cells with fungal extracts (AMtw3, AMtf15, AMf6, AMf4, and AMsb23) caused cell lysis at all tested concentrations significantly after 4 h of incubation. Extracts from *F. waltergamsii* AMtw3 (70.95%, 62.25%) and *Aspergillus* sp. AMtf15 (61.9%, 54.01%) exhibited the highest bacteriolytic activity at 4 MIC against *K. oxytoca* and *S. aureus*. The reduction in the bacterial population was more pronounced against *K. oxytoca*, with relative absorbance percentages varying from 37.75–100%.

Effect of extracts on the permeability of the outer cell membrane. The capacity of endophytic extracts to permeabilise the outer cell membrane was determined throughout 24 h at seven different concentrations. Fig 3 shows that the permeabilisation activity of extracts was concentration dependent. Overall, all extracts resulted in a decrease in the optical density with increasing concentrations of extracts. This trend, therefore, could indicate the loss of intracellular ingredients by the chelation of divalent cations. It can also be noted that the destabilization power of the outer membrane of both bacteria by the different extracts was generally not significantly different ($p>0.05$) from that of ciprofloxacin at their corresponding concentrations.

Bacterial growth curves. The growth kinetics of bacterial strains exposed to three different concentrations (MIC, 2MIC and 4MIC values) of extracts for 24 h were compared to bacterial growth without antibiotic treatment (Fig 4). As revealed by the optical densities at 620 nm, all extracts at all tested concentrations significantly inhibited the growth of the two bacterial pathogens compared to the negative control. Globally, although there was an initial increase in the number of viable cells during the first 2–3 hours of incubation, a gradual decrease in the bacterial population was noted from the 3rd to the 24th hour depending on the extracts and the microorganism (Fig 4). The maximum bactericidal effect was noted after 24 h of incubation, with a decrease in the bacterial population by 1.78–7.42 times for *S. aureus* ATCC 43300 and 1.80–7.76 times for *K. oxytoca*. The extract from *F. waltergamsii* AMtw3 was the most efficient in killing *K. oxytoca* at all tested concentrations, with 5.80-, 6.14- and 7.76-fold reductions in the bacterial population at MIC, 2 MIC and 4 MIC, respectively. Against *S. aureus* ATCC 43300, the extract from *T. annesophieae* AMsb23 with 4.49-, 4.56-, and 7.42-fold reductions in the bacterial population at MIC, 2 MIC, and 4 MIC, respectively, was the most active. The activity of the most active extracts against both pathogens was significantly higher ($p\leq0.05$) than that of the positive control (ciprofloxacin) at MIC, which is known to exert a bactericidal effect (S3 Table in S1 File).

Effect of endophytic extracts on biofilm formation. *Biofilm quantification*. Biofilm production by *K. oxytoca* and *S. aureus* ATCC 43300 in NB medium supplemented with 2% glucose after 48 hours of incubation was expressed in terms of the mean fluorescence values from independent assays performed in triplicate. Fig 5 shows that after 48 hours of incubation, both *K. oxytoca* and *S. aureus* ATCC 43300 could form a significant amount of biofilm materialised by the mean relative fluorescence units (RFU) (λ_{Ex} 530 nm and λ_{Em} 590 nm) from two independent assays over 10000. There was no significant difference ($P\leq0.05$) between the amounts of biofilm formed by the two bacteria.

Biofilm formation inhibition

Bacterial biofilms play a significantly important role in urinary tract infections (UTIs), which are responsible for persistent infections causing relapses and acute prostatitis [28]. Bacterial

