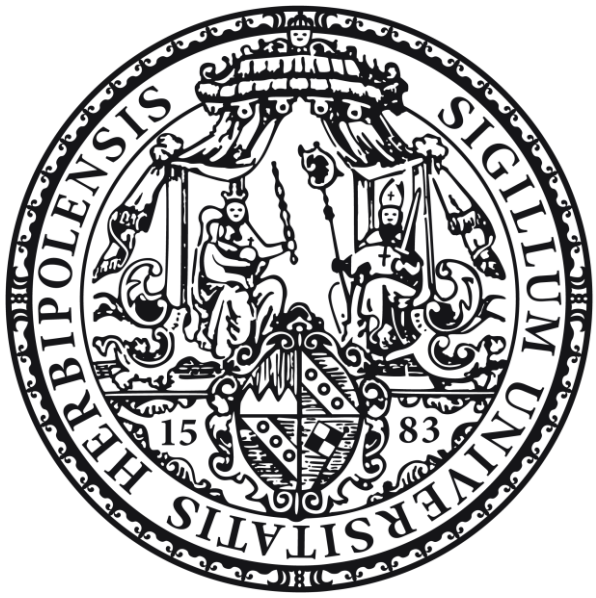

The Search for Novel Effective Agents Against Multidrug-Resistant Enterobacteriaceae

Die Suche nach neuen wirksamen Wirkstoffen gegen multiresistente Enterobacteriaceae

Doctoral thesis for a doctoral degree
at the Graduate School of Life Sciences,
Julius-Maximilians-Universität Würzburg,
Section: Infection and Immunity

submitted by

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‘For in him we live and move and have our being.’

Acts 17: 28a NIV

To my family

Acknowledgements

This work was carried out at the Institute for Pharmacy and Food Chemistry and the Institute for Molecular Infection Biology of the Julius-Maximilians University of Wuerzburg under the main Supervision of

Prof. Dr. Ulrike Holzgrabe

to whom I am very much thankful for the acceptance to join her working group, the implicit trust, the insightful guidance, the remarkable mentorship, as well as the cordial support throughout my doctoral studies. “Asante sana”.

I would also like to extend my profound appreciation to

PD. Dr. Knut Ohlsen and **Prof. Dr. Dr. Lorenz Meinel**
for their supervision and acceptance to form my thesis committee. I am very grateful for their practical guidance, mind-opening discussions, and for directing their valuable resources towards this work.

My sincere thanks also go to Prof. Dr. Knut Kirmse for his readiness and acceptance to be the Chairperson of my thesis committee

The German Academic Exchange Service (DAAD) is much appreciated for the financial support through the research grant for doctoral programmes in Germany (57381412) offered to me in 2018.

I also deeply thank the Government of the United Republic of Tanzania, through the Muhimbili University of Health and Allied Sciences (MUHAS), for the administrative and financial support during my studies. My sincere appreciation to the Head of the Department of Medicinal Chemistry Dr. Vicky Manyanga and all staff of the School of Pharmacy at MUHAS for their unwavering support.

“Asante sana” to Prof. Dr. Eliangiringa Kaale and Dr. Joseph Sempombe for their introduction, reference, and guidance during the scholarship application and their steadfast mentorship and inspiration.

I highly acknowledge the technical, administrative, material, and moral support from Prof. Dr. Christoph Schoen, Jun. Prof. Oliver Scherf-Clavel Dr. Gerd Vogg, Dr. Jens Schmitz, Dr. Ludwig Höllein, Dr. Curd Schollmayer, Dr. Tobias Hertlein, Drs. Rory and Merle Arrowsmith, Dr. Heiner Arzet, Dr. Huruma Nkone, Dr. Barnabas Mtokambali, Dr. Martin Kohler, Christoph Mahler, Liana Pogorelaja, Liane Dreher, Lieselotte Möhler, Martina Wecklein-Weidinger, Christine Schneider, Christoph Keßler, Mattias Völker, Karl Vollmuth, and all staff of the Graduate School of Life Sciences (GSLS).

Many thanks to my former and current colleagues whom I was very privileged to get a chance to work with:
Adrian L., Alexander B., Antonio F., Christine H.,Cristian L., Daniela V., Florian G., Hayfa G., Jonas U., Jonas W., Jushua W., Laura B., Liling T., Lukas K., Marie H., Marco B., Markus G., Markus Z., Mohamed M., Niclas F., Nicolas S., Patrick N., Paul G., Rasmus W., Reuben P., Sebastian S., Sylvia K., and Theresa L.

Heartfelt thanks to my lovely wife Ivone for her passionate love, care, prayers, understanding, and very dedicated support throughout my studies. Thanks to our beloved son Haki for his cheerful and friendly mood, for warmly welcoming me home, and for always wanting to play with me regardless of my mood or exhaustion.
Sincere thanks to my Mother Veronica and my Father *Mwalimu* M.E:N. Lumalanja , my siblings, extended family, and friends, who kept me so close despite the long distances, and who never stopped wishing the best for me. “Asanteni sana”

Last but not least,
I am overly thankful to the almighty God for his abundant grace and enduring mercies

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AA	amino acid
AAC	aminoglycoside N-acetyltransferase
ABA	ATP-binding cassette
AIM	Australian imipenemase
ALKBH	alpha-ketoglutarate-dependent dioxygenase
AMEs	Aminoglycoside-Modifying Enzymes
AmpC	*Amp* gene-derived cephalosporinase
AMR	Antimicrobial Resistance
ANT	aminoglycoside nucleotidyltransferase
APH	Aminoglycoside phosphotransferase
ARR	ADP-ribosylation of rifamycin (enzyme)
AST	Antimicrobial susceptibility testing
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
ATR	Attenuated Total Reflectance
BPC	Base Peak Chromatogram
BSI	Blood Stream Infections
CARB	carbenicillin active beta-lactamase
CAT	chloramphenicol acetyltransferase
CAU	*Caulobacter crescentus* isolated beta-lactamase
Cbz	benzyl chloroformate
CFU	Colony Forming Units
CHDLs	Carbapenem-hydrolysing class D beta-lactamases
CIP	Center of Institut Pasteur
ClogP	calculated partition coefficient between n-octanol and water
CLSI	Clinical Laboratory Standards Institute
CMY	cephamycins active beta-lactamase
CNS	Central Nervous System
COSY	COrrelation SpectroscopY
CphA	Carbapenem hydrolyzing first (A) from *Aeromonas hydrophila*
CRAB	Carbapenem-Resistant *Acinetobacter baumannii*
CRE	Carbapenem-Resistant Enterobacteriaceae

CRPA	Carbapenem-Resistant *Pseudomonas aeruginosa*
CTMAB	cetyltrimethylammonium bromide
CTX-M	Cefotaxime-Munich
DCE	dichloroethane
DEPT-135	Distortionless Enhancement by Polarization Transfer-135
DGU	Degassing Unit
DHA	beta-lactamase discovered in Dhahran, Saudi Arabia
DIM	Dutch IMipenemase
DIPEA	N, N-Diisopropylethylamine
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EHEC	Enterohemorrhagic *Escherichia coli*
EMA	European Medicines Agency
EPS	Extracellular Polymeric Substrates
Ere	erythromycin esterase
ESBLs	Extended-spectrum beta-lactamases
ESI–MS	Electrospray Ionization–Mass Spectrometry
EUCAST	European Committee for Antimicrobial Susceptibility Testing
FDA	Food and Drug Administration
FEZ	*Fluoribacter gormanii* derived Zn-beta-lactamase
Fos	fosfomycin thiol transferase enzyme
FT-IR	Fourier Transform Infrared
GC-MS	Chromatography-Mass Spectrometry
GC-MS	Gass Chromatography-Mass Spectrometry
GES	Guiana-extended spectrum
GIM	German imipenemase
GIT	Gastro-Intestinal Tract
GOB	*Chryseobacterium meningosepticum* class B beta-lactamase
HBTU	2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
hERG	human Ether-à-go-go-Related Gene
HMBC	Heteronuclear Multiple-Bond Correlation
HPLC–MS	High-Performance Liquid Chromatography-Mass Spectrometry

HR-ESI-MS	High Resolution-Electrospray Ionization-Mass spectrometry
HSQC	Heteronuclear Single-Quantum Correlation
HTS	High Throughput Screening
IMI	Imipenem-hydrolyzing β-lactamase
ImiS	Imipenemase from *Aeromonas veronii* bv. Sobria
IND	*Chryseobacterium (Flavobacterium) indologenes* derived beta-lactamase
INT	iodonitrotetrazolium chloride
IPA	isopropyl alcohol
KHM	Kyorin University Hospital imipenemase
KPC	*Klebsiella pneumoniae* Carbapenemase
LB	Lysogeny Broth
LC-MS	Liquid Chromatography-Mass Spectrometry
Lpp	lipoprotein
LPS	lipopolysaccharides
MAE	Microwave Assisted Extraction
MATE	Multidrug and toxic-compound extrusion
MBC	Minimum Bactericidal Concentration
MDR	Multidrug-Resistance
MFS	Major Facilitator Superfamily
MIC	Minimum Inhibitory Concentration
MKLS	macrolides, ketolides, lincosamides and streptogramins antibiotics
MOE	Molecular Operating Environment
MPHA	macrolide phosphotransferase
MRC	Minimum Regrowth Concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
MTCC	Microbial Type Culture Collection
NCCLS	National Committee for Clinical Laboratory Standards
NCIM	National Collection of Industrial Microorganisms
NDM	New Delhi metallo-beta-lactamase
NMC	Not Metalloenzyme Carbapenemase
NMR	Nuclear Magnetic Resonance
nOHNH	number of O-H and N-H bonds

nON	number of oxygen and nitrogen atoms
OD600	Optical Density at 600 nm
Ole	Oleandomycin glycosyltransferase
OM	Outer Membrane
OmpA	Outer Membrane Protein A
OXA	oxacillin active beta-lactamase
PBP	Penicillin Binding Protein
PBS	Phosphate Buffer Saline
PCA	Principal Component Analysis
PCC	pyridinium chlorochromate
PDB	Protein Data Bank
PDR	Pan-Drug-Resistance
PG	peptidoglycan
PMAE	Pressurized Microwave-Assisted Extraction
PSE	Pseudomonas-Specific Enzyme
PTP	Protein Tyrosine Phosphatase
Q-TOF	Quadrupole Time of Flight
RGT	Enzyme with RTG (arginine, threonine, glycine) triad
RNA	ribonucleic acid
RND	Resistance Nodulation Division
RoX	rifampicin monooxygenase
RP	Reversed Phase
RPH	rifampicin phosphotransferase
RP-HPLC	Reversed Phase-High Performance Liquid Chromatography
RT	Retention Time
RTI	Respiratory Tract Infections
SFC	*Serratia fonticola* Carbapenemase
SFE	Supercritical Fluid Extraction
Sfh	*Serratia fonticola* carbapenem hydrolase
SHV	Sulfhydryl Reagent Variable
SIM	Seoul imipenemase
SMP	Sao Paulo metallo-β-lactamase
SMR	Small Multidrug Resistance
STAB	sodium triacetoxyborohydride

TEA	Triethyl Amine
TEM	Named after the patient (Temoneira) providing the first sample
TetX	tetracycline 11a-monooxygenase
Tol	Translocation Protein
Pal	peptidoglycan-associated protein
THF	tetrahydrofuran
TLC	Thin Layer Chromatography
tPSA	total Polar Surface Area
UAE	Ultrasonic-Assisted Extraction
UTI	Urinary Tract Infections
UV	Ultraviolet
UV-Vis	Ultraviolet-Visible
VIM	Verona integron-encoded metallo-β-lactamase
VRE	Vancomycin-Resistant Enterococci
WHO	World Health Organization
XDR	eXtensive Drug Resistance

Chapter I

Introduction

1. INTRODUCTION

The World Health Organization (WHO) defines Antimicrobial Resistance (AMR) as the change of bacteria, viruses, fungi and other parasites over time making antibiotics and other antimicrobial medicines ineffective and causing infections to become increasingly difficult or impossible to treat [1]. The increasing rate of AMR is an imminent threat to global public health. While projected to cause about 10 million deaths globally by the year 2050, approximately 4.95 deaths were associated with AMR in 2019 [2, 3]. This makes AMR one of the leading causes of death globally, with the highest burden found among low-income countries [3-8]. Apart from high mortalities, AMR causes high morbidities, increased healthcare burdens, and economic losses [2-4].

Although the development of AMR among bacteria is a natural phenomenon, several human-related behaviours/activities are greatly contributing to its development and spreading [9]. These include unethical and irrational prescribing and dispensing of antibiotics causing their overuse and/or misuse, extensive use of antibiotics in livestock farming, the circulation of substandard and falsified antibiotics, as well as improper disposal of antibiotics, among other factors [4, 6, 7, 10-13]. These practices have added to the antibiotic selective pressure on bacteria, hence leading to AMR [14].

1.1. Intrinsic, adaptive, and acquired resistance to antibacterial agents

Resistance to antibacterial agents can occur via intrinsic, adaptive, or acquired means. The presence of these distinct possibilities of resistance confers an increased ability of bacteria to resist various treatments. Since the three means are not mutually exclusive, their co-existence within many bacteria species highly intensifies the AMR challenge.

Intrinsic resistance refers to an inherent or innate ability of bacteria to withstand different antimicrobial treatments. This is represented by the presence of an outer membrane (OM) in Gram-negative bacteria cell envelopes which acts as a significant barrier to the permeability of antibiotics. The OM is known to only allow a slow passive diffusion of lipophilic molecules. On the other hand, intrinsic resistance is granted by the absence of non-specific porin channels which could allow the permeation of antimicrobial agents [15]. Porins typically allow the passage of only small hydrophilic molecules, hence making the passage of large hydrophilic molecules through the OM or porins almost inevitable. Moreover, the presence of a constitutively expressed range of efflux pumps serves as another intrinsic gateway to AMR. Such pumps make the accumulation of antibacterial agents to effective levels within the bacterial cells less attainable. Finally, intrinsic resistance can be realised through a natural lack of target(s) for a particular antibacterial agent in some bacteria

species. This is exemplified by the failure of daptomycin to act against Gram-negative bacteria despite its enormous success against critical Gram-positive bacteria [15, 16].

Adaptive AMR on the other hand is characterized by the ability of bacteria to adapt and hence survive stress conditions via implementing rapid changes in their transcriptomes following certain signs from their surroundings. Through these adaptations, bacteria can overcome stress resulting from exposure to antibacterial agents or harsh environmental factors such as temperature and nutrients. This can be exemplified by the formation of bacterial sub-populations which adopt a state of non-active growth (persisters), turning to alternative sources of nutrients, and/or production of matrices of Extracellular Polymeric Substrates (EPSs) (biofilms) [15, 16].

Finally, acquired AMR results from the acquisition of genes encoding resistance mechanism(s) via mutations, as well as chromosomal or horizontal gene transfer mechanisms. Depending on the acquired genetic information, the ultimate resistance can occur in terms of expression of antibiotics degrading or modifying enzymes, expression of Multidrug-Resistance (MDR) efflux pumps, modification of the porin channels, and/or target modifications, among other mechanisms [15]. The potential of horizontal gene transfers to occur even between non-closely related bacterial species and its relatively faster occurrence makes it the most problematic mode of resistance acquisition [4, 17]. The sharing of genetic materials can occur either through the uptake of those materials from the environment (transformation), direct physical contact between bacteria (conjugation), or a transfer by bacteriophages (transduction). In all cases, plasmids, transposons, and integrons are the typical carriers of the resistant genes [15, 16].

1.2. Bacterial mechanisms of AMR

Bacteria express resistance to antibiotics through five main mechanisms. One is the antibiotic's target modification hence preventing their interaction and intended action. Changes in penicillin-binding proteins in response to penicillin exposure are a typical example of this mechanism.

Second is the inactivation or modification of the antibiotics following the action of bacterial enzymes like beta-lactamases, transferases, and other bacterial enzymes [4, 12, 18, 19]. Through these mechanisms, several major antibiotic classes such as beta-lactam antibiotics, macrolides, aminoglycosides, and tetracyclines have faced major resistance-related drawbacks. **Table 1** summarizes selected aspects and examples of key antibiotic-destroying/inactivating enzymes [20-24].

Table 1: Main types and functional mechanisms of antibiotic destroying or modifying enzymes.

Enzyme type/class	Sub-classes	Description	Examples of enzymes
Beta lactamases			
Ambler Class A	Classical-narrow spectrum	Bush-Jacoby-Medeiros functional classification: Group 2 (serine-beta-lactamase)	PSE, CARB
	Extended-spectrum beta-lactamases (ESBLs)		TEM, SHV, CTX-M
	Class A carbapenemases		SHV-38, KPC, GES, IMI/NMC-A, SFC-1
Ambler Class B	Subclass B1	Bush-Jacoby-Medeiros functional classification: Group 3 (Metallo-beta-lactamases)	VIM-1, NDM-1, GIM-1, SIM-1, IND-1, IMP-1, SMP, KHM-1, DIM-1
	Subclass B2		CphA, Sfh-I, ImiS
	Subclass B3		GOB-1, AIM-1, FEZ-1, CAU-1
Ambler Class C	Amp C	Bush-Jacoby-Medeiros functional classification: Group 1 (cephalosporinases)	AmpC
	Extended spectrum AmpC (ESAC)		CMY -10, CMY -19, CMY-37
Ambler Class D	Extended-spectrum beta-lactamases (ESBLs) (Cephalosporinases)	Bush-Jacoby-Medeiros functional classification: Group 2 (serine-beta-lactamase)	OXA-10, OXA-11, OXA-15
	Oxacillinases Carbapenem-hydrolysing class D beta-lactamases (CHDLs)		OXAs (1-5) OXA-23, OXA-48
Macrolide esterases		catalyse the hydrolysis of 14- and 15-membered macrolides (e.g., erythromycin and azithromycin)	Erythromycin esterases (EreA, EreB)
Transferases	Aminoglycoside-Modifying Enzymes (AMEs)	perform acetylation, phosphorylation or adenylation of aminoglycosides at different positions	AAC, ANT, APH
	Chloramphenicol and analogues-modifying enzymes	catalyse acetylation or phosphorylation of chloramphenicol and analogues	CATs
	Enzymes modifying macrolides, ketolides, lincosamides and streptogramins (MKLS)	catalyse phosphorylation, glycosylation, and acetylation, of MKLS antibiotics	MPHA, MPHB, OleI, OleD, streptogramin A-O-acetyltransferase
	Fosfomycin-modifying enzymes	perform inactivation of Fosfomycin via Glutathione S-transferase activity	FosA3, FosC2
	Rifamycin-modifying enzymes	catalyse ribosylation, glycosylation, and phosphorylation of rifamycin	ARR, RPH, RGT
Monooxygenases	Tetracyclines modifying enzymes	catalyses the monohydroxylation of all tetracyclines,	TetX
	Rifamycin-inactivating enzymes	catalyses the oxidation of rifamycins	RoX

Furthermore, the third mechanism involves the decreased penetration of antibiotics through the bacteria due to the structural modification of porin channels. This mechanism is known to limit the entry of quinolones, tetracyclines, and beta-lactam antibiotics [25]. The fourth mechanism is the bypass of one antibiotic target through

the development of other targets capable of carrying out similar functions but non-susceptible (has low affinity) to the respective antibiotic. This can be demonstrated by the acquisition of methicillin resistance among *Staphylococcus aureus* using the Penicillin Binding Protein 2a (PBP 2a), as well as resistance to rifamycin due to the production of an antibiotic-resistant RNA polymerase [26, 27].

Lastly, the activation of efflux pumps leading to the removal of antibiotics from the bacterial cells is another major contributor to antibiotic resistance among both Gram-positive and Gram-negative bacteria [19]. The most common substrates of bacterial efflux pumps are penicillins, cephalosporins, quinolones, macrolides, aminoglycosides and tetracyclines [19, 28, 29]. Families, mechanisms and substrates of the known efflux pumps are indicated in **Figure 1** [29].

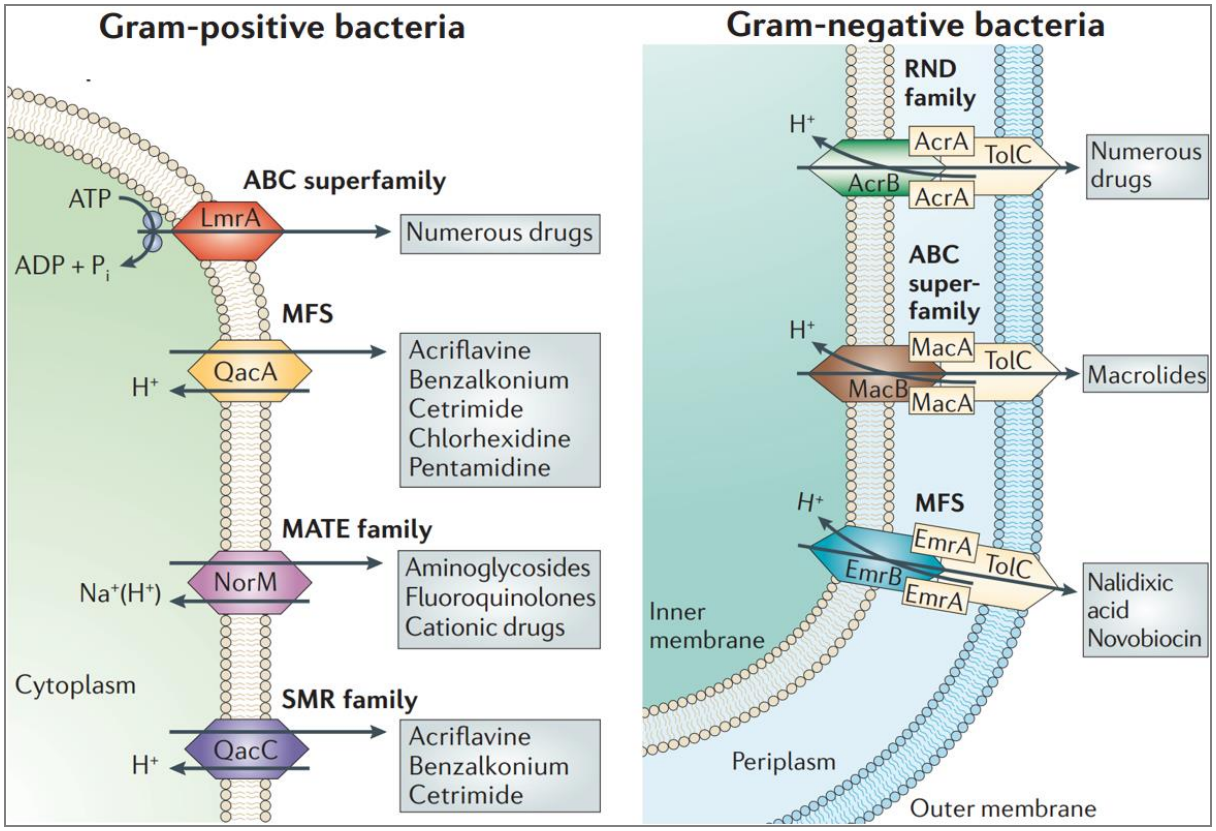

Figure 1: Five families of multidrug-resistance efflux pumps: the ATP-binding cassette (ABC) superfamily, the major facilitator superfamily (MFS), the multidrug and toxic-compound extrusion (MATE) family, the small multidrug resistance (SMR) family and the resistance nodulation division (RND) family. Also shown are a representation of their structures, position on the bacterial envelope, as well as antibiotics and other substrates for each class. Adopted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature, Nature Reviews Microbiology, Multidrug-resistance efflux pumps? not just for resistance, Piddock L.J.V., 2006 [29].

1.3. Resistance to multiple antibiotics and prioritization of pathogens

Worsening the challenge of AMR is the rise of bacteria resistant to a larger number of antibiotics within and across the available classes of antibacterial agents. Such phenotypes have further been classified as Multidrug-Resistance (MDR, an acquired non-susceptibility to at least one agent in three or more antimicrobial classes), eXtensive Drug Resistance (XDR, non-susceptibility to all but one or two categories of antimicrobials), Pan-Drug-Resistance (PDR, non-susceptibility to all agents in the antimicrobials category) [30, 31]. Although the definitions for these categories might vary, those stated above are broadly used. The presence of pathogens across all these categories, therefore, poses a significant threat to humanity and pushes towards the blink of the '*post-antibiotic'* era.

Based on the necessity to adequately address pathogens posing different threat levels, a priority pathogen list to guide antibiotics' research and development was put forward by the WHO [32]. In addition to mycobacteria whose global priority is already well established, three Critical priority pathogens are topping the list. These include the Carbapenem-Resistant *Acinetobacter baumannii* (CRAB), Carbapenem-Resistant *Pseudomonas aeruginosa* (CRPA), and the third-generation cephalosporin-/Carbapenem-Resistant Enterobacteriaceae (CRE) [3, 5, 32]

Following them are the High priority pathogens which include vancomycin-resistant *Enterococcus faecium*, clarithromycin-resistant *Helicobacter pylori*, fluoroquinolone-resistant Salmonella species, vancomycin- and methicillin-resistant *Staphylococcus aureus*, fluoroquinolone-resistant Campylobacter species, and third-generation cephalosporin- and fluoroquinolone-resistant *Neisseria gonorrhoeae*. Concluding the list is the medium priority category composed of penicillin-non-susceptible *Streptococcus pneumoniae*, ampicillin-resistant *Haemophilus influenzae*, and fluoroquinolone-resistant Shigella species [3, 32].

The prioritization of pathogens in the above categories was based on the ability of the bacteria to cause multiple systems infection, the global spread of their resistance, their high healthcare burden, transmissibility, and mortality levels, as well as their low preventability, treatability, and pipeline volumes [32]. Based on these highly inclusive criteria, the list serves as a crucial guide for the allocation of resources and efforts towards addressing the AMR problem [3-5].

The family Enterobacteriaceae, among other critical priority pathogens, is composed of Gram-negative rod-shaped, facultative anaerobes, capable of fermenting sugars to produce lactic acid among other products [33]. Bacteria in this family include *Escherichia coli*, *Klebsiella pneumoniae*, Enterobacter, Salmonella, Citrobacter, Proteus, Shigella, and Serratia species, among others. Among these bacteria are the top causative agents of Urinary Tract Infections (UTIs), Blood Stream Infections (BSI), Respiratory Tract Infections (RTIs), and infection of the Gastro-Intestinal Tract

(GIT). In that, *E. coli* and *K. pneumoniae* are not only among the commonest BSI-causing pathogens globally [5, 6] but also among the most problematic pathogens associated with the highest rates of infections, mortalities and ability to evade antibiotics via various resistance mechanisms [3-5, 34].
Furthermore, the development of resistance to third-generation cephalosporins and carbapenems by *E. coli* and *K. pneumoniae* has amplified the challenges posed by these bacteria. Those agents have for a long time remained the cornerstone for the management of disease conditions caused by these bacteria, including those resistant to other antibiotics [5, 10, 12]. The hope, which came with the re-introduction of colistin in the 2000s as the last resort antibiotic for the treatment of CRE infections, was rather short-lived. This was due to the identification of horizontally (plasmid coded) transmissible colistin-resistant genes (*mcr 1–10)* in 2015 [11, 35, 36]. These genes encode phosphoethanolamine transferases capable of modifying the structure of lipopolysaccharides (LPS) on the outer membrane of the Gram-negative bacteria [37].

In addition to the described unfolding of resistance among the *E. coli* and *K. pneumoniae*, their Gram-negative nature represents an intrinsic limitation towards the successful development of novel agents against them, making them more difficult to treat (**Figure 2**) [15, 16, 38]. Partly, this is due to the roles played by the bacterial outer cell membrane, which poses a significant challenge towards the penetration of antibiotics through the bacterial envelope [39].

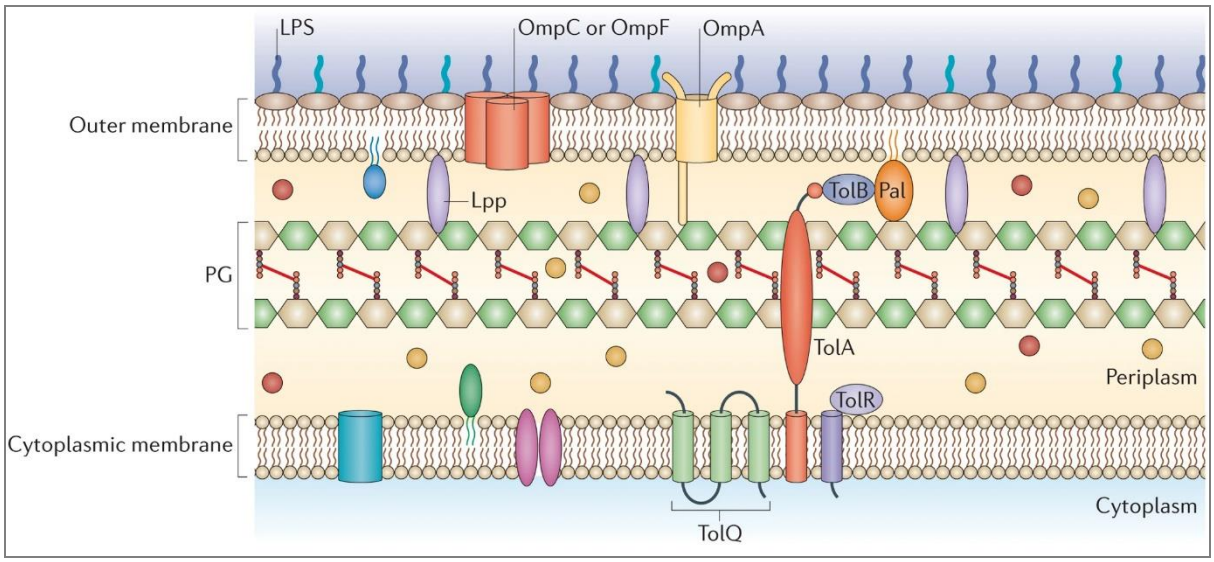

Figure 2: The two-membrane composition of the Gram-negative bacteria cell envelope. The inner/cytoplasmic membrane is made of a phospholipid bilayer, while the outer membrane is made of an interior single layer of phospholipids whose exterior counterpart is composed of lipopolysaccharides (LPS). The periplasmic space is composed of a peptidoglycan (PG) layer as well as periplasmic proteins. The stability of the envelope is derived from the covalent crosslinking involving Braun's lipoprotein (Lpp), non-covalent interactions between the outer-membrane protein (OmpA) and the PG and between the PG and the peptidoglycan-associated

lipoprotein (Tol-Pal) complex. Reprinted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature, Nature Reviews Microbiology, Outer-membrane vesicles from Gram-negative bacteria: biogenesis and functions, Schwechheimer C et al., 2015 [38].

1.4. Strategies to overcome AMR

Effective addressing of the AMR challenge necessitates highly inclusive and crosscutting measures at individual, community, institutional, and global levels. An array of the key strategies towards addressing this challenge can be summarized under the following aspects:

i. Actively preventing infections by abiding by the recommended hygienic lifestyles, increasing access to clean water, and ensuring hygienic food preparation practices [10, 12].
ii. Ensuring ethical and informed prescription and dispensing of antibiotics, along with encouraging rational use of antibiotics among individuals [40].
iii. Developing and implementing national and regional plans, policies, programmes, and AMR surveillance systems [6].
iv. Ensuring constant training of healthcare workers and provision of public health education on antibiotic stewardship [41].
v. Limiting the use of antibiotics in agriculture, avoiding the use of antibiotics for growth promotion, and employing alternative means for infection prevention and treatment in livestock [40, 42].
vi. Encouraging extensive vaccination, use of alternative agents to antibiotics, adjuvant traditional remedies, and combination therapies [43].
vii. Strengthening of medicines regulatory systems to avoid access to substandard and falsified antibiotics, along with proper disposal of expired or remaining antibiotics [13].
viii. Investing and encouraging the discovery and development of novel antibacterial agents, vaccines, and diagnostics [4].

The diverse nature of these approaches underscores the complex nature of the AMR challenge which requires collective efforts from individual to a global level to address. Considering the negative impacts associated with AMR, the presence of multiple mitigating strategies as highlighted above is necessary.

1.5. Discovery and development of antibacterial agents

The development and fast spread of resistance against colistin, the last resort antibiotic for some serious Gram-negative infections, necessitates continuous efforts to bring other effective agents to the clinic [35-37]. This is also in line with addressing the Central Nervous System (CNS) and kidney toxicities associated with colistin, which caused its abandonment in the 1970s, only to return in the 2000s due to otherwise untreatable MDR infections [11, 40].

1.5.1. Outlook of the antibiotic development pipeline

A look at the antibiotic development pipeline indicates 12 new antibacterial agents were approved between 2017 and 2021 by the United States Food and Drug Administration (FDA), the European Medicines Agency (EMA), or both. Among these, 10 agents are derivatives of the existing antibiotic classes or combinations of old antibiotics with resistance-modifying agents. Only lefamulin and meropenem-varbobactam were noted to represent new chemical classes, while inconclusive data on the absence of cross-resistance was obtained for meropenem-varbobactam, lefamulin and cefiderocol [44]. Belonging to pleuromutilins, lefamulin is a semi-synthetically prepared antibiotic originating from the fungus *Clitopilus passeckerianus*. Its analogue retapamulin was approved by the FDA and EMA in 2007 for the treatment of bacterial skin infections. The other two members tiamulin and valnemulin have found use in veterinary medicine [45]. Furthermore, while 5 agents were reported to target CRE, only cefiderocol targets all three WHO-critical priority pathogens (CRAB, CRPA and CRE) [44, 46].

On the other hand, the clinical antibiotic pipeline was reported to contain 45 traditional antibacterial agents and 32 non-traditional ones in different phases of development. Among the traditional agents, 6 are targeting the WHO priority pathogens and host at least one of the WHO innovation criteria (having a new target, a new mode of action, a new chemical class, and absence of cross-resistance), whereby, only 2 were active against at least one of the critical priority pathogens. Interestingly, 28% of the 127 agents in the pre-clinical pipeline are reported to target key Enterobacteriales, but their success in the clinical pipeline is faced by about one-third annual discontinuation rate of their respective development programmes [44].

Generally speaking, the recently approved agents as well as those in different stages of development are inadequate to address the rates of emergence and spread of antibiotic-resistant bacteria. The reflected almost empty antibiotic development pipeline necessitates supporting the continuous efforts towards an increased rate of filling it with novel effective agents [7, 10, 44].

1.5.2. Approaches in the discovery and development of antibacterial agents

Most of the antibiotics currently in use were discovered during the golden age of antibiotic discovery (the 1940s – 1960s) (**Figure 3**). During this era, a large proportion of antibiotics were derived from fungi and bacteria among other natural sources [7, 17]. However, the rise of genomics, combinatorial chemistry, High Throughput Screening (HTS) and rational drug designing, saw the shifting of interest and efforts away from searching for antibacterial agents from natural products. This shift was also influenced by the higher rediscovery rates following the over-mining of the few frequently explored fungal and bacterial species [47]. Nevertheless, using synthetic approaches, only a handful of antibacterial agents have so far been developed (**Figure 3**). Typical examples include sulfonamides, diaminopyrimidines, sulfones, pyridinamides, quinolones, and oxazolidinones. Among these, only

fluoroquinolones are exhibiting highly potent and broad-spectrum antibacterial activities [17, 47]

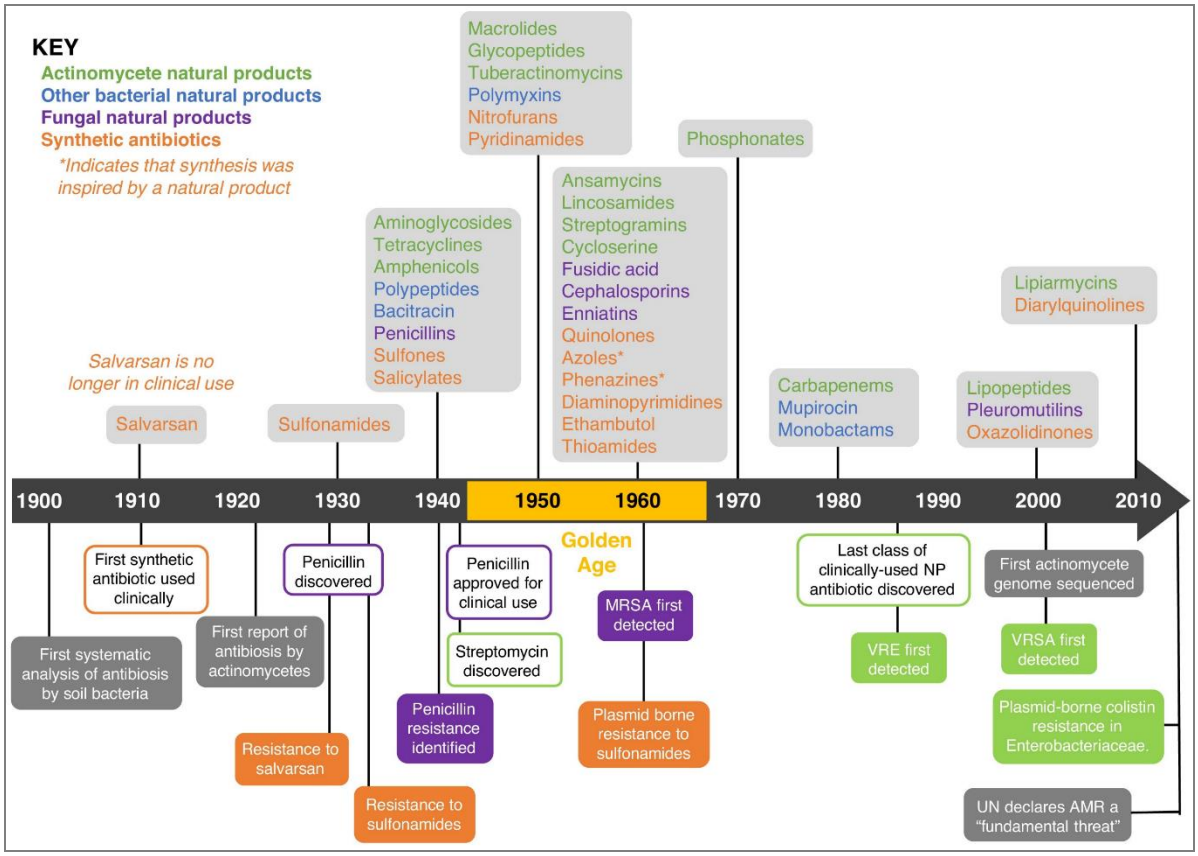

Figure 3: The antibiotics discovery timeline to 2010 showing their sources, times of entry to the clinic and highlights on the development of resistances against them. Reprinted under the CC BY open access licence from Elsevier Ltd, Current Opinion in Microbiology, Antibiotics: past, present, and future, Hutchings M. I. et al., 2020 [48].