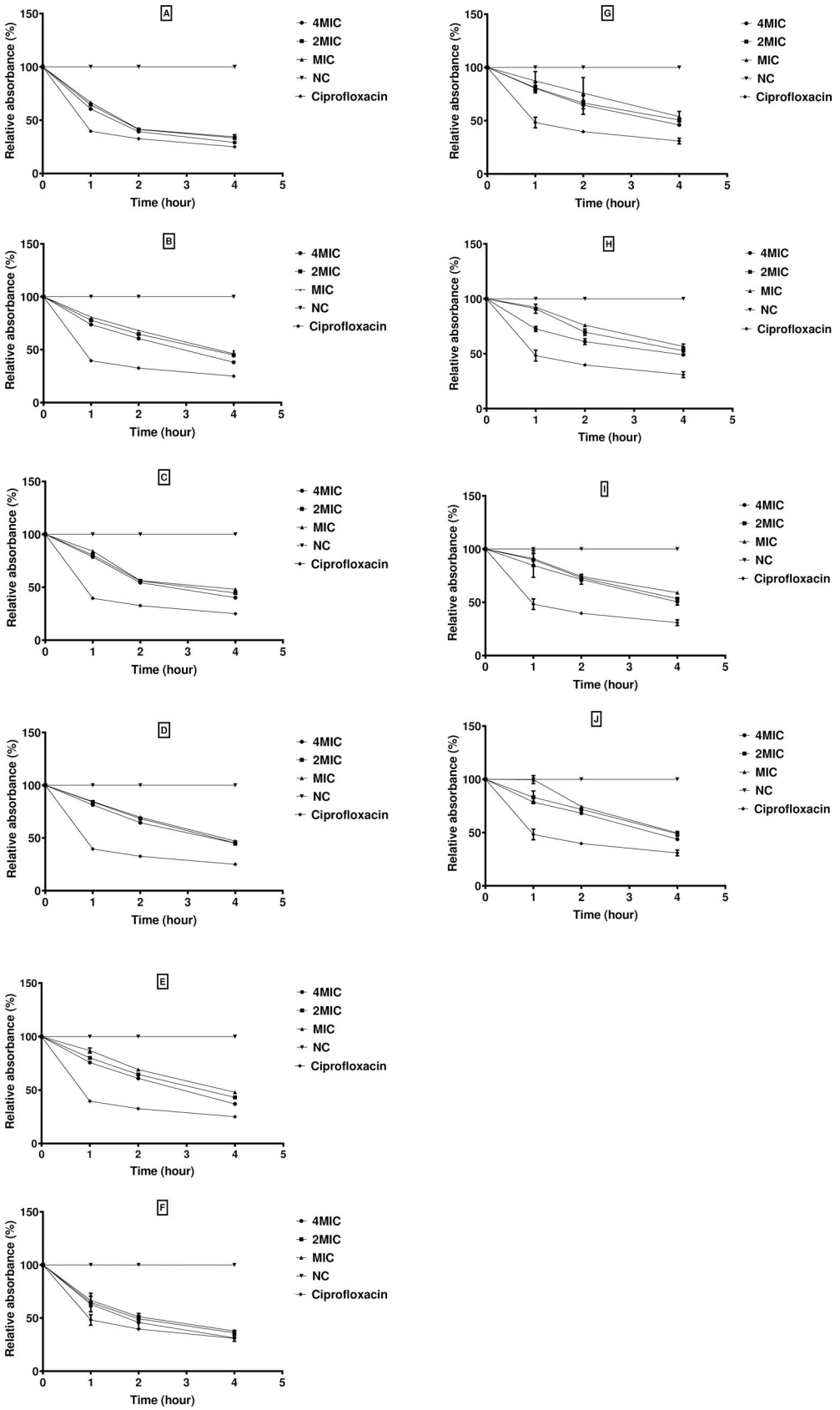
A
B
C
D
E
F
G
H
I
J
Relative absorbance (%)
Time (hour)
4MIC
2MIC
MIC
NC
Ciprofloxacin

Fig 2. The bacteriolytic activity of endophytic fungi extracts on *S. aureus* ATCC 43300, and *K. oxytoca*. (A) AMtw3 on *K.oxytoca*; B) AMtf15 on *K. oxytoca*; (C) AMf6 on *K. oxytoca*; (D) AMf4 on *K. oxytoca*; (E) AMsb23 on *K. oxytoca*; (F) AMtw3 on *S. aureus*; (G) AMtf15 on *S. aureus*, (H) AMf6 on *S. aureus*; (I) AMf4 on *S. aureus*; (J) AMsb23 on *S. aureus*. AMf: *A. muricata* fruit, AMtf: *A. muricata* thorn of fruit, AMtw: *A. muricata* twigs, AMsb: *A. muricata* stem bark, MIC: Minimal Inhibitory Concentration, NC: Negative Control. Data are expressed as the mean±SD.

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biofilm formation is difficult to eradicate due to several factors, including persistent cells showing reduced metabolism that leads to higher levels of antimicrobial resistance and the natural resistance conferred to bacteria by the biofilm structure [28]. Therefore, the search for new therapeutic agents is necessary. In this respect, the selected active extracts were also evaluated for their potential to inhibit biofilm formation by *S. aureus* ATCC 43300 and *K. oxytoca*. Determining the inhibitory concentration 50 (IC_{50}), defined as the concentration of extract required to reduce biofilm formation by half, revealed that extracts exhibited different degrees of inhibition against both bacterial pathogens (Table 4). The IC_{50} values ranged from 0.25–11.86 μg/mL against *K. oxytoca* and 0.36–11.08 μg/mL depending on the extract and the microorganism. The extract from *F. waltergamsii* AMtw3 was consistently the most potent against the two bacteria (IC_{50} 0.24 and 0.36 μg/mL), followed by *Aspergillus* sp. AMtf15 ($IC_{50,}$ 0.73 and 1.903 μg/mL). *K. oxytoca* was more sensitive than *S. aureus*. Extracts from AMf6, AMtf15, AMtw3, and AMsb23 were 1.81, 2.60, 1.44 and 1.27 times more potent against biofilm formation by *K. oxytoca* than *S. aureus*.

Antioxidant activity of fungal extracts

DPPH radical scavenging activity. The ability of the 17 antibacterial extracts to scavenge the free radical DPPH was measured at an absorbance of 517 nm. From the results, the radical scavenging activity of the tested extracts ranged from 146.05 to > 1000 μg/mL. Globally, among the 17 extracts tested, only 7 showed radical scavenging activity 50 (RSA_{50}) at less than 1000 μg/mL (Table 5). Although their activity was significantly lower ($p \leq 0.05$) than that of ascorbic acid used as a standard (RSA_{50} 8.92 μg/mL), the extract from *T. clemensii* AMtf5 exhibited the highest scavenging activity (RSA_{50} 146.05 μg/mL), followed by an extract from *Aspergillus* sp. AMr9 (RSA_{50} 176.9 μg/mL) obtained from the thorns of fruits and roots of *A. muricata*, respectively. Interestingly, although *Aspergillus* spp. from roots displayed good potency, another *Aspergillus* isolate from the thorn of fruits was weakly active (RSA_{50} 663.35 μg/mL). Similarly, while the extract from *P. citrinum* AMsb1 from stem bark was potent (RSA_{50} 211.30 μg/mL), the *P. citrinum* AMf6 from fruits was the less active (RSA_{50} 799.75 μg/mL) of the tested extracts. The same difference in activity could be observed with the *Talaromyces* species from the thorn of fruits (RSA_{50} 146.05 μg/mL) and stem bark (RSA_{50} 536.15 μg/mL). These observations could suggest that the organ of isolation of endophytic isolates could substantially influence the potency of each microbial species.