Generally, these trends indicate the limited chances of obtaining antibacterial compounds by purely chemical synthetic means inspired by the screening of libraries containing random synthetic compounds [47]. Although it has been possible to identify the inhibitors of different targets through *in vitro* and *in silico* screenings, ensuring penetration and accumulation of those inhibitors particularly within the Gram-negative has been challenging [39, 47]. Based on these limitations, the antibiotic discovery approaches employing target-based designing and screening of synthetic chemical libraries have not yet provided any new antibacterial agent [8, 18, 49].

Low success rates in approaches not involving natural products and recent advancements in genomics, proteomics, and metabolomics, have largely contributed to the revisiting of nature as a potential and proven source of novel antibiotics [18]. The existence of such modern techniques offers a great contribution towards faster and more objective identification of new antibiotics from diverse natural sources. This

way, the frequency of isolating already discovered molecules or classes with known antimicrobial potentials is minimized and more attention can be given to about 99% of natural sources yet to be explored [7, 17].

1.6. Plants as sources of antibacterial agents

A large proportion of the global population is reported to be relying on plants as their primary source of healthcare in the treatment of various disease conditions [50, 51]. Traditional plant-based therapies are generally regarded as readily available, less expensive, do not require modern expertise and facilities for administration, and have lesser side effects than conventional therapies [10, 52, 53]. The success in ethnomedical/traditional practices has prompted many studies aimed at generating scientific evidence on the claimed plants' biological potentials, as well as isolation, characterization, and further development of the bioactive compounds.

While the choices of plants for screening of antibacterial activities are commonly influenced by their traditional uses, Eloff J.N. observed no difference in the frequency of antibacterial activities between randomly collected plant species and those used by traditional healers to treat different infections [54]. Moreover, a large proportion of studies investigate extracts from a few plant parts, prepared from a limited number of extracting solvents, and tested against a very narrow selection of bacteria [17]. These limitations leave many potential plant species only slightly explored, hence a higher likelihood of missing other promising activities. Put together, this necessitates the creation of focused and diverse libraries highlighting potential plant species, parts, extracts, tested bacteria, and observed activities. While reducing the rates of rediscovery and providing concrete guidance for further research, such libraries can minimize the loss of resources in studying known inactive extracts.

Furthermore, despite widespread and long-standing efforts to study plants for antibacterial and other biological potentials, the field is still faced with several crucial challenges. One of them is the limited likelihood of reproducing the biological potentials of a particular plant reported elsewhere. For a long time, these discrepancies have been almost exclusively linked to the variabilities in the geographical, ecological, and biological factors related to the studied plants. However, given the large number of other involved experimental variables and associated reporting practices, this challenge necessitates a detailed evaluation and coordinated mitigation strategies [55].

Additionally, newer methods for the preparation of plant extracts which require lesser extraction time, minimize solvent use, utilize minimum plant materials, and provide high yields are highly needed. The availability of such methods will be crucial in replacing the less efficient, as well as materials and other resources intensive conventional extraction techniques. In that, more studies are needed to explore and demonstrate the suitability of newer extraction techniques such as Microwave

Assisted Extraction (MAE), Ultrasonic-Assisted Extraction (UAE), and Supercritical Fluid Extraction (SFE) in a broader range of applications [56].

Furthermore, isolation and characterization are other crucial steps towards the complete identification of new antibacterial compounds from plant sources. These steps are usually dominated by laborious and time-consuming multiple steps of fractionation and purification guided by tedious biological assays. Recently, the use of bioautographic techniques has demonstrated a great potential to enable fast identification and more focus on antibacterial sub-fractions within complex crude mixtures. However, the widespread use of this valuable technique is yet to be realized [57].

Screening for biological activities from plant sources has revealed many crude extracts and isolated phytochemicals with antibacterial activities, beneficial synergies with modern antibiotics, and the potential to modify or eliminate key resistance mechanisms [19, 47, 58]. Broad differences in antibacterial potentials, targets, and modes of action have been reported among compounds within and across different phytochemical classes (**Figure 4**) [19, 59, 60]. However, despite the broad efforts to explore the antibacterial potentials of phytochemicals, no plant-derived antibiotics are yet to find their way to the clinic [47]. The limited levels of efficacy, low target selectivity, chemical complexity, poor solubility, low stability, and challenging pharmacokinetic profiles are among the factors attributed to the low desirability of phytochemicals as conventional antibiotics [17, 47, 59].

Nevertheless, the success of plants in traditional uses, the chemical diversity and novelty of phytochemicals, and their diverse modes of action necessitate the continued exploration of plants for novel antibacterial agents (**Figure 4**) [59, 60]. Therefore, while aiming at harnessing novel antibacterial scaffolds of plant origin, their associated shortcomings can be addressed using the available synthetic means.

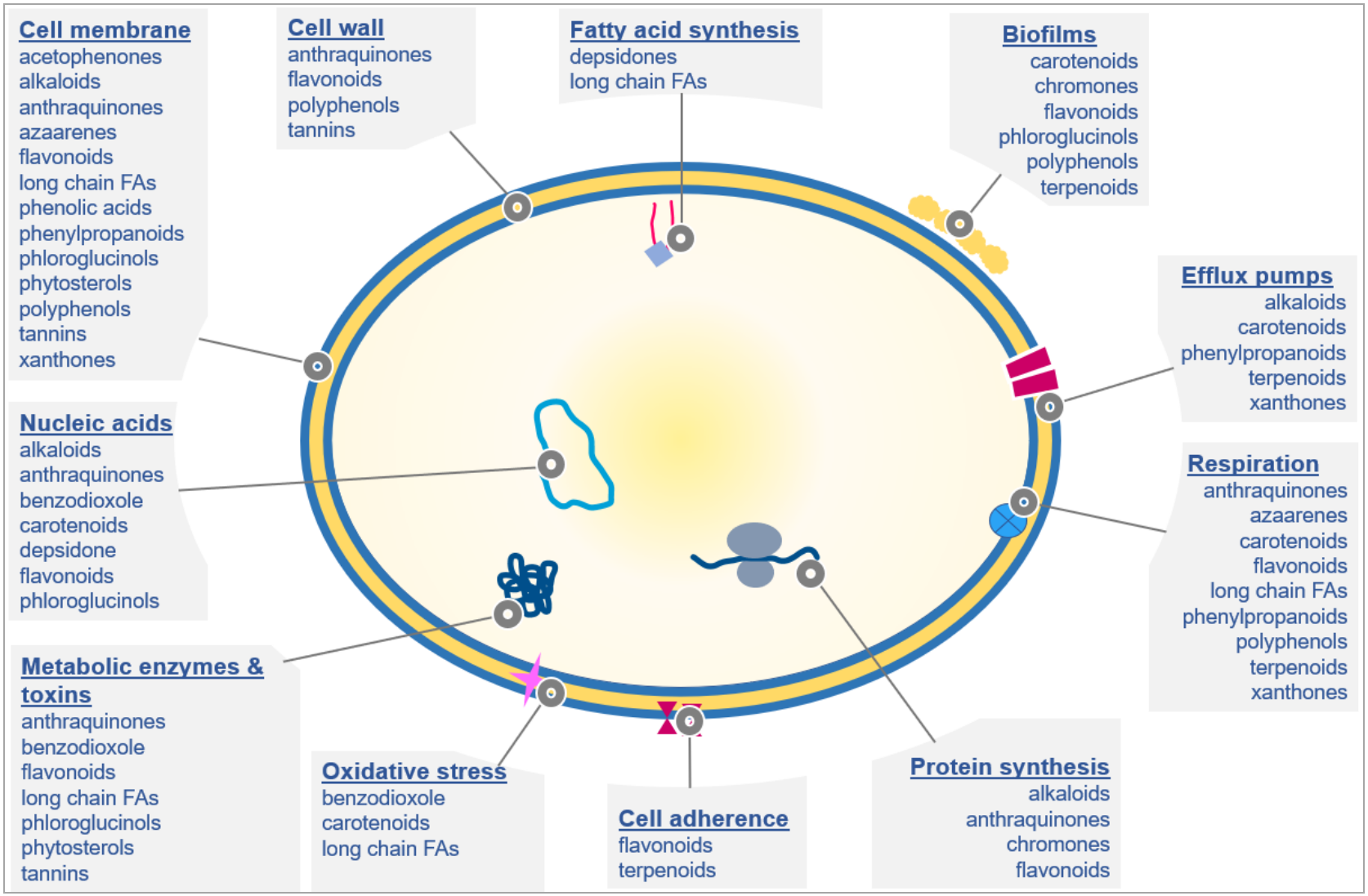

Figure 4: Bacterial targets of actions of various types of phytochemicals.

1.7. Synthetic modifications of natural compounds

As highlighted above, the development of antibiotics from purely synthetic methods has to date witnessed low success rates which contributed to the regaining of interest towards natural products' proven potential of providing new antibiotics [8, 17, 18, 47].

However, the necessity of employing synthetic means to address the shortfalls in nature-derived compounds gave rise to semi-synthetic approaches for optimizing nature-derived scaffolds [8, 18]. Using synthetic means, the exploration of broader chemical spaces around natural scaffolds, while addressing their toxicity, solubility, stability, pharmacokinetic, and pharmacodynamic profiles can be achieved [7, 17, 47]. Generally, between 200 – 300 drugs are approximated to have been prepared using semi-synthetic means so far. These numbers are far beyond the current clinically available purely synthetic antibiotics [17]. Common examples of those agents are amoxicillin, azithromycin, minocycline, plazomycin, telavancin and tigecycline, a few to mention [8, 17].

Furthermore, the creation and updating of libraries of nature-derived compounds with antibacterial potentials are important in replacing the libraries containing random synthetic compounds during target-based screening for antibacterial agents. The use of such libraries is key in informing the ongoing efforts in establishing the rules of penetration and accumulation of antibacterial agents within Gram-negative bacteria. On the other hand, the libraries serve as a readily available source of ideas and inspirations for novel natural scaffolds to be further studied using semi-synthetic means, hence improving their activities and/or optimising other related profiles [39, 47].

Together with the opportunities in the nature-inspired synthesis of antibacterial agents, the approach is as well limited in several ways. Among the key challenges is the occurrence of active but structurally complex natural products hence making their syntheses and/or modifications incredibly challenging. Other aspects include the low access to some natural products due to high costs, limited distribution of producing species, difficulty to attain culturing/growth conditions, as well as complex isolation and purification processes. Nevertheless, the increasing use of genomic, proteomic, and metabolomic approaches in the exploration of antibacterial agents from nature is promising towards addressing some of these challenges [17, 47, 61-63].

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Chapter II

Aims of the Thesis

2. AIMS OF THE THESIS

The main goal of this thesis was to search for new effective antiinfectives against Multidrug-Resistant (MDR) strains of *Escherichia coli* and *Klebsiella pneumoniae*. This goal was further divided into five specific aims:

Firstly, it was aimed to develop libraries of plant species and phytochemicals with potential antibacterial activities against *E. coli* and *K. pneumoniae* using the data available in the literature. It was further intended to determine the existing relationships between the antibacterial activities and aspects of plant families, parts, and extracting solvents, as well as phytochemicals classes, drug-likeness, and determinants of compound accumulation within Gram-negative bacteria.

The second goal was to evaluate the efficiency and selectivity of a Microwave Assisted Extraction (MAE) technique when performed under a combination of high-pressure and low-temperature conditions. Here, it was aimed to determine the suitability of using this extraction technique in place of conventional techniques requiring more energy, solvents, and plant materials, as well as longer extraction durations. Additionally, the possibility of avoiding the common use of high extraction temperatures under MAE hence sparing heat-sensitive compounds and avoiding cross-reactions was investigated.

Thirdly, the thesis aimed at evaluating the likelihood of reproducing antibacterial activities previously reported among plant extracts, as a key step towards the conduction of further or follow-up studies on previously identified potential species. Moreover, types and frequencies, and possible effects of aspects most likely to impair reproducibility were evaluated while aiming at proposing possible ways to mitigate them.

The fourth goal was to determine the structures of compounds responsible for the antibacterial activity against *E. coli* and *K. pneumoniae* observed in the crude extract of *Paeonia officinalis* L. (Paeoniaceae). Also intended was the exploration of the suitability of bioautography-guided assays in the isolation compound active against the two bacteria alongside the *in vivo* and *in vitro* potentials of the isolated compounds against susceptible and MDR strains hosting diverse resistance phenotypes.

Finally, it was aimed to investigate the antibacterial potentials of glucovanillin derivatives prepared via synthetic modifications of one of the compounds from the created library of phytochemicals. Here, the exploration of a broader chemical space around a natural scaffold through the inclusion of natural and synthetic moieties alongside testing for antibacterial potential against the two bacteria was intended.

Chapter III

Review

3. Searching for new agents against Enterobacteriaceae from nature: Approaches, potential plant species, isolated compounds, and their respective properties

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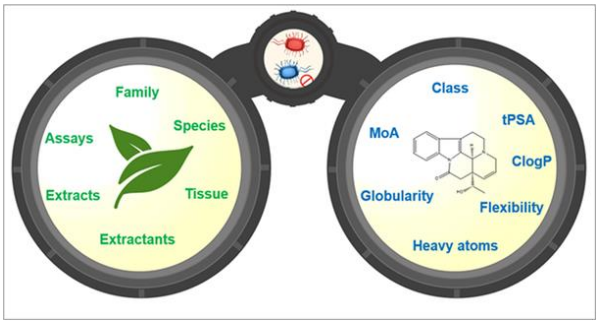
Manuscript submitted to Phytochemistry Reviews



Abstract

The rising trend of antibiotic-resistant infections around the world and the low antimicrobials development pipeline volume are necessitating continued efforts in the search for novel treatment options. Following the discovery of penicillin, a big proportion of other antibiotics currently in clinical use have been discovered from many fungi and bacterial sources. This prominent success has long motivated widespread efforts in the search for antibacterial compounds from other natural sources including plants. Favourably, plant-derived compounds have the potential to host novel chemical scaffolds, hence increasing the likelihood to hit new bacterial targets while exhibiting new modes of action. In this prospect, the current review aimed at appraising approaches and outcomes from studies commissioned to evaluate the antibacterial activities of crude plant extracts and the isolation of compounds effective against *E. coli* and *K. pneumoniae*. Moreover, highlights on plant species, crude extracts, and isolated compounds with good antibacterial profiles against both bacteria and their respective properties, as well as key aspects from the varying experimental approaches are presented and discussed. Additionally, statistical analyses were conducted to substantiate relevant patterns observed among the selected plant- and phytochemicals-related aspects in relation to their antibacterial activities. It is anticipated that the shared insights will impart the ongoing efforts with improved experimental designs, inspire ideas for further studies and contribute to the successful hunting for new antibacterial chemical scaffolds via in silico approaches.

Keywords: *Escherichia coli*; *Klebsiella pneumoniae*; crude extract; isolation; chemoinformatics; compounds library.

Introduction

The ever-increasing rates of antimicrobial-resistant infections warrant continuous efforts in the search for new treatment options. Among others, nature is a potential source of novel effective antimicrobial agents. Numerous bacteria and fungi species have already contributed to the existing arsenal of antibiotics. However, the natural development of Antimicrobial Resistance (AMR) among bacteria, augmented with factors like misuse of antibiotics and extensive utilization of antibiotics in agriculture has rendered most antibiotics less useful (Anand et al., 2019; Hoffman, 2020; Murray et al., 2022).

Antibiotic-resistant infections due to gram-negative bacteria are generally more difficult to treat, hence posing a more serious public health threat. The World Health Organization (WHO) categorizes carbapenem-resistant and third-generation cephalosporins-resistant Enterobacteriaceae as pathogens of critical priority against which new antibiotics are urgently needed. This is mainly due to their global spread of resistance, high healthcare burden, low treatability, and pipeline volume among other factors (WHO, 2017, 2021)

Although plants are yet to contribute to any of the antibiotics currently available on the market, studies on the antibacterial properties of plant extracts and the isolation of antibacterial compounds are extremely common. As a result, the literature on reports of plant extracts or isolated compounds with a diverse range of antibacterial activities is increasingly rich. The outcomes of such studies are partly meant to inform further efforts in isolation, biological screening, syntheses, and optimization of biological and pharmacokinetic activities among others. However, these follow-up approaches are usually occluded by various factors including difficulty in the screening of the bulky literature for plant species with targeted activities, the limited geographical distribution of potential plant species, and low reproducibility of previous findings due to numerous factors (Masota et al., 2021)

Moreover, plants host a great potential to deliver compounds with a high degree of chemical novelty which lower changes for the rapid development of cross-resistance while increasing the likelihood to hit new bacterial targets and modes of action. Reports on antibacterial plant extracts and the responsible phytochemicals are therefore crucial to the entire community of researchers in the discovery and development of antibiotics. In addition to advocating for the development of traditional ways of treating bacterial infections, a rich diversity of antibacterial phytochemicals can potentially inspire further approaches in screening, designing, and syntheses of novel antibiotics (Anand et al., 2019; Katz & Baltz, 2016; Newman & Cragg, 2016).

To this end, the availability of concrete information on plants with high potential for delivering active antibacterial compounds is key. Additionally, the creation and updating of specialized libraries of phytochemicals with experimentally proven

antibacterial potentials are essential for bridging the gap between initial crude extracts screening or isolation studies and further stages down the line of antibiotics development.

Furthermore, such studies must be conducted following the most reliable approaches to avoid high failure rates in subsequent studies. Variations of phytochemical profiles due to geographical, ecological, and climatic differences are inevitable. In return, more efforts should be placed on streamlining and adhering to the standard and up-to-date experimental approaches, to ensure increased reliability of the resulting data.

This review highlights the different aspects and approaches in the screening of plant extracts for antibacterial activities, specifically against the gram-negative *Escherichia coli* and *Klebsiella pneumoniae*. Highlights are provided on common motivations and objectives in studying plants for their antibacterial potentials, the nature and essence of traditional practices around studied plant species, as well as selected aspects in the preparation of crude extracts and testing of their antibacterial activities. Furthermore, the accounts on recently isolated compounds with high activity against the two bacteria, including their chemical and drug-likeness characterization and relevant statistical analyses are included.

Methods

Peer-reviewed research articles from studies on antibacterial plant extracts were obtained from Google scholar, PubMed and Web of Science scholarly databases using the search string: (antibacterial OR antimicrobial) AND (plant OR crude OR extract*) AND ("*Escherichia coli*" OR "*Klebsiella pneumonia**") AND ("broth" AND ("microdilution" OR "macro dilution")). Further filters were placed to remove studies which involved essential or volatile oils, algae, lichen, propolis, as well as nanoparticle formulations of crude plant extracts. The search included articles published between January 2010 and December 2021 in English language and no limitations on the types of journals were put in place.

Retrieved articles were further screened for relevance using their titles, abstracts and the main bodies in that order. All articles reporting the antibacterial activities of essential or volatile oils and that exclusively used disc or well diffusion to determine the Minimum Inhibitory Concentrations (MICs) were thereafter excluded. Only articles reporting the use of broth microdilution or macro dilution assays and with the crude extract's MIC values of $\leq$128 µg/mL against *E. coli* and/or *K. pneumoniae* were included.

Concerning isolated compounds, an independent search was done on the same set of scholarly databases using the search string: (*isolat* OR characteriz*) AND (compound* OR agent* OR phytochemical*) AND (antibacterial OR antimicrobial) AND plant AND ("Escherichia coli" OR "Klebsiella pneumonia*") AND ("broth" AND ("microdilution" OR "macro dilution"))*. Articles published in English between January 2010 and December 2020 were retrieved, regardless of their respective journals.

Similarly, further screening was done to include only plant-isolated compounds with MIC values of ≤100 µg/mL against *E. coli* or *K. pneumoniae* as determined by broth dilution methods.

The MIC cut-off points employed in this review were aimed at highlighting only the plant extracts and isolated compounds with a high magnitude of antibacterial activities. Ultimately, selected data was extracted from each article was populated in an MS Excel sheet. Further analyses to determine the relationships between reported MIC values and plant families, plant parts, extracting solvents, molecular weights, total polar surface area, ClogP, number of hydrogen bonds acceptors and donors, Kernel density, molecular flexibility and globularity, as well as number of heavy atoms was carried out using WordCloud, Origin®, ChemDraw®, Molinspiration and Molecular Operating Environment (MOE) software.

Common motivations for screening for antibacterial potentials of plants

Various factors were observed to motivate the continued efforts on searching for antibacterial compounds from plants. Among other factors, the existing broad traditional usage of plant-derived materials in the management of different diseases was crucial. Here it was commonly stated that about 80% of the world population is estimated by the WHO to rely wholly or partially on natural remedies as their primary source of health care (Adigüzel et al., 2005; Ayaz et al., 2016; Gbedema et al., 2010; Hassan et al., 2009; Kuete, Kamga, et al., 2011; Voukeng et al., 2017). Traditional remedies were reported to be regarded as cheap, readily available, more acceptable and associated with lesser side effects (Hassan et al., 2009; Panghal et al., 2011). These features have contributed to their more extensive favourability in the face of modern medicines which are challenged by their limited availability, high costs, requirements for expertise, being less trusted and being more associated with adverse drug reactions and side effects (Hassan et al., 2009; Madureira et al., 2012; Rashed & Butnariu, 2014; Singh et al., 2010).

Further, the rise in antibiotic-resistant infections on top of the existing high burden of infectious diseases in developing countries was noted to prominently stir the ongoing efforts in the search for new accessible and effective treatment options (Camacho-Corona et al., 2015; Fankam et al., 2014; Hossan et al., 2018; Kouitcheu Mabeku et al., 2006; Singh et al., 2010; Tekwu et al., 2012; Voukeng et al., 2017). Based on the broad availability and extensive uses of traditional medicines, their inclusion in the arsenal for fighting AMR was regarded to be essential (Chatterjee et al., 2009).

Nature has contributed to about 60% of the available antimicrobial agents (Madureira et al., 2012), a majority of them being from bacterial and fungi sources (Hoffman, 2020; Katz & Baltz, 2016). Further, it was estimated that only 6% of plant species have been screened for different biological activities, with a phytochemical evaluation conducted in only about 15% of them (Verpoorte, 2000). Based on plants' potential to

synthesize compounds with possible ideal features for novel antibiotics (Hossan et al., 2018; Ustun et al., 2016), continued studies in this direction are highly encouraged.

Similarly, the choices of plant species to be studied were noted to be influenced by the existing traditional practices (Noundou et al., 2016; Panghal et al., 2011; Singh et al., 2010; Tankeo et al., 2016; Venkata Ratnam & Venkata Raju, 2009), previous reports on antimicrobial or cytotoxic activities (Djeussi et al., 2013; Kuete, Kamga, et al., 2011; Voukeng et al., 2012; Wilson et al., 2005), as well as the quest to explore the antibacterial potential of other plant parts (Rashed & Butnariu, 2014).

Core objectives in screening for antibacterial activities of crude plant extracts

Based on the prevailing motivations, a range of core objectives in executing the respective studies was realized. While validation or provision of scientific evidence for the claimed antibacterial properties of traditional remedies was a frequent goal (Gbedema et al., 2010; Rigano et al., 2007; Ruiters et al., 2016; Singh et al., 2008; Siwe Noundou et al., 2014), other typical goals included: general ascertainment of antimicrobial activities (Adigüzel et al., 2005; Arif et al., 2009; Chatterjee et al., 2009; Madureira et al., 2012; Tekwu et al., 2012), evaluating crude extracts' potency against MDR bacterial strains (Fankam et al., 2014; Ordonez et al., 2009; Voukeng et al., 2017), and determination of synergistic effects between the crude plant extracts and conventional antibiotics (Chatterjee et al., 2009; Hossan et al., 2018; Noumedem et al., 2013; Seukep et al., 2016).
Additionally, aiming at determining the phytochemical compositions of crude extracts (Rashed & Butnariu, 2014), as well as ascertaining the antimicrobial potential of crude extracts' fractions and isolated compounds (Kuete et al., 2012; Ngameni et al., 2009; Noundou et al., 2016; Tankeo et al., 2016) were prominent.

The portrayed broad scope of the underlying objectives in studying the antibacterial properties of crude plant extracts is likely influenced by the differences in core research interests, backgrounds, and skills, in addition to the availability of the needed resources. While it is essential to ensure thorough investigations are conducted on each studied plant extract, a balance between the number of pursued objectives and the quality of the produced data should always be sought.

Traditional practices around plant species studied for antimicrobial properties

Accounting for known traditional uses of the studied plant species was frequently portrayed upon the provision of general plants' descriptions. The studied plant species were commonly described to be used in the traditional management of different types of both infectious and non-infectious diseases (Bitchagno et al., 2015b; Madureira et al., 2012; Mbosso Teinkela et al., 2016; Ruiters et al., 2016; Sahoo et al., 2008), along with usages in wounds treatments, as antidotes (Fankam

et al., 2014; Noumedem et al., 2013; Voukeng et al., 2017), and as antiseptics (Canales et al., 2016). Moreover, some plants were indicated to be used as parts of diet or food additives (Ordonez et al., 2009; Rao et al., 2010; Siwe Noundou et al., 2014).

The traditional remedies were mostly prepared as decoctions, macerates, infusions, pastes, tonics, diluted latex, sap, or heated bandages (Ayaz et al., 2016; Chatterjee et al., 2009; Panghal et al., 2011; Ruiters et al., 2016; Singh et al., 2007; Siwe Noundou et al., 2014). In cases where prior extraction was needed, water and alcohol were the most implicated extractants (Djeussi et al., 2013; Rigano et al., 2007; Siwe Noundou et al., 2014). Although the information on the route of administration was scarcely provided, oral and topical routes are commonly used in many traditional remedies (Adigüzel et al., 2005).

The knowledge of the associated traditional practices prominently informed studies aimed at validating different traditionally claimed biological potentials. Importantly, reports on traditional practices guided the conception and designing of some studies (Gbedema et al., 2010; Rigano et al., 2007; Ruiters et al., 2016; Singh et al., 2008; Siwe Noundou et al., 2014). Studying and documenting the existing ethnomedical knowledge and practices in various societies is therefore of great relevance. This is essential in guiding the choice of plant species, plant parts, extraction techniques as well as bacterial species to be targeted during the follow-up studies.

Preparation of crude plant extracts

Extraction as a crucial step in studying of biological activities of plant-derived samples was observed to host many variables which can influence the composition of the recovered extract(s). Maceration (Arif et al., 2009; Canales et al., 2016; Kuete et al., 2006; Noundou et al., 2016), Soxhlet (Dhiman et al., 2011; Korukluoglu et al., 2010; Uzun et al., 2004) and percolation (Ordonez et al., 2009; Rigano et al., 2007; Singh et al., 2008) were noted to be the techniques of widespread usage. Further, methanol, ethanol and acetone were markedly the extensively used solvents across different extraction techniques. Additionally, a good number of studies involved the use of multiple extracting solvents in a parallel or sequential manner (Arif et al., 2009; Canales et al., 2016; Madureira et al., 2012; Noundou et al., 2016; Orhan et al., 2009; Sahoo et al., 2008)

Apparently, the choice of an extraction technique is influenced by factors like the availability of resources, skills and the need to reproduce previous protocols. Although the inclusion of extraction temperatures in techniques requiring heating was uncommon, it should be considered necessary. Carrying out extractions using multiple solvents leads to the recovery of compounds across different polarity ranges. However, this approach is more beneficial when sequential rather than parallel extractions are conducted. Following a sequential approach, a more selective extraction based on the phytochemicals' polarities can be achieved which might result in better MIC values in case the antibacterial compounds are present.

In other studies, the indication of the exact feeds-to-solvent ratio (Adigüzel et al., 2005; Fankam et al., 2014; Gbedema et al., 2010; Ustun et al., 2016), the overall duration of extraction (Fankam et al., 2014; Noundou et al., 2016; Siwe Noundou et al., 2014) and techniques for removal of the solvent after extraction were reported. Among the drying techniques were rotary evaporation under vacuum (Ayaz et al., 2016; Tankeo et al., 2016), open-air drying (Noundou et al., 2016) freeze-drying/lyophilization (Chatterjee et al., 2009; Panghal et al., 2011; Singh et al., 2008; Siwe Noundou et al., 2014), and nitrogen gas spraying (Gbedema et al., 2010) were commonly applied.

Although rotary evaporation under vacuum was a widely used technique, the equipment is commonly unavailable in resource-limited settings, and the same applies to upper-end techniques like lyophilization. In such cases, the drying of crude extracts can solely rely on approaches like open-air drying or air blowing. These techniques are prone to result in higher quantities of residual solvents within the 'dried extract', which depending on the solvent may influence the observed antibacterial activity in addition to introducing errors in the weighed amounts. Furthermore, while most traditional practices use water as an extractant, its use in many laboratory settings is highly limited by the common lack of powerful drying techniques.

Remarkable aspects of handling dried crude extracts included ensuring proper storage conditions and sterilization of crude extracts before further studies were conducted. Some specified storage conditions for crude extracts included freezing or refrigeration at -80 °C to 4 °C (Adigüzel et al., 2005; Camacho-Corona et al., 2015; Fankam et al., 2014; Madureira et al., 2012; Noumedem et al., 2013). Interestingly, sterilization of crude extracts using UV light (200 – 400 nm) over a 24 h duration was also described (Chatterjee et al., 2009), in that case, the attainment of extract's sterility was confirmed by repeated streaking on agar plates.
Efforts to ensure the sterility of crude extracts are nevertheless not common. This is perhaps because there are other sterility checkpoints down the road of antimicrobial testing such as filter sterilization of extracts' test solutions or via the inclusion of crude extracts' solutions sterility control(s) in the experiment (Masota et al., 2021).

Antimicrobial susceptibility testing

General aspects and use of AST guidelines

Antimicrobial susceptibility testing (AST) stays at the core of determining the antibacterial activities of crude plant extracts under investigation. Ensuring sterility of the crude extracts' test solution was highly regarded in some studies, whereby it was achieved through the use of sterilization filters with a pore diameter of 0.22 – 0.45 µm (Orhan et al., 2009; Ozcelik et al., 2010; Sahoo et al., 2008; Tekwu et al., 2012; Ustun et al., 2016). The use of agar diffusion assays to quickly screen for activities of large quantities of crude extracts before MIC determination by broth dilutional assays was observed (Ayaz et al., 2016; Karsha & Lakshmi, 2010; Panghal et al., 2011; Wilson et al., 2005). Besides, discrepancies between the activities determined by

diffusion and broth dilution assays were observed (Kouitcheu Mabeku et al., 2006; Sahoo et al., 2008; Ustun et al., 2016; Uzun et al., 2004). In such cases, bacteria found less susceptible using diffusion methods were more susceptible during broth dilution assays, and *vice versa*.

Among the cited standard AST guidelines were those provided by the National Committee for Clinical Laboratory Standards (NCCLS) (Orhan et al., 2009; Singh et al., 2008; Uzun et al., 2004) or its subsequent organization, the Clinical Laboratory Standards Institute (CLSI) (Ayaz et al., 2016; Canales et al., 2016; Chatterjee et al., 2009; Madureira et al., 2012). Depending on the pursued guideline, the total incubation time at 37° C varied between 18 and 24 hours (Ordonez et al., 2009; Orhan et al., 2009; Ozcelik et al., 2010; Rigano et al., 2007; Wilson et al., 2005). While the number of replications per test and repetitions of the respective experimental sets is crucial, this data was relatively scarce. Though, the inclusion of three replicates and repeating the experiments twice was highlighted in some studies (Ngameni et al., 2009; Panghal et al., 2011; Tekwu et al., 2012; Venkata Ratnam & Venkata Raju, 2009)
These methodological disparities are most likely due to the lack of detailed and streamlined guidelines particularly dedicated to the AST of crude extracts from natural sources. Consequently, the scientific community in this field is compelled to use methods previously reported in other studies or standard guidelines primarily intended for AST of conventional antibiotics (Masota et al., 2021). Regardless of the success attained through this approach, several challenges are eminent as further discussed below.

Crude extracts' test concentration ranges

What range of crude extracts test solutions' concentration should be applied during the screening of their antibacterial activities, remains to be a question open for further discussion. While the maximum tested concentration of 1000 µg/mL was noted to be common (Ayaz et al., 2016; Ordonez et al., 2009; Rigano et al., 2007; Singh et al., 2008), some studies reported concentrations above 10,000 µg/mL (Kouitcheu Mabeku et al., 2006; Ratnam & Raju, 2008).

Different scholars have previously attempted to categorize the potency of crude extracts based on the MIC values exhibited. For example, Kuete et al., classified extracts with MICs below 100 µg/mL as significantly active, between 100 and 625 µg/mL as moderately active and above 625 µg/mL as weakly active (Kuete, 2010). Other categorizations by Rios and Rcio regarded extracts with MICs below 100 µg/mL as interesting and those with MICs above 1000 µg/mL as inactive (Rios & Recio, 2005). On the other extreme, Farby et al. regarded crude extracts' MIC value below 8000 µg/mL as active (Fabry et al., 1998).

Based on the observed variations in the used test concentrations and the attempts to categorize the crude extracts' potencies, there is an outstanding need to streamline the categorization criteria. This will provide much-needed guidance and help

researchers objectively decide on the extracts' concentration range to be tested. On the other hand, it will ease the comparison of crude extracts' antibacterial activities and optimize the usage of valuable resources for testing for concentrations beyond the commonly agreeable ranges (Madureira et al., 2012). This is partly because follow-up studies are more likely to prioritize plant extracts with reasonably high activities, in order to increase the chances of ultimately isolating compounds with higher activity profiles.

Exploring biological activities beyond MIC values

Although the common determination of MIC values serves a big purpose in highlighting the extract's antibacterial potential, exploration of other related potentials was observed. Closely related to MIC was the determination of Minimum Bactericidal Concentration (MBC), especially on extracts with observed inhibitory activity against the particular bacteria (Dhiman et al., 2011; Ngameni et al., 2009; Tekwu et al., 2012; Voukeng et al., 2017). Others included time-kill assays (Chatterjee et al., 2009; Gbedema et al., 2010; Hossan et al., 2018), mode of action studies (Karsha & Lakshmi, 2010), as well as the determination of toxicity or cytotoxicity profiles of the crude extracts (Kuete et al., 2006; Ozcelik et al., 2010). Likewise, studies on synergistic effects between the crude extracts and conventional antibiotics ((Chatterjee et al., 2009; Hossan et al., 2018; Noumedem et al., 2013; Seukep et al., 2016), as well as the action of the crude extract on bacterial efflux pumps (Kuete et al., 2012; Noumedem et al., 2013; Seukep et al., 2016) were observed.

Generally, the availability of such data adds great value with particular respect to informing the ongoing traditional practices and the usage of the respective herbal preparations or finished herbal products. On the other hand, due to the intrinsic complexity of plant extracts, the observed outcomes on bacterial survival times, mode of action, toxicity and synergistic effects cannot be exclusively linked to the constituent compounds exhibiting the observed bacterial inhibition. Therefore, whether or not any other biological activities should be explored at the crude extract level, depends much on the intended applications and conclusions to be drawn.

Reported antibacterial activities, plant species, parts and extracting solvents

The level of antimicrobial activity MIC≤128 µg/mL against *E. coli* and/or *K. pneumoniae* was reported in among crude extracts of 128 plant species originating from a total of 56 families (**Table 1**). A broad range of bacteria comprising standard reference- and clinical isolates as well as susceptible and multi-drug resistant strains were reported to be inhibited by crude extracts from across all indicated plant parts and species (**Table 1**).

Table 1: Plants species, families, parts and extracting solvents with reported antibacterial activities of ≤ 128 µg/mL against different strains of E. *coli* and/or *K. pneumoniae*

	Plant species and authority(ies)	**Plant family**	**Part with the reported activity**	**Extracting solvent(s) with the reported activity**	**MIC (µg/mL) against *E. coli* (Strain number)**	**MIC (µg/mL) against *K. pneumoniae* (Strain number)**	**Ref.**
1	*Acacia arabica* (Lam.) Wild.	Mimosaceae	leaves	water	31.4 (O157 EHEC)	30.1 (nd)	(Hassan et al., 2009)
2	*Acacia nilotica* (L.) Delile	Leguminosae	leaves, barks	ethanol 50% v/v, ethanol 90% v/v	19.5 (ATCC 25922)	9.75 (ATCC 7008030)	(Khan et al., 2009)
3	*Acalypha indica* Linn	Euphorbiaceae	leaves	methanol	125 (nd)	125 (nd)	(Gopalakrishnan et al., 2000)
4	*Adiantum capillus-veneris* L.	Adiantaceae	nd	methanol	0.48 (MTCC 443)		(Singh et al., 2008)
5	*Adiantum venustum* D. Don	Adiantaceae	nd	methanol	15.62 (MTCC 443)	7.81 (MTCC 109)	(Singh et al., 2008)
6	*Agrimonia pilosa* Ladeb	Rosaceae	herb	water	7.81 (ATCC 25922)		(McMurray et al., 2020)
7	*Albizia gummifera* (J.F.Gmel) C.A.Sm	Leguminosae	leaves	methanol	128 (LMP0101U)		(Tekwu et al., 2012)
8	*Alchornea cordifolia* (Schumach. And Thonn.) Muell. Arg	Euphorbiaceae	leaves	ethanol, methanol, ethyl acetate (Ec), Chloroform (Kp)	63 (ATCC 25922)	63 (ATCC 13883)	(Noundou et al., 2016)
9	*Alchornea cordifolia* (Schumach. and Thonn.) Müll. Arg	Euphorbiaceae	leaves, stem barks	methanol & ethanol (Ec), chloroform (Kp)	63 (ATCC 25922)	63 (ATCC 13883)	(Noundou et al., 2016)
10	*Alchornea floribunda* Müll. Arg.	Euphorbiaceae	leaves (Ec, Kp), roots (Kp)	methanol (leaves), chloroform (roots)	70 (ATCC 25922)	63 (ATCC 13883)	(Noundou et al., 2016)
11	*Alhagi mannifera* Jaub. & Spach	Fabaceae	leaves	petroleum ether	1.25 (ATCC 25922)	0.325 (ATCC 13883)	(Jaradat et al., 2021)
12	*Allium sativum* L.	Amaryllidaceae	cloves	ethanol 95% (v/v)	65 (clin. isol)		(Karuppiah & Rajaram, 2012)

13	*Alpinia galanga* L.	Zingiberaceae	rhizomes	methanol	40 (MTCC 1563)		(Rao et al., 2010)
14	*Andrographis echiodes* Nees	Acanthaceae	whole plant	chloroform	70 (NCIM 2065)	50 (NCIM 2957)	(Umadevi et al., 2003)
15	*Anemone chinensis* Bunge	Ranunculaceae	root	water	125 (ATCC 25922)		(McMurray et al., 2020)
16	*Annona squamosa* L.	Annonaceae	leaves	methanol		78 (ATCC 4552)	(Campos et al., 2021)
17	*Artocarpus communis* J.R, &G. Forst.	Moraceae	roots	methanol	64 (ATCC 8739)	128 (ATCC 11296)	(Kuete, Ango, et al., 2011)
18	*Asparagus racemosus* Wild	Liliaceae	tubers	acetone	125 (clin. isol)	31 (clin. isol)	(Panghal et al., 2011)
19	*Asphodelus tenuifoliu* Cav	Liliaceae	fruits	water	62.5 (clin. isol)		(Panghal et al., 2011)
20	*Balanites aegyptiaca* L	Balanitaceae	fruits	methanol		31 (clin. isol)	(Panghal et al., 2011)
21	*Barringtonia acutangula* (L.) Gaertn.	Lecythidaceae	twigs	chloroform (Kp), ethanol (Ec)	125 (nd)	125 (nd)	(Sahoo et al., 2008)
22	*Beilschmiedia obscura* (Staph). Engl.	Lauraceae	fruits	methanol	16 (ATCC 8739)	64 (ATCC 11296)	(Fankam et al., 2014)
23	*Beilshmiedia acuta* Kostem	Lauraceae	barks (Ec), leaves (Kp), fruits (Kp)	methanol	64 (ATCC 10536)	128 (ATCC 11296 & KP63), 32 (KP55),	(Tankeo, Tane, et al., 2015)
24	*Berberis aristata* DC.	Berberidaceae	roots	aqueous alcohol 50%	0.31 (MTCC 443)	0.62 (MTCC 109)	(Singh et al., 2007)
25	*Berberis asiatica* Roxb. Ex DC.	Berberidaceae	stem	aqueous alcohol 50%	2.5 (MTCC 443)	0.62 (MTCC 109)	(Singh et al., 2007)
26	*Berberis chitria* Buch.-Ham. ex Lindl.	Berberidaceae	stem	aqueous alcohol 50%	2.5 (MTCC 443)	0.62 (MTCC 109)	(Singh et al., 2007)
27	*Berberis lycium* Royle	Berberidaceae	stem	aqueous alcohol 50%	0.62 (MTCC 443)	0.31 (MTCC 109)	(Singh et al., 2007)
28	*Berginia ciliata* (Haw.) Sternb. Revis.	Saxifragaceae	roots	methanol	125 (nd)		(Neupane & Lamichhane,

	Saxifrag. suppl.						2020)
29	*Bolusanthus speciosus* (H. Bolus) Harms	Fabaceae	leaves	acetone	80 (nd)		(Elisha et al., 2017)
30	*Bridellia micrantha* (Hochst.) Baill	Euphorbiaceae	stem barks	methanol	50 (ATCC 25922)		(Kathare et al., 2021)
31	*Caesalpinia bonduc* (L.) Roxb.	Fabaceae	Seed coats	chloroform (Ec), methanol (Ec, Kp)	44 (MTCCB 1662)	88 (MTCCB 109)	(Arif et al., 2009)
32	*Calpurnia aurea* (Aiton) Benth spp. aurea	Fabaceae	leaves	acetone	40 (nd)	80 (nd)	(Elisha et al., 2017)
33	*Cassia abbreviata* Oliver	Fabaceae	stem barks	methanol		125 (ATCC 9997)	(Madureira et al., 2012)
34	*Cerbera manghas* L.	Apocynaceae	leaves	ethanol 80% (v/v)	4 (ATCC 25922)		(Frankova et al., 2021)
35	*Chenopodium ambrosioides* L.	Amaranthaceae	aerial parts	DCM		125 (ATCC 9997)	(Madureira et al., 2012)
36	*Cichorium intybus* L.	Asteraceae	pods	ethanol 80%	32.9 (O157 EHEC)	14.3 (nd)	(Hassan et al., 2009)
37	*Cinnamomum cassia* (L.) J.Presl	Lauraceae	barks	n-hexane	46.8 (Ec ATCC 25922)	46.8(clin. isol)	(Hossan et al., 2018)
38	*Cinnamomum zeylenicum* Linn Cor.	Lauraceae	leaves	methanol	64 (ATCC 8739)		(Voukeng et al., 2012)
39	*Cistus laurifolius* L.	Cistaceae	leaves	ethanol	32 (ATCC 35218)	32 (RSKK 574)	(Ustun et al., 2016)
40	*Clerodendron splendens* G. Don	Verbenaceae	leaves	methanol	128 (NCTC 9002)	128 (NCTC 418)	(Gbedema et al., 2010)
41	*Clerodendrum viscosum* Vent.	Lamiaceae	leaves	ethanol		128 (nd)	(Oly et al., 2011)
42	*Cremaspora triflora* (Thonn.) K.Schum	Rubiaceae	leaves	acetone	80 (nd)	80 (nd)	(Elisha et al., 2017)
43	*Crinum purpurascens* Herb.	Amaryllidaceae	leaves	methanol	128 (ATCC 8739)		(Voukeng et al., 2017)

44	*Curcuma malabarica* Vel.	Zingiberaceae	tubers	n-hexane, acetone		10 (NCIM 2957)	(Wilson et al., 2005)
45	*Curcuma zedoaria* Rosc	Zingiberaceae	tubers	n-hexane, acetone		10 (NCIM 2957)	(Wilson et al., 2005)
46	*Cylicodiscus gabunensis* Harms	Mimosae	Stem bark	ethyl acetate	3.12 (clin.isol)		(Kouitcheu Mabeku et al., 2006)
47	*Datura stramonium* L.	Solanaceae	seeds	petroleum ether	39.1 (ATCC 8739)		(Uzun et al., 2004)
48	*Dioscorea bulbifera* L. var sativa	Dioscoreaceae	bulbils	methanol	64 (ATCC 8739 & AG 100A), 128 (AG102)	64 (ATCC 11296), 128 (KP55 & KP63)	(Kuete et al., 2012)
49	*Dorstenia psilurus* Welwitch	Moraceae	roots	methanol	128 (ATCC 10536)		(Voukeng et al., 2012)
50	*Dorstenia turbinata* Engl.	Moraceae	twigs	methanol	19.53 (LMP701)	78.12 (LMP803)	(Ngameni et al., 2009)
51	*Echinops giganteus* A. Rich.	Asteraceae	roots	methanol		32 (K24)	(Fankam et al., 2011)
52	*Elaeodendron croceum (*Thunb) DC.	Celastraceae	leaves	acetone	80 (nd)		(Elisha et al., 2017)
53	*Emblica officinalis* Gaertn.	Phyllanthaceae	fruits	water	25.5 (O157 EHEC)	16.5 (na)	(Hassan et al., 2009)
54	*Embothrium coccineum* J.R. Forst.& G. Forst	Proteaceae	leaves	dichloromethane (Ec), ethyl acetate (Kp)	31.125 (clin. isol)	125 (clin. isol)	(Canales et al., 2016)
55	*Eruca sativa* (L.) Mill.	Brassicaceae	seeds	petroleum ether	65 (clin. isol)	68 (clin. isol)	(Gulfraz et al., 2011)
56	*Erythrina sigmoidea* Hua	Fabaceae	barks	methanol	32 (ATCC 8739), 16 (AG100)	64 (ATCC 11296), 16 (KP63), 128 (KP24), 64 (K2)	(Djeussi et al., 2013)
57	*Euphorbia hirta* L.	Euphorbiaceae	leaves	ethanol	100 (ATCC25922)		(Upadhyay et al., 2010)
58	*Euphorbia prostrata* Ait	Euphorbiaceae	whole plant	methanol	128 (AG100)		(Voukeng et al., 2017)
59	*Faraga tessmanii* Eng.	Rutaceae	roots	methanol	128 (ATCC10536), 16 (AG 100)	16 (ATCC11296)	(Tankeo, Damen, et al., 2015)

60	*Feijoa sellowiana* (O.Berg) O.Berg	Myrtaceae	fruits	water	4 (ATCC 11229)	16 (ATCC 10031)	(Vuotto et al., 2000)
61	*Ficus bubu* Warb.	Moraceae	barks	methanol	39.1 (clin. isol)		(Mbosso Teinkela et al., 2016)
62	*Ficus exasperata* Vahl.	Moraceae	leaves	methanol	128 (LMP0101U)		(Tekwu et al., 2012)
63	*Ficus polita* Vahl.	Moraceae	roots	methanol	64 (ATCC 8739)	128 (ATCC 11296)	(Kuete, Kamga, et al., 2011)
64	*Garcinia smeathmanii* Oliver	Clusiaceae	stem barks	methanol	39.06 (nd)	78.12 (nd)	(Kuete, Komguem, et al., 2007)
65	*Harungana madagascariensis* Lam.ex Pior	Hypericaceae	barks	methanol	<8 (ATCC 10536), 64 (AG100), 32 (AG 102 & AG100A), 64 (AG100 tet), 128 (MC4100), <8 (W3110)	16 (ATCC 11296 & KP63),64 (KP55), 128 (KP24)	(Tankeo et al., 2016)
66	*Helicanthus elastica* (Desr.) Danser	Loranthaceae	fresh parts	ethanol		62.5 (ATCC15380)	(Sunil Kumar et al., 2014)
67	*Heteromorpha arborescens* (Spreng.) Chan. & Schltdl	Apiaceae	leaves	acetone	80 (nd)		(Elisha et al., 2017)
68	*Hypericum roeperianum* G.W. Schimp.ex A. Rich var roeperianum	Hypericaceae	leaves	acetone	80 (nd)		(Elisha et al., 2017)
69	*Hyptis albida* Kunth	Lamiaceae	aerial parts	DCM:methanol (1:1 v/v)		100 (ATCC 700603)	(Camacho-Corona et al., 2015)
70	*Iris domestica* (L.) Goldblatt and Mabb	Iridaceae	rhizome	water	62.5 (ATCC 25922)		(McMurray et al., 2020)
71	*Lycopodium complanatum* L.	Lycopodiaceae	nd	petroleum ether, chloroform, methanol	32 (ATCC 35218), 64 (clin.isol)	16 (RSKK 574), 32 (clin. isol)	(Orhan et al., 2009)

72	*Malva oxyloba* Boiss.	Malvaceae	leaves	methanol	78 (ATCC 25922)		(Shadid et al., 2021)
73	*Markhamia tomentosa* K. Schum.	Bignoniaceae	leaves, barks	methanol	128 (AG100 & AG100A)	128 (K2)	(Voukeng et al., 2017)
74	*Marrubium globosum* Montbr. Et Auch. Ex Benth. spp. Libanoticum	Lamiaceae	aerial parts	methanol	32 (ATCC 11229)	16 (ATCC 27736)	(Rigano et al., 2007)
75	*Mentha arvensis* L.	Lamiaceae	leaves	n-hexane	11.7 (Ec ATCC 25922)		(Hossan et al., 2018)
76	*Moringa oleifera* Lam	Moringaceae	leaves	methanol	2.5 (ATCC 25922)		(Begum et al., 2021)
77	*Morus mesozygia* Stapf ex A. Chev	Moraceae	leaves	acetone	80 (nd)	80 (nd)	(Elisha et al., 2017)
78	*Murraya koenigii* (L.) Spreng.	Rutaceae	leaves	benzene (Ec), acetone (Kp)	125 (clin. isol)	62.5 (clin. isol)	(Panghal et al., 2011)
79	*Nauclea latifolia* Smith	Rubiaceae	stem bark (Ec), leaves (Kp)	methanol	32 (LMP0101U)	64 (LMP 0210U)	(Tekwu et al., 2012)
80	*Nauclea pobeguinii* (Pobég. ex Pellegr.) Merr. ex E.M.A.	Rubiaceae	barks (Ec, Kp), leaves (Kp)	methanol	32 (ATCC 10536), 64 (AG100)	128 (KP55)	(Seukep et al., 2016)
81	*Newbouldia laevis* (P. Beauv.) Seem.	Bignoniaceae	root barks (Ec), leaves (Kp)	methanol	78.12 (LMP0101U), 128 (ATCC10536)	128 (ATCC 11296)	(Kuete, Eyong, et al., 2007; Tankeo, Tane, et al., 2015)
82	*Nymphaea lotus* L.	Nymphaeaceae	flowers	water	32.9 (O157 EHEC)	24.3 (na)	(Hassan et al., 2009)
83	*Ocimum basilicum* L.	Lamiaceae	aerial parts	ethanol	125 (clin. isol)		(Adigüzel et al., 2005)
84	*Ocimum gratissimum* L.	Lamiaceae	leaves	methanol	62.5 (clin. isol)		(Prasannabalaji et al., 2012)
85	*Olea europaea* L.	Oleaceae	leaves	acetone	60 (UUMF-ST07)	25 (UUMF-KP16)	(Korukluoglu et al., 2010)

86	*Parkinsonia aculeate* L.	Fabaceae	aerial parts	n-hexane, DCM, ethyl acetate & methanol		125 (ATCC 9997)	(Madureira et al., 2012)
87	*Pedalium murex* L.	Pedaliaceae	fruits	acetone	125 (clin. isol)		(Panghal et al., 2011)
88	*Peperomia pellucida* (L.) Kunth	Piperaceae	aerial parts	ethanol 80% (v/v)	4 (ATCC 25922)		(Frankova et al., 2021)
89	*Phlomis armeniaca* Benth.	Lamiaceae	nd	petroleum ether, methanol	128 (ATCC 35218)	64 (RSKK574)	(Ozcelik et al., 2010)
90	*Phlomis bourgaei* Boiss.	Lamiaceae	nd	petroleum ether, methanol	128 (ATCC 35218)	64 (RSKK574)	(Ozcelik et al., 2010)
91	*Phlomis leucophracta* P.H.Davis& Hub.-Mor.	Lamiaceae	nd	petroleum ether, methanol	128 (ATCC 35218)	64 (RSKK574)	(Ozcelik et al., 2010)
92	*Phlomis lunariifolia* Sm.	Lamiaceae	nd	petroleum ether, methanol	128 (ATCC 35218)	64 (RSKK574)	(Ozcelik et al., 2010)
93	*Phlomis lycia* D. Don	Lamiaceae	nd	petroleum ether, methanol	128 (ATCC 35218)	64 (RSKK574)	(Ozcelik et al., 2010)
94	*Phlomis pungens* (var hirta & Pungens)	Lamiaceae	nd	petroleum ether, methanol	128 (ATCC 35218)	64 (RSKK574)	(Ozcelik et al., 2010)
95	*Picrorhiza kurroa* Royle ex Benth.	Plantaginaceae	roots	methanol	125 (nd)		(Neupane & Lamichhane, 2020)
96	*Piper nigrum* L.	Piperaceae	fruits	methanol, acetone, DCM	128 (ATCC 8739 & AG100A), 125 (NCIM 2089)	125 (NCIM 2957)	(Karsha & Lakshmi, 2010; Noumedem et al., 2013)
97	*Pithecellobium dulce* (Roxb.) Benth	Fabaceae	stem barks	ethanol 50% (v/v)		80 (MTCC 109)	(Singh et al., 2010)
98	*Pittosporum viridiflorum* Sims	Pittosporaceae	leaves	acetone	80 (nd)		(Elisha et al., 2017)
99	*Polygonum hydropiper* L.	Polygonaceae	aerial parts	methanol 80% v/v	64 (MTCC 739)	53.3 (ATCC 700603)	(Ayaz et al., 2016)
100	*Polyscias fulva (Hiern)* Harms.	Araliaceae	leaves, roots (Kp)	methanol	128 (W 3110)	128 (ATCC 11296 & KP63),	(Tankeo, Tane, et al., 2015)
101	*Psidium guajava* L.	Myrtaceae	leaves	methanol	0.78 (na)		(Dhiman et al., 2011)

102	*Psychotria sycophylla* (K. Schum) Petit	Rubiaceae	aerial parts	methanol	128 (AG100ATET)	128 (KP55)	(Demgne et al., 2021)
103	*Rheum australe* D. Don	Polygonaceae	roots	methanol	62.5 (nd)	125 (ATCC 13883)	(McMurray et al., 2020)
104	*Rheum emodi* Wall	Polygonaceae	rhizome	methanol	62.5 (ATCC 25922)		(Rolta et al., 2020)
105	*Ricinus communis* L.	Euphorbiaceae	seeds	methanol		31 (clin. isol)	(Panghal et al., 2011)
106	*Salvadora persica* L.	Salvadoraceae	leaves	n-hexane & methanol		125 (ATCC 9997)	(Madureira et al., 2012)
107	*Salvia euphratica* Montbret, Aucher & Rech. F. var. Euphratica	Lamiaceae	aerial parts	ethanol 96%	125 (ATCC 25923)		(Guzel et al., 2019)
108	*Sechium edule* (Jacq.)Sw.	Cucurbitaceae	leaves	ethanol 80% (v/v)	20 (ATCC 35218 & ATCC 25922), 20-40 (clin. isol)	40 (clin. isol)	(Ordonez et al., 2009)
109	*Silybum marianum* (L.) Gaertn.	Compositae	seeds	water (Ec), ethanol 80% (Kp)	38.2 (O157 EHEC)	20.0 (na)	(Hassan et al., 2009)
110	*Smilax acutifolia* Schltdl.	Smilacaceae	roots	DCM:methanol (1:1 v/v)	100 (ATCC 259222)		(Camacho-Corona et al., 2015)
111	*Smilax cordifolia* Humb. & Bonpl. ex Willd.	Smilacaceae	roots	DCM:methanol (1:1 v/v)	100 (ATCC 259222)		(Camacho-Corona et al., 2015)
112	*Smilax glabra* Roxb	Smilacacaeae	tuber	water	125 (ATCC 25922)		(McMurray et al., 2020)
113	*Smilax invenusta* Kunth	Smilacaceae	roots	DCM:methanol (1:1 v/v)	100 (ATCC 259222)		(Camacho-Corona et al., 2015)
114	*Smilax schiedeana* Kunth	Smilacaceae	roots	DCM:methanol (1:1 v/v)	100 (ATCC 259222)		(Camacho-Corona et al., 2015)
115	*Sphaeranthus hirtus* Willd.	Compositae	seeds	water	39.4 (O157 EHEC)	19.4 (nd)	(Hassan et al., 2009)

116	*Syzigium samarangese* (Blume) Merr. & L.M.Perry	Myrtaceae	fruits	ethyl acetate (Ec), petroleum ether & methanol (Kp)	125 (MTTC 1687)	125 (MTCC 109)	(Ratnam & Raju, 2008)
117	*Tecrium africanum* Thunb.	Lamiaceae	leaves	methanol: DCM (1:1 v/v)	125 (Ec ATCC8739)		(Ruiters et al., 2016)
118	*Tectona grandis* Linn.	Verbenaceae	fruits	ethanol	64 (ATCC8739)	128 (ATCC 1148)	(Bitchagno et al., 2015b)
119	*Terminalia bellirica* (Gaertn.) Roxb.	Combretaceae	fruits	ethanol	23.4 (Ec ATCC 25922)	93.7 (clin. isol)	(Hossan et al., 2018)
120	*Trachystemon orientalis* (L.) G. Don	Boraginaceae	whole plant	ethanol	39.1 (ATCC 8739)		(Uzun et al., 2004)
121	*Trichilia emetia* Vahl	Meliaceae	leaves	n-hexane		125 (ATCC 9997)	(Madureira et al., 2012)
122	*Tridesmostemon omphalocarpoides* Engl.	Sapotaceae	stem bark	methanol	78.12 (LMP010U)		(Kuete et al., 2006)
123	*Trigonella foenum-graecum* L.	Fabaceae	leaves	benzene	31 (clin. isol)	31 (clin. isol)	(Panghal et al., 2011)
124	*Vangueria spinosa* Roxb.	Rubiaceae	leaves	ethanol	32.2 (MTCC 739)	25.5 (MTCC 432)	(Chatterjee et al., 2009)
125	*Vitis vinifera* L.	Vitaceae	fruits	methanol	19.74 (clin. isol)		(Guessaibia et al., 2019)
126	*Xylopia aethiopica* (Dunal) A. Rich.	Annonaceae	fruits	methanol	64 (ATCC 10536)	64 (KP 63)	(Fankam et al., 2011)
127	*Zingiber officinale* Roscoe	Zingiberaceae	rhizomes	ethanol 95% (v/v)	75 (clin. isol)		(Karuppiah & Rajaram, 2012)
128	*Zuccagnia puncata* Cav.	Leguminosae	leaves	ethanol 96% (v/v)	100 (ATCC 25922)		(Zampini et al., 2005)

nd = data not declared; clin.isol = clinical isolate; Ec = Escherichia coli; Kp = Klebsiella pneumoniae, DCM = dichloromethane

Antibacterial activity across plants' families

To substantiate the observed patterns between most frequently studied plant families and their antibacterial potentials against *E. coli* and *K. pneumoniae*, statistical analyses involving frequencies, means, and quantiles (1–3 quartiles), were conducted. Moreover, similar analyses were performed for other parameters including types of plant tissues, nature of the solvents used for extraction and classes of phytochemicals.

The plants studied were noted to belong to a total of 51 plant species, among them, Lamiaceae, Moraceae, Fabaceae, Euphorbiaceae and Rubiaceae formed the 5 most studied families (**Fig. 1, Table 1**). Moreover, **Figure 2** indicates the ranking of antibacterial potentials for families with at least 4 plant species studied, in which Berberidaceae, Fabaceae, Lauraceae and Euphorphiaceae were the most active families against both bacteria. Nevertheless, MIC values of ≤10 µg/mL were reported in plants from the rather less represented families of Apocynaceae, Adiantaceae, Mimosaceae, Moringaceae, Myrtaceae, Piperaceae, Rosaceae and Verbenaceae (**Table 1**).

Figure 1: Word cloud diagrams representing the type and frequency (based on font size) of the families of plant species with reported MIC values of 128 or lower against either *E. coli* or *K. pneumoniae* or both

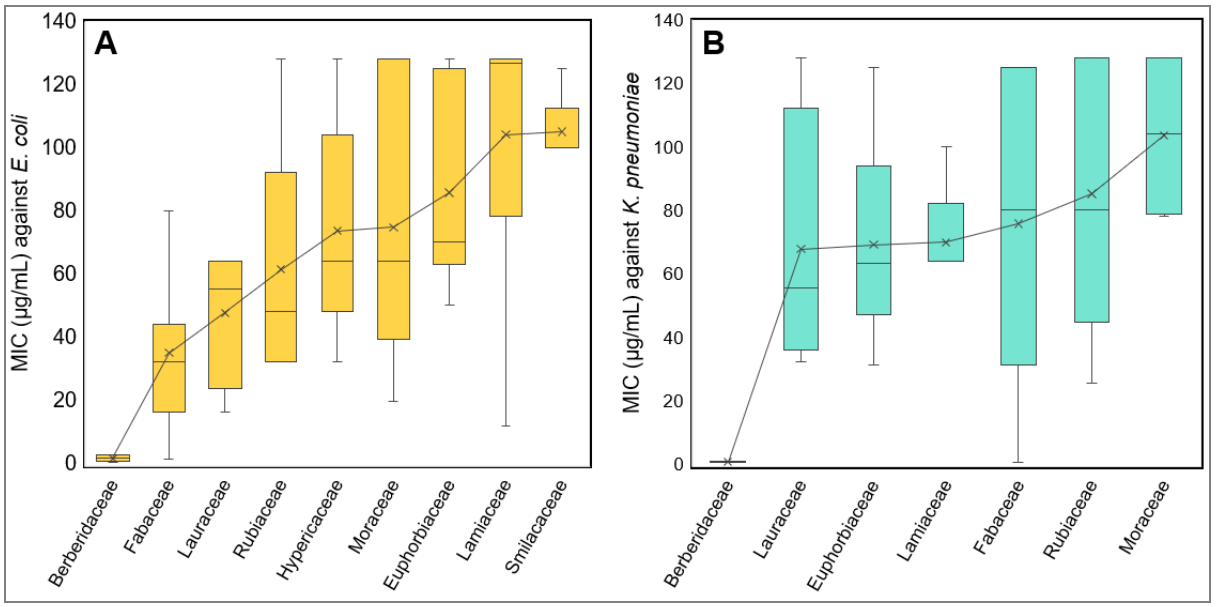


Figure 2: Box-Whisker plot showing the average distribution of MIC values against *E. coli* (A) and *K. pneumoniae* (B) by plants from across the families with at least 4 studied species (x = mean value, whiskers' span shows the highest and lowest values).

The large differences between the lowest and highest MIC values in a given family could be due to the data coming from different laboratories, differences in plant species, parts and extracting solvents. However, all activities were determined via the same experimental procedure (broth dilution assay). The antimicrobial potentials of Berberidaceae were linked to the presence of berberine and other isoquinoline alkaloids like chenabine, jhelumine, sindamine, karakoramine, punjabine, and hilgitine (Khan et al., 2016; Srivastava et al., 2015). Moreover, the presence of different lupine and quinolizidine alkaloids, in addition to an array of flavonoids was attributed to the antimicrobial potentials of the family Fabaceae (Ahmad et al., 2016; Krishna et al., 2012; Orni et al.). Similarly, the family Lauraceae is known for high compositions of antimicrobial essential oils among other terpenoids, in addition to alkaloids, flavonoids, lignans, and steroids (Cao et al., 2015; Custódio & Florêncio da Veiga Junior, 2014; Damasceno et al., 2019; Wan Salleh & Ahmad, 2017). Furthermore, the antimicrobial activities of plants from the family Euphorbiaceae were linked to the presence of terpenoids, flavonoids, saponins, tannins, and alkaloids, among other secondary metabolites (Bijekar & Gayatri, 2014; Mwine & Van Damme, 2011).

Based on such diverse phytochemical compositions, it is difficult to ascertain if the observed higher antibacterial potentials of those families are functions of a particular class of compounds, a synergistic role of several classes or both. Nevertheless, these findings highlight and provide guidance on the plant families with a higher likelihood of hosting compounds against Enterobacteriaceae and possibly other Gram-negative bacteria. On the other hand, they emphasize the need for deeper and extensive exploring of antibacterial activities from among the less-frequently studied,

yet highly potential families. Similar findings by Chassagne et al., 2021, showed higher activities across a wide range of Gram-negative bacteria exhibited by plants from the families of Apiaceae, Combretaceae, Fabaceae, Lauraceae, Rutaceae, Rubiaceae, and Zingiberaceae (Chassagne et al., 2020). Moreover, there is a need for further reviews/studies on the comparison of antimicrobial potentials of different plant families/genera with a focus on more related groups of bacteria.

Antibacterial activities across plant tissues

Leaves, barks, roots, fruits, and aerial parts were noted to be the most used plant tissues among the reviewed studies in the screening for antimicrobial activities of different plants. Among them, the activities from the seeds, barks, rhizomes, and fruits extracts were consistently higher against both bacteria. On the other end, extracts from aerial parts, roots, and leaves were noted to be of lower potencies (**Figure 3**). These findings are similar to those reported by Chassagne et al., 2021, whereby extracts from rhizome, fruits, seeds, and stem barks showed higher potentials across a range of Gram-negative bacteria (Chassagne et al., 2020).

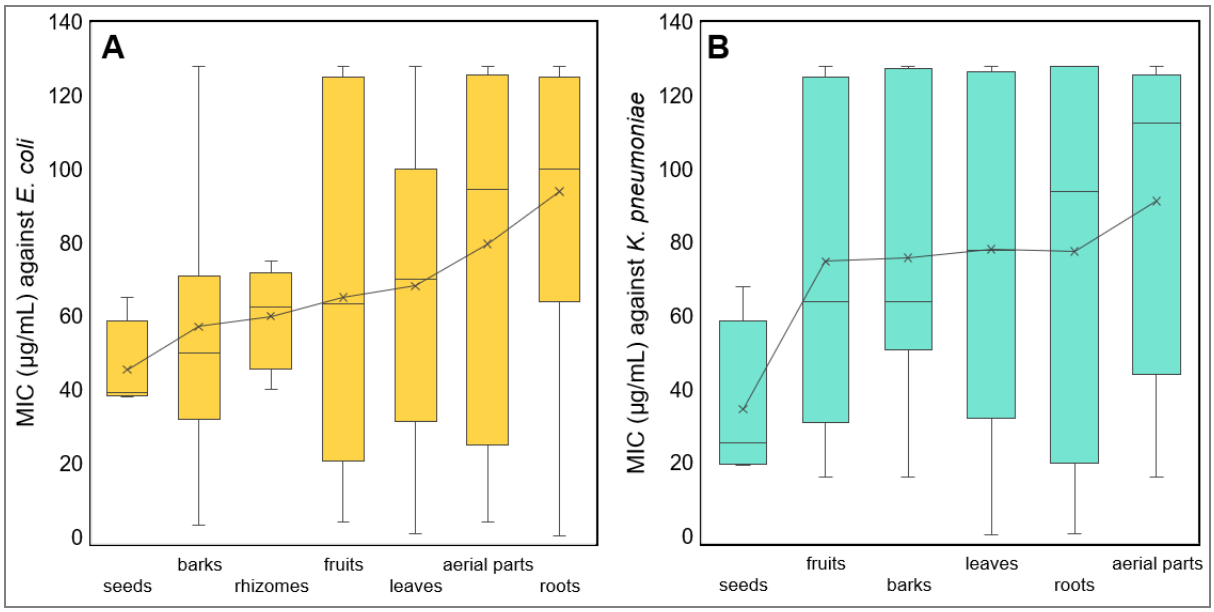


Figure 3: Box-Whisker plots showing distributions of MIC values against *E. coli* (A) and *K. pneumoniae* (B) by extracts from different plant tissues with at least 4 studied samples (x = mean value, whiskers' span shows the highest and lowest values).

The observed differences in antibacterial activities across various plant tissues might be related to the differences in types and quantities of phytochemicals available in each tissue as driven by genetical, seasonal and ecological factors (Drabińska et al., 2021; Lavola et al., 2017). Generally, these findings lend higher preferences to extracts from seeds, barks, rhizomes, and fruits in the screening for activities against Gram-negative bacteria, in cases where choices are to be made.

Antibacterial activities across extracting solvents

The current review has indicated methanol, ethanol, acetone, water and petroleum ether and chloroform to be the most frequently used solvents in the extraction of plant

materials towards screening of their antibacterial potentials. As revealed in **Figure 4**, water, chloroform, and ethanol extracts were generally the most potent against *E. coli*, as it was for water, chloroform, and acetone against *K. pneumoniae*. These findings are partly different from previous reports of higher potentials of acetone and methanol extracts among Gram negative bacteria (Chassagne et al., 2020).

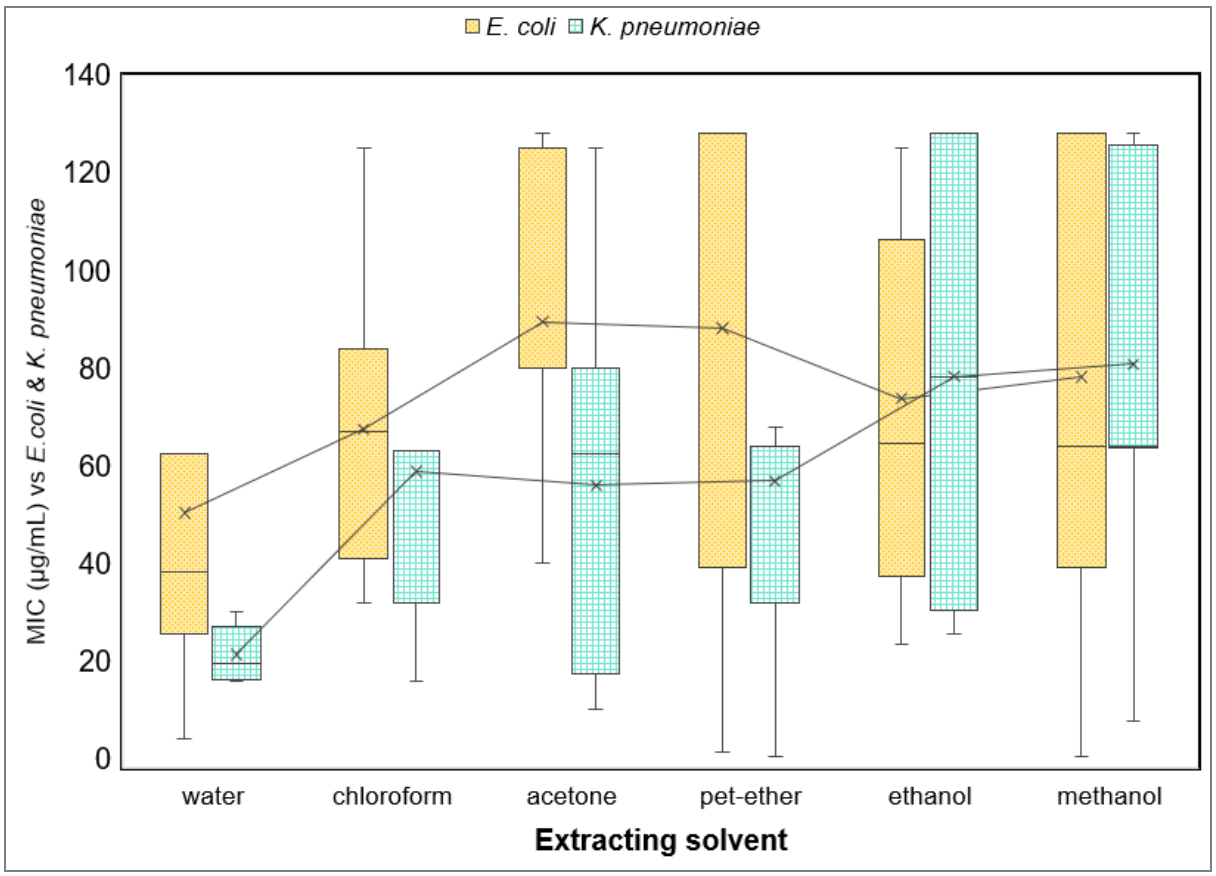


Figure 4: Box-Whisker plot showing distributions of MIC values against *E. coli* and *K. pneumoniae* by extracts from different solvents with at least 5 studied samples (x = mean value, whiskers' span shows the highest and lowest values).

The nature of the extracting solvents is crucial in determining the ultimate polarities of the extracted phytochemicals. The observed higher prevalence and activities of less-polar solvents reflect the higher potentials of more lipophilic phytochemicals, particularly against Gram-negative bacteria (Hatano et al., 2005; Melliou et al., 2005; Merkl et al., 2010). Moreover, this aspect is discussed further in the following sections of this review. Conversely, the observed highest potentials exhibited by water extracts are of interest. This is particularly because of a clear break in the trend of observed activities with an increase in solvents' polarities. Partly, this might be explained by the synergistic effects from many highly polar compounds present in water extracts, which ultimately exhibit lower potentials upon their isolation (Paluch et al., 2021).

Screening for phytochemicals present in crude extracts

In addition to the provision of accounts on classes of phytochemicals which were previously ascertained in the plants studied (Bitchagno et al., 2015b; Hassan et al., 2009; Ordonez et al., 2009; Rigano et al., 2007), the screening for classes of phytochemicals present in the investigated crude extracts was broadly conducted. Most of such experiments involved semi-quantitative or qualitative approaches using classical methods for the identification of phytochemicals (Bitchagno et al., 2015b; Dhiman et al., 2011), and in some cases, the use of simplified techniques like Thin Layer Chromatography (TLC) profiling followed by spray reagents was portrayed (Madureira et al., 2012).

The frequently reported phytochemical classes were flavonoids (Dhiman et al., 2011; Kouitcheu Mabeku et al., 2006; Ordonez et al., 2009; Rigano et al., 2007), phenolic compounds (Madureira et al., 2012; Noumedem et al., 2013; Ordonez et al., 2009; Voukeng et al., 2012), alkaloids (Fankam et al., 2014; Kuete et al., 2006; Orhan et al., 2009; Voukeng et al., 2012), steroids (Bitchagno et al., 2015b; Dhiman et al., 2011; Fankam et al., 2014; Panghal et al., 2011) and anthraquinones (Bitchagno et al., 2015b; Kuete et al., 2006; Noumedem et al., 2013; Panghal et al., 2011). Others included terpenoids (Bitchagno et al., 2015b; Madureira et al., 2012; Rashed & Butnariu, 2014; Voukeng et al., 2012), carbohydrates (Dhiman et al., 2011; Kouitcheu Mabeku et al., 2006; Rashed & Butnariu, 2014), tannins (Dhiman et al., 2011; Fankam et al., 2014; Gbedema et al., 2010), saponins (Fankam et al., 2014; Kuete et al., 2006; Voukeng et al., 2012) and essential oils (Ruiters et al., 2016).

Moreover, the tendency of attributing several observed phytochemical classes to either the observed antibacterial activities (Karsha & Lakshmi, 2010; Rigano et al., 2007; Singh et al., 2010) or possible antibacterial mode(s) of action (Dhiman et al., 2011) of the investigated extract was observed. Nevertheless, the objectivity of such conclusions is limited. This is because primarily, the observed activities are not necessarily the functions of the most abundant phytochemicals within the crude extract, and also the high likelihood of synergizing and overlapping activities of different classes. In the absence of the required resources for the successful isolation and characterization of respective antibacterial compounds, one could more objectively identify the phytochemical class of the active spot(s) on a TLC profile after ascertaining their activities by bioautography techniques (Madureira et al., 2012; Noundou et al., 2016).