Reduction of Fe^{3+} ions by ortho-phenanthroline. The seventeen extracts were also screened for their ability to reduce Fe^{3+} to Fe^{2+}. The results from Table 6 show a significant correlation between the concentration of the extracts and their reducing power. By projecting the optical densities of the extracts on a concentration-activity curve of NH_2OH used as standard, the results showed that only extracts from five endophytic fungi, including AMtf15, AMf6, AMs3, AMf4 and AMf1, exhibited activity (12.03, 12.28, 11.97, 12.35 and 12.37 μg equivalent NH_2OH/g, respectively) at 1000 μg/mL. Moreover, at concentrations of 1000, 500, 250 and 125 μg/mL, the ferric ion reducing capacities of all five extracts were not significantly different ($P > 0.05$).

Out of the seventeen extracts screened for antioxidant activity, extracts from *Aspergillus* sp. AMtf15 and *P. citrinum* AMf6 exhibited DPPH radical scavenging and ferric ion reducing

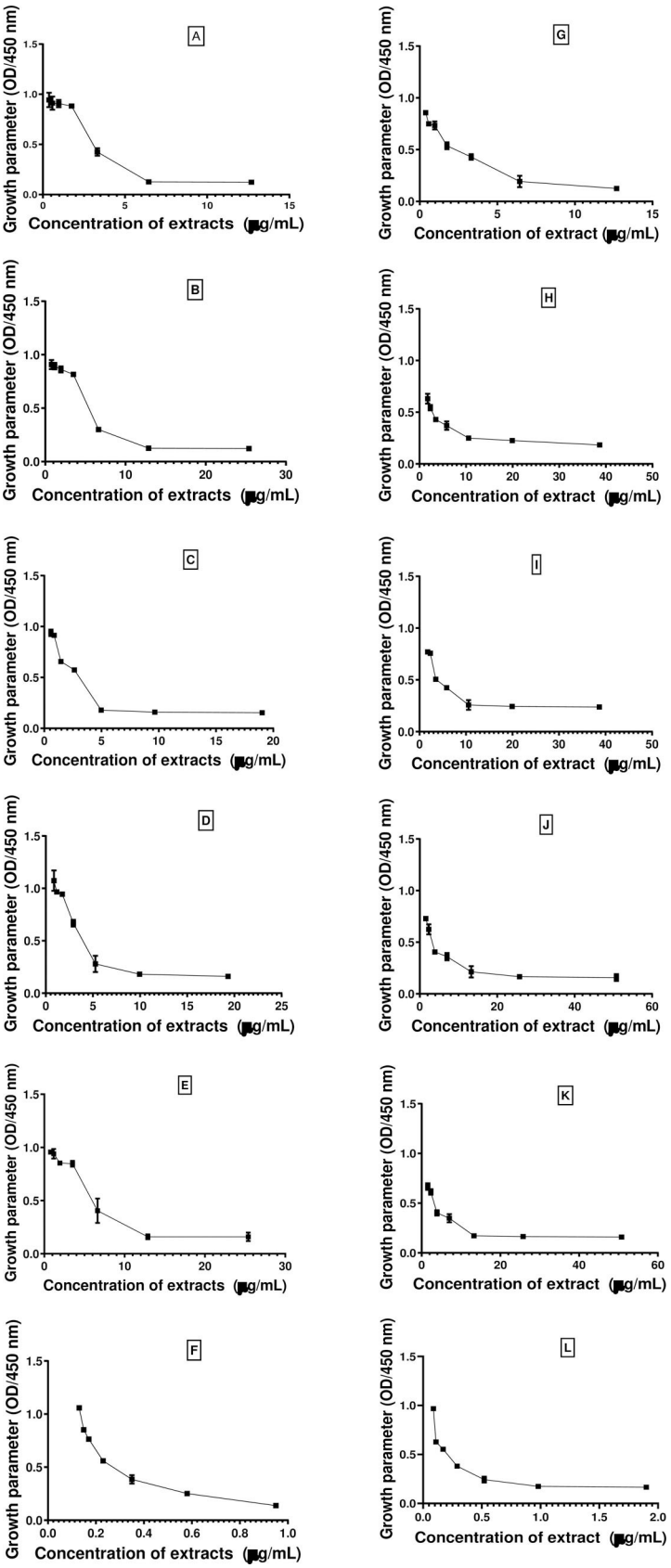
A
Growth parameter (OD/450 nm)
Concentration of extracts (µg/mL)
G
Growth parameter (OD/450 nm)
Concentration of extract (µg/mL)
B
Growth parameter (OD/450 nm)
Concentration of extracts (µg/mL)
H
Growth parameter (OD/450 nm)
Concentration of extract (µg/mL)
C
Growth parameter (OD/450 nm)
Concentration of extracts (µg/mL)
I
Growth parameter (OD/450 nm)
Concentration of extract (µg/mL)
D
Growth parameter (OD/450 nm)
Concentration of extracts (µg/mL)
J
Growth parameter (OD/450 nm)
Concentration of extract (µg/mL)
E
Growth parameter (OD/450 nm)
Concentration of extracts (µg/mL)
K
Growth parameter (OD/450 nm)
Concentration of extract (µg/mL)
F
Growth parameter (OD/450 nm)
Concentration of extracts (µg/mL)
L
Growth parameter (OD/450 nm)
Concentration of extract (µg/mL)