Identification, isolation, and characterization of antibacterial compounds

Efforts to establish the identity of compounds responsible for the observed activities were generally portrayed in two main aspects. The first approach involved the use of Gas Chromatography-Mass Spectrometry (GC-MS) through which the masses and relative abundances of a large number of compounds present within the extracts were determined (Canales et al., 2016; Dhiman et al., 2011; Orhan et al., 2009; Rao

et al., 2010). Further identification of those compounds with the help of Mass spectral databases was implicated (Kuete, Kamga, et al., 2011; Orhan et al., 2009).

This approach has the potential to give hints on the identities of a large number of compounds present within the crude extracts within a relatively short time. Moreover, an analysis of the novelty of the present compounds and any previously reported biological activities can be conducted without the need to pre-isolate the bulk of compounds. Still, the approach is restricted to cases where compounds present in the crude extract were previously isolated and their respective data are retrievable from the reference databases. Further, the observed antibacterial activities are not necessarily the functions of the most abundant compound(s) within the extract (Rao et al., 2010).

The second modality involved a series of methods aimed at isolating and fully characterizing compounds exhibiting the observed antibacterial activities. Unlike the previous approach, more focus and prioritization were required to reduce the workload and minimize the utilization of available resources. To enable this, the use of bioassay-guided fractionation and isolation was reported (Kuete et al., 2012; Tankeo et al., 2016). In addition to the common preparation of sub-fractions using silica gel packed open column chromatography (Kuete, Ango, et al., 2011; Kuete, Kamga, et al., 2011; Tankeo et al., 2016; Zampini et al., 2005), other techniques employing vacuum column chromatography and gel filtration with cross-linked dextran (Sephadex LH-20) were presented (Kuete, Ango, et al., 2011; Ngameni et al., 2009).

Furthermore, the widespread utilization of spectrometric and spectroscopic technologies like UV-Vis spectroscopy (Bitchagno et al., 2015b; Rashed & Butnariu, 2014), IR spectroscopy (Bitchagno et al., 2015b; Kuete et al., 2012; Noundou et al., 2016), Mass spectrometry (Bitchagno et al., 2015b; Korukluoglu et al., 2010; Rashed & Butnariu, 2014; Tankeo, Damen, et al., 2015)), Nuclear Magnetic Resonance (NMR) Spectroscopy (Bitchagno et al., 2015b; Noundou et al., 2016; Zampini et al., 2005) was observed. Other determined characteristics included melting points (Bitchagno et al., 2015b; Kuete et al., 2012) and optical rotation properties of the isolated compounds (Kuete et al., 2012; Ngameni et al., 2009). Despite lacking in the reviewed studies, the use of Quadrupole Time of Flight (Q-TOF) mass spectrometry in the determination of accurate masses and hence chemical formulas of phytochemicals within plant extracts before their actual isolation is increasingly popular (Raju et al., 2015; Yang et al., 2021).
Generally, carrying out isolation and characterization of the antibacterial compounds from plant extracts following the establishment of their antibacterial properties was noted to be less frequent among the reviewed studies. Among other factors, this may be caused by the overall requirements for more sophisticated expensive equipment expertise usually associated with those experiments. On the other hand, the majority of the authors are likely in favour of reporting such findings in separate subsequent articles. While gaining more publications might motivate this tendency, the resulting

gaps complicate the follow-up and application of the subsequent outcomes by the readers.

Prospects from evaluation of crude plant extracts

A number of studies were observed to emphasize the contribution of the reported findings toward supporting the ongoing traditional uses of the investigated plant species (Gbedema et al., 2010; Madureira et al., 2012; Noundou et al., 2016; Ratnam & Raju, 2008). Moreover, a number of determined activities were claimed to be reported for the first time (Kouitcheu Mabeku et al., 2006; Ozcelik et al., 2010; Ustun et al., 2016; Uzun et al., 2004), hence underscoring the existence of many yet-to-be-discovered antibacterial potentials hosted among largely unexplored plant biodiversity (Verpoorte, 2000).

The shared opinion that screening for antibacterial compounds among plant-derived extracts is of valuable contribution in the search for new antibiotics was realized (Ayaz et al., 2016; Bitchagno et al., 2015b; Tekwu et al., 2012). Moreover, many authors were quick to recommend the need for conducting further studies aimed at isolating the active compounds (Camacho-Corona et al., 2015; Chatterjee et al., 2009; Kouitcheu Mabeku et al., 2006; Orhan et al., 2009; Sahoo et al., 2008; Sunil Kumar et al., 2014; Voukeng et al., 2017) as well as determining the underlying modes of action (Noundou et al., 2016), and toxicity profiles (Chatterjee et al., 2009; Hassan et al., 2009; Noumedem et al., 2013). Recommendations on follow-up studies by other investigators are, however, commonly limited by factors such as the limited availability of plant species of interest along with low rates of success in reproducing findings reported elsewhere (Masota et al., 2021).

Plant-isolated compounds effective against *E. coli* and *K. pneumoniae*

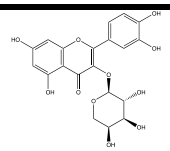
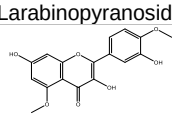
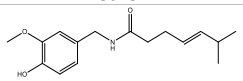
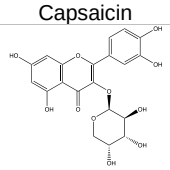
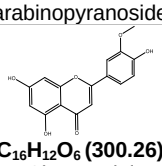
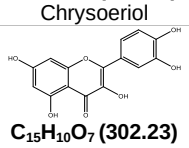
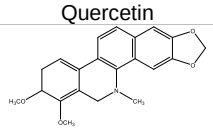
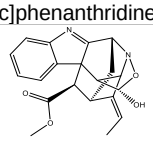
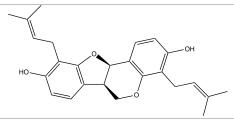
A total of 122 compounds active against *E. coli* and/or *K. pneumoniae* (MIC≤100 µg/mL) isolated and from crude plant extracts were retrieved from a literature search between 2010 and 2020 (**Table 2**). The reported MIC values were determined through broth dilution assays.

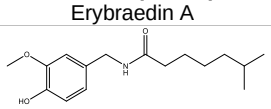
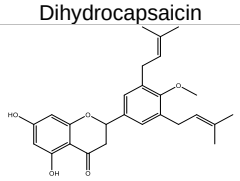
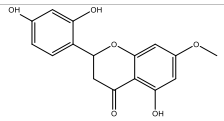
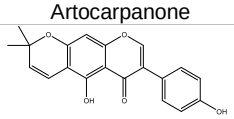
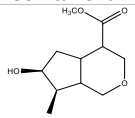
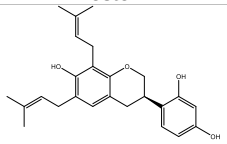
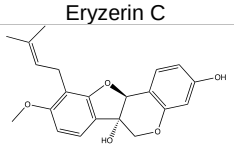
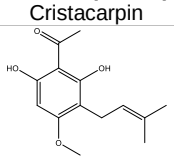
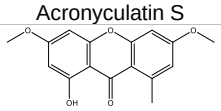
The corresponding molecular formula, molecular weight and ClogP values were determined using ChemDraw® software, whereas the number of hydrogen bond donors (nON) and acceptors (nOHNH), as well as the total polar surface areas (tPSA), were calculated on a Molinspiration chemoinformatics software (**Table 2**). These properties were chosen in the quest of assessing the retrieved compounds in line with Lipinski's rule of 5 (Pollastri, 2010)

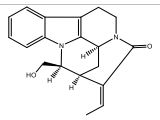
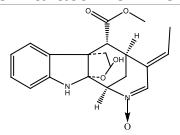
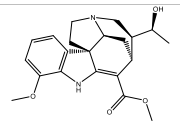
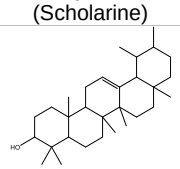
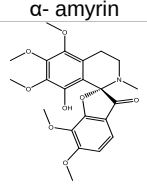
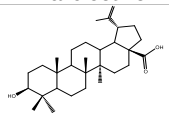
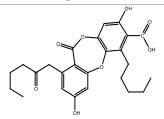
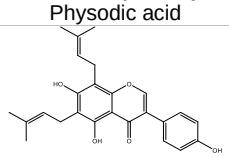
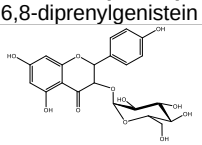
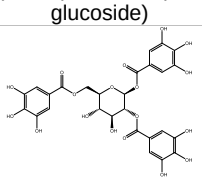
An account of the structures, names, MIC values, and other selected properties for each compound is provided in **Table 2**. Whenever possible the simple common names preferably those stated by the authors were indicated. However, in cases

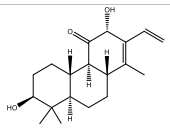
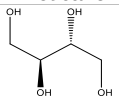
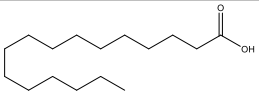
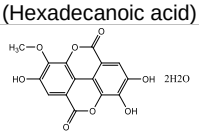
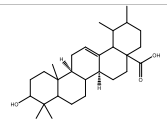
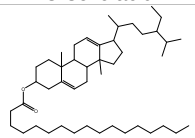
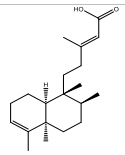
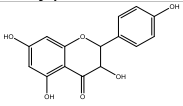
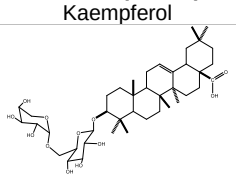
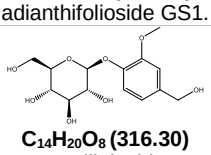
where no names were provided, the indicated names were generated on the ChemDraw® software.

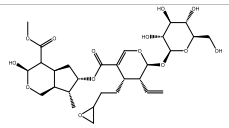
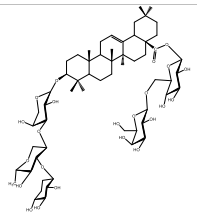
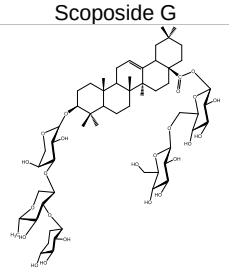
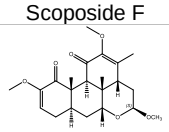
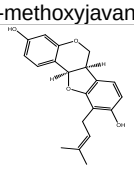
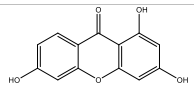
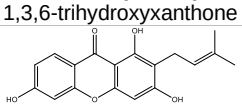
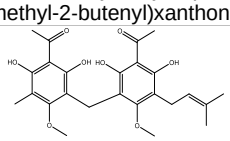
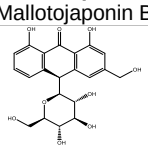
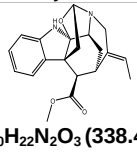
Table 2: Plant-isolated compounds (122) active against *E. coli* (Ec) and *K. pneumoniae* (Kp) (MIC ≤ 100 µg/mL). Compounds are arranged in order of increasing MIC values based on *E. coli* followed by *K. pneumoniae*.

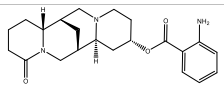
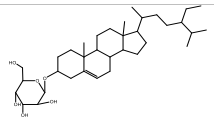
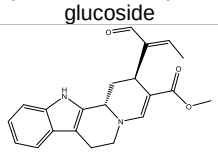
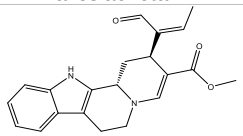
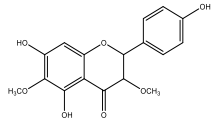
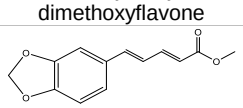
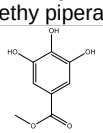
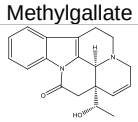
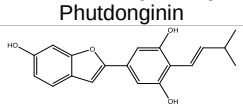
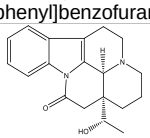
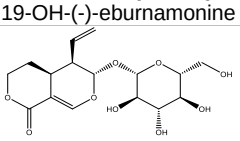
SN	Chemical structure, molecular formula and name	Class (Subclass)	MIC (µg/mL)		ClogP	nON; nOHNH	tPSA	Reference
			Ec	Kp				
1	 **$C_{20}H_{18}O_{11}$ (434.35)** Quercetin-3-O-α-Larabinopyranoside	Flavonoid (Flavonol glycoside)	0.187		-0.303	1;7	186.37	(Metwally et al., 2010)
2	 **$C_{17}H_{14}O_7$ (330.29)** Quercetin 5,4'-dimethyl ether	Flavonoid (Falvonol)		0.49	1.693	7;3	105.45	(Elkady et al., 2020)
3	 **$C_{16}H_{23}NO_3$ (277.36)** Capsaicin	Phenylpropanoid (Capsaicinoid)	5	0.6	2.692	4;2	58.56	(do Nascimento et al., 2014)
4	 **$C_{20}H_{18}O_{11}$ (434.35)** Quercetin-3-O-β-D-arabinopyranoside	Flavonoid (Flavonol glycoside)	0.093		-0.303	11;7	186.37	(Metwally et al., 2010)
5	 **$C_{16}H_{12}O_6$ (300.26)** Chrysoeriol	Flavonoid (Flavone)	0.06	0.25	2.749	6;3	96.22	(do Nascimento et al., 2014)
6	 **$C_{15}H_{10}O_7$ (302.23)** Quercetin	Flavonoid (Flavonol)	1.25		1.503	7;5	127.45	(Metwally et al., 2010)
7	 **$C_{21}H_{21}NO_4$ (351.40)** 1,2-Dimethoxy-12-methyl-2,3,12,13-tetrahydro-[1,3]dioxolo[4',5':4,5]benzo[1,2-c]phenanthridine	Azaarene (Phenantridine)	16	1.97	3.767	5;0	40.16	(Tantapakul et al., 2012)
8	 **$C_{20}H_{20}N2O_4$ (352.39)** Nareline	Alkaloid (Indole alkaloid)	50	1.56	1.771	6;1	71.36	(Liu et al., 2015)
9		Flavanoid (Isoflavonoid/ Pterocarpan)	2		6.259	4;2	58.92	(Sadgrove et al., 2020)

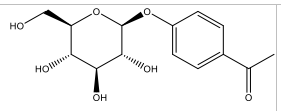
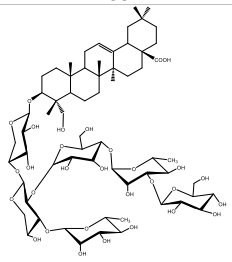
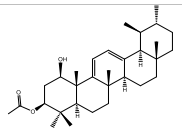
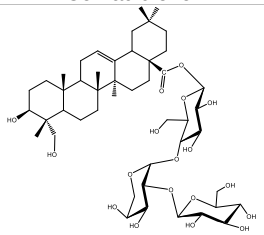
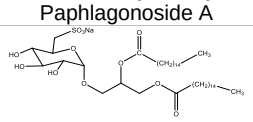
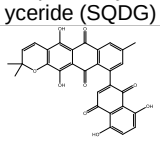
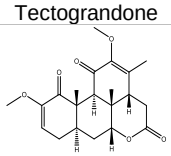
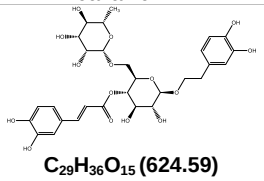
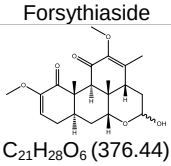
	$C_{25}H_{28}O_4$ (392.49) Erybraedin A							
10	 **$C_{16}H_{25}NO_3$ (279.38)** Dihydrocapsaicin	Phenylpropanoid (Capsaicinoid)	5	2.5	58.56	4;2	3.1762	(do Nascimento et al., 2014)
11	 **C26H30O5 (422.52)** Abyssione-V 4′-O-methyl ether	Flavonoid (Flavanone)	3.9	3.9	7.032	5;2	75.99	(Chukwujekwu et al., 2011)
12	 **$C_{16}H_{14}O_6$ (302.23)** Artocarpanone	Flavonoid (Flavanone)	3.9	-	2.250	6;3	96.22	(Septama & Panichayupakaranant, 2017)
13	 **$C_{20}H_{16}O_5$ (336.34)** Alpinumisoflavon	Flavonoid (Isoflavonoid)	3.9	3.9	4.469	5;2	75.99	(Chukwujekwu et al., 2011)
14	 **$C_{16}H_{12}O_7$ (316.27)** Isorhamentin	Flavonoid (Flavonol)		3.9	1.217	7;4	116.45	(Elkady et al., 2020)
15	 **$C_{11}H_{18}O_4$ (214.26)** Cyclopenta(c) pyran-4-carboxylic acid, octahydro-3,6-dihydroxy-7-methyl ester	Terpenoid (Monoterpenoid/ Iridoid)	4	64	-0.145	4;1	55.76	(Sarıkahya et al., 2011)
16	 **$C_{25}H_{30}O_4$ (394.51)** Eryzerin C	Flavonoid (Isoflavonoid)	5		6.044	4;2	69.92	(Sadgrove et al., 2020)
17	 **$C_{21}H_{22}O_5$ (354.40)** Cristacarpin	Flavonoid (Isoflavonoid/ Pterocarpan)	6		3.896	5;2	68.15	(Sadgrove et al., 2020)
18	 **$C_{14}H_{18}O_4$ (250.29)** Acronyculatin S	Acetophenone	6.25		3.388	4;2	66.76	(Tchangoue et al., 2020)
19	 **$C_{16}H_{14}O_5$ (286.28)** Lichenxanthone	Xanthone	6.25		4.089	5;1	64.99	(Tchangoue et al., 2020)

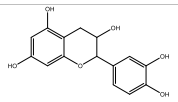
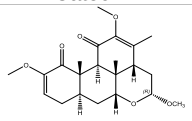
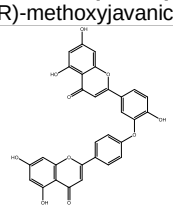
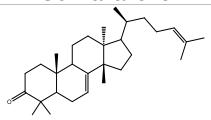
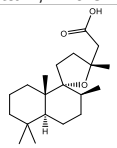
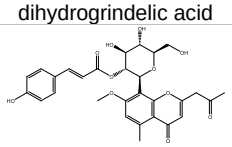
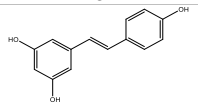
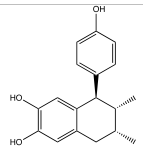
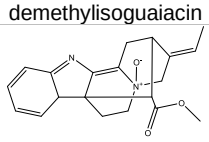
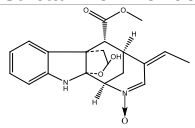
20	 **$C_{19}H_{20}O_2$ (308.381)** Normavacurine-21-one	Alkaloid (Monoterpenoid indole alkaloid)	100	6.25	1.599	4;1	43.78	(Liu et al., 2015)
21	 **$C_{20}H_{22}N_2O_5$ (370.40)** 5-hydroxy-19, 20-E-alschomine.	Alkaloid (Monoterpenoid indole alkaloid)	100	6.25	0.441	7;2	93.59	(Liu et al., 2015)
22	 **$C_{21}H_{26}N_2O_4$ (370.44)** 12-methoxyechitamidine (Scholarine)	Alkaloid	100	6.25	1.980	6;2	71.03	(Liu et al., 2015)
23	 **$C_{30}H_{50}O$ (426.38)** α- amyrin	Terpenoid (Pentacyclic triterpenoid)	6.25		10.66	1;1	20.23	(Kaur, 2015)
24	 **$C_{22}H_{25}NO_8$ (431.41)** Thalicfoetine	Alkaloid (Spirobenzylisoquinoline alkaloid)	6.25		1.900	9;1	95.92	(Ding et al., 2019)
25	 **$C_{30}H_{48}O_3$ (456.71)** JFE	Terpenoid (Lupane-type terpenoid)	6.25	25	8.477	3;2	57.53	(Joshua et al., 2020)
26	 **$C_{25}H_{28}O_8$ (456.49)** Physodic acid	Depsidone		7.5	6.397	8;3	130.36	(Kosanic et al., 2013)
27	 **$C_{25}H_{26}O_5$ (406.47)** 6,8-diprenylgenistein	Flavonoid (Isoflavone)	7.8	7.8	6.257	5;3	86.99	(Chukwujekwu et al., 2011)
28	 **$C_{21}H_{22}O_{11}$ (450.39)** Astragalin (Kaempferol-3-O-β-d-glucoside)	Flavonoid (Flavonol glycoside)	-	7.81	0.327	11;7	186.37	(Elkady et al., 2020)
29	 **$C_{27}H_{24}O_{18}$ (636.47)** 1,2,6-tri-O-galloyl-b-D-glucopyranose	Tannin (Hydrolysable tannin)	12.1 - 97.5	24.3 - 97.5	0.111	18;11	310.66	(Bag et al., 2013)

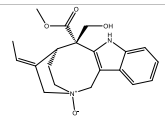
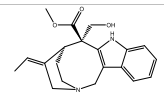
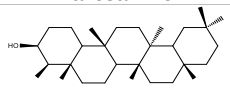
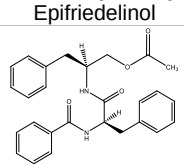
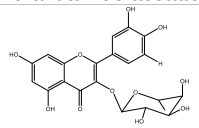
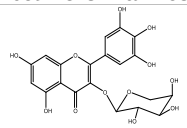
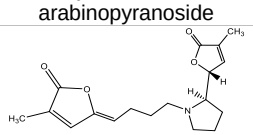
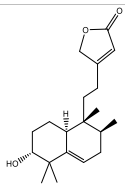
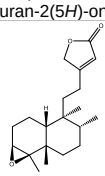
30	 **$C_{19}H_{28}O_3$ (304.43)** Niloticane	Terpenoid (Cassane diterpene)	33	16	2.739	3;2	57.53	(Eldeen et al., 2010)
31	 **$C_4H_{10}O_4$ (122.12)** Meso-Erythritol	Sugar alcohol	12.5		-1.707	4;4	80.92	(Mbosso et al., 2010)
32	 **$C_{16}H_{32}O_2$ (256.43)** Palmitic acid (Hexadecanoic acid)	Long-chain fatty acid	12.5		7.212	2;1	37.3	(Kaur, 2015)
33	 **$C_{15}H_8O_8$ (316.22)** 3-O-methyl ellagic acid dihydrate (ellagic acid derivative)	Tannin (Hydrolyzable tannin)	12.5		0.589	8;3	122.52	(Parveen et al., 2015)
34	 **$C_{28}H_{55}O_3$ (428.65)** Ursolic acid	Terpenoid (Pentacyclic triterpenoid)	[50]	12.5 [100]	7.589	3;2	57.53	(Srinivasan et al., 2017; Wolska et al., 2010; Zhu et al., 2015)
35	 **$C_{46}H_{80}O_2$ (665.14)** 14-methyl-12, 13-dehydro-sitosterol-heptadeconate	Phytosteroid	12.5		18.845	2;0	26.3	(Kaur, 2015)
36	 **$C_{20}H_{32}O_2$ (304.47)** (E)-5-[(1R,2S,4aR,8aS)-1,2,4a,5-tetramethyl-2,3,4,7,8,8a-hexahydronaphthalen-1-yl]-3-methylpent-2-enoic acid	Terpenoid	15.6	93.8	5.38	2;1	7.482	(Du et al., 2015)
37	 **$C_{15}H_{12}O_6$ (286.23)** Kaempferol	Flavonoid	16		1.367	6;4	107.22	(Teffo et al., 2010)
38	 **$C_{41}H_{66}O_{12}$ (750.96)** adianthifolioside GS1.	Terpenoid (Saponin/triterpene glycoside)	16		6.120	12;7	195.6	(Sonfack et al., 2021)
39	 **$C_{14}H_{20}O_8$ (316.30)** Vanilloloside	Phenolic glycoside	16	32	-1.320	8;5	128.84	(Sarıkahya et al., 2011)

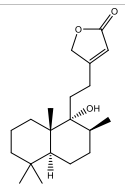
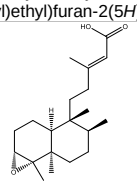
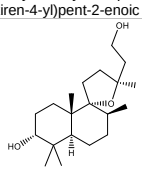
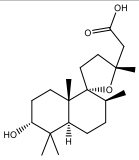
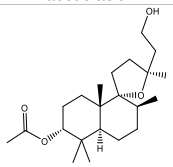
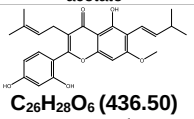
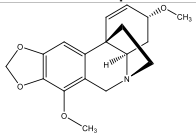
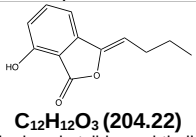
40	 **$C_{29}H_{42}O_{14}$ (614.64)** Laciniatoside-I	Terpenoid (Monoterpenoid/ Iridoid glucoside	16	32	-0.698	14;5	-0.43	(Sarıkahya et al., 2011)
41	 **$C_{59}H_{96}O_{23}$ (1173.39)** Scoposide G	Terpenoid (Saponin/triterpene glycosides)	16	32	4.492	23;12	363.13	(Sarıkahya et al., 2011)
42	 **$C_{59}H_{96}O_{23}$ (1173.39)** Scoposide F	Terpenoid (Saponin/triterpene glycosides)	16	32	4.492	23;13	363.13	(Sarıkahya et al., 2011)
43	 **$C_{22}H_{30}O_6$ (390.47)** (16S)-methoxyjavanicin B	Quassinoid (degredaded triterpene)		19.56	1.755	6;0	71.06	(Prema et al., 2019)
44	 **$C_{20}H_{20}O_4$ (324.37)** Phaseollidin	Flavonoid (Isoflavonoid /Pterocarpan)	20		4.308	4;2	5.92	(Sadgrove et al., 2020)
45	 **$C_{13}H_8O_5$ (244.20)** 1,3,6-trihydroxyxanthone	Xanthone	25		2.427	5;3	86.99	(Panthong et al., 2013)
46	 **$C_{18}H_{16}O_5$ (312.32)** 1,3,6-trihydroxy-2-(3-methyl-2-butenyl)xanthone	Xanthone	25		4.328	5;3	86.99	(Panthong et al., 2013)
47	 **$C_{25}H_{30}O_8$ (458.50)** Mallotojaponin B	Phloroglucinols	25.0		5.100	8;4	133.52	(Tchangoue et al., 2020)
48	 **$C_{21}H_{22}O_9$ (418.39)** 7-O-Methylaloeresin A	Chromone glycoside	25.1		0.954	9;7	167.91	(Asamenew et al., 2011)
49	 **$C_{20}H_{22}N_2O_3$ (338.40)** Picrinine	Alkaloid (Akuammiline alkaloid)	50	25	1.614	5;1	50.8	(Liu et al., 2015)

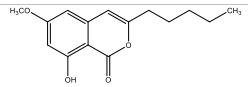
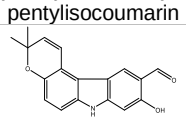
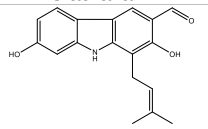
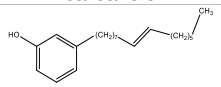
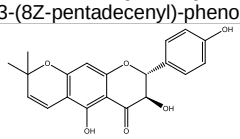
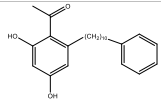
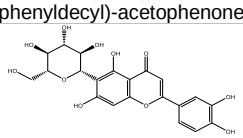
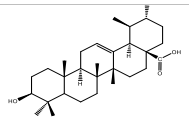
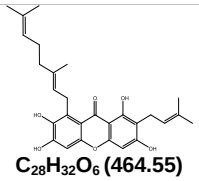
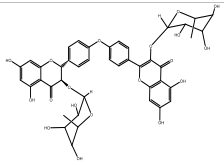
50	 **$C_{22}H_{29}N_3O_3$ (383.49)** Lanatine A	Alkaloid (Quinolizidine alkaloid)	25-50	25-50	2.162	6;2	75.87	(Neto et al., 2011)
51	 **$C_{35}H_{60}O_6$ (576.85)** β-sitosterol-3-O-β-D-glucoside	Steroidal glycoside	50	25	9.486	6;4	99.38	(Njinga et al., 2016)
52	 **$C_{21}H_{22}N_2O_3$ (350.41)** Vallesiachotamin	Alkaloid (Monoterpene indole alkaloid)	100	25	2.978	5;1	58.64	(Liu et al., 2015)
53	 **$C_{21}H_{22}N_2O_3$ (350.41)** Isovallesiachotamine	Alkaloid (Monoterpene indole alkaloid)	100	25	2.978	5;1	58.64	(Liu et al., 2015)
54	 **$C_{17}H_{16}O_7$ (332.30)** 5, 7, 4'-trihydroxy-3, 6-dimethoxyflavone	Flavonoid (Dimethoxy flavone)	26		1.462	7;3	105.45	(Teffo et al., 2010)
55	 **$C_{13}H_{12}O_4$ (232.24)** Methy piperate	Benzodioxole	30		2.884	4;0	44.76	(Khaing, 2019)
56	 **$C_8H_8O_5$ (184.14)** Methylgallate	Phenolic compound (Galloyl ester)	30 (78)	(78)	0.931	5;2	86.99	(Madikizela et al., 2013; Oladosu et al., 2019)
57	 **$C_{19}H_{20}N_2O_2$ (308.38)** Phutdonginin	Alkaloid (Monoterpene indole alkaloid)	32		2.525	4;1	43.78	(Cheenpracha et al., 2014)
58	 **$C_{19}H_8O_4$ (310.34)** 2-[(3,5-dihydroxy)-(Z)-4-(3-methylbut-1-enyl)phenyl]benzofuran-6-ol	Flavonoid (Arylbenzofuran flavonoid)	32		4.98	4;2	69.92	(Kuete, Ango, et al., 2011)
59	 **$C_{19}H_{22}N_2O_2$ (310.39)** 19-OH-(-)-eburnamonine	Alkaloid (Indole alkaloid)	32		2.809	4;1	43.78	(Cheenpracha et al., 2014)
60	 **$C_{16}H_{22}O_9$ (358.34)** Sweroside	Terpenoid (Monoterpenoid/Iridoid glucoside)	32	32	-1.598	9;4	134.91	(Sarıkahya et al., 2011)

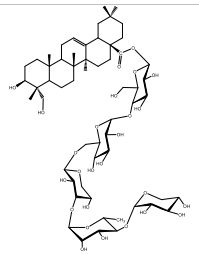
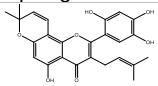
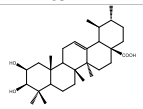
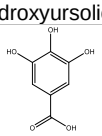
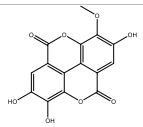
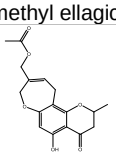
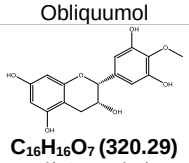
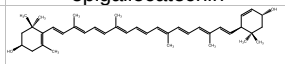
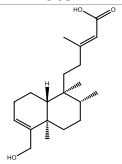
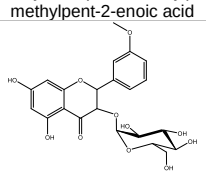
61	 **$C_{14}H_{18}O_7$ (298.29)** Picein	Phenolic glycoside	32	64	-0.281	7;4	116.45	(Sarıkahya et al., 2011)
62	 **$C_{64}H_{104}O_{30}$ (1353.50)** Isacoside	Terpenoid (Saponin/triterpene glycosides)	64	32	0.558	30;17	471.74	(Sarıkahya et al., 2011)
63	 **$C_{31}H_{48}O_3$ (468.72)** Conrauidienol	Terpenoid (triterpene)	32-64	64	9.197	3;1	46.53	(Kengap et al., 2011)
64	 **$C_{47}H_{76}O_{18}$ (929.10)** Paphlagonoside A	Terpenoid (Saponin/triterpene glycosides)	32	128	2.287	18;11	294.98	(Sarıkahya et al., 2011)
65	 **$C_{41}H_{77}NaO_{12}S$ (817.09)** Sulfonoquinovosyldiacylglyceride (SQDG)	Glycolipid	32-64	256	-99.99	12;3	175.12	(Bharitkar et al., 2014)
66	 **$C_{30}H_{20}O_9$ (524.48)** Tectograndone	Anthraquinone (Naphtoquinone)	32	>256	6.632	9;4	158.43	(Bitchagno et al., 2015a)
67	 **$C_{21}H_{26}O_6$ (374.43)** Javanicin F	Quassinoid (Degredaded triterpene)		37.44	0.849	6;0	78.9	(Prema et al., 2019)
68	 **$C_{29}H_{36}O_{15}$ (624.59)** Forsythiaside	Polyphenol (Phenylethanoid glycoside)	38.33		-0.942	15;9	245.29	(Qu et al., 2012)
69	 $C_{21}H_{28}O_6$ (376.44) Javanicin B	Quassinoid (Degredaded triterpene)		37.64	1.151	6;1	82.06	(Prema et al., 2019)

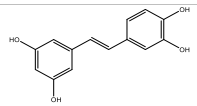
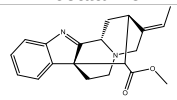
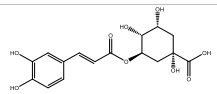
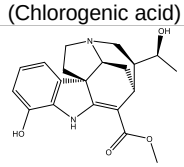
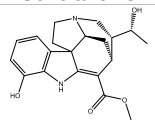
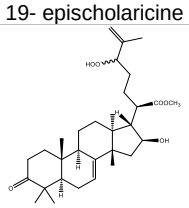
70	 $C_{15}H_{14}O_6$ (290.271) Catechin	Flavonoid (Flavanol)	39	39	0.533	6;5	110.38	(Oladosu et al., 2019)
71	 **$C_{22}H_{30}O_6$ (390.47)** (16R)-methoxyjavanicin B	Quassinoid (Degredaded triterpene)		39	1.755	6;0	71.06	(Prema et al., 2019)
72	 **$C_{30}H_{18}O_{10}$ (538.46)** Ochnaflavone	Flavonoid (Biflavone)	41.6		5.262	10;5	162.98	(Makhafola et al., 2012)
73	 **$C_{30}H_{48}O$ (424.71)** Lanosta-7,24-dien-3-one	Terpenoid (Triterpenoid)		44.07	10.191	1;0	17.07	(Prema et al., 2019)
74	 **$C_{20}H_{34}O_3$ (322.48)** rel-8S,13R-dihydrogrindelic acid	Terpenoid (Diterpene)		46.9	6.294	3;1	46.53	(Du et al., 2015)
75	 **$C_{29}H_{30}O_{11}$ (554.54)** Aloin	Anthraquinone glycoside	49.9		0.846	11;4	169.05	(Asamenew et al., 2011)
76	 **$C_{14}H_{12}O_3$ (228.24)** Resveratrol	Polyphenol (phytoalexin)	50		2.833	3;3	60.69	(Kusumaningtyas et al., 2020)
77	 **$C_{18}H_{20}O_3$ (284.35)** 3′-demethoxy-6-O-demethylisoguaiacin	Lignin	50		4.379	3;3	60.69	(Favela-Hernandez et al., 2012)
78	 **$C_{20}H_{22}N_2O_3$ (338.40)** Strictamine N^4-oxide	Alkaloid (Akuammiline alkaloid)	50	50	3.189	5;0	338.407	(Liu et al., 2015)
79	 **$C_{20}H_{22}N_2O_5$ (370.40)** 5-hydroxy-19,20-Z-alschomine	Alkaloid (Indole alkaloid)	50	50	0.441	7;2	93.59	(Liu et al., 2015)

80	 **$C_{20}H_{24}N_2O_4$ (356.42)** Vallesamine N^4-oxide	Alkaloid (Valessaman alkaloid)	50	50	2.609	6;2	81.62	(Liu et al., 2015)
81	 **$C_{20}H_{24}N_2O_3$ (340.42)** Vallesamine	Alkaloid (Valessaman alkaloid)	50	50	2.174	5;2	61.8	(Liu et al., 2015)
82	 **$C_{30}H_{52}O$ (428.74)** Epifriedelinol	Terpenoid (Triterpenoid)	50	50	11.147	1;1	20.23	(Kannathasan et al., 2019)
83	 **$C_{27}H_{28}N_2O_4$ (444.531)** Aurantiamide acetate	Phenylalanine (Modified dipeptide)	50	50	4.18	6;2	84.5	(Tamokou et al., 2012)
84	 **C21H20O11 (448.38)** Myricetin-3-O-rhamnoside	Flavonoid (Flavonol glycoside)	60		0.215	11;7	186.37	(Madikizela et al., 2013)
85	 **$C_{20}H_{18}O_{12}$ (450.35)** Myricetin-3-O-arabinopyranoside	Flavonoid (Flavonol glycoside)	60		-0.969	12;8	206.6	(Madikizela et al., 2013)
86	 **$C_{18}H_{23}NO_4$ (317.38)** Pandamarilactonine-A	Alkaloid (Butenolide)	62.5		1.922	5;0	55.84	(Laluces et al., 2015)
87	 **$C_{20}H_{30}O_3$ (318.45)** 4-(2-((1*R*,2*S*,6*R*,8a*S*)-1,2,5,5,6-pentamethyl-1,2,3,5,6,7,8,8a-octahydronaphthalen-1-yl) ethyl) furan-2(5*H*)-one	Terpenoid (Isolabdane diterpenoid)		62.5	3.968	3;1	46.53	(Du et al., 2015)
88	 **$C_{20}H_{30}O_3$ (318.45)** 4-(2-((1a*S*,3a*R*,4*S*,5*R*,7a*S*,7b*R*)-4,5,7a,7b-tetramethyldecahydronaphtho[1,2-*b*] oxiren-4-yl)ethyl)furan-2(5*H*)-one	Terpenoid (Clerodane diterpenoid)		62.5	4.345	3;0	38.83	(Du et al., 2015)

89	 **$C_{20}H_{32}O_3$ (320.47)** 4-(2-((1*R*,2*S*,4a*S*,8a*S*)-1-hydroxy-2,4a,5,5,8a-pentamethyldecahydronaphthalen-1-yl)ethyl)furan-2(5*H*)-one	Terpenoid (Labdane diterpenoid)		62.5	4.452	3;1	46.53	(Du et al., 2015)
90	 **$C_{20}H_{32}O_3$ (320.47)** (*E*)-3-methyl-5-((1a*R*,3a*S*,4*R*,5*S*,7a*R*,7b*S*)-4,5,7a,7b-tetramethyldecahydronaphtho[1,2-*b*]oxiren-4-yl)pent-2-enoic acid	Terpenoid (Clerodane diterpenoid)		62.5	5.772	3;1	49.83	(Du et al., 2015)
91	 **$C_{20}H_{36}O_3$ (324.50)** (2*R*,2'*S*,4a'*S*,5*S*,6'*R*,8a'*S*)-5-(2-hydroxyethyl)-2',5,5',5',8a'-pentamethyldecahydro-2'*H*,3*H*-spiro[furan-2,1'-naphthalen]-6'-ol	Terpenoid (Labdane diterpenoid)		62.5	4.026	3;2	49.69	(Du et al., 2015)
92	 **$C_{20}H_{34}O_4$ (338.488)** 2-((2*R*,2'*S*,4a'*S*,5*R*,6'*R*,8a'*S*)-6'-hydroxy-2',5,5',5',8a'-pentamethyldecahydro-2'*H*,3*H*-spiro[furan-2,1'-naphthalen]-5-yl) acetic acid	Terpenoid (Labdane diterpenoid)		62.5	4.207	4;2	66.76	(Du et al., 2015)
93	 **$C_{22}H_{38}O_4$ (366.54)** (2*R*,2'*S*,4a'*S*,5*S*,6'*R*,8a'*S*)-5-(2-hydroxyethyl)-2',5,5',5',8a'-pentamethyldecahydro-2'*H*,3*H*-spiro[furan-2,1'-naphthalen]-6'-yl acetate	Terpenoid (Labdane diterpenoid)		62.5	4.972	4;1	55.76	(Du et al., 2015)
94	 **$C_{26}H_{28}O_6$ (436.50)** Artocarpin	Flavonoid (3- prenylated flavone)	62.5		6.409	6;3	96.22	(Septama & Panichayupakaranant, 2017)
95	 **$C_{18}H_{21}NO_4$ (315.36)** Buphanidrine	Alkaloid (Morphine alkaloid)	63		1.790	5;0	40.16	(Cheesman et al., 2012)
96	 **$C_{12}H_{12}O_3$ (204.22)** 7-hydroxybutylidene phthalide	Phthalide	64		3.412	3;1	46.53	(Miran et al., 2020)

97	 **$C_{15}H_{18}O_4$ (262.30)** 8-hydroxy-6-methoxy-3-n-pentylisocoumarin	Coumarin (Isocoumarin)	64		4.216	4;1	55.76	(Taechowisan et al., 2019)
98	 **$C_{18}H_{15}NO_3$ (293.32)** Clauraila D	Alkaloid (Carbazole alkaloid)	64		4.736	4;2	58.56	(Maneerat et al., 2012)
99	 **$C_{18}H_{17}NO_3$ (295.33)** 2,7-dihydroxy-3-formyl-1-(3′-methyl-2′-butenyl) carbazole	Alkaloid (Carbazole alkaloid)	64		4.589	4;3	69.56	(Maneerat et al., 2012)
100	 **$C_{21}H_{34}O$ (298.29)** 3-(8Z-pentadecenyl)-phenol	Phenolic compound	64		8.896	1;1	20.23	(Taechowisan et al., 2019)
101	 **$C_{20}H_{18}O_6$ (354.358)** Conraui flavonol	Flavonoid (Conrauiflavonol)	64	64	3.368	6;3	96.22	(Kengap et al., 2011)
102	 **$C_{24}H_{32}O_3$ (368.51)** 2,4-dihydroxy-6-(10-phenyldecyl)-acetophenone	Acetophenone	64		8.087	3;2	57.53	(Taechowisan et al., 2019)
103	 **$C_{21}H_{20}O_{11}$ (448.38)** Isoorientin	Flavonoid (Flavone)	64	64	0.209	11;8	197.37	(Sarıkahya et al., 2011)
104	 **$C_{30}H_{48}O_3$ (456.71)** Ursolic acid	Terpenoid (Pentacyclic triterpenoid)	64	64	8.627	3;2	57.53	(do Nascimento et al., 2014)
105	 **$C_{28}H_{32}O_6$ (464.55)** Norcowanin	Xanthone (8-prenylated xanthone)	64		7.724	6;4	107.22	(Siridechakorn et al., 2012)
106	 **$C_{42}H_{40}O_{19}$ (848.76)** Ericoside	Flavonoid (Biflavonoid glycoside)	64		3.282	18;10	291.82	(Bitchagno et al., 2016)

107	 **$C_{58}H_{94}O_{26}$ (207.36)** Paphlagonoside B	Terpenoid (Triterpenoid glycoside)	64	64	0.423	26;15	412.82	(Sarıkahya et al., 2011)
108	 **$C_{25}H_{24}O_7$ (436.46)** Artonin E	Flavonoid (Prenylated flavonoid)	64	128	5.110	7;4	116.45	(Kuete, Ango, et al., 2011)
109	 **$C_{30}H_{48}O_4$ (472.35)** 2β-hydroxyursolic acid	Terpenoid (Pentacyclic triterpenoid)	128	64	7.392	4;3	77.76	(Bitchagno et al., 2016)
110	 **$C_7H_6O_5$ (170.12)** Gallic acid	Phenolic acid (Gallic acid)	78	78	0.425	5;4	97.99	(Oladosu et al., 2019)
111	 **$C_{15}H_8O_8$ (316.22)** 3-O-methyl ellagic acid	Tannin (Hydrolysable tannin)	80		0.589	8;3	122.52	(Jain et al., 2018)
112	 **$C_{17}H_{18}O_6$ (318.32)** Obliquumol	Chromone	80	-	3.001	6;1	82.06	(Ramadwa et al., 2019)
113	 **$C_{16}H_{16}O_7$ (320.29)** 4′-O-methyl epigallocatechin	Flavonoid (Flavanol)	90	-	0.117	7;5	119.61	(Khumalo et al., 2019)
114	 **$C_{39}H_{54}O_2$ (554.85)** Lutein	Carotenoid	90		10.709	2;2	40.46	(Songca et al., 2012)
115	 **$C_{20}H_{32}O_3$ (320.47)** (*E*)-5-((1*S*,2*R*,4a*R*,8a*S*)-5-(hydroxymethyl)-1,2-dimethyl-1,2,3,4,4a,7,8,8a-octahydronaphthalen-1-yl)-3-methylpent-2-enoic acid	Terpenoid (Clerodane diterpenoid)		93.8	5.695	3;2	57.53	(Du et al., 2015)
116	 **$C_{22}H_{24}O_{11}$ (464.42)** Isorhamentin 3-O-glucoside	Flavonoid (Flavonoid glycoside)		1.95	0.913	11;6	175.37	(Elkady et al., 2020)

117	 **$C_{14}H_{12}O_4$ (170.12)** Piceatannol	Polyphenol (Stillbenoid)	100		2.236	4;4	80.92	(Kusumaningtyas et al., 2020)
118	 **$C_{20}H_{22}N_2O_2$ (322.40)** Strictamine	Alkaloid (Akuammiline alkaloid)	100		2.803	4;0	41.9	(Liu et al., 2015)
119	 **$C_{16}H_{18}O_9$ (354.31)** 3-O-caffeoylquinic acid (Chlorogenic acid)	Polyphenol (Quinic acid derivative)	100		-1.879	9;6	164.75	(Xia et al., 2011)
120	 **$C_{20}H_{24}N_2O_4$ (356.42)** Scholaricine	Alkaloid	100	100	1.210	6;3	82.03	(Liu et al., 2015)
121	 **$C_{20}H_{24}N_2O_4$ (356.42)** 19- epischolaricine	Alkaloid	100	100	1.210	6;3	82.03	(Liu et al., 2015)
122	 **$C_{31}H_{48}O_6$ (516.71)** Toosentanin A	Terpenoid (Lanosan-type triterpenoid)		100	5.171	6;2	93.06	(Zhu et al., 2015)

ClogP = calculated partition coefficient between n-octanol and water; nON = number of hydrogen bonds acceptors (number of oxygen and nitrogen atoms in a molecule); nOHNH = number of hydrogen bonds donors (number of O-H and N-H bonds in a molecule); tPSA = calculated total polar surface area of the molecule.

Antibacterial activities and modes of action across phytochemical classes of isolated compounds

An evaluation of the isolated compounds revealed a majority of them to belong to the terpenoids, flavonoids and alkaloids classes (**Table 2, Fig. 5**). Among these classes, average highest activities were observed among flavonoids against both bacteria. Moreover, terpenoids exhibited higher potentials against *E. coli*, whereas alkaloids were more potent against *K. pneumoniae* (**Fig. 5**). However, MIC values $\leq$ 10 µg/mL were observed among compounds belonging to acetophenones, azaarenes, depsidones and xanthones (**Table 2**).

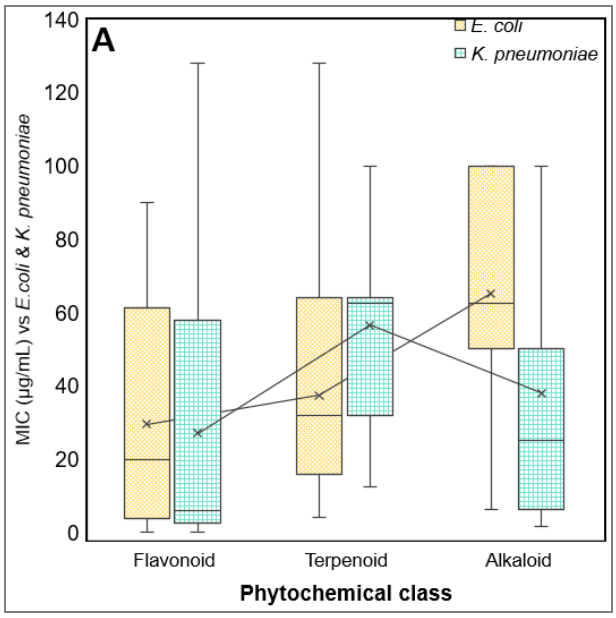


Figure 5: Distributions of MIC values observed among *E. coli* and *K. pneumoniae* against flavonoids, terpenoids and alkaloids as the three most frequent classes of the isolated compounds

The antibacterial potentials of flavonoids, terpenoids, alkaloids and other presented classes of phytochemicals are functions of a number of known modes of action, the account of which is provided on **Table 3**. It is evident that most of the phytochemical classes are proven to exhibit their antibacterial potentials via several modes of action.

Notably, the mechanisms targeting bacterial cell membrane and cell wall, the syntheses of nucleic acids and proteins, electron transport chains and efflux pumps, as well as selected bacterial enzymes were noted to be distributed across many classes. On the other hand, modes targeting inhibition of cell division, inhibition of oxygen uptake, disruption of oxidative phosphorylation, deprivation of essential nutrients/substrates and lowering of extracellular pH were only characteristic of selected classes (**Table 3**). Moreover, the fact that most phytochemicals act by disruption of the bacterial cell membranes is of interest, given that very few of the antibiotics currently in clinical use are known to act by this mechanism (e.g., colistin and daptomycin) (Elias et al., 2021; Taylor & Palmer, 2016).

Table 3: Modes of action of some classes of phytochemicals hosting isolated compounds indicated in **Table 2**.

SN	Phytochemical class	Reported Modes of action	Ref.
1	Acetophenone	○ Disruption of bacterial cell membrane integrity/permeabilization	(Santander et al., 2015)
2	Alkaloids	○ Disruption of bacterial cell membrane integrity/permeabilization ○ Inhibition of nucleic acids synthesis (Inhibit dihydrofolate reductase) ○ Inhibition of protein synthesis ○ Inhibition of cell division ○ Disruption of bacterial homeostasis ○ Inhibition of efflux pumps	(Cushnie & Lamb, 2005; Khameneh et al., 2019)
3	Anthraquinones	○ Disruption of bacterial cell membrane	(Alves et al.,

		integrity/permeabilization o Inhibition of cell wall synthesis o Inhibition of nucleic acids synthesis o Inhibition of protein synthesis o Interfere respiratory chain on bacterial membranes o Inhibition of essential bacterial enzymes	2004; Haraguchi et al., 2014; Malmir et al., 2017)
4	Azaarenes	o Inhibition of bacterial respiratory chain o Disruption of bacterial cell membrane integrity/permeabilization o Decrease in cellular ATP synthesis	(Catallo & Portier, 1992)
5	Benzodioxole	o Inhibition of protein tyrosine phosphatase o Oxidation of redox thiol o DNA binding o Inhibition of RNAIII promotor activation	(Gordon et al., 2013; Gupta et al., 2016; White et al., 2021)
6	Carotenoids	o Inhibition of oxygen uptake o Modulation of efflux pumps o Inhibition of quorum sensing and biofilm formation o Oxidative damage of membranes, DNA, proteins, and lipids o Disruption of oxidative phosphorylation o Anti-virulence activity	(Karpinski et al., 2021)
7	Chromones	o Inhibition of protein synthesis o Inhibition of biofilm formation	(Diwakar et al., 2011; Salem et al., 2013; Zhan et al., 2021)
8	Depsidone	o Inhibition of protein tyrosine phosphatase 1B (PTP1B) o Inhibition of DNA repair and maintenance (RecA) enzyme o Inhibition of bacterial Fatty acid synthesis	(Seo et al., 2009; Urena-Vacas et al., 2022)
9	Flavonoids	o Disruption of bacterial cell membrane integrity/ permeabilization o Inhibition of cell wall/envelope synthesis o Inhibition of bacterial nucleic acid synthesis o Inhibition of protein synthesis o Inhibition of electron transport chain and ATP synthesis o Inhibition of bacterial toxins o Reduction of cell attachment o Inhibition of biofilm formation o Inhibition of porins	(Cushnie & Lamb, 2005; Farhadi et al., 2019; Górniak et al., 2018; Khameneh et al., 2019; Xie et al., 2015)
10	Long chain fatty acids	o Disruption of bacterial cell membrane integrity/permeabilization o Interference with oxidative phosphorylation o Formation of peroxidation or auto-oxidation products o Inhibition of nutrients uptake o Enzymes' inhibition o Fatty acid biosynthesis inhibition o Induction of autolysis/cell lysis o Disruption of the electron transport chain	(Desbois & Smith, 2010)
11	Phenolic acids	o Lowering of extracellular pH (Hyper-acidification at plasma membrane interphase) causing disruption of cell membrane integrity/permeabilization	(Borges et al., 2013; Cueva et al., 2010; Pernin et al., 2019)

12	Phenylpropanoids	○ Disruption of bacterial cell membrane/permeabilization ○ Interference of aerobic metabolism ○ Inhibition of efflux pumps	(Álvarez-Martínez et al., 2021; Nogueira et al., 2021)
13	Phloroglucinols	○ Disruption of bacterial cell membrane/ permeabilization ○ Cell membrane depolarization ○ DNA damage ○ Inhibition of metabolic enzymes ○ Inhibition of biofilm formation	(Celaj et al., 2020; Khan et al., 2021)
14	Phthalides	○ Inhibition of dihydrofolate reductase ○ Antiadhesive activity	(Grube et al., 2019; Ibraheem et al., 2022)
15	Phytosteroids	○ Disruption of bacterial cell membrane ○ Topoisomerase I inhibition ○ Prevention of transpeptidation by inhibition of cell surface protein, Sortase	(Das et al., 2021; DoĞAn et al., 2017)
16	Polyphenols	○ Disruption of bacterial cell membrane/ permeabilization ○ Disruption of bacterial cell wall ○ Cell membrane depolarization ○ /Inhibition of ion channels ○ Inhibition of biofilm formation ○ Inhibition of cell membrane-based receptors ○ Reduction of intracellular ATP concentration	(Álvarez-Martínez et al., 2020; Daglia, 2012; Jia et al., 2021; Xu et al., 2019)
17	Tannins	○ Disruption of bacterial cell membrane/ permeabilization ○ Damaging activity of bacterial cell wall ○ Inhibition of extracellular microbial enzymes ○ Inhibition of metabolic enzymes ○ Deprivation of essential substrates	(Buzzini et al., 2008; Daglia, 2012; Scalbert)
18	Terpenoids	○ Disruption of bacterial cell membrane/ permeabilization ○ Inhibition of efflux pumps ○ Alteration of oxidative phosphorylation ○ Inhibition of oxygen uptake ○ Inhibition of biofilm formation and quorum sensing ○ Reduction of cell adherence	(Khameneh et al., 2019; Mahizan et al., 2019; Moo et al., 2021)
19	Xanthones	○ Disruption of bacterial cell membrane/ permeabilization ○ Reduction of intracellular ATP ○ Inhibition of efflux pumps	(Durães et al., 2021; Koh et al., 2016; Sivaranjani et al., 2019)

Analysis of observed antibacterial potentials versus drug-likeness of isolated compounds

The Principal Component Analysis (PCA) of the data performed here showed that the compounds' MICs were not collated to the total polar surface area, molecular weight, as well as the number of hydrogen bond donors and acceptors (angles between their respective vectors ~ 90°) (**Fig. 6a**). On the other hand, a weak negative correlation (angles close to 180°) was observed between the MICs and the compounds' ClogP values (**Fig. 6a**).

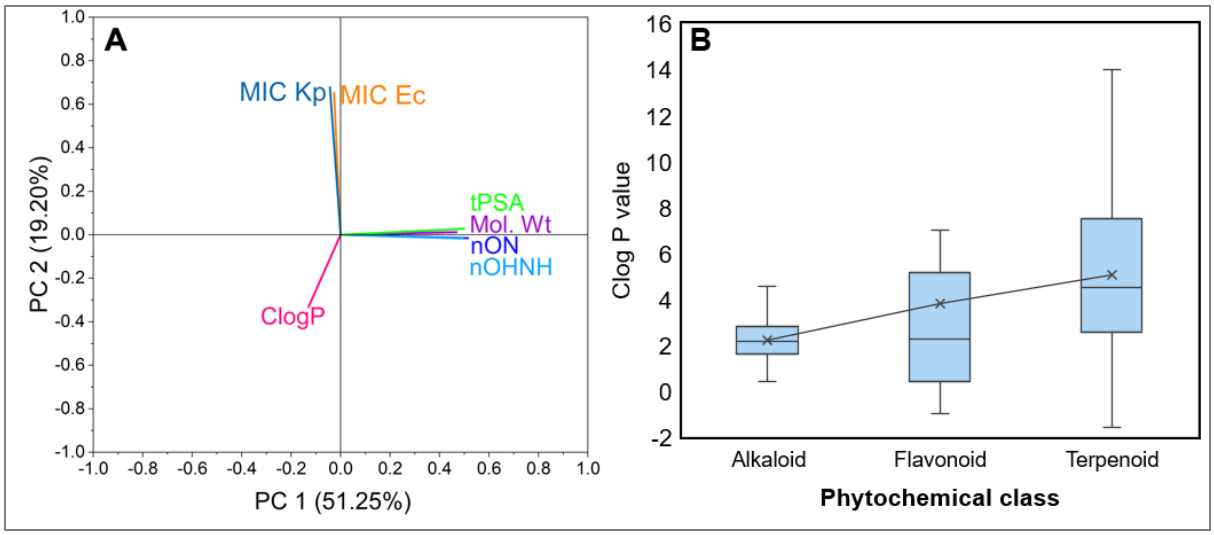


Figure 6: (A) Principal component analysis for the correlations between the MIC values against *E. coli* and *K. pneumoniae* and the calculated total polar surface area (tPSA), molecular weight (Mol. wt), number of hydrogen donors (nON), number of hydrogen bond acceptors (nOHNH) and Clog P. (B) Distribution of Clog P values across three most frequent phytochemical classes of isolated compounds.

Lower MIC values (high antibacterial activity) were therefore fairly linked to higher ClogP (more lipophilic) values of the isolated compounds. On the other hand, compounds belonging to flavonoids and terpenoids showed higher average ClogP values (**Fig. 6b**), which was consistent with their higher activities against both bacteria as compared to alkaloids (**Fig. 5**).

These observations underline the influence of compounds' lipophilicity on their antibacterial activities against Gram-negative bacteria. Among other prospects, high lipophilicity might yield better interactions between the compounds and components of the outer bacterial cell membranes in Gram-negative bacteria, thus facilitating the exhibition of other antibacterial mechanisms (Podunavac-Kuzmanovic et al., 2008). Other studies have indicated higher antibacterial potentials in compounds of different nature when their lipophilicities were increased through formation of corresponding esters, ethers, prenylation, or substitutions with longer alkylation chains (Hatano et al., 2005; Khameneh et al., 2019; Melliou et al., 2005; Merkl et al., 2010).

Nevertheless, lipophilicity of drugs is a key factor determining of the ultimate drug's target selectivity (Lewis et al., 2004). The selected classes of antibacterial compounds were indicated to lose their selectivity with increase in lipophilicities. For instance, increasing lipophilicity of the Novel Bacterial Topoisomerase inhibitors (NBTIs) was reported to yield higher potency against Gram-negative bacteria at the expense of considerable inhibition of the human Ether-à-go-go-Related Gene (hERG) (Kolaric et al., 2021). Furthermore, higher lipophilicities among a number of peptide antibiotics were found to result in haemolysis, as a result of developing poor selectivity between bacterial and mammalian cell membranes (Henriksen et al., 2014; Liu et al., 2020).

Molecular weights distribution of isolated compounds in relation to their MIC values

Molecular weights of the isolated compounds were portrayed to be densely distributed within the range of 250 – 500 g/mol (**Fig. 7**). Similar to the outcomes of the PCA analysis described above, no particular patterns were observed between the molecular weights and the MIC values against both *E. coli* and *K. pneumoniae*. That is to say, the observed MIC values were rather fairly distributed across the stated range of molecular weights (**Fig. 7**).

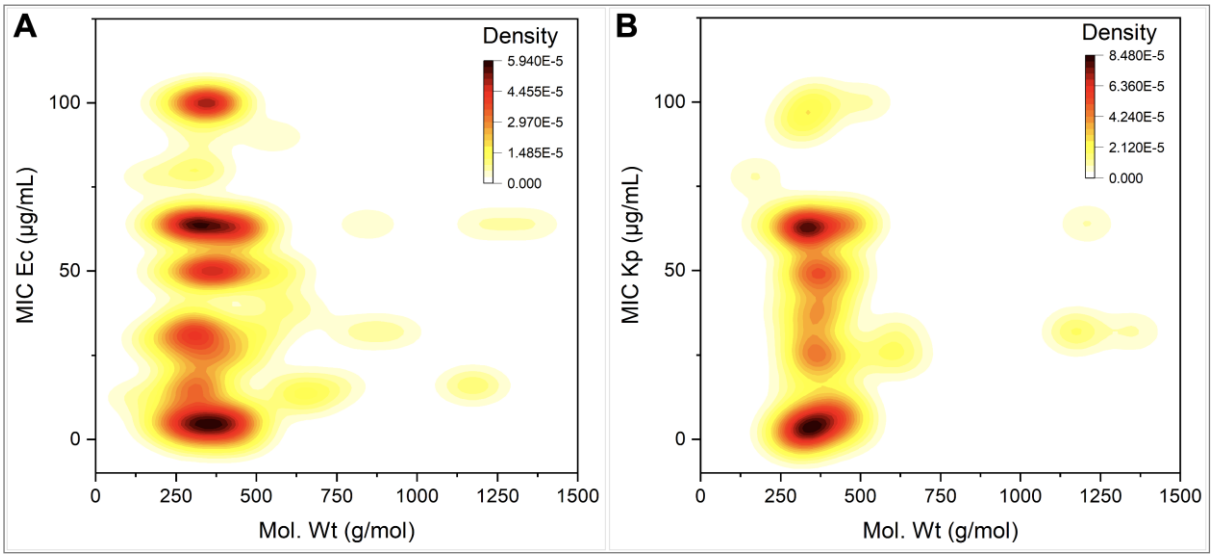


Figure 7: Kernel density plots showing the density distribution of isolated compounds' molecular weights with their corresponding MIC values against *E. coli* (A) and *K. pneumoniae* (B).

The understanding that the molecular weights of antibiotics are unlikely to be linked to their ultimate antibacterial properties is common. However, different outcomes can be expected in cases where an increase in molecular weight leads to a significant rise in the compounds' polarity. Moreover, while the influence of a compound's molecular weight does not highly impact the conduct of *in vitro* antibacterial studies, compounds with higher molecular weights might demand different routes and modes of administration in studies involving higher animals.

Distribution of MIC values with molecular flexibility, globularity, and number of heavy atoms in the isolated compounds

In line with the report from the laboratory of P.J. Hergenrother on the roles of molecular flexibility and globularity on the accumulation of compounds particularly within bacteria, the above library of 122 compounds was further evaluated based on these criteria (Richter et al., 2017). In that higher mean MICs against *E. coli* and *K. pneumoniae* were noted with an increase in the number of rotatable bonds within the phytochemicals (**Fig. 8a**). Further, compounds with molecular globularities between 0.05 – 0.08 were observed to exhibit the lowest mean MICs against both bacteria with a notable gradual decrease in activities above and below this range **(Fig. 8b**). An evaluation based on the number of heavy atoms showed a trend of decreasing MIC

values with an increase in the number of heavy atoms (**Fig. 8c**). Generally, 84% of the phytochemicals had low globularities of ≤0.2, whereas and 70% of them had ≤4 rotatable bonds and were therefore densely populated within these two boundaries (**Fig. 8d**).

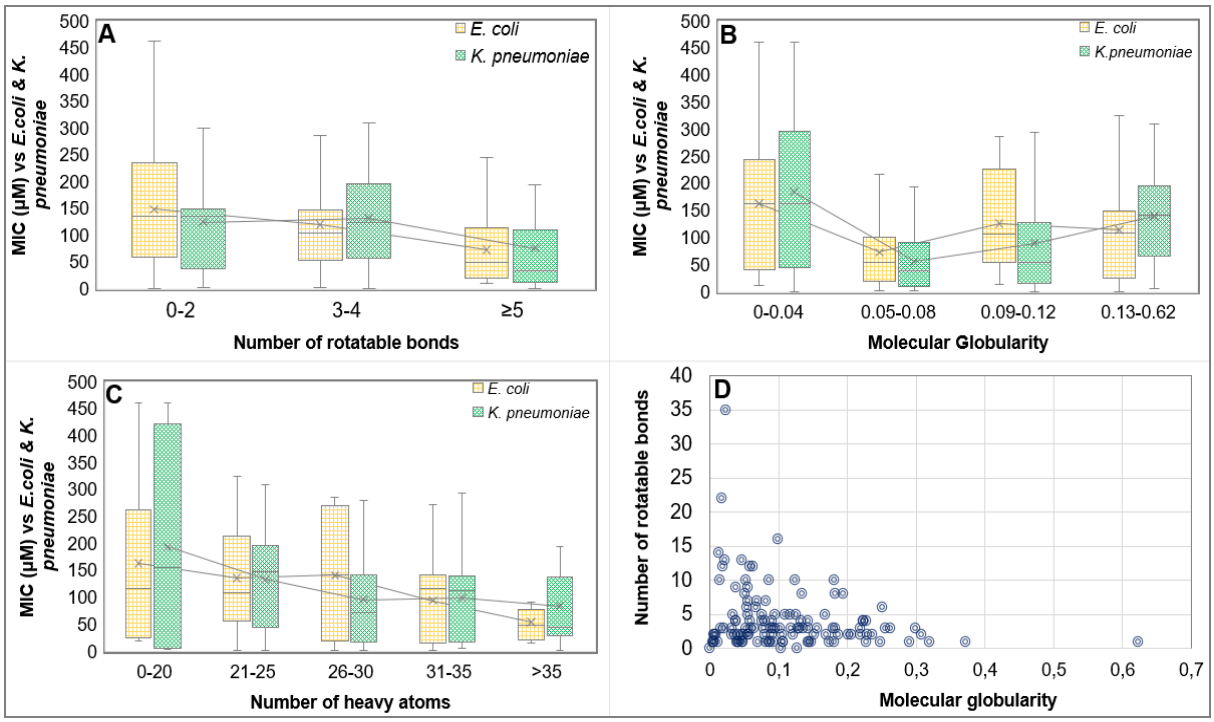


Figure 8: Distribution of MIC values with molecular flexibility (A), globularity (B), number of heavy atoms (C), as well as between molecular flexibility and globularity (D) among the phytochemicals presented in Table 2.

The high activities exhibited by the phytochemicals against the two Gram-negative bacteria can hence be linked to their respective flexibility, globularity, and the number of heavy atoms present. These observations are in agreement with those from the Hergenrother Lab in terms of a high proportion of the phytochemicals active (MIC ≤ 100 µg/mL) against both bacteria showing low globularity (84%) and flexibility (70%), as well as a general increase in mean MICs with an increase in globularity moving from 0.05 to 0.62 (Richter et al., 2017).

However, the noted differences with respect to compounds' molecular flexibility and globularity of <0.04 in relation to their exhibited activities. Among other factors, these discrepancies may be described by the possible lack of direct correlations between the likelihood of phytochemicals to accumulate and their ultimate antibacterial activities. This can be brought about by the general tendency of many phytochemical classes to act by multiple modes of action, which commonly involve the disruption of bacterial cell membrane integrity (**Table 3**). Even so, these observations underscore the essence of considering these parameters in the design of compounds targeting Gram-negative bacteria.

Conclusion and future perspectives

The current review has highlighted the big, highly valuable, and long-standing efforts in the search for antibacterial compounds against *E. coli* and *K. pneumoniae* from numerous plant species. Diverse approaches were noted in aspects of preparing crude extracts, antimicrobial susceptibility testing, as well as isolation and characterization of antibacterial compounds, among others. While many positive lessons from those approaches were communicated, there is an eminent need for more streamlining of numerous approaches in this field.

Plant species and extracts with reported high antimicrobial activities against numerous susceptible and MDR strains of *E. coli* and *K. pneumoniae* presented in this review can provide a valuable contribution towards further research works on the same or related plant species. Additionally, it is anticipated that the provided overview of approaches undertaken by others is useful towards attaining improved design of experiments and evading the common pitfalls.

Furthermore, this review has provided an account of plant-isolated antibacterial compounds, highlighting various aspects of their chemical natures in relation to the exhibited antibacterial potentials against *E. coli* and *K. pneumoniae*. Notably, higher activities against both bacteria were fairly related to the higher lipophilic character of the isolated compounds, although this character might as well signify their low target selectivity. On the other hand, molecular weight, total polar surface area as well as the number of hydrogen bond donors and acceptors were not correlated to the observed MIC values. Additionally, the descriptors of molecular flexibility, globularity and number of heavy atoms were observed to influence the resulting MIC values in various ways. The presented compounds and associated descriptions might contribute to further studies on antibacterial phytochemicals, as well as efforts on hunting for new antibacterial chemical scaffolds among other aspects of antibiotics design and development.

The global rise of antimicrobial resistance necessitates recruiting all available options in the search for viable solutions. Plants like other natural products are proven to host a valuable potential for the discovery of novel antibiotics. Ongoing efforts on ascertaining potential plant species and isolation of promising antibacterial compounds are therefore highly credible.

Acknowledgements

The authors would like to thank the financial support of the German Academic Exchange Service (DAAD) offered to NEM through research grants for Doctoral Programmes in Germany, 2018/2019 (57381412).

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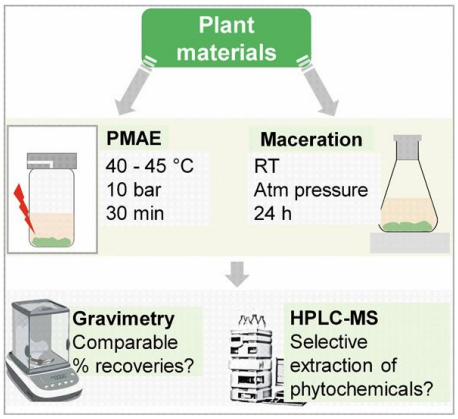
Chapter IV

Results

4. RESULTS

4.1. Comparison of extraction efficiency and selectivity between low-temperature pressurized microwave-assisted extraction and prolonged maceration

Nelson E. Masota, Gerd Vogg, Eberhard Heller, and Ulrike Holzgrabe



Abstract

Extraction is a key step in studying compounds from plants and other natural sources. The common use of high temperatures in Pressurized Microwave-Assisted Extraction (PMAE) makes it unsuitable for the extraction of compounds with low or unknown thermal stability. This study aimed at determining the suitability of low-temperature, short-time PMAE in attaining yields comparable to those of prolonged maceration at room temperature. Additionally, we explored the phytochemical differences of the extracts from both techniques. Maceration at room temperature for 24 hr and PMAE at 40–45°C and 10 bar for 30 min were carried out on 18 samples from 14 plant species at a solvent-to-feeds ratio of 10. The PMAE yields of 16 out of 18 samples were within the proportions of 91–139.2% as compared with the respective extracts from maceration. Varying numbers of nonmatching peaks were noted in MS chromatograms of five extract pairs, indicating selective extraction of some compounds. Low-temperature PMAE can attain reasonable extraction efficiency with the added value of sparing compounds of low thermal stability. The method can also enable the recovery of compounds distinct from those obtained by maceration.

Keywords: extraction, HPLC–MS, maceration, pressurized microwave-assisted extraction

1 INTRODUCTION

The search for bioactive compounds for the treatment of diseases, among other applications, is key to ensure the continuous availability of viable treatment options. A number of approaches can be used in the discovery of new medicines. These include the screening of natural products or chemical libraries, in silico designing, and modification of existing medicines, to mention a few. Extraction is an important step toward obtaining phytochemicals of interest from plant materials. Outcomes of an extraction process are influenced by extraction duration, temperature, pressure, solvent's polarity, and acidity of the extraction medium, among other factors.[1]

Different extraction methods have been reported. Maceration, percolation, infusion, decoction, and Soxhlet extraction are among the most employed techniques. This is mainly due to their less requirement for modern equipment and other infrastructure. However, other modern methods are currently in place. Most of them aim at attaining higher yields, reduced solvent use, and shorter extraction time.[1]

Microwave-assisted extraction (MAE) involves the use of microwaves (300 MHz to 300 GHz) to generate thermal energy through rotation or vibration of dipoles or ionic conduction.[2] However, commercially available laboratory microwave units commonly use a frequency of 2.45 GHz, as other frequencies can interfere with telecommunication and radar systems. In MAE, both heat and mass transfer are directed toward the solvent.[3] The rapid heating generated by MAE causes sudden evaporation of residual water or solvent molecules in plant cells. This results in a build-up of high internal pressure and rupturing of the cells.[3] These events are, thus, in favor of higher rates of desorption, diffusion, and partition of the phytochemicals from the plant matrix into the extracting solvent.[4]

Attaining high recovery rates using conventional methods is a challenging task. Studies have indicated the necessity for longer extraction time and higher temperatures as possible modifications of these methods to boost their efficiency.[5-7] Besides prolonged exposure to atmospheric oxygen, thermal, oxidative, or enzymatic degradations, as well as cross-reactions among the phytochemicals, can occur.[3, 8]

There are two possible equipment modes of carrying out an MAE. In the open mode, the equipment operates at an atmospheric pressure, commonly associated with a refluxing mechanism.[9-11] Modification of domestic microwaves to suit this mode is also a common approach.[8, 12] On the contrary, the closed mode offers the choice of operating at a high pressure. The pressure is built up by the pumping of inert gas into the extraction chamber. Nevertheless, a degree of pressure may be generated by vapor pressure during heating of the extraction mixture.[9, 13]

The use of pressure enables the heating of the solvents above their boiling points. Depending on the phytochemicals of interest, this can result in higher yields and an overall decrease in extraction time.[13, 14] Furthermore, the application of pressure is in

line with the working principle of pressurized liquid extraction, whereby, besides enabling heating of the solvent above the boiling point, high pressure improves the permeation of the solvent through the plant matrices, hence favoring the desorption process.[10, 15] The combination of pressure in MAE is also termed as pressurized microwave-assisted extraction (PMAE).[13-16]

Current reports on the use of MAE indicate a broad use of rather high extraction temperatures, mostly in the range of 60–120°C.[4, 5, 17-19] This approach has the benefit of achieving good yields using a few seconds to <10 min. However, it is not suitable for the extraction of heat-sensitive compounds or when the compounds of interest are unknown.

In the current study, we aimed at exploring the usefulness of PMAE when conducted at low temperatures and moderate time duration. Besides evaluating the recovery efficiency of PMAE in comparison to maceration, we also wanted to determine if the obtained extracts differed in phytochemical profiles.

2 RESULTS

A total of 18 plant samples were obtained from 14 plant species (**Table 1**). The plants were selected on the basis of a parallel study aimed at evaluating the antimicrobial activities of these plants.

Table 1. Details of the studied plants

Name	Part(s) studied	Internal accession number
Acacia melanoxylon R.Br.	leaves	XXXX-399-E-80
Alpinia purpurata (Vieill.) K. Schum.	leaves	2010-88-B-70
Asparagus densiflorus (Kunth) Jessop	leaves	XXXX-284-P-80
Asparagus officinalis L.	stem	XXXX-660-G-80
Artocarpus heterophyllus Lam.	leaves	1986-42-B-80
Cinnamomum verum J.Presl	leaves, stem	2010-90-B-80
Erythrina crista-galli L.	bark, stem	1982-348-E-80
Ficus carica L.	bark, stem	XXXX-220-G-80
Garcinia spicata Hook. f.	leaves	1977-306-D-80
Garcinia tinctoria (DC.) W. Wight	leaves	XXXX-74-B-80
Olea europaea L.	leaves	XXXX-64-P-20
Paeonia officinalis L.	leaves	2013-11-S-10
Prunus sargentii Rehder cv. Rancho	leaves, bark	2005-137-M-80
Zingiber officinale Roscoe	rhizome	Charge 329272 (Kraeuter Mix, Germany)

2.1 Quantitative comparison of the extract compositions

Of the 18 tested samples, 16 were found to provide MAE yields with the proportions >90% as compared with maceration (**Figure 1a**). Moreover, 13 samples provided

yields within 100 ± 10% of the maceration, with 79% and 139% being the lowest and highest proportions, respectively.