Fig 3. Effect of endophytic fungi extracts on the outer membrane permeability of *S. aureus* ATCC 43300 and *K. oxytoca*. (A) AMtw3 on *K. oxytoca*. B) AMtf15 on *K. oxytoca*; (C) AMf6 on *K. oxytoca*; (D) AMf4 on *K. oxytoca*; (E) AMsb23 on *K. oxytoca*; (F) Ciprofoxacin on *K. oxytoca*; (G) AMtw3 on *S. aureus*; (H) AMtf15 on *Staphylococcus aureus*; (I) AMf6 on *S. aureus*; (J) AMf4 on *S. aureus*; (K) AMsb23 on *S. aureus*; (L) Ciprofloxacin on *S. aureus*. AMf: *A. muricata* fruit, AMtf: *A. muricata* thorn of fruit, AMtw: *A. muricata* twigs, AMsb: *A. muricata* stem bark. Data are expressed as the mean±SD.

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capacities. However, extracts from AMtf5, AMtw3, AMr9, AMsb23 and AMsb1 displayed DPPH radical scavenging activity and very weak ferric ion reducing capacities, while AMs3, AMf4 and AMf1 only showed ferric ion reducing capacities.

Discussion

Antimicrobial resistance is one of the most critical global public health threats of the 21st century. Faced with this reality, the need for action, particularly the search for and development of new antibiotics to avert a developing global health care crisis, is imperative [29]. Natural products, mainly from microbial origins with their widely divergent chemical structures, have been a prolific source and an inspiration for numerous antibiotic agents [30]. Over the last decades, endophytic fungi inhabiting various medicinal plants have gained tremendous attention due to their ability to produce novel bioactive compounds exhibiting various biological properties, including antibacterial properties [12, 15, 16]. Moreover, antibacterial compounds produced by endophytes have been shown to occupy a broad spectrum of structural classes, such as alkaloids, peptides, steroids, terpenoids, phenols, quinines, and flavonoids [31, 32]. The extensive tropical rainforests of Cameroon rich with thousands of medicinal plants can be an excellent bioresource for plant-associated microorganisms with the potential to produce novel and highly potent antimicrobial compounds [18, 23]. Therefore, the current study was designed to investigate the antibacterial and antioxidant potential of extracts from endophytic fungi associated with *Annona muricata*, a plant traditionally used in Cameroon to treat microbial infections.

Crude ethyl acetate extracts from forty-one (41) endophytic fungi isolated from distinct organs of *A. muricata* were screened for their ability to inhibit *Escherichia coli* ATCC 25922, *Klebsiella oxytoca*, and *Staphylococcus aureus* ATCC 43300. Of the 41 extracts tested, 17 (41.46%) exhibited activity against at least one bacteria. Our previous screening of 56 extracts from endophytic fungi isolated from the three Cameroonian medicinal plants, *Terminalia mantaly*, *Terminalia catappa*, and *Cananga odorata*, against seven bacterial strains revealed that approximately 13% were very active against all tested bacterial strains [23]. Another previous screening of 152 extracts from endophytic fungi from *A. muricata* against *Plasmodium* parasites revealed that over 17% of isolates exhibited activity [18]. These results indicate that many endophytes inhabiting Cameroonian plants could produce active metabolites to inhibit various human pathogenic microbes. The seventeen active endophytes identified by sequencing their ITS1-5.8S rRNA-ITS2 region belonged to the *Aspergillus*, *Curvularia*, *Fusarium*, *Meyerozyma*, *Penicillium*, and *Talaromyces* genera. Among them, 11 (64.70%) were identified at the genus level, while only six (35.29%) were identified at the species level. Given the inability of the ITS sequence to give precise identification of our endophytic isolates, further analysis, such as the sequencing of additional genes followed by multilocus phylogenetic analysis, will be conducted to achieve the precise identification of these isolates [33–35]. However, the great diversity of fungal genera identified from the present study indicates that *A. muricata* hosts a large group of microorganisms capable of producing antibacterial metabolites. The extracts from the seventeen identified isolates were submitted to a dose–response study for MIC determination. All extracts exhibited activity against all tested bacteria, with MIC values ranging

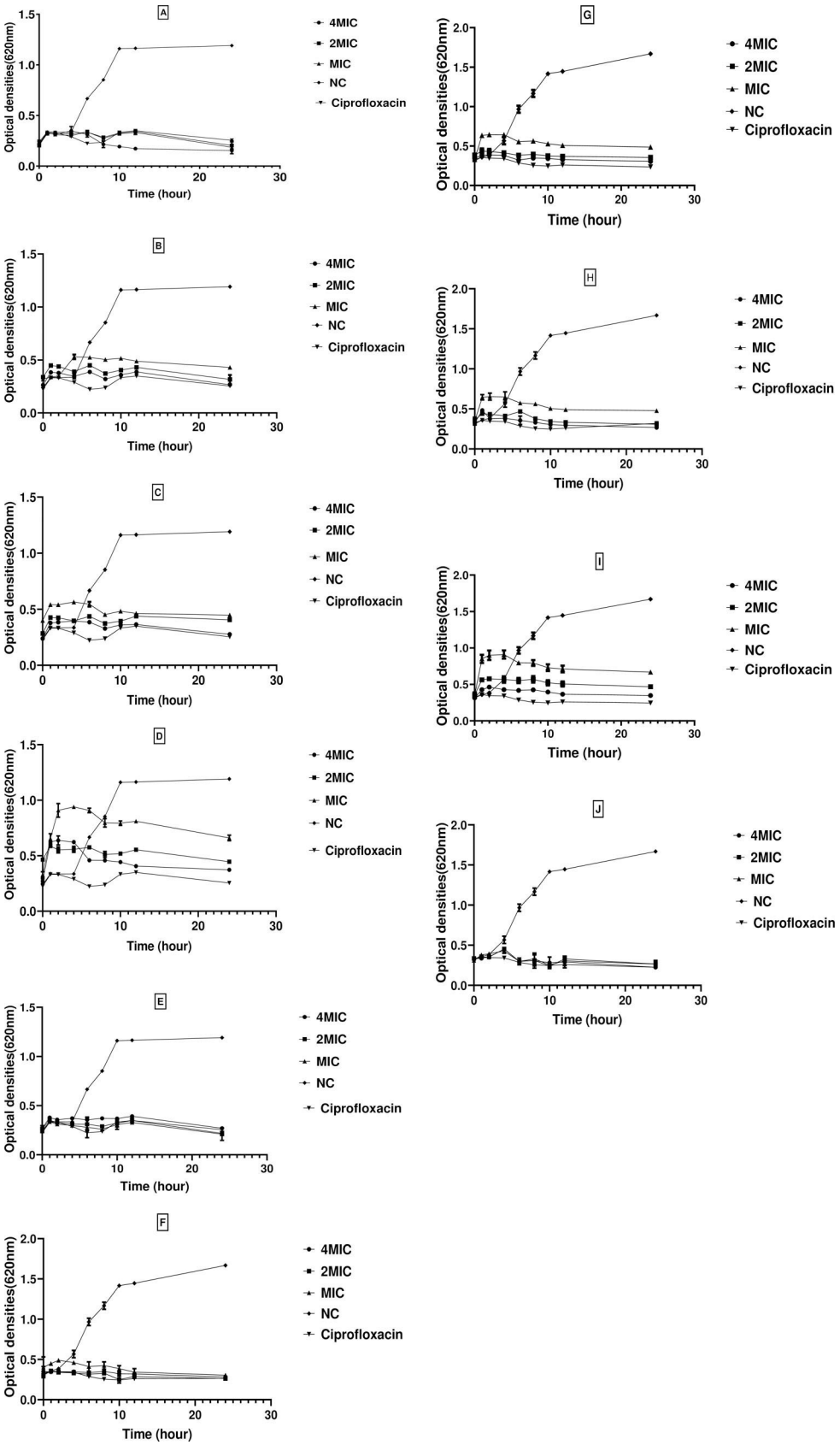
A
B
C
D
E
F
G
H
I
J
Optical densities(620nm)
Time (hour)
4MIC
2MIC
MIC
NC
Ciprofloxacin

Fig 4. Activity kinetics of extracts on *S. aureus* ATCC 43300 and *K. oxytoca*. (A) AMtw3 on *K. oxytoca*. B) AMtf15 on *K. oxytoca*; (C) AMf6 on *K. oxytoca*; (D) AMf4 on *K. oxytoca*; (E) AMsb23 on *K. oxytoca*; (F) AMtw3 on *S. aureus*; (G) AMtf15 on *S.aureus* ATCC; (H) AMf6 on *S. aureus*; (I) AMf4 on *S. aureus*; (J) AMsb23 *on S. aureus*. AMf: *A. muricata* fruit, AMtf: *A. muricata* thorn of fruit, AMtw: *A. muricata* twigs, AMsb: *A. muricata* stem bark, MIC: Minimal Inhibitory Concentration; NC: Negative Control. Data are expressed as the mean±SD.