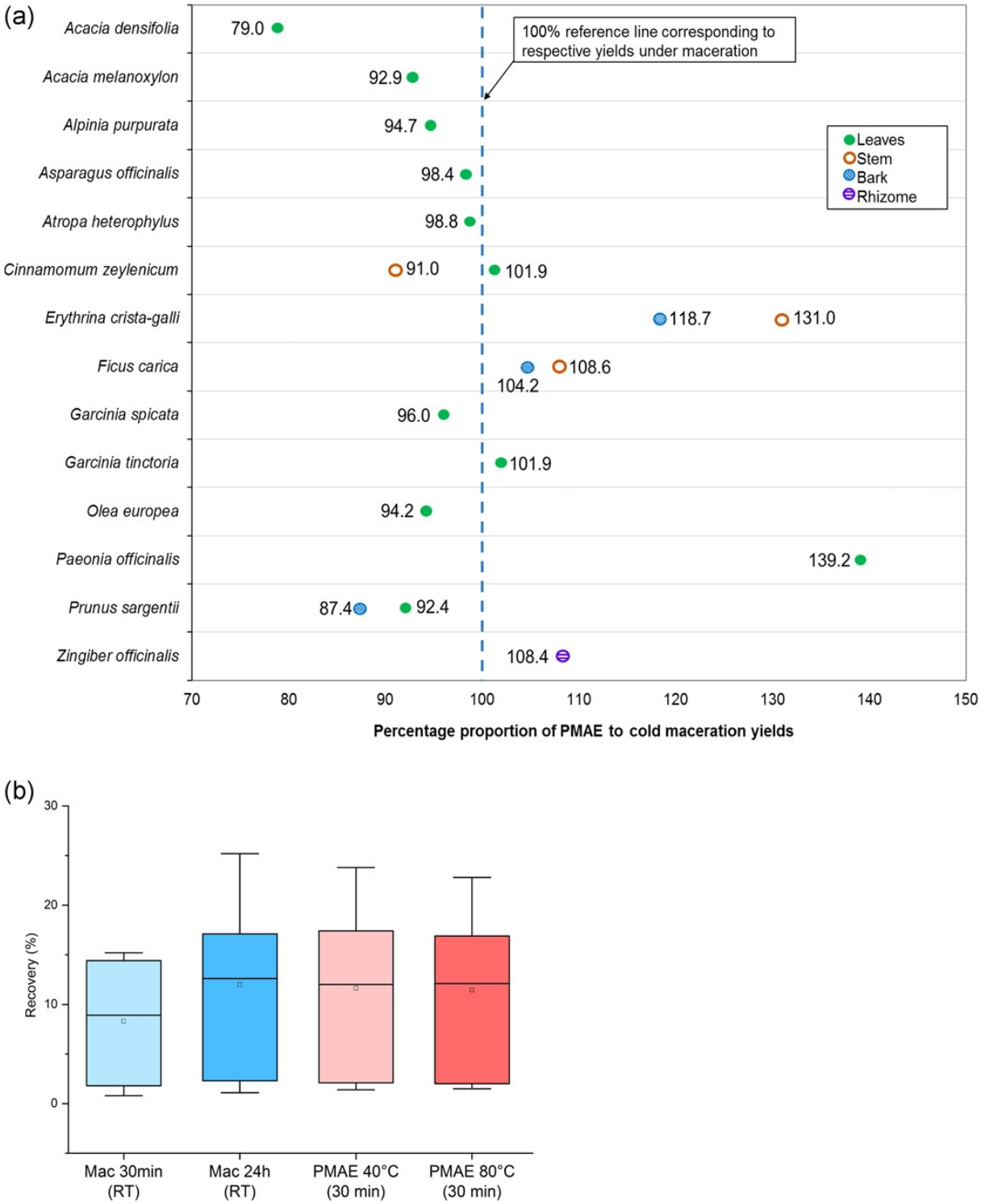


Figure 1: (**a**) The percentage proportion of gravimetric recoveries obtained from pressurized microwave-assisted extraction (PMAE) at 40°C (30 min) to maceration at room temperature (24 hr). (**b**) Box and whisker plot comparing median values, means, and ranges of percentage recoveries obtained by maceration at room temperature for 30 min and 24 hr (blue) and PMAE at 40°C and 80°C for 30 min (red)

Evaluation based on the plant's part tested revealed that 7 of the 10 leaves samples showed PMAE yields lower (79–99%) than those of maceration. On the contrary, out of the four and three samples from stems and barks, respectively, two of each showed higher PMAE yields (104–131%) than those of maceration (**Figure 1a**).

Maceration carried out for the same duration as PMAE (30 min) resulted in recoveries lower than those observed under 24-h maceration and 30-min PMAE at 40°C. As displayed by box and whisker plots in **Figure 1b**, recoveries from PMAE experiments conducted at a higher temperature (80°C) were not superior to those obtained when PMAE was conducted at 40°C.

2.2 Semiquantitative comparison by peak intensities in UV and mass spectrometry (MS) chromatograms

Normalization of the chromatograms was ensured by injecting the same volume and concentration of the sample, as well as maintaining all other chromatographic parameters. Notable differences in peak intensities were observed in all or some of the peaks in corresponding chromatograms of all samples. In 10 out of 18 samples, there were higher intensities of peaks in chromatograms of the extracts prepared from PMAE. The differences in intensities ranged from small (**Figure 2**) to well notable ones (**Figure 3a**). A similar pattern was observed in MS base peak chromatograms (BPCs; **Figure 3b**). Additional figures under this group are shown in Section 2 of the Supporting Information file.

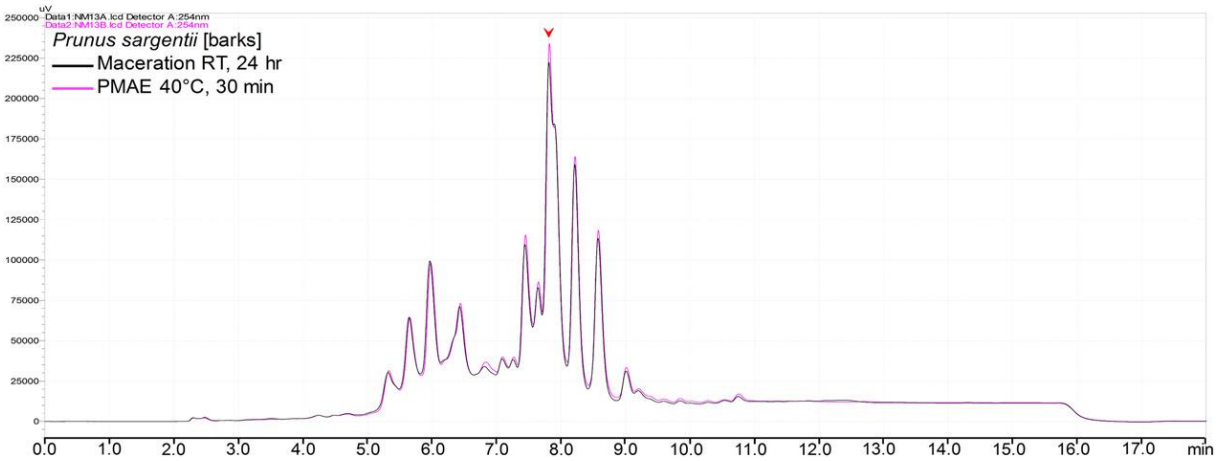


Figure 2: Slightly higher intensities of peaks in chromatograms of *Prunus sargentii* barks extract obtained under pressurized microwave-assisted extraction (PMAE; pink) as compared with maceration (black)

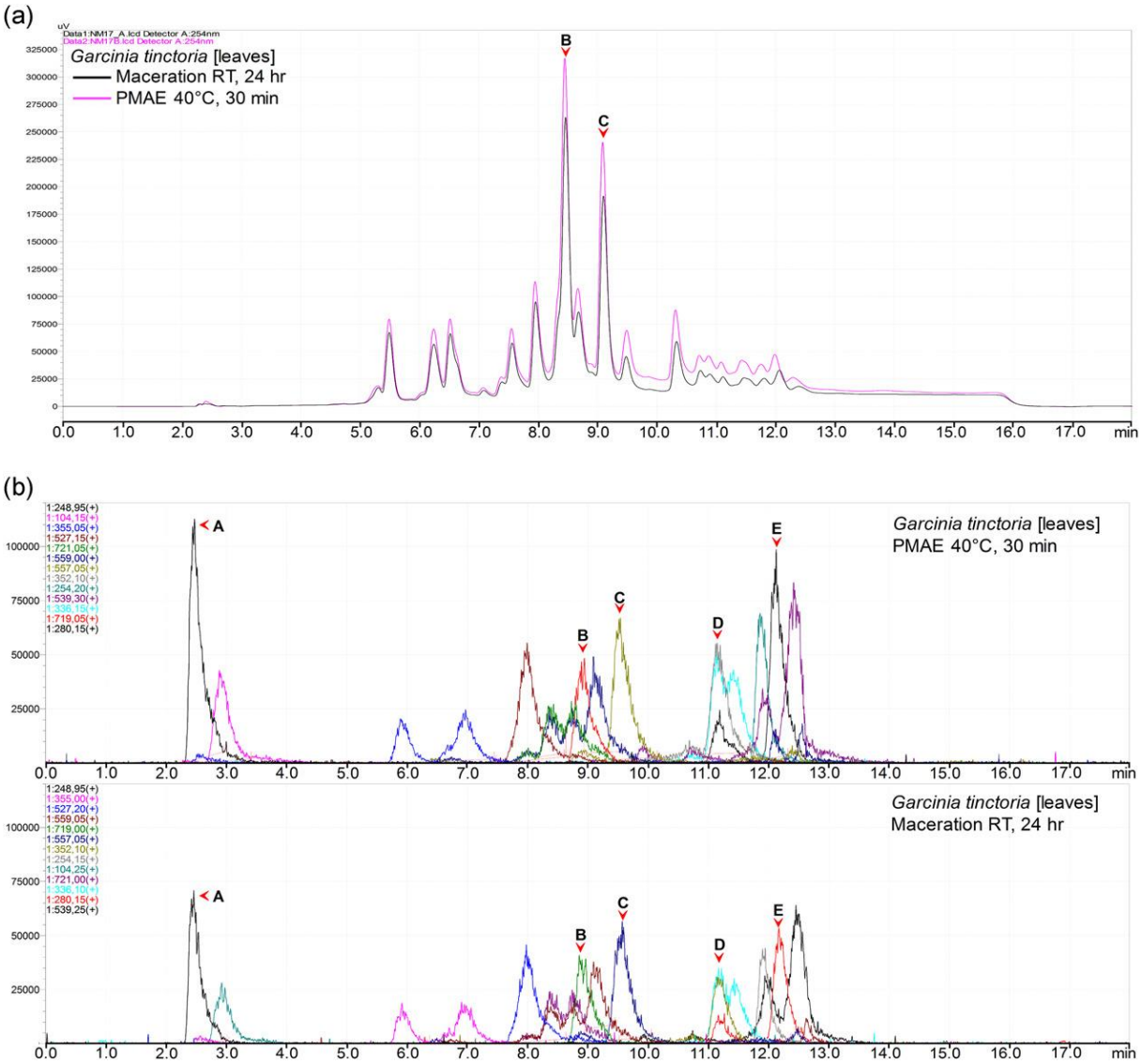


Figure 3: (**a**) Prominent higher intensities of peaks in chromatograms of *Garcinia tinctoria* leaves extract obtained under pressurized microwave-assisted extraction (PMAE; pink) as compared with maceration (black). (**b**) Base peak chromatograms of *G. tinctoria* leaves extract showing higher intensities of peaks B and C in PMAE (top) than in maceration (bottom), as observed in (a). Higher intensities in PMAE are also observed in peaks A, D, and E

However, in 2 of the 18 samples, the chromatogram of a sample extracted by maceration generally revealed higher intensities of peaks as compared with those of the sample obtained under PMAE (**Figure 4a**). However, this was not consistent with the intensities observed in the BPCs of *Zingiber officinale* extracts (**Figure 4b**) in which peaks A and B had equivalent intensities and peaks C and D were not observable (marked x) due to a possible poor or no ionization, which makes the UV and MS detection hardly comparable.

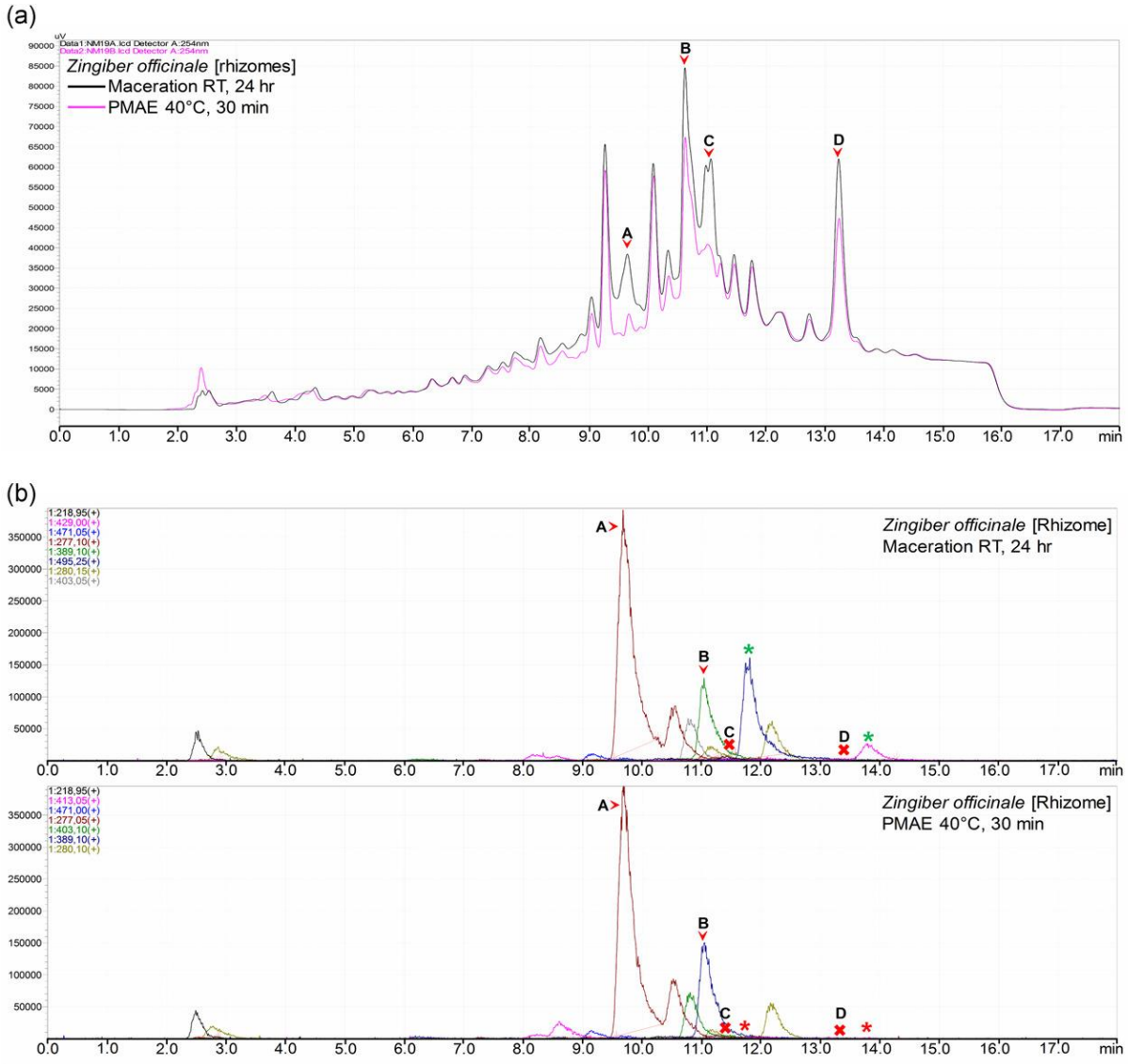


Figure 4: (**a**) Prominent higher intensities of peaks in chromatograms of *Zingiber officinale* rhizomes extract obtained under maceration (black) as compared with pressurized microwave-assisted extraction (PMAE; pink). (**b**) Base peak chromatograms (BPCs) of *Z. officinale* rhizome extracts showing equivalent intensities of peaks A and B in both PMAE and maceration, as opposed to higher intensities for maceration observed in the UV chromatograms (a). UV peaks C and D were not found in the BPCs (x). Two additional peaks/compounds (*) are observed in the BPC from maceration (above). Green "*" indicate the present peaks. Red "*" indicate missing peaks

Furthermore, 6 of the 18 samples showed a combination of the above two scenarios. In these samples, peaks from both extraction methods appeared to have relatively higher intensities at different regions of the UV chromatograms (**Figure 5a**) and the corresponding BPCs (**Figure 5b**). Comparable results are given in Section 2 of the Supporting Information.

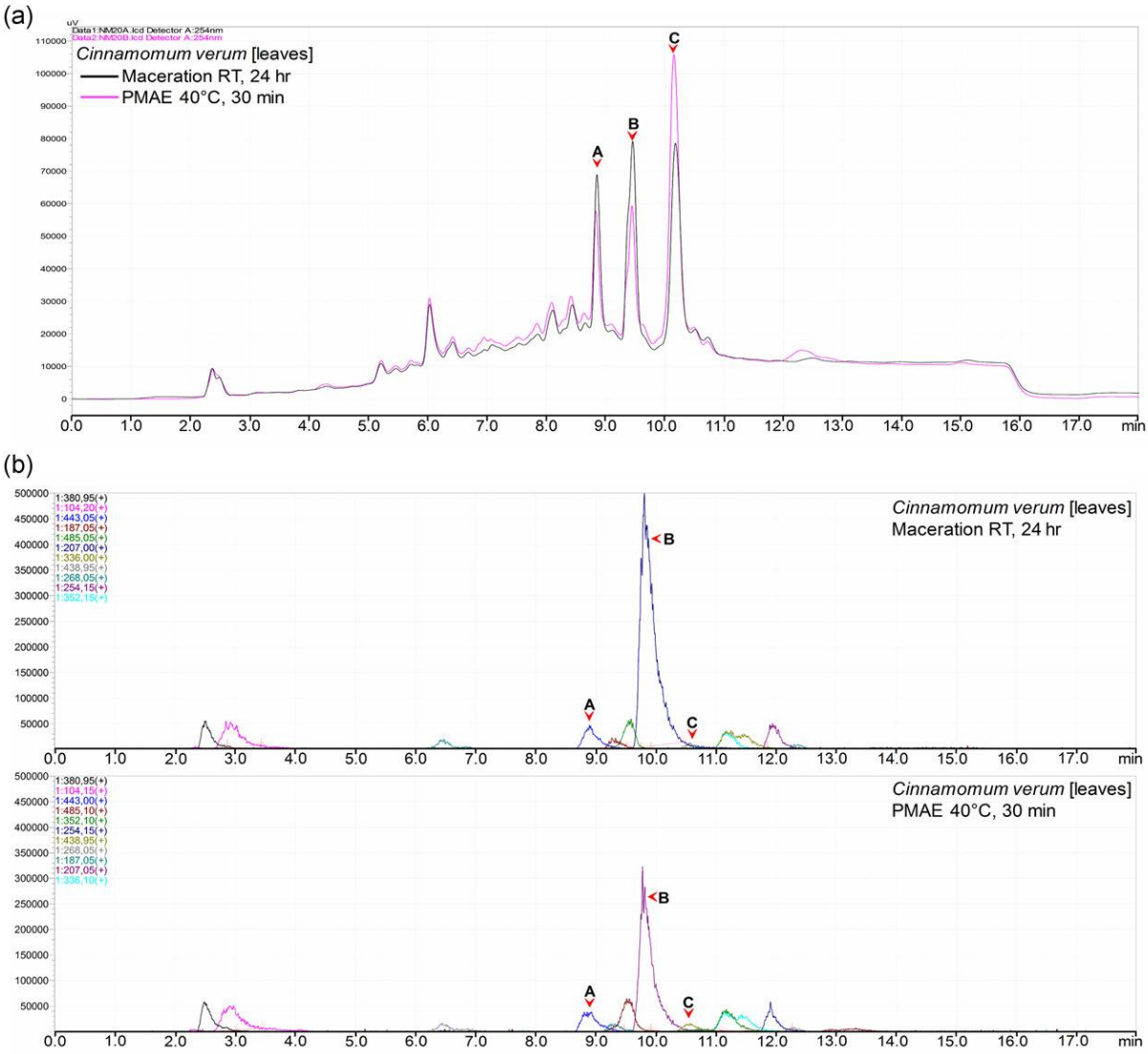


Figure 5: (**a**) Mixed higher peak intensities at different regions of the chromatograms of *Cinnamomum verum* leaves extract obtained under pressurized microwave-assisted extraction (PMAE; pink) as compared with maceration (black). (**b**) Base peak chromatograms for *C. verum* leaves extract showing higher intensities of peaks A and B and lower intensity for peak C under maceration (above) than in PMAE (below), conforming to the pattern observed in the UV chromatograms (a)

2.3 Qualitative comparison of the extract compositions

The search for additional/nonmatching peaks on the UV chromatograms indicated that 4 out of the 18 chromatograms contained at least one additional peak within the extracts obtained by both methods. The additional peaks varied in intensities from small, as shown in **Figure 6**, to notably large, as exemplified in **Figure 7a** (see also Section 1 of the Supporting Information).

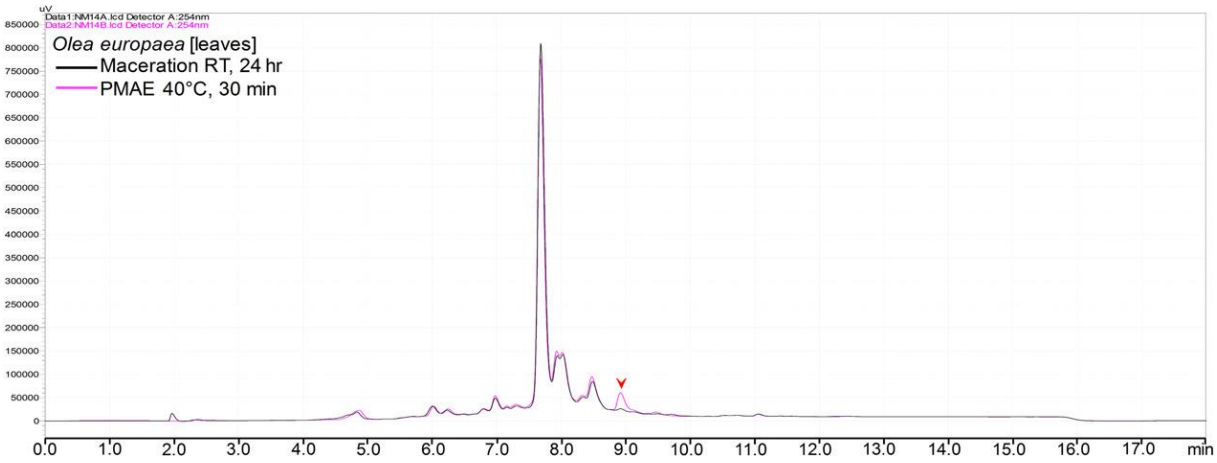
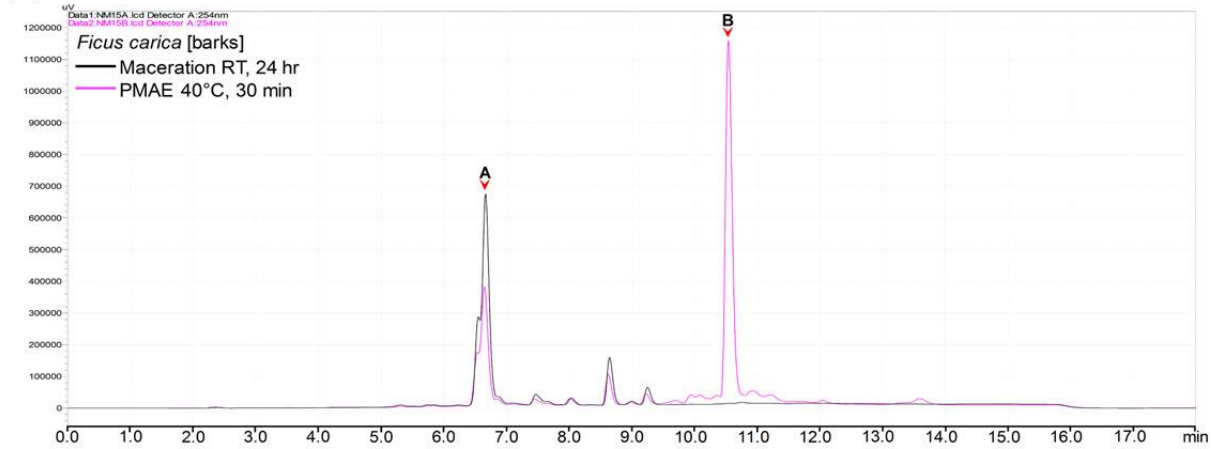


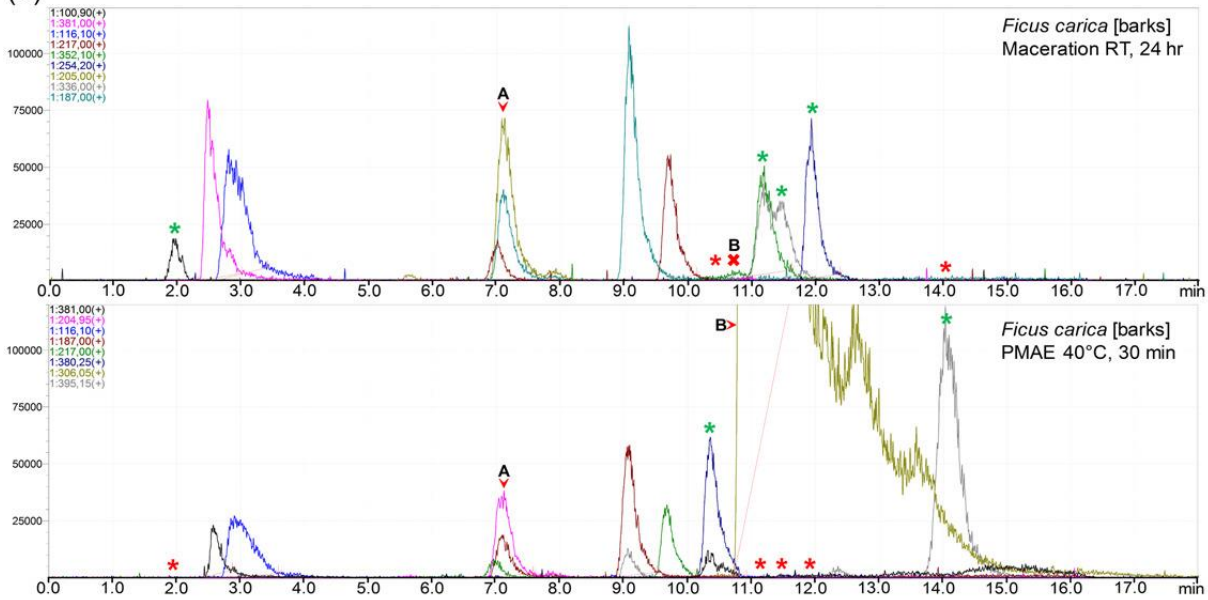
Figure 6: An additional peak (constituent) in the UV chromatogram of *Olea europaea* leaves extract under pressurized microwave-assisted extraction. PMAE, pressurized microwave-assisted extraction

The BPC corresponding to the notable additional peak (B) seen on the UV chromatogram of PMAE extract of *Ficus carica* barks (**Figure 7a**) was not found in the corresponding MS BPC of the extract prepared by maceration (**Figure 7b**). **Figure 7c** shows the absence of the *m/z* signal in the maceration extract, corresponding to that observed in the PMAE extract.

(a)


Ficus carica [barks]
Maceration RT, 24 hr
PMAE 40°C, 30 min
A
B
min

(b)


Ficus carica [barks]
Maceration RT, 24 hr
Ficus carica [barks]
PMAE 40°C, 30 min
A
B
min

(c)

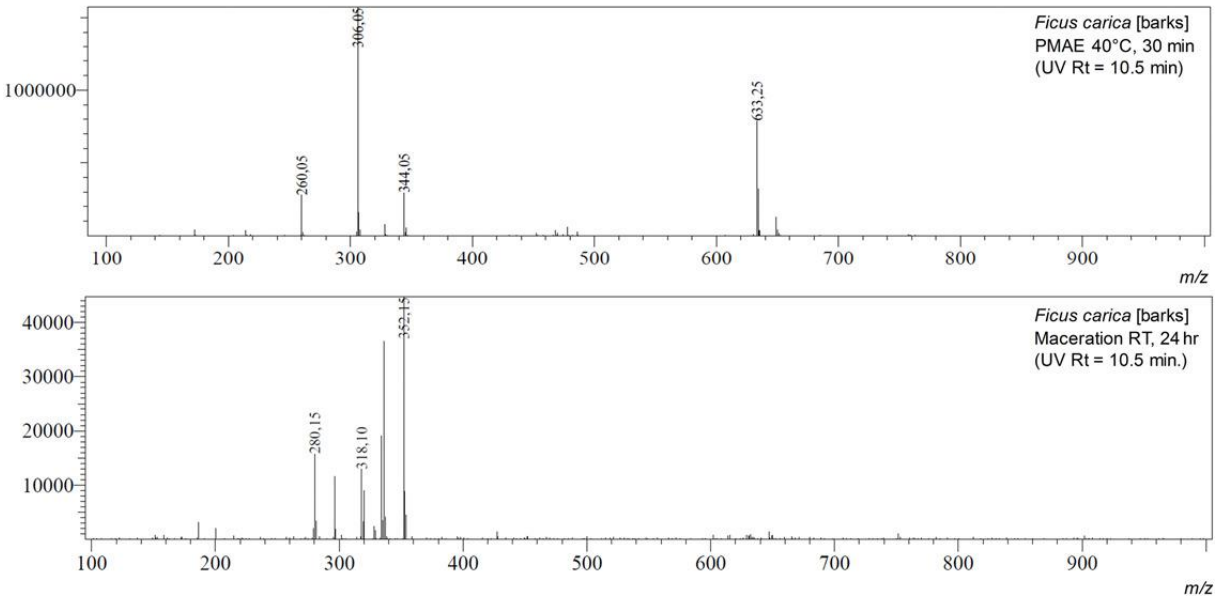

Ficus carica [barks]
PMAE 40°C, 30 min
(UV Rt = 10.5 min)
306,05
633,25
260,05
344,05
m/z
Ficus carica [barks]
Maceration RT, 24 hr
(UV Rt = 10.5 min.)
352,15
280,15
318,10
m/z

Figure 7: (**a**) An additional peak (B) in the UV chromatogram of *Ficus carica* barks extract obtained under pressurized microwave-assisted extraction (PMAE). A peak with a higher intensity (A) is observed in the maceration extract. (**b**) Base peak chromatograms of *F. carica* bark extract showing a large peak (B) in the PMAE extract, which is absent in the maceration extract (x). As observed in (a), peak A has a higher intensity in maceration extract. Also, peaks present and missing in both extracts are marked (*). (**c**) Comparison of the *m/z* signals corresponding to the additional peak (B) at RT = 10.5 min in the UV chromatograms of *F. carica* bark in (a) (*m/z* signals were obtained under averaged spectrum 4.2 s wide in positive ion mode)

However, although we observed nonmatching peaks in the UV chromatograms of *Olea europaea* leaves (**Figure 6**), *F. carica* stem, and *Alpinia purpurata* leaves extract (Supporting Information), all the peaks observed under MS BPCs for these samples were found to be matching in extracts from both methods (Supporting Information). As displayed in **Figure 8**, the mean and median number of additional BPCs (compounds) found in maceration extracts were slightly higher than those in PMAE extracts.

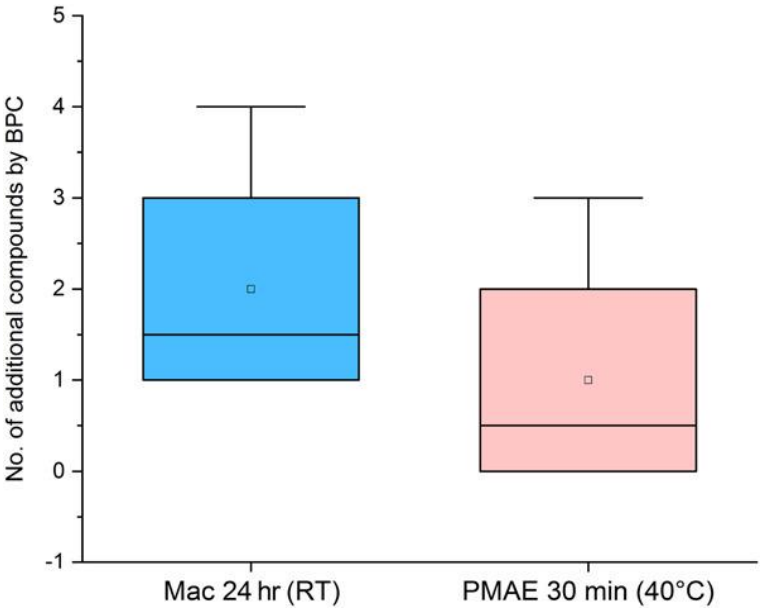


Figure 8: A box and whisker plot of the number of additional base peak chromatograms (BPCs; compounds) observed in five pairs of the tested plant samples between maceration (blue) and pressurized microwave-assisted extraction (PMAE; red) extracts

3 DISCUSSION

Pressurized MAE in a relatively short time (30 min.) and at low temperature can achieve recoveries comparable to those in maceration under prolonged duration (24 hr). The two methods are likely to extract different types of compounds, which may affect the magnitude of biological activity under investigation.

Maceration-like yields of crude extracts can still be obtained using relatively lower temperatures during PMAE. Moreover, the quantity of yields obtained is likely to be affected by the nature of the plant matrix.

This is evident based on 16 out of 18 of PMAE extracts showing crude extract with proportions of above 90% in comparison to those of maceration. The majority of PMAE of plant materials is currently in favor of using relatively high temperatures (60–120°C) and short extraction times.[4, 5, 18] Nevertheless, the findings obtained here highlight the possibility of achieving maceration-like outcomes at lower temperatures and moderate extraction time. The lower PMAE yields in the majority of samples of leaves underscore previous reports on the impact of plant matrix on PMAE. This is linked to the presence of residual amounts of moisture in the matrix, which increases MAE efficiency.[3, 20] Moreover, as seen in Figure 1b, higher recoveries can be obtained when maceration is carried out at prolonged durations. The use of high temperature (80°C) in PMAE did not result in superior yields than those obtained at 40°C.

We studied the role of low microwave power and pressure by maintaining other key factors (plant matrix, solvent-to-matrix ratios, and extracting solvent). The observed outcomes are, therefore, independent of these factors. However, the small number of samples from stems and barks may limit the generalizations of the above-drawn conclusion on the role of plant matrices. Moreover, the lower recoveries observed when maceration was carried for 30 min indicate the lack of attainment of extraction equilibrium before this time point. However, the findings have indicated that the use of higher temperatures like 80°C used as a control set in this study does not always guarantee higher recoveries. This is caused by the possible destruction of the plant matrices at higher temperatures, which impair the mass transfer of phytochemicals into the extracting solvent.[19] The need for using high temperatures in PMAE should, therefore, necessarily involve experiments to predetermine the temperature for optimal recoveries.[8, 18]

Low-temperature PMAE can, therefore, be considered as a very useful approach in attaining good yields of phytochemicals. This is more essential when there is no information on the thermostability of the phytochemicals responsible for the activity of interest, because it avoids possible degradations or a cross-reaction of phytochemicals occurring at high temperature and prolonged extraction times.[4, 19]

The use of PMAE can recover higher amounts of all or some of the phytochemicals from the plant matrix. Moreover, a degree of selectivity most likely based on the nature of phytochemicals and their solubility at different temperatures and time conditions is possible.[5, 21] This is demonstrated by the higher intensities of UV and BPC peaks observed in chromatograms of PMAE extracts in 8 out of 14 samples that had no additional UV peaks (**Figure 3a,b** and Supporting Information). However, as superimposed chromatograms of other 5 out of 18 samples showed a mix in higher intensity peaks, other factors may be in play (**Figure 5a,b**). Other studies have also

indicated differences in selectivity of extracted phytochemicals when MAE was compared with other extraction methods.[4-6]

This can be caused by the nature of phytochemicals present in the matrix and their dependence on temperature and duration of extraction.[3, 22] The magnitudes of peaks' intensities can be directly related to the quantities of respective phytochemicals. This is because, for each sample, the same sample concentration and injection volume were used during high-performance liquid chromatography–mass spectrometry (HPLC–MS) analysis.

When the phytochemicals of interest are known, the application of PMAE at low temperatures is a valid option for a selective increase of their recovery from the plant matrix. This can particularly be beneficial when these compounds are sensitive to high temperatures. However, high heat stability of phytochemicals of interest warrants the use of high temperatures, with further benefits of shorter extraction times and even higher yields.[2, 8, 18]

Differences in profiles of recovered phytochemicals may be imposed by the selected extraction method. Determination of the actual degree of additional compounds is nevertheless subject to the applied detection method.
Our evaluation of HPLC chromatograms under a UV detector showed only 4 (*O. europaea leaves*, *F. carica* barks, *F. carica* stem, and *A. purpurata* leaves) out of 18 samples to possess new/additional peaks in extracts from at least either one of the extraction methods. Moreover, upon cross-examination of these peaks with the respective mass spectra, only one of them (peak B in **Figure 7a**) was confidently noted to be additional (**Figure 7b,c**). The compound corresponding to peak A at the RT of 6.6 min in UV chromatograms had a molecular peak at *m/z* 815.10 and was likely a caricaflavonol diester A.[23] However, on the basis of literature and library search, we could not ascertain the identity of the compound corresponding to an additional peak B at RT = 10.5.

Varying numbers of additional peaks/compounds were noted in five pairs of extracts from both methods when the MS BPCs were evaluated independent of the UV chromatograms (**Figure 8**). This enabled us to arrive at a different conclusion in this aspect. Maceration extracts had a slightly higher mean and median numbers of additional BPCs as compared with PMAE (**Figure 8**). Therefore, these findings show chances of the prospect of recovering completely different types of phytochemicals, based on the method of extraction.[14, 24, 25]

Apart from the detection method, other factors such as method selectivity due to other chromatographic conditions play a role. Through their particular influence on sensitivity and selectivity of the method, these factors are prone to affect a clear observation of additional compounds in generated chromatograms.[7] For example, this study employed a single UV detection wavelength of 254 nm; hence, compounds having chromophores with maximum absorbance at other wavelengths or

compounds that lack a chromophore may be missed. These factors may explain the non-UV detection of other compounds, which were detected on the Electrospray Ionization–Mass Spectrometry (ESI–MS) detector (**Figure 3a,b**).

Moreover, poor or lack of ionization of some compounds might have contributed to the observed missing MS BPCs corresponding to some peaks in UV chromatograms (**Figure 4a,b**). There are low chances that these additional compounds are the products of degradation or cross-reactions among phytochemicals in the crude extracts. This is based on the relatively low temperature and moderate extraction durations used in the study.[3, 18]

In addition to the selection of a suitable extraction method, we underline the need for using more versatile detection methods for evaluation of plant extracts, whenever possible, which may include the use of a tandem arrangement of two or more detectors, if possible. This decreases the necessity of developing specific methods for each plant sample, especially when many samples are to be routinely analyzed.

The common utilization of high temperatures in MAE and PMAE at the expense of losing heat-unstable compounds can be avoided. This study has demonstrated that the use of low temperatures in PMAE has the potential of attaining recovery rates comparable to prolonged maceration and offers a different profile of extracted phytochemicals. Taken together, these can improve the magnitude and range of activities under observation.

In line with previous findings, the nature of plant materials was observed to bear an influence on the quantity of recovered extracts under PMAE as compared with maceration. Among other factors, this is influenced by the ability of the plant material to hold residual moisture even after prolonged drying.

Likewise, fairly higher amounts of individual phytochemicals can be obtained using PMAE as compared with prolonged maceration. This selectivity may be a result of differences in solubility properties of a given phytochemical in relation to extraction temperature and time. Apart from the solvent-related factors, the extraction method used plays a role in determining the quantity and type of phytochemicals extracted. This bears implications on the range and magnitude of prospective activities to be observed.

4 EXPERIMENTAL

4.1 Plant materials, reagents, and experimental conditions

Leaves, stems, and barks of 14 plant species (see **Table 1**) were collected from the botanical garden of the University of Wuerzburg, Germany. Ginger rhizomes were obtained from Kraeuter Mix GmbH, Germany. Fresh plant materials were chopped into small pieces and air-dried at room temperature (26–27°C) for 2 weeks. Dried

plant materials were reduced into coarse powders using a laboratory electric blender (Braun, Germany).

Maceration was carried out by soaking plant materials in methanol in a solvent-to-matrix ratio of 10 ml/g. Extraction was done over 24 hr (and over 30 min for 12 control samples) at room temperature under constant stirring by magnetic stirrers. Pressurized MAE was done over 30 min using the same solvent-to-matrix ratio of 10 ml/g, followed by automated stirring, in a microwave reaction system (synthWAVE; MLS GmbH, Germany).[14] Temperature, microwave energy, and pressure were set at 40°C, 150 W, and 10 bar, respectively. The pressure was generated by argon gas. These settings resulted in a temperature–time profile of ambient to 40°C (3 min), 40–45°C (1 min), 45–40°C (7 min), and 40°C (19 min). Automated intermittent microwave irradiation (0–150 W) ensured the maintenance of temperature within the stated limits. Control PMAE experiments at 80°C for 30 min were conducted for 12 control samples in which the temperature rose from ambient to 80°C in 1 min at the microwave energy of 300 W.

After both maceration and PMAE extractions, the crude extracts were filtered (Whatman No. 1) and concentrated under vacuum at 40°C. The resultant semisolid extracts were further freeze-dried for 12 hr, affording dry powders, which were weighed and stored at −15°C.

HPLC analysis was done on an LCMS-2020 (Shimadzu, Japan) equipped with a DGU-20A3R degassing unit, a LC20AB liquid chromatograph, and an SPD-20A UV/Vis detector. Mass spectra were obtained by an LCMS-2020 with a Synergi 4U fusion-RP (150 × 4.6 mm) column as a stationary phase, nebulizing and drying gas (N2) flow rates of 1.5 and 15.0 l/min, respectively, desolvation line temperature of 250°C, and heat block temperature of 400°C. Both systems were controlled by the Shimadzu LabSolutions software. Stock solutions of 20 mg/ml for each extract were prepared using methanol as a diluent. Working solutions at 2 mg/ml were then obtained by further diluting stock solutions using methanol. A method used for routine analyses was applied. The chromatographic conditions entailed a reversed-phase column (RP C18; 4 µm, 150 × 4.6 mm; Agilent Technology) and a mobile phase, solvent A: water + 0.1% formic acid and solvent B: methanol + 0.1% formic acid. Moreover, a gradient elution profile was applied: 5–100% B (0–12 min), 100% B (12–17 min), and 100–5% B (17–18 min). An injection volume of 20 µl was used and UV detection was performed at 254 nm and a tandem ESI–MS operating in positive ionization mode.

4.2 Data analysis

The yields were compared by observing the percentage proportions at which the yield from PMAE differed from those of maceration. This was carried out independently for each sample. For each sample, the UV chromatograms from both methods were superimposed and examined for differences in numbers and

intensities of corresponding peaks. 18 MS BPC pairs of corresponding extracts were compared for the presence of the same peaks and the number of additional peaks.

The identity of additional/nonmatching peaks observed in UV chromatograms was cross-checked on the corresponding MS spectra. The peak was regarded as representing an additionally extracted compound if it was not observed in the BPCs of the corresponding extract. The BPCs of each pair of extracts showing additional/nonmatching peaks were comparatively analyzed using box and whisker plots.

ACKNOWLEDGMENTS

The authors would like to acknowledge the technical assistance of Florian Geyer and Christine Heinz. The study was supported by funding from the Deutscher Akademischer Austauschdienst (DAAD) offered to N. E. M. through research grants for Doctoral programmes in Germany, 2018/19 (57381412). Open access funding enabled and organized by Projekt DEAL.

CONFLICTS OF INTERESTS

The authors declare that there are no conflicts of interests.

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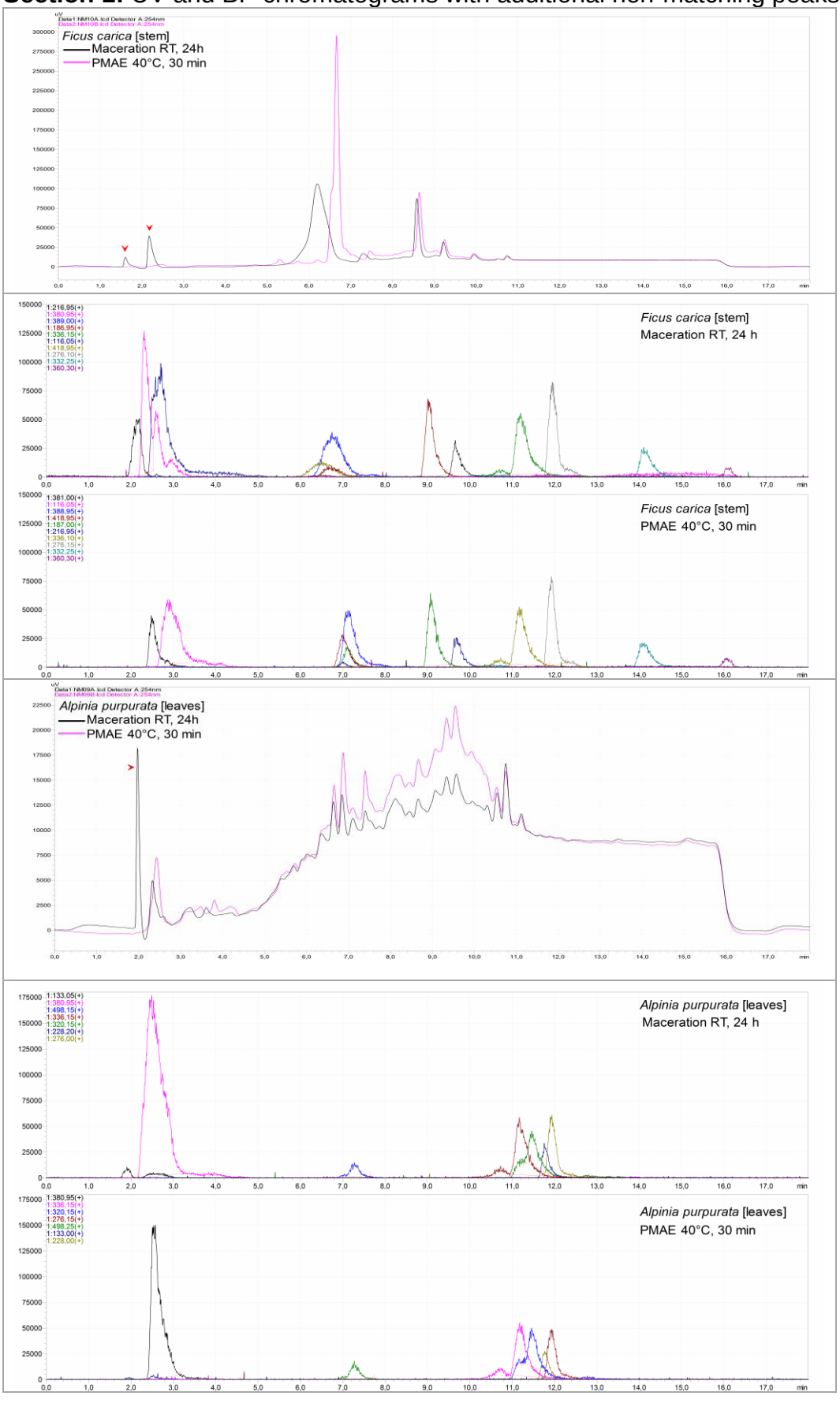
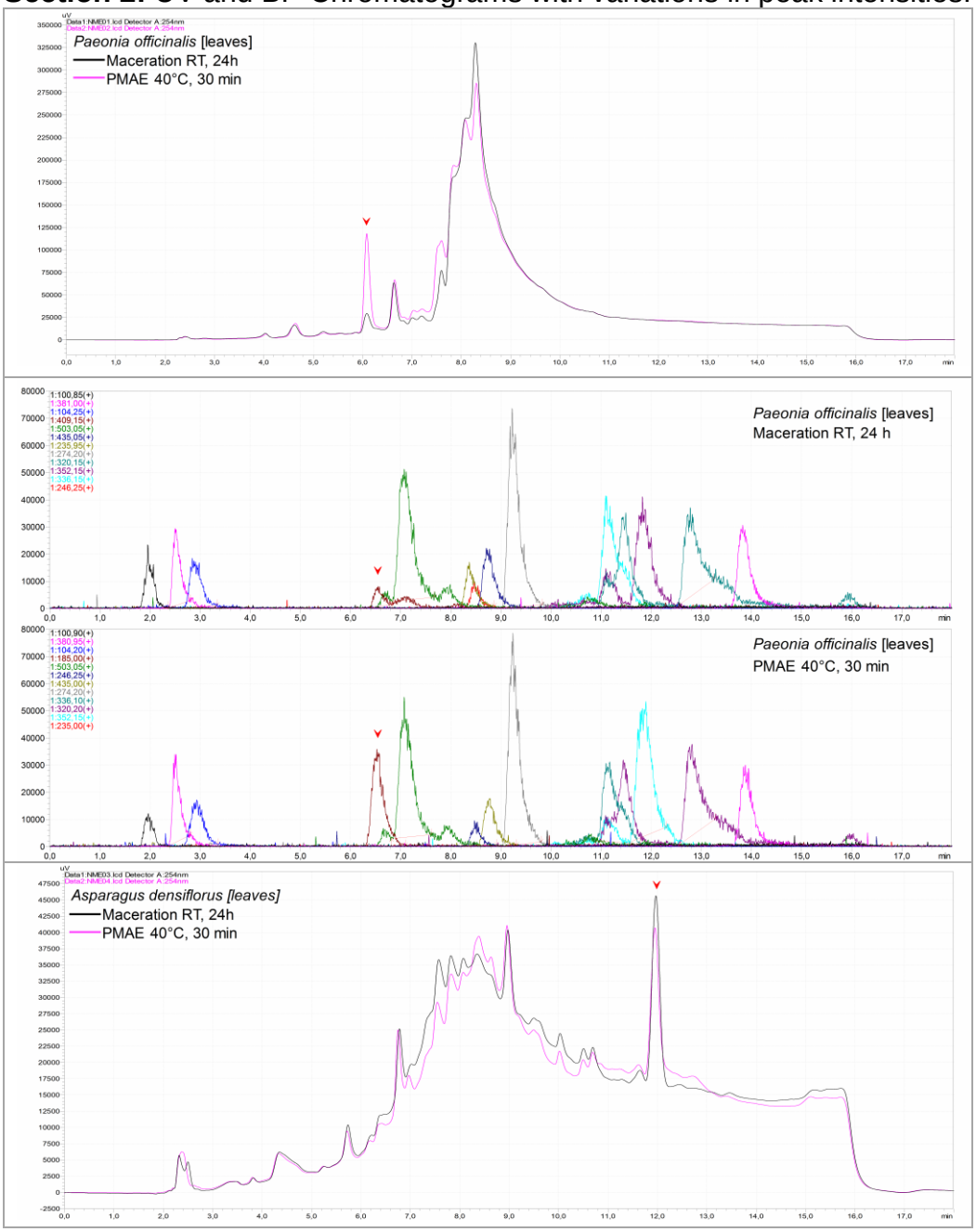
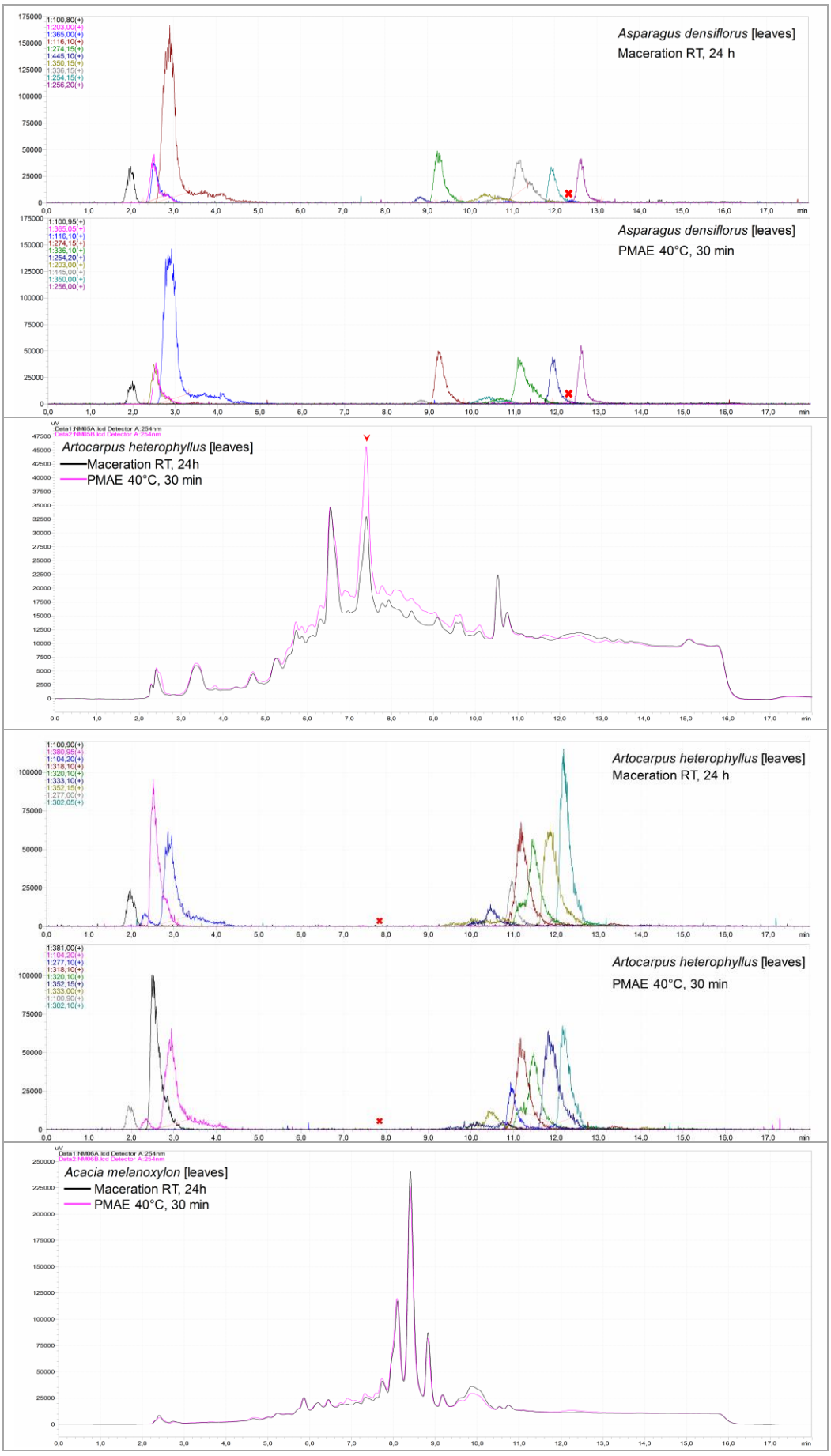
SUPPORTING INFORMATION

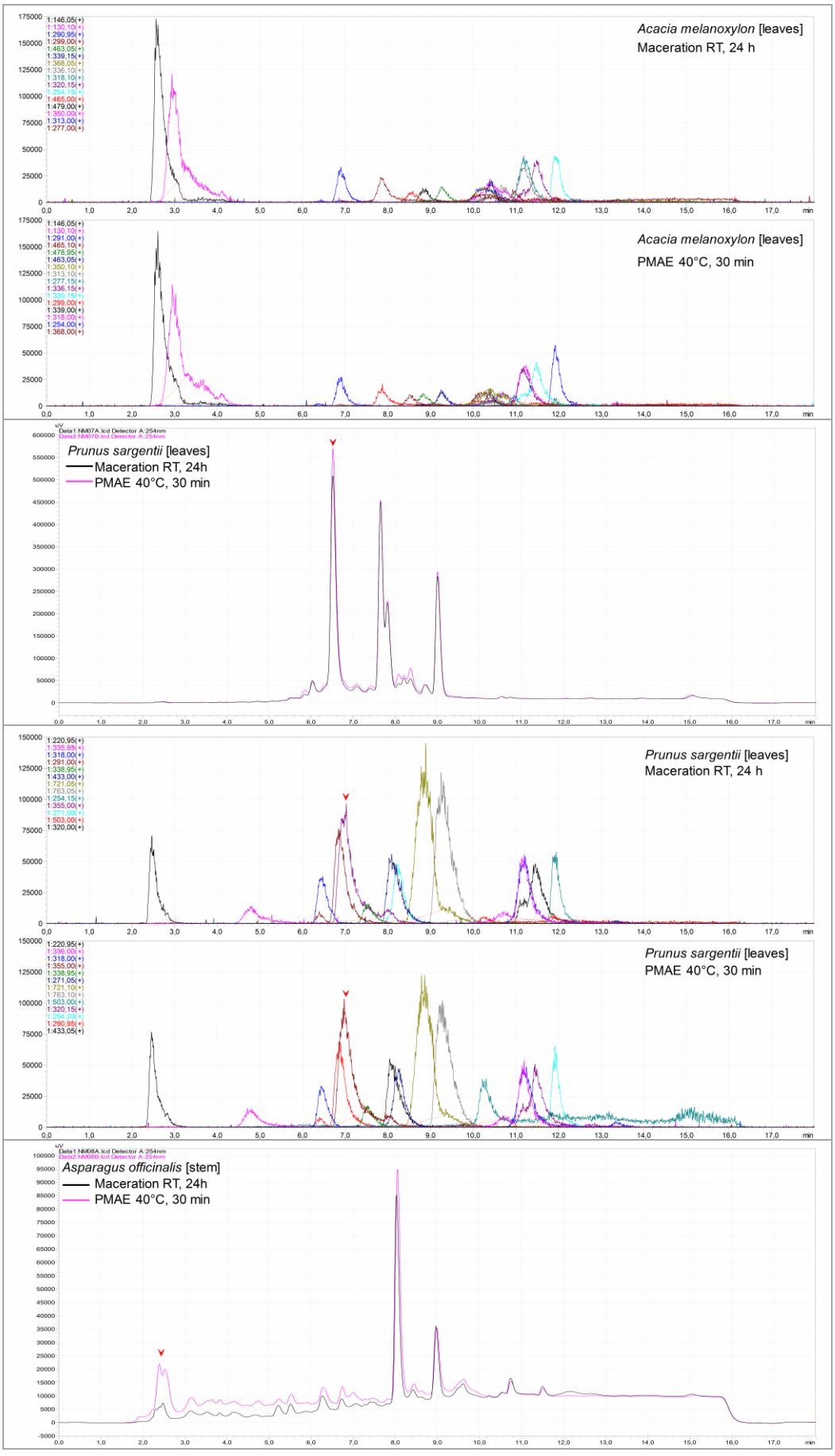
Section 1: UV and BP chromatograms with additional non-matching peaks.

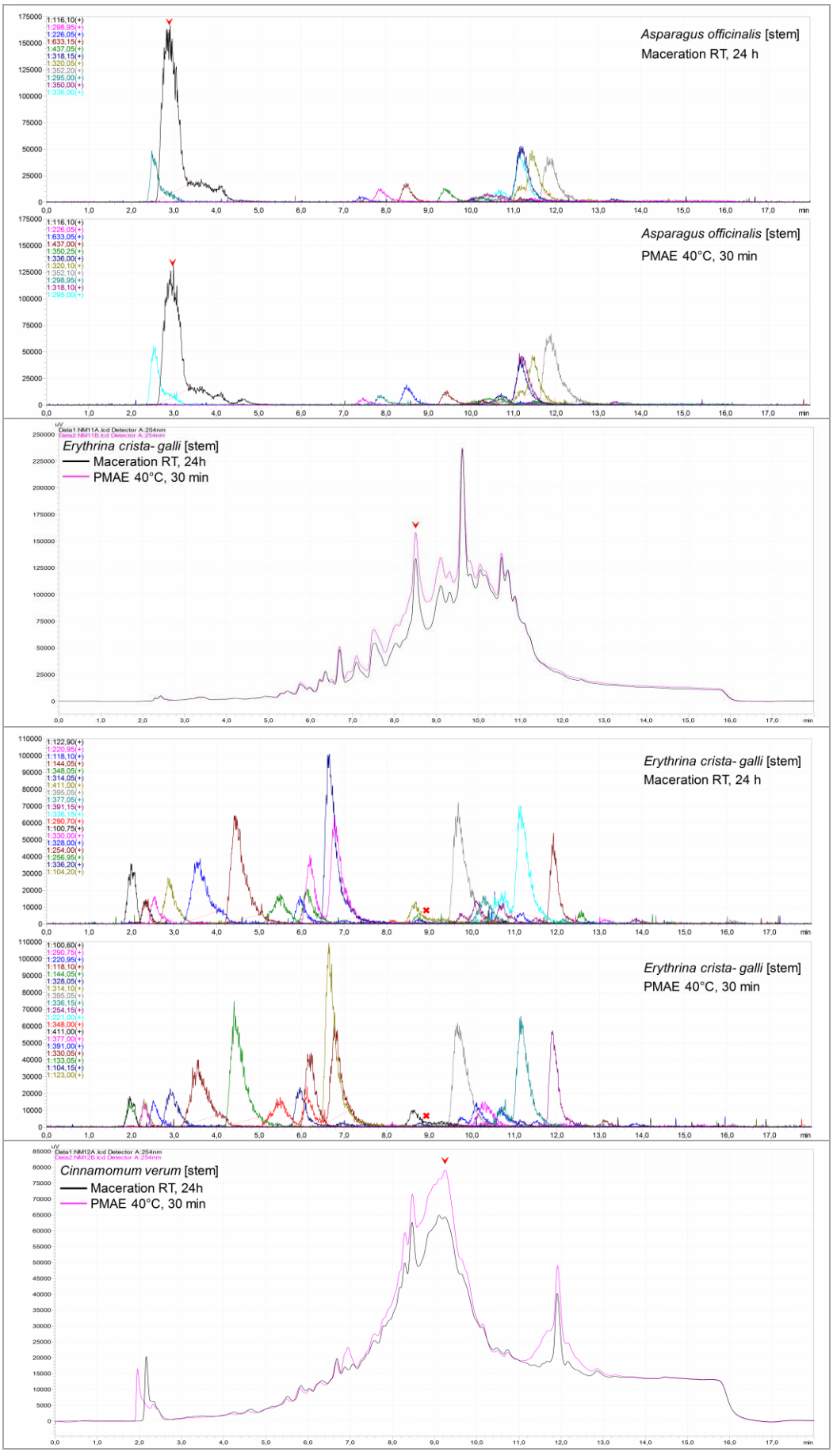


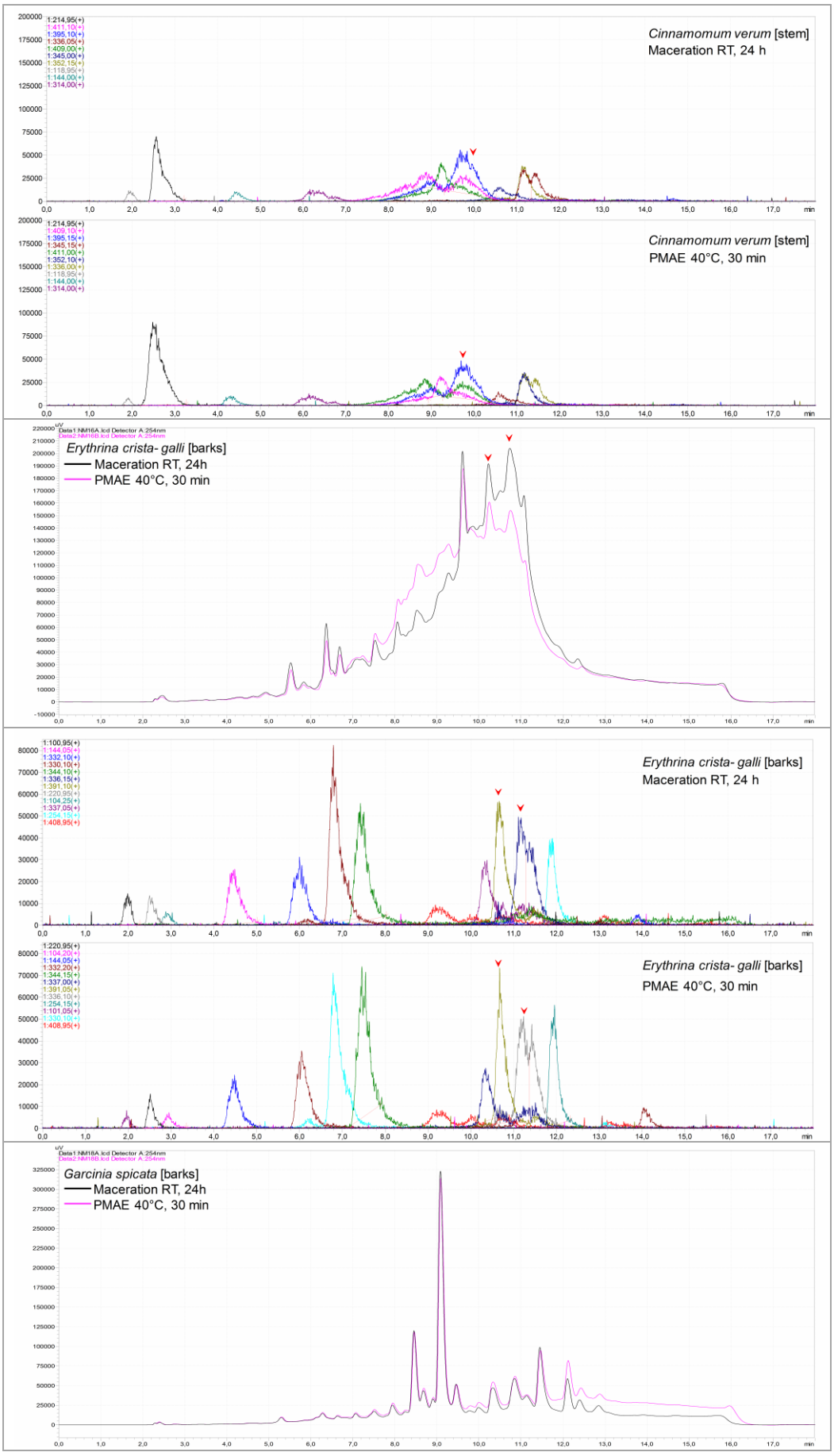
Section 2: UV and BP Chromatograms with variations in peak intensities.

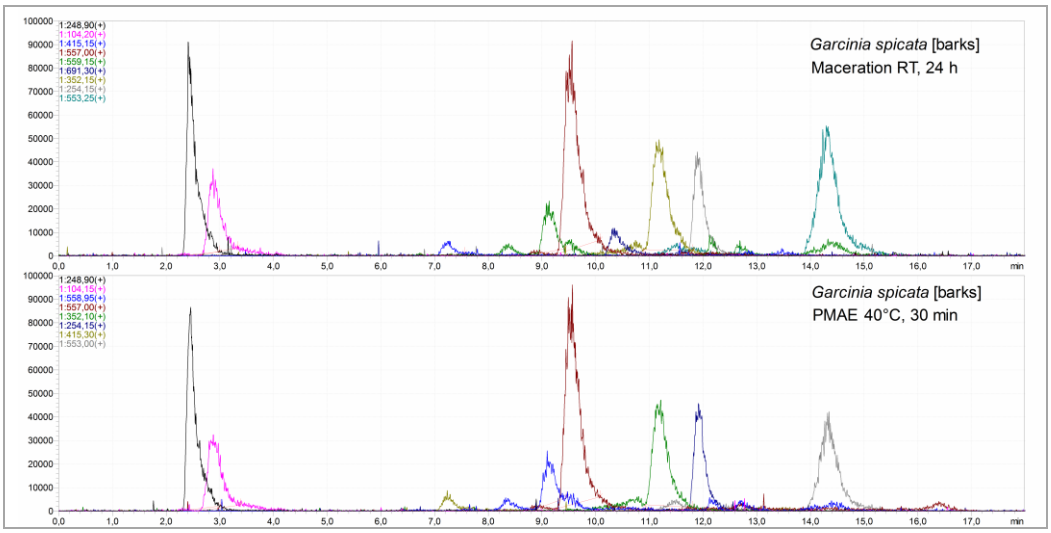



Asparagus densiflorus [leaves]
Maceration RT, 24 h
Asparagus densiflorus [leaves]
PMAE 40°C, 30 min
Artocarpus heterophyllus [leaves]
Maceration RT, 24h
PMAE 40°C, 30 min
Artocarpus heterophyllus [leaves]
Maceration RT, 24 h
Artocarpus heterophyllus [leaves]
PMAE 40°C, 30 min
Acacia melanoxylon [leaves]
Maceration RT, 24h
PMAE 40°C, 30 min


Acacia melanoxylon [leaves]
Maceration RT, 24 h
Acacia melanoxylon [leaves]
PMAE 40°C, 30 min
Prunus sargentii [leaves]
Maceration RT, 24h
PMAE 40°C, 30 min
Prunus sargentii [leaves]
Maceration RT, 24 h
Prunus sargentii [leaves]
PMAE 40°C, 30 min
Asparagus officinalis [stem]
Maceration RT, 24h
PMAE 40°C, 30 min


Asparagus officinalis [stem]
Maceration RT, 24 h
Asparagus officinalis [stem]
PMAE 40°C, 30 min
Erythrina crista- galli [stem]
Maceration RT, 24h
PMAE 40°C, 30 min
Erythrina crista- galli [stem]
Maceration RT, 24 h
Erythrina crista- galli [stem]
PMAE 40°C, 30 min
Cinnamomum verum [stem]
Maceration RT, 24h
PMAE 40°C, 30 min


Cinnamomum verum [stem]
Maceration RT, 24 h
Cinnamomum verum [stem]
PMAE 40°C, 30 min
Erythrina crista- galli [barks]
Maceration RT, 24h
PMAE 40°C, 30 min
Erythrina crista- galli [barks]
Maceration RT, 24 h
Erythrina crista- galli [barks]
PMAE 40°C, 30 min
Garcinia spicata [barks]
Maceration RT, 24h
PMAE 40°C, 30 min


Garcinia spicata [barks]
Maceration RT, 24 h
Garcinia spicata [barks]
PMAE 40°C, 30 min
min

4.2. Reproducibility challenges in the search for antibacterial compounds from nature

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ABSTRACT

Background: Reproducibility of reported antibacterial activities of plant extracts has long remained questionable. Although plant-related factors should be well considered in serious pharmacognostic research, they are often not addressed in many research papers. Here we highlight the challenges in reproducing antibacterial activities of plant extracts.

Methods: Plants with reported antibacterial activities of interest were obtained from a literature review. Antibacterial activities against *Escherichia coli* and *Klebsiella pneumoniae* were tested using extracts' solutions in 10% DMSO and acetone. Compositions of working solutions from both solvents were established using LC-MS analysis. Moreover, the availability of details likely to affect reproducibility was evaluated in articles which reported antibacterial activities of studied plants.

Results: Inhibition of bacterial growth at MIC of 256–1024 μg/mL was observed in only 15.4% of identical plant species. These values were 4–16-fold higher than those reported earlier. Further, 18.2% of related plant species had MICs of 128–256 μg/mL. Besides, 29.2% and 95.8% of the extracts were soluble to sparingly soluble in 10% DMSO and acetone, respectively. Extracts' solutions in both solvents showed similar qualitative compositions, with differing quantities of corresponding phytochemicals. Details regarding seasons and growth state at collection were missing in 65% and 95% of evaluated articles, respectively. Likewise, solvents used to dissolve the extracts were lacking in 30% of the articles, whereas 40% of them used unidentified bacterial isolates.

Conclusion: Reproducibility of previously reported activities from plants' extracts is a multi-factorial aspect. Thus, collective approaches are necessary in addressing the highlighted challenges.

Introduction

The discovery of novel antibiotics is urgently needed due to the ongoing challenge of antimicrobial resistance (AMR). Approaches in the search for new antibiotics include modifications of existing antibiotics, *in silico* target-based designing and synthesis of new molecules, as well as screening chemical libraries and nature. All approaches are mainly driven by the need for achieving novel antibacterial agents with novel chemical structure, target, and mode(s) of action, as well as with the absence of cross-resistance to existing antibiotics [1, 2]. Moreover, the search for compounds targeting different bacterial virulence mechanisms (pathoblockers) is a promising approach which offers lower possibilities for resistance development [3].

Nature is a potential source of hit compounds with antibacterial activity. More than half of the antibiotics currently in use are of fungi and bacterial origins. However, compounds from plants have not yet contributed to any of the antibiotics currently available on the market [4, 5]. Nevertheless, research works ranging from documentation of plants' ethnobotanical uses to isolation and optimization of lead compounds from plants are common [4, 5]. Hence, this constitutes an important part of the search for new antibacterial compounds. These studies report on plant species, parts, nature of the extract, and bacteria species on which antibacterial activity was observed. In the case of a positive outcome, the follow-up studies typically aim at isolating, characterizing, and even optimizing the active compounds towards a lead compound, which is suitable for pre-clinical studies [6–8].

Since reporting of initial findings on antibacterial activity of plant extracts aims at providing a base for supporting further studies, a reasonably good level of reproducibility of the reported findings is crucial for preparing larger amounts of the crude extract of interest for more investigations as well as for other laboratories, who want to add other studies. Nevertheless, several factors may limit the attainment of good reproducibility level of results from Antimicrobial Susceptibility Testing (AST) of plant extracts. These include factors related to climate, soil, collection and drying practices, extraction methods as well as nature of the test bacteria [9–13]. Specifically, the determination of the anti-infective activity of extracts and the subsequently produced fractions is often performed inaccurately [13, 14], or follow old procedures, which are scientifically no longer acceptable, e.g. the use of agar diffusion assays.

This study aimed at reproducing previously reported antibacterial activities of plant extracts active against selected gram-negative bacteria from the family of Enterobacteriaceae, because we urgently need new antibiotics in this field. Further, we assessed the possibility of obtaining comparable outcomes upon the use of related plant species. Additionally, we evaluated the availability of key details regarding the plants, bacteria, and selected experimental aspects as reported in the articles from which the studied plants were obtained.

Materials and methods

Identification of plants with antibacterial activities

Literature search was conducted using the search string: '*Plant OR extract AND antibacterial OR antimicrobial OR activity AND Escherichia coli OR Klebsiella pneumoniae*'. The search was done on PubMed®, Web of Science™ and Google Scholar databases, targeting full research articles published between 1948 and 2018 in English.

Target plant species were identified by virtue of having crude extracts with moderate to high activities (Minimum Inhibitory Concentrations (MIC) of ≤256 µg/mL) against *E. coli* or *K. pneumoniae*, as determined by broth dilution assays [15].

Materials

Acetone, dimethyl sulfoxide (DMSO), ethanol, ethyl acetate, n-hexane, methanol, petroleum ether, iodonitrotetrazolium chloride (INT) were purchased from Sigma-Aldrich Chemie, (Schnelldorf, Germany); Mueller Hinton Broth (MHB), Lysogeny Broth (Lennox) (LB) and agar were purchased from Carl Roth (Karlsruhe, Germany); gentamicin sulfate from AppliChem (Darmstadt, Germany) and demineralized water.