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from 3.125 to 100 μg/mL depending on the extracts and microorganisms. The most active extracts against the three bacterial pathogens were consistently from the *Fusarium* and *Penicillium* genera, with *F. waltergamsii* AMtw3 being the most active, followed by *P. citrinum* AMf6. These findings agree with previous studies reporting the potential of endophytic fungi belonging to these genera as sources of active antimicrobial metabolites [15, 16]. Fortunately, these active endophyte extracts were noncytotoxic against Vero cells at concentrations as high as 100 μg/mL using the MTT colorimetric method. These results suggest that the antibacterial activity of extracts reported in the present study could not be related to the toxicity effect; therefore, antibacterial compounds produced by these endophytes could have good selectivity against mammalian cells. On the other hand, crude ethyl acetate extracts from endophytes belonging to these genera and inhabiting other Cameroonian medicinal plants were previously reported for their weak cytotoxicity against normal cell lines such as HEK239T mammalian

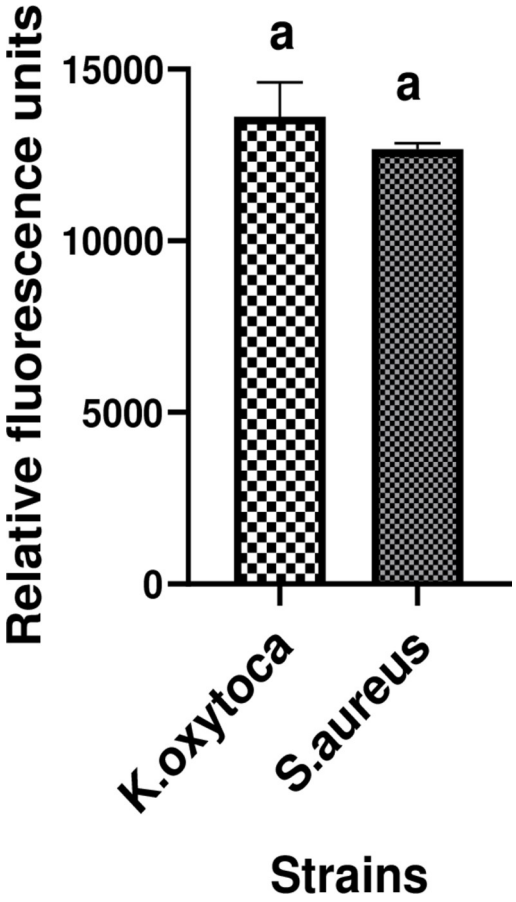

Fig 5. Biomass of biofilms produced by K.oxytoca and S.aureus ATCC 43300 after 48hours in Nutrient broth (NB) medium supplemented with 2% glucose. The experiment was performed twice in triplicate, and data are expressed as the mean±SD. Values with the same letter express no significant difference at $p>0.05$.

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Table 4. Inhibitory concentration 50 (IC_{50}) values of endophytic fungal extracts against *K. oxytoca* and *S. aureus* biofilm formation.

Fungal extracts	IC_{50} (μg/mL± SD)	
	K. oxytoca	*S. aureus* ATCC 43300
***F. waltergamsii* AMtw3**	0.25±0.02[a]	0.36±0.00[a]
***Aspergillus* sp. AMtf15**	0.73±0.09[a]	1.90±0.38[b]
***P. citrinum* AMf6**	3.52±0.36[b]	6.39±0.13[c]
***Curvularia* sp. AMf4**	11.68±0.17[c]	11.08±0.73[d]
***T. annesophieae* AMsb23**	3.91±0.69[b]	4.98±0.03[e]
Ciprofloxacin	0.03±0.00[a]	0.07±0.00[a]

AMf: *A. muricata* fruit, AMtf: *A. muricata* thorn of fruit, AMtw: *A. muricata* twigs, AMsb: *A. muricata* stem bark, IC_{50}: Inhibitory Concentration 50; along the column, values carrying the same letter superscripts are not significantly different ($p>0.05$), and values carrying different letters are significantly different ($P<0.05$). The experiment was performed twice in duplicate.

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cells [18, 36, 37]. However, this noncytotoxicity of crude metabolites produced by these endophytes could also be related to the culture conditions. We previously found that extracts from *Fusarium* sp. N240 and *Xylaria* sp. N120 cultured in potato dextrose broth were cytotoxic against HEK239T cells but were nontoxic when the fungi were grown in Czapek Dox medium [37].

Fungal extracts can exert their antimicrobial effect on bacteria via one or various mechanisms of action. Therefore, several model assays were used to investigate the mode of antibacterial action of the five most potent extracts (AMtw3, AMtf15, AMf6, AMf4, and AMsb23) against *S. aureus* ATCC43300 and *K. oxytoca*. The five extracts inhibited the production of catalase by both pathogens. The ability of bacteria to produce catalase contributes to their pathogenicity by detoxifying the oxygen-dependent microbicidal products of phagocytic cells. The large concentration of H_2O_2 may overwhelm an organism's defenses and may prove fatal to microorganisms [38]. Therefore, the loss of the ability to produce catalase by *S. aureus* and *K. oxytoca* indicates their inability to deactivate antimicrobials by catalase production [39]. These five extracts also demonstrated their ability to affect both the inner and outer membranes of bacteria. The results from the bacteriolysis assay indicate that extracts cause gross membrane

Table 5. DPPH radical scavenging parameters of the seven active endophytic fungal extracts.

Extracts	RSA_{50} (μg/mL± SD)	CE_{50}	ARP
***Aspergillus* sp. AMtf15**	663.35±4.73[a]	3.31 x 10^{4a}	3.02 x 10^{-5a}
***T. clemensii* AMtf5**	146.05±4.31[b]	0.73 x 10^{4b}	13.69 x 10^{-5b}
***P. citrinum* AMf6**	799.75±11.66[c]	3.99 x 10^{4c}	2.50 x 10^{-5c}
***F. waltergamsii* AMtw3**	282.30±0.98[d]	1.41 x 10^{4d}	7.09 x 10^{-5d}
***Aspergillus* sp AMr9**	176.90±0.84[e]	0.88 x 10^{4e}	11.36 x 10^{-5e}
***T. annesophieae* AMsb23**	536.15±38.39[f]	2.68 x 10^{4f}	3.73 x 10^{-5e}
***P.citrinum* AMsb1**	211.30±1.13[g]	1.05 x 10^{4g}	9.52 x 10^{-5f}
Vitamin C	8.92±1.065[h]	0.0446 x 10^{4h}	224.21 x 10^{-5h}

RSA_{50}: radical scavenging activity 50, EC_{50}: efficient concentration 50, ARP: antiradical power, NA: not applicable, AMr: *A. muricata* root, AMf: *A. muricata* fruit, AMtf: *A. muricata* thorn of fruit, AMtw: *A. muricata* twigs, AMsb: *A. muricata* stem bark. Along the column, values carrying the same letter superscripts are not significantly different ($p>0.05$), and values carrying different letters are significantly different ($P<0.05$).

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Table 6. Quantitative evaluation of Fe^{3+} reducing power by endophytic extracts of *A. muricata*.

Code of extracts	µg equivalent NH_2OH/g of extract± SD						
Concentrations (µg/mL)	**1000**	**500**	**250**	**125**	**62.5**	**31.25**	**15.625**
***Aspergillus* sp. AMtf15**	12.03±0.59^{a}	11.46±0.16^{ab}	11.45±0.40^{abc}	11.36±0.34^{bc}	7.96±0.20^{d}	7.65±0.07^{de}	7.28±0.20^{de}
***Penicillium* sp AMrb1**	2.98±0.10^{g}	1.99±0.06^{f}	1.45±0.01^{e}	1.06±0.02^{a}	0.91±00^{ab}	0.79±0.2^{bc}	0.68±0.11^{bc}
***P. citrinum* AMf6**	12.28±0.36^{a}	11.88±0.12^{ab}	11.77±0.43^{abc}	11.72±0.38^{abc}	8.25±0.50^{d}	7.88±0.09^{de}	7.70±0.07^{de}
***P.citrinum* AMs3**	11.97±0.36^{a}	11.70±0.16^{ab}	11.57±0.16^{abc}	11.48±0.06^{abc}	7.84±0.09^{e}	4.12±0.35^{d}	3.76±0.43^{d}
***Meyerozyma* sp.AMf3**	1.06±0.00^{a}	0.95±0.08^{ab}	0.84±0.03^{abc}	0.83±0.06^{abcd}	0.82±0.06^{abcde}	0.79±0.10^{abcdef}	0.67±0.01^{abcdef}
***Penicillium* sp. AMrb11**	1.06±0.02^{a}	0.87±0.06^{ab}	0.82±0.19^{abc}	0.79±0.11^{ab}c	0.74±0.21^{abcde}	0.74±0.16^{abcde}	0.65±0.03^{abcde}
***Curvularia* sp.AMf4**	12.35±0.42^{a}	11.74±0.25^{ab}	11.53±1.06^{abc}	11.30±0.74^{abcd}	11.15±0.95^{abcde}	10.28±0.00^{abcdef}	8.47±0.43^{f}
***Talaromyces* sp.AMtf5**	3.92±0.18^{e}	2.67±0.08^{d}	1.76±0.08^{c}	1.26±0.03^{a}	0.95±0.07^{ab}	0.84±0.02^{b}	0.81±0.13^{b}
***Aspergillus* sp.AMb7**	1.10±0.00^{a}	1.08±0.01^{ab}	0.87±0.02^{abc}	0.80±0.02^{abcd}	0.76±0.00^{bcde}	0.73±0.06^{cdef}	0.69±0.20^{cdef}
***Aspergillus* sp AMsb23**	4.47±0.26^{f}	2.86±0.05^{e}	1.71±0.07^{a}	1.32±0.28^{ab}	1.01±0.15^{abc}	0.95±0.20^{bcd}	0.81±0.14^{bcd}
***F. waltergamsii* AMtw3**	2.26±0.00^{e}	1.61±0.01^{d}	1.41±0.02^{a}	1.29±0.06^{a}	1.08±0.06^{b}	1.01±0.01^{b}	0.69±0.02^{c}
***P.citrinum*AMf1**	12.37±0.02^{a}	11.83±0.16^{ab}	11.72±0.25^{abc}	11.70±0.22^{abc}	8.11±0.37^{d}	7.95±0.17^{d}	4.35±0.32^{e}
***Aspergillus* sp AMr9**	3.79±0.19^{f}	2.68±0.12^{e}	1.86±0.00^{d}	1.36±0.09^{a}	1.15±0.05^{ab}	0.89±0.03^{abc}	0.89±0.19^{abc}
***Aspergillus* sp.AMrb9**	1.09±0.01^{e}	0.94±0.00^{d}	0.84±0.03^{a}	0.81±0.00^{a}	0.74±0.01^{b}	0.68±0.00^{bc}	0.67±0.00^{c}
***Aspergillus* sp AMsb1**	4.90±0.26^{e}	3.27±0.13a	2.20±0.09^{a}	1.57±0.03^{ab}	1.2±0.07^{abc}	1.06±0.22^{abcd}	0.79±0.00^{acd}
***Aspergillus* sp.AMr10**	1.43±0.03^{f}	1.20±0.00^{a}	1.16±0.02^{ab}	1.05±0.11^{abc}	0.95±0.02^{bcd}	0.91±0.05^{cde}	0.72±0.02^{e}

AMr: *A. muricata* root, AMrb: *A. muricata* root bark, AMf: *A. muricata* fruit, AMs: *A. muricata* seed, AMtf: *A. muricata* thorn of fruit, AMtw: *A. muricata*, AMb: *A. muricata* bark, AMsb: *A. muricata* stem bark; along the line, values carrying the same letter are not significantly different (P>0.05), and values carrying different letters are significantly different (P<0.05).

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damage and provoke whole-cell lysis. This property of endophytic fungal extracts was previously reported by Mbekou *et al.* [23]. The outer membrane permeability assay results showed a gradual decrease in optical densities with increasing extract concentration, which indicates membrane destabilization. Lethal injury of microbial cell membranes may alter their permeability and affect the membrane's ability to osmoregulate the cell adequately or exclude toxic materials [40]. This antibacterial action can result in membrane expansion, increased membrane fluidity and permeability, disturbance of membrane-embedded proteins, inhibition of respiration, and alterations in the ionic homeostasis between intracellular and extracellular compartments of bacteria, eventually leading to cell death [41].

All five fungal extracts also exert a bactericidal effect on both pathogens as materialised by reducing the bacterial population, as shown by the graph of optical densities versus incubation time. Bactericidal agents are sought after to fight against resistant bacteria because they attack and kill bacteria outright, preventing these cells from causing further damage within the body [42]. Among bacterial therapeutic targets, biofilms remain the main virulence factor contributing to the pathogenesis and resistance of microbial pathogens [4]. Therefore, finding antibiotics with the ability to inhibit both planktonic cells and bacterial biofilm structure is of paramount importance. Our investigation revealed that extracts from AMtw3, AMtf15, AMf6, AMf4, and AMsb23 strongly inhibited *S. aureus* ATCC43300 and *K. oxytoca* biofilm formation (IC_{50} 0.25 to 11.86 µg/mL). The inhibition of biofilm formation by extracts from endophytic fungi has also been reported by Dawande *et al.* [43] and Kaur *et al.* [44]. The antibiofilm-guided investigation of these extracts could identify very potent antibiofilm metabolites, a potential starting point for new drug discovery.

The potential of extracts to prevent oxidative stress resulting from excess nonneutralized reactive species in the body was also investigated through DPPH radical scavenging and FRAP reducing power assays. Antioxidants are tremendously important substances that can protect

the body from damage caused by free radicals. The scavenging activity of the fungal extracts was measured by discoloration to yellow following the formation of a nonradical (2,2-diphenyl-1-hydrazine) molecule [45]. Fungal extracts AMtf15, AMf6, AMtw3, AMr9, AMsb1, AMsb23 and AMf4 exhibited good DPPH radical scavenging activity (RSA_{50} 146.05 to 799.75 μg/mL). The metal chelating capacity may also indicate its potential antioxidant activity [46]. The iron-chelating activity of all fungal extracts was determined by reaction with orthophenanthroline. The results showed that five extracts at the highest concentration tested, including AMtf15, AMf6, AMs3, AMf4, and AMf1, exhibited iron-chelating activity (12.03, 12.28, 11.97, 12.35 and 12.37 μg equivalent NH_2OH/g, respectively). Overall, two extracts from *Aspergillus* sp. AMtf15 and *P. citrinum* AMf6 exhibited DPPH radical scavenging and ferric ion reducing capacities. The antioxidant activities of these fungal extracts observed in the present study could be related to their phytochemical content. Our previous compositional analysis of ethyl acetate extracts from endophytic fungi from *Annona muricata*, *Aspergillus* sp. AMb7 and *P. citrinum* AMrb23 revealed the presence of 44 and 38 different secondary metabolites, with isolongifolene (30.18%) and octadecyl-3,5-di-tert-butyl-4-hydroxydrocinnamate (19.62%) being the most abundant in AMb7 and AMrb23, respectively [18]. Therefore, endophytic fungi from *Annona muricata* can produce a significant number of metabolites with the potential to act as both antimicrobials and antioxidants.

Conclusion

The present study was designed to provide insight into the antibacterial, antioxidant and mode of action of crude metabolites from endophytic fungi inhabiting *Annona muricata* growing in Cameroon. Our investigation showed that a high percentage of isolates (41.46%) belonging to six different genera (*Aspergillus*, *Curvularia*, *Fusarium*, *Meyerozyma*, *Penicillium*, and *Talaromyces*) exhibited potent antibacterial activity against the causative agent of UTIs. The more potent displayed various modes of antibacterial action and antioxidant activity. These results suggest that each of the active endophytes identified from the present study can produce antibacterial molecules. Further antibacterial-guided fractionation is currently ongoing to purify and identify active compounds that may serve as a starting point for developing new pharmacological agents.

Supporting information

S1 File.
(DOCX)

Acknowledgments

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