Preparation of crude extracts

All materials were collected from fully matured plants in March, May and August 2018 from the Botanical garden of the University of Wuerzburg. Collected plant materials were kept in open paper bags and transported to the laboratory within 2 hours. The materials were then chopped into small pieces and air-dried under shade at room temperature for one to two weeks.

Dried plant materials were size reduced into coarse powders using an electric blender (Braun, Germany). Extraction was performed using 72 h maceration at room temperature and magnetic stirring. Extraction solvent types and sequences were reproduced as reported in the cited articles as indicated in **Tables 2** and **3**. Crude extracts solutions were obtained upon filtration and were dried under vacuum at 40 °C. Extracts obtained from solvents composed of alcohols and water (80% v/v) were further dried in a freeze dryer (Martin Christ Gefriertrocknungsanlagen, Germany) at -60 °C and 0.03–0.13 mbar. Dried extracts were weighed and stored at -15 °C until they were further used.

Preparation of stock and working extract test solutions

Two sets of stock solutions were prepared by dissolving the extracts in 10% dimethyl sulfoxide (DMSO) and acetone, respectively, followed by ultrasonication (Bandelin electronic, Germany) for 15 minutes. Clear solutions were then obtained by centrifugation of the sonicated samples at 13000 RPMs for 10 minutes (Heraus pico 17, Thermo Fisher Scientific, Germany). 2 mL of working solutions were prepared at concentrations equivalent to 2048 µg/mL by diluting the respective volumes of stock

solutions with MHB media. Following the dilutions, working solutions were mixed for 10 minutes on a lateral shaker, followed by centrifugation for 10 minutes. The concentrations of DMSO and acetone in the working solutions were 2.048 and 20.48% (v/v), respectively.

Qualitative phytochemical screening

Qualitative phytochemical screening was done on extracts from identical plant species whose phytochemical profiles were reported in the respective publications. Qualitative tests for the presence of alkaloids, terpenes, sterols, flavonoids, phenols and tannins were carried out as per methods described by Harbone [16]. Moreover, we used LC-MS to study the profiles of an extract which phytochemical profile was established using GC-MS and LC-MS techniques. The procedure for LC-MS analysis is described in the section below.

Solubility testing and comparative LC-MS analysis

The extents to which the extracts had dissolved in the respective solvents were semi-quantitatively evaluated based on the amount sediments at the bottom of the Eppendorf tubes after each centrifugation, as described above.

LC-MS analysis was done on selected extracts to further investigate the differences in compositions between the working solutions prepared using acetone and DMSO. Sampling of the extracts was done to include extracts with better solubility in acetone (*C. longa* and *G. tinctoria*) as well as in 10% DMSO (*F. carica*) (**Table 4**).

In that, stock solutions equivalent to 10 mg/mL were prepared from the extracts in acetone and 10% DMSO, followed by 10 min. ultrasonication and centrifugation at 13000 RPMS for 10 min. To prepare working solutions, 1 mL of the stock solutions diluted with 4 mL of each distilled water, with subsequent 10 min ultrasonication and centrifugation at 13000 RPMS for 10 min.

To remove the solvents, 2 mL of the supernatant from each working solution were transferred to 10 mL preparation glasses and were freeze dried for 24 h. Solution for LC-MS analysis were thereafter obtained by reconstituting the dried residues with 1 mL of methanol. Analysis was conducted by injecting 10 µL of each obtained solution into an LCMS-2020 (Shimadzu, Japan) system, using previously reported chromatographic conditions [17].

Antimicrobial susceptibility testing

Since a broad range of bacterial strains were used in the referred studies, we opted to use reference *Escherichia coli* (ATCC 25922) and *Klebsiella pneumoniae* (ATCC 10031) strains. The two strains have no resistance mechanisms to antibiotics and were obtained from the American Type Culture Collection (ATCC).

Overnight bacteria cultures were prepared by suspending one colony from LB agar plates into 2 mL of autoclave sterilized LB broth followed by 24 h incubation at 37 °C

under lateral shaking (Edmund Bühler GmbH, Germany). Fresh cultures were then prepared by transferring 200 µL of the overnight culture into 20 mL of LB broth with a subsequent incubation for 6 h at 37 °C under lateral shaking. The number of Colony Forming Units (CFU) in the fresh cultures was determined using the optical densities (Eppendorf BioPhotometer Plus, Eppendorf AG, Germany) of ten-times diluted fresh cultures and Newman's correlation curve. Bacterial suspensions containing 10^6 CFU/mL of each bacteria were prepared by drawing the appropriate volume of the fresh culture into the corresponding amount of MHB media. These suspensions were used within 30 minutes from their preparation [18, 19].

Antimicrobial susceptibility testing was done using broth microdilution assays on 96 wells microtiter plates (Greiner Bio-One, Austria). Using a multichannel pipette (Eppendorf AG, Germany), 100 µL of autoclave MHB media was added in triplicates into wells in rows 2 to 12, followed by 200 µL of the extract test solution in the first row. A serial two-fold dilution was then done by drawing 100 µL of the extract solution from the first row and mixing with 100 µL of MHB media in the second row. The procedure was repeated to the last row, whereby the final 100 µL was discarded [18, 19].

Following the serial dilution, 100 µL of 10^6 CFU/mL bacteria suspension in MHB media were added into the respective wells on the microtiter plate in triplicates. Each extract was tested in a range of 0.5–1024 µg/mL. The highest concentration of DMSO and acetone, respectively in the first wells were 1.024 and 10.24%(v/v). Gentamicin sulfate was used as a positive control in a range of 0.0156–32 µg/mL. Negative control involved solutions of DMSO and acetone, respectively, in MHB media at 1.28%(v/v) and 10.24%(v/v). Other controls in place included sterility controls for crude extracts and MHB media, as well as bacteria growth controls. All extracts and controls were tested in triplicates and the experiments were repeated twice in accordance to references [13, 20, 21].

The loaded plates were incubated for 18 h at 37 °C (Hera Cell incubator, Heraeus, Germany); then 40 µL of 0.2 mg/mL solution of iodonitrotetrazolium chloride (INT) was added into all wells and further incubated for 30 minutes. The MICs were determined by visual observation for wells with no formation of pinkish coloration, indicating the absence of actively diving bacteria.

Results

From the literature search, 204 plant species were identified to meet our set criteria of having either crude extracts or volatile/essential oils with MICs ≤256 µg/mL against *E. coli* or *K. pneumoniae*, determined by broth dilution assays. Upon excluding studies, which tested volatile or essential oils, 40 species were noted to be available in the Botanical garden of the University of Wuerzburg as either identical or related species. Moreover, due to the limited availability of parts like nuts, fruits and pericarps, matching plant parts were obtained from only 13 identical and 11 related

species (**Tables 1–3**). The growing conditions for the plants corresponded to their natural habitats with regard to temperature and relative humidity (**Table 1**).

Table 1. Plants studied.

SN	Name (Family)	Plant part	Internal accession number	Growth area
1	*Castanea sativa* Mill. (Fagaceae)	Leaves	XXXX-539-H-60	outdoor
2	*Cinnamomum verum* L. (Lauraceae)	Leaves	2010-90-B-80	Glasshouse—tropical[a]
3	*Datura stramonium* L. (Solanaceae)	Seeds	XXXX-868-G-74	outdoor
4	*Juniperus oxycedrus* L. (Cupressaceae)	Leaves	XXXX-925-G-80	outdoor
5	*Murraya koenigii* (L.) Spreng. (Rutaceae)	Leaves	2004-70-B-80	Glasshouse—tropical[a]
6	*Olea europaea* L. (Oleaceae)	Leaves	XXXX-954-G-80	Glasshouse—Mediterranean[b]
7	*Piper betle* L. (Piperaceae)	Leaves	1990-349-D-80	Glasshouse—tropical[a]
8	*Ricinus communis* L. (Euphorbiaceae)	Seeds	XXXX-1004-G-74	outdoor
9	*Salvia officinalis* L. (Lamiaceae)	Leaves	XXXX-1015-G-80	outdoor
10	*Satureja hortensis* L. (Lamiaceae)	Aerial parts	XXXX-596-G-70	outdoor
11	*Silybum marianum* (L.) Gaertn. (Asteraceae)	Seeds	XXXX-1080-G-74	outdoor
12	*Viscum album* L. (Loranthaceae)	Leaves	XXXX-1072-H-70	outdoor
13	*Zingiber officinale* Rosc. (Zingiberaceae)	Rhizomes	Charge 329272 (Kraeuter Mix, Germany)	outdoor
14	*Acacia melanoxylon* R.Br. (Fabaceae)	Leaves	XXXX-399-E-80	Glasshouse—cultivation area[c]
15	*Acacia retinoides* Schltdl. (Fabaceae)	Leaves	XXXX-106-P-70	Glasshouse—cultivation area[c]
16	*Adiantum raddianum* C.Presl. (Adiantaceae)	Whole plant	2001-62-B-80	Glasshouse—cultivation area[c]
17	*Alpinia purpurata* (Vieill.) K. Schum. (Zingiberaceae)	Leaves	2010-88-B-70	Glasshouse—tropical[a]
18	*Curcuma longa* L. (Zingiberaceae)	Rhizome	2004-25-D-80	Glasshouse—tropical[a]
19	*Erythrina crista-galli* L. (Fabaceae)	Bark	1982-348-E-80	Glasshouse—Mediterranean[b]
20	*Ficus carica* L. (Moraceae)	Bark	XXXX-220-G-80	outdoor
21	*Garcinia spicata* Hook.f. (Clusiaceae)	Leaves	1977-306-D-80	Glasshouse—tropical[a]
22	*Garcinia tinctoria* (DC.) W. Wight (Clusiaceae)	Leaves	XXXX-74-B-80	Glasshouse—tropical[a]
23	*Paeonia officinalis* L. (Paeoniaceae)	Leaves	2013-11-S-10	outdoor
24	*Satureja montana* L. (Lamiaceae)	Aerial parts	XXXX-1068-K-80	outdoor

[a] temperature = 18–25 °C, relative humidity = 70–90%;
[b] temperature = 4–25 °C, relative humidity = 50–70%;
[c] temperature = 8–25 °C, relative humidity = 50–70%.

https://doi.org/10.1371/journal.pone.0255437.t001

Table 2. Minimum inhibitory concentrations (MICs) of 10% DMSO and acetone dissolved extracts of plant species identical to those reported in the literature.

Studied plants and extractants			Minimum inhibitory concentration (μg/mL)						
			10% DMSO dissolved extract		acetone dissolved extracts		Previously reported antibacterial activities		
Sn	Plant's name	Extracting solvent	Ec (ATCC 25922)	Kp (ATCC 10031)	Ec (ATCC 25922)	Kp (ATCC 10031)	Ec	Kp	Ref.
1	*Castanea sativa* Mill.	ethyl acetate	>1024	>1024	>1024	>1024	256	256	[25]
2	*Cinnamomum verum* L.	methanol	>1024	**1024**	>1024	**1024**	64	256	[23]
3	*Datura stramonium* L.	petroleum ether	>512	>512	>512	>512	39.1	-	[26]
4	*Juniperus oxycedres* L.	methanol	>1024	>1024	>1024	>1024	250	-	[27]
5	*Murraya koenigii* (L.) Spreng.	acetone (benzene*)	>1024	>1024	>1024	>1024	125	250	[28]
6	*Olea europaea* L.	acetone	>1024	>1024	>1024	>1024	60	25	[24]
7	*Piper betle* L.	ethanol	>1024	>1024	>1024	**1024**	250	250	[29]
8	*Ricinus communis* L.	methanol	>1024	>1024	>1024	>1024	250	31	[28]
9	*Salvia officinalis* L.	acetone	>1024	>1024	>1024	>1024	-	156	[30]
10	*Satureja hortensis* L.	methanol	>1024	>1024	>1024	>1024	250	-	[31]
11	*Silybum marianum* (L.) Gaertn.	ethanol-water 8:2 v/v	>1024	>1024	>1024	>1024	41.2	20	[32]
12	*Viscum album* L.	methanol	>1024	>1024	>1024	>1024	256	256	[22]
13	*Zingiber officinale* Rosc.	ethanol	>512	>512	>512	>512	75.6	185.5	[33]

* Solvent used in the reference article but avoided in this study due to toxicity concerns

https://doi.org/10.1371/journal.pone.0255437.t002

Table 3. Minimum inhibitory concentrations (MICs) of 10% DMSO and acetone dissolved extracts of plant species reported to those reported in the literature.

Studied plants and extractants				Minimum inhibitory concentration (μg/mL)						
				DMSO dissolved extract		acetone dissolved extract		Previously reported antibacterial activities		
Sn	Studied species	Species reported in literature	Extracting solvent	Ec (ATCC 25922)	Kp (ATCC 10031)	Ec (ATCC 25922)	Kp (ATCC 10031)	Ec	Kp	Reference
1	*Acacia melanoxylon* R.Br.	*Acacia nilotica*	ethanol 80%	>1024	>1024	>1024	>1024	19.5	9.75	[34]
2	*Acacia retinoides* Schltdl.	*Acacia nilotica*	ethanol 80%	>1024	>1024	>1024	>1024	19.5	9.75	[34]
3	*Adiantum raddianum* C. Presl.	*Adiantum venustum*	methanol	>1024	>1024	>1024	>1024	15.6	7.81	[35]
4	*Alpinia purpurata* (Vieill.) K. Schum.	*Alpinia galanga* (L.) Wild	methanol	>1024	>1024	>1024	>1024	80	160	[36]
5	*Curcuma longa* L.	*Curcuma malabarica*	n-hexane	>1024	>1024	>1024	**1024**	-	10	[37]
6	*Erythrina crista-galli* L.	*Erythrina sigmoidea*	methanol	>1024	>1024	>1024	>1024	16	64	[38]
7	*Ficus carica* L.	*Ficus bubu* Warb.	methanol	>1024	>1024	>1024	>1024	39.1	-	[39]
8	*Garcinia spicata* Hook.f.	*Garcinia smeathmannii* Oliver	methanol	>1024	**512**	>1024	**512**	39.1	78.1	[40]
9	*Garcinia tinctoria* (DC.) W. Wight	*Garcinia smeathmannii* Oliver	methanol	>1024	>1024	>1024	>1024	39.1	78.1	[40]
10	*Paeonia officinalis* L.	*Paeonia broteroi* Boiss. & Reut.	acetone (Multiple*)	**256**	**128**	**256**	**128**	-	250	[41]
11	*Satureja montana* L.	*Satureja hortensis* L.	methanol	>1024	>1024	>1024	>1024	250	-	[31]

* Five extraction solvents of varying polarities were used in the reference article, all giving the same MIC value.

https://doi.org/10.1371/journal.pone.0255437.t003

Plants' details such as taxonomy, location and season of collection, growth state at collection, and parts studied were obtained from the reviewed articles as far as reported. Furthermore, we gathered information on solvents used for extraction, type of the bacterial strains studied, reference AST methods used, bacteria growth visualizing techniques as well as positive and negative controls applied.

We observed a very low reproducibility of antibacterial activities in extracts from plant species identical to those previously reported (**Table 2**). Only extracts from *Cinnamomum verum* L. and Piper betle L. showed MICs within the tested concentration (1024 μg/mL) against at least one of the bacteria tested.

Among the related species, extracts from *Garcinia spicata* Hook. f. showed MICs of 512 μg/mL against *K. pneumoniae*, whereas *Paeonia officinalis* L. inhibited the growth of both E. coli and K. pneumoniae at MICs of 256 μg/mL and 128 μg/mL, respectively (**Table 3**). All MICs are rather high.

Generally, the screening for phytochemicals present in the extracts studied was observed to be uncommon in literature. In our study, we could obtain results on qualitative phytochemical screening in only 3 out of 13 compared identical plant species, whereas no quantitative screening was reported This hindered a large qualitative and quantitative comparison in this aspect.

However, the methanol leaf extract of *V. album* was reported to contain alkaloids, terpenes, sterols, flavonoids and polyphenols, whereas a similar extract in our study did not contain sterols [22]. Moreover, our results were similar to those reported in the methanol leave extract of *C. verum*, which contained alkaloids, flavonoids,

phenols, sterols and tannins [23]. The observed antibacterial activities in *V. album* and *C. verum* were not directly associated to a specific type of phytochemicals found to be present in the extracts. Korukluoglu et al. used GC-MS and LC-MS to screen for compounds present in an acetone extract of *O. europaea* leaves. Similar to their findings, our LC-MS analysis of the corresponding extract showed the presence of vanillic acid (*m/z* 312), syringic acid (*m/z* 342), p-coumaric acid (*m/z* 308), ferulic acid (*m/z* 338), and oleuropein (*m/z* 540). Moreover, the presence of three other compounds each with a mass to charge ratio 282 (4-hydroxybenzoic acid, veratric acid and protocatecuic acid) could not be verified with certainty since we observed only one peak corresponding to *m/z* 282, whereas caffeic acid (*m/z* 396) was not present. However, the referred study reported inhibitory effect of caffeic acid against *E. coli* and *K. pneumoniae*, among other bacteria [24].

Upon preparing stock and working solutions, most crude extracts dissolved to a greater extent in acetone as compared to 10% DMSO. This observation, however, did not result in notable differences in the observed antibacterial activities of the solutions (**Table 4**).

Table 4. Semi-quantitative evaluation of solubility of crude extracts in 10% DMSO and acetone.

Sn	Plant's name	Part	Extracting solvent	Dissolving extent of crude extract in:			
				10% DMSO		acetone	
				stock	working	stock	working
1	*Castanea sativa* Mill.	Leaves	ethyl acetate	+	++	++	+
2	*Cinnamomum verum* L.	Leaves	methanol	+	++	++	++
3	*Datura stramonium* L.	Seeds	petroleum ether	+	+++	+++	+++
4	*Juniperus oxycedres* L.	Leaves	methanol	++	++	++	++
5	*Murraya koenigii* (L.) Spreng.	Leaves	acetone	+	+++	++	++
6	*Olea europaea* L.	Leaves	acetone	+	+++	+++	+
7	*Piper betle* L.	Leaves	ethanol	++	++	++	++
8	*Ricinus communis* L.	Seeds	methanol	+	+++	++	+++
9	*Salvia officinalis* L.	Leaves	acetone	+	+++	++	+
10	*Satureja hortensis* L.	Aerial parts	methanol	+	++	++	++
11	*Silybum Marianum* (L.) Gaertn.	Seeds	ethanol 80%	+	+++	++	+++
12	*Viscum album* L.	Leaves	methanol	+	++	++	+
13	*Zingiber officinale* Rosc.	Rhizomes	methanol, ethanol	+	+++	+++	++
14	*Acacia melanoxylon* R.Br.	Leaves	ethanol 80%	+++	++	++	++
15	*Acacia retinoides* Schltdl.	Leaves	ethanol 80%	+	+++	++	++
16	*Adiantum raddianum* C.Presl.	Whole plant	methanol	++	++	++	++
17	*Alpinia purpurata* (Vieill.) K. Schum.	Leaves	methanol	+++	++	++	++
18	*Curcuma longa* L.	rhizome	n-hexane	+	++	+++	+++
19	*Erythrina crista-galli* L.	Bark	methanol	+	+++	++	++
20	*Ficus carica* L.	Bark	methanol	++	+++	+	++
21	*Garcinia spicata* Hook.f.	Leaves	methanol	++	++	++	+++
22	*Garcinia tinctoria* (DC.) W. Wight	Leaves	methanol	+	++	++	+++
23	*Paeonia officinalis* L.	Leaves	acetone	+	++	+++	++
24	*Satureja montana* L.	Aerial parts	methanol	+	++	++	+

+++ = Soluble; ++ = sparingly soluble; + = slightly soluble

https://doi.org/10.1371/journal.pone.0255437.t004

LC-MS analysis of working solutions prepared using acetone and 10% DMSO showed that the extracts were qualitatively similar to one another. However, the solutions had some quantitative differences. For example, higher quantities of phytochemical were observed in n-hexane rhizome extract of *C. longa* dissolved in

acetone compared to 10% DMSO (**Fig 1**), because the extract showed better solubility in acetone (**Table 4**). In addition, additional peaks were noted in the low polarity region of the acetone dissolved extract's chromatogram (**Fig 1**).

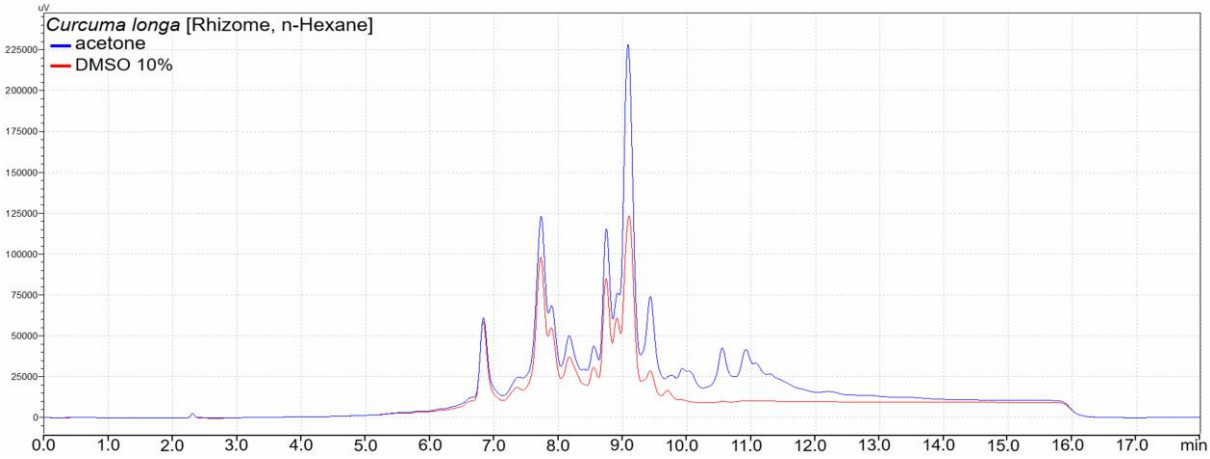
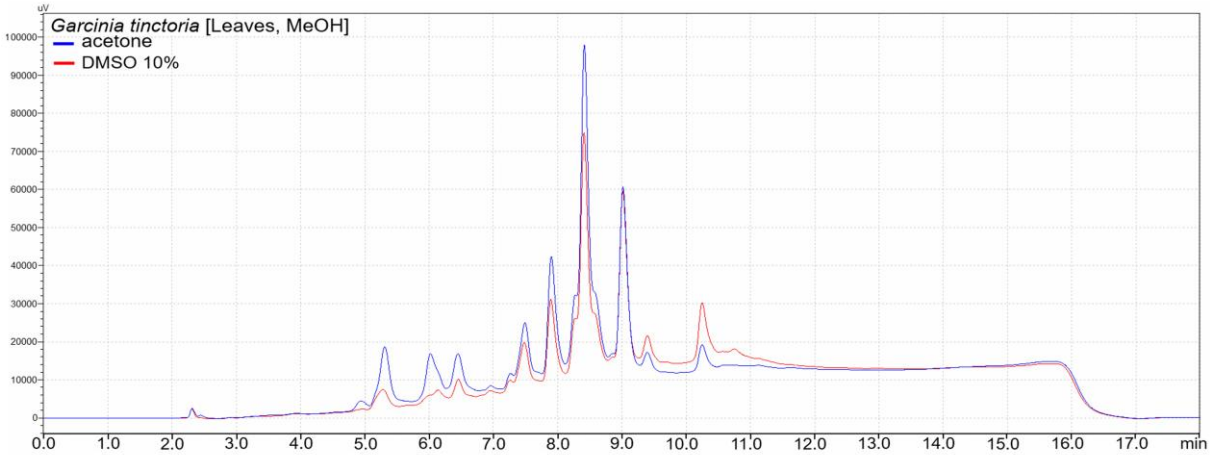


Fig 1. An overlay of UV chromatograms of acetone (blue) and DMSO (red) based working solutions of an n-hexane rhizomes extract of *C. longa*. Qualitative similarity and higher quantities of phytochemicals observed in the acetone based working solution, which had better solubility compared to that of DMSO.

Moreover, when dissolved in acetone, *G. tinctoria* leaves extract from a more polar extracting solvent (methanol) showed higher quantities of phytochemicals in the polar region of the chromatogram, as compared to the less polar region (**Fig 2a**) (**Table 4**). The Base Peak Chromatograms (BPC) of the same solutions indicated the presence of at least 3 additional compounds in the less polar region of the acetone based working solution (**Fig 2b**).

(a)



(b)

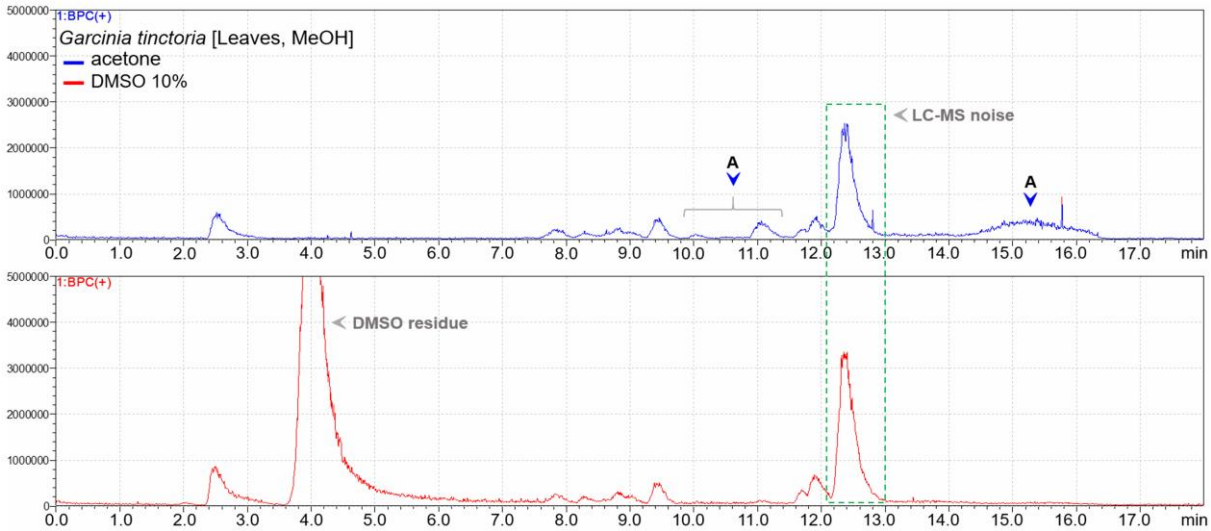
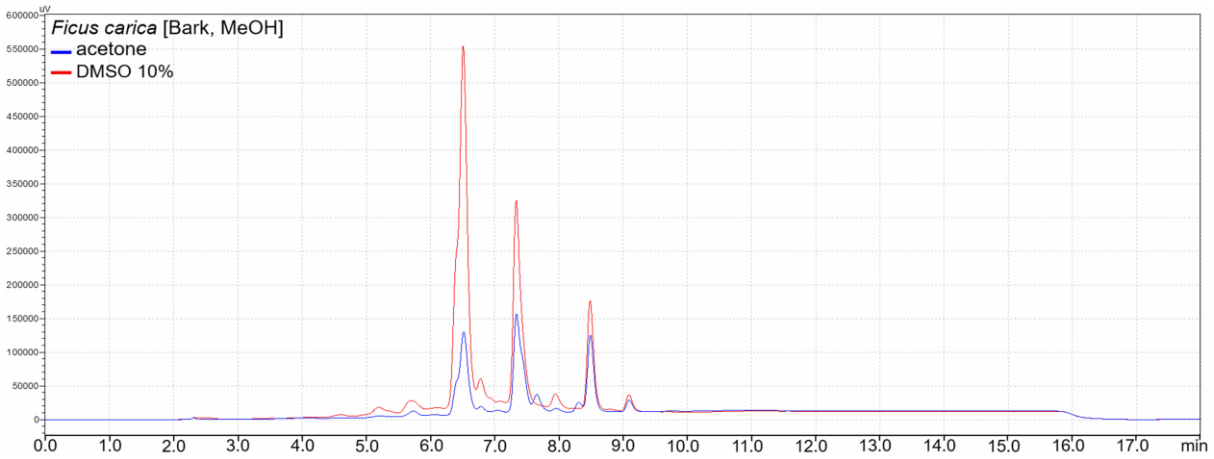


Fig 2. **a**. An overlay of UV chromatograms of acetone (blue) and DMSO (red) based working solutions of a methanol leaves extract of *G. tinctoria*, which had better solubility in acetone. Higher quantities of phytochemicals are observed in the acetone based working solution towards a less polar region of the chromatogram. **b**. Comparison of base peak chromatograms of acetone (blue) and DMSO (red) based working solutions of a methanol leaves extract of *G. tinctoria*, which had a better solubility in acetone. At least three additional compounds (marked A) are visible in the less polar region of acetone based working solution's chromatogram.

Furthermore, **Fig 3a** exemplifies the observation of higher quantities of phytochemicals in a DMSO dissolved working solution of the *F. carica* bark extract with higher solubility in 10% DMSO. The differences are particularly higher towards the more polar region of the chromatogram. The BPC of the same pair of working solutions showed 4 additional compounds as compared to 1 in acetone and DMSO based working solutions, respectively (**Fig 3b**). Noteworthy is also the lesser polarity of additional compounds in acetone as compared to DMSO based working solutions.

(a)



(b)

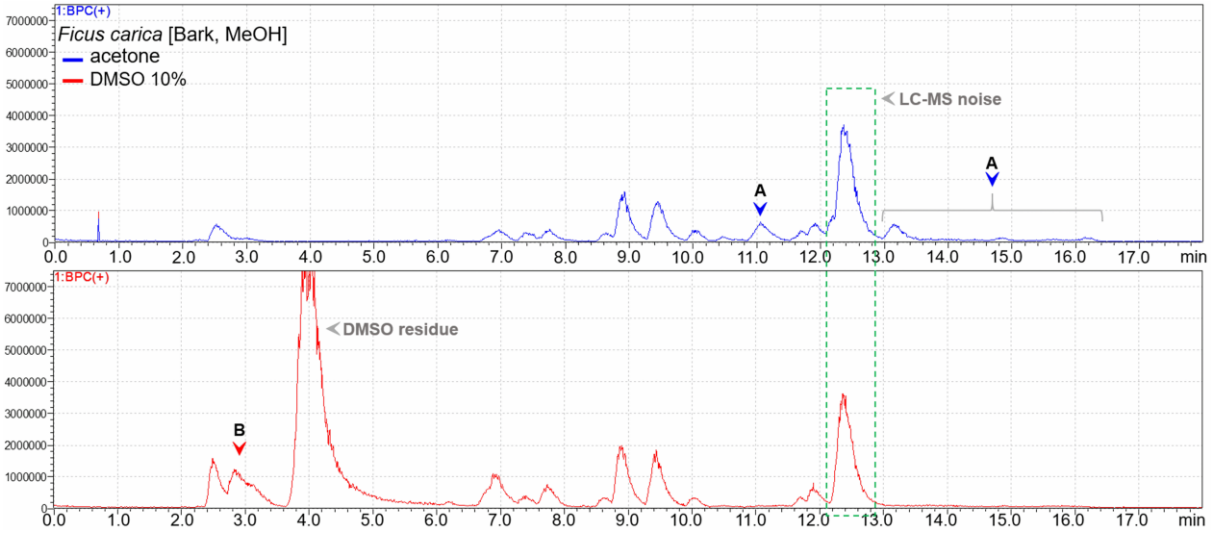


Fig 3. **a**. An overlay of UV chromatograms of acetone (blue) and DMSO (red) based working solutions of a methanol barks extract of *F. carica*, which had a better solubility in 10% DMSO. Higher quantities of phytochemicals are seen in the in a DMSO based working solution. **b**. Comparison of base peak chromatograms of acetone (blue) and DMSO (red) based working solutions of a methanol barks extract of *F. carica*, which had a better solubility in 10% DMSO. At least 4 (marked A) and 1 (marked B) additional compound(s) are visible in the chromatograms of acetone and DMSO based working solutions, respectively.

Accounting for aspects likely to affect reproducibility

Upon evaluation of selected aspects in the articles used to determine our choices of the studied plants, several inconsistencies were observed:

1. On aspects related to practices during the collection of plant materials; 13 out of 20 articles did not specify the time of the year/season in which the collection was done. Additionally, the growth state of the plant at the time of collection (e.g. maturity, flowering) was not indicated in all articles. On the other hand, 18

out of 20 articles adhered well to the reporting of the location(s) from which the studied plant(s) were collected (**Fig 4a**).

2. Moreover, 11 out of 20 articles indicated to have used reference bacterial strains from sources such as the American Type Culture Collection (ATCC), Microbial Type Culture Collection (MTCC), National Collection of Industrial Microorganisms (NCIM), Center of Institut Pasteur (CIP) or PMFKg. Also, 5 out of 20 articles reported the use of only clinical isolates. Further, 4 out of 20 articles did not specify the sources of the bacteria used in conducting the AST studies (**Fig 4b**).

3. Regarding methodological aspects in the conduction of antimicrobial susceptibility tests; 6 out of 20 articles did not specify the solvent(s) used in dissolving the crude extracts before testing. Only 5 out of 20 articles cited the Clinical and Laboratory Standards Institute (CLSI), formerly called the National Committee for Clinical Laboratory Standards (NCCLS). The remaining articles (16 out of 20) indicated to have cited other journal articles or a textbook as a reference for the applied test methods (**Fig 4b**).

4. Furthermore, we noted varying methods used in evaluating the MIC values of the studied extracts. These included unaided visual observation for turbidity, colored indicator-aided visual observation, and the use of spectrophotometric devices. Additionally, the positive and/or negative control(s) used in the AST experiments were missing in 8 out of 20 evaluated articles (**Fig 4b**).

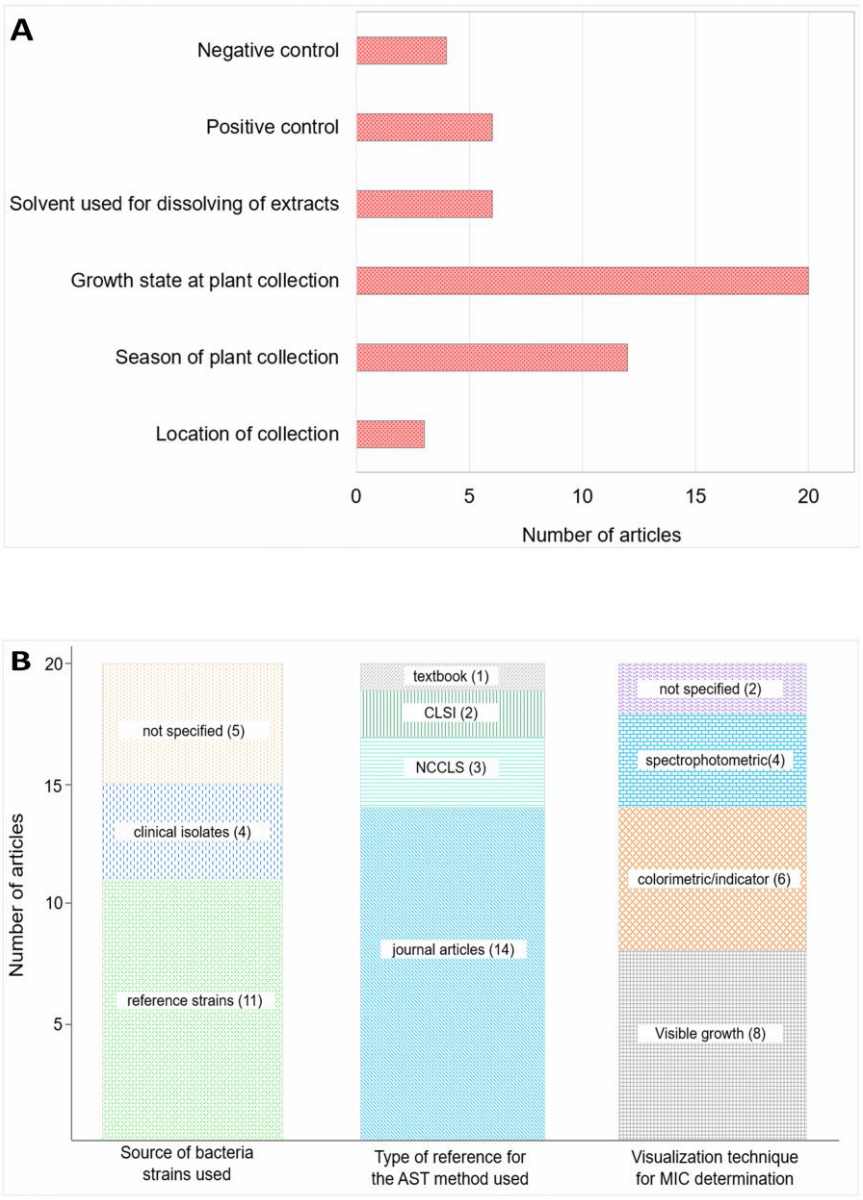


Fig 4. **a**. Frequency of missing key information/aspects in the referred articles (n = 20). **b**. Variations in methodological approaches in antimicrobial susceptibility testing with respect to identities of studied bacteria, origins of test methods and visualization techniques for ascertaining the MIC values (n = 20).

Discussion

Antibacterial activity of identical plant species

Low chances of reproducing previously reported antibacterial activities can be anticipated even upon ensuring the use of the same plant, bacteria species, extraction solvents, studied plant parts, and testing methods. We could not reproduce any of the previously reported MICs of the tested plants against the tested bacteria. The MICs we observed in *Cinnamomum verum* L. and *Piper betle* L. were at least four folds higher than those previously reported (**Table 2**). This was irrespective of ensuring the use of same plant species and extracting solvents, using recommended solvents for dissolving extracts, and employing reference bacterial strains with no

known resistance mechanisms. With some variations in the extraction and MIC reading methods, a small degree of variation in the MIC values was expected. However, our results showed relatively high deviations from those previously reported (**Table 2**).

On the one hand, reproducibility challenges are due to solubility issues of crude extracts and varying composition of phytochemicals due to differences in geographical locations, sampling, climatic conditions, and ecological factors [9, 10, 42, 43]. On the other hand, this might be due to unstated plant-related and experimental details such as antibacterial testing methods and used bacterial strains [13, 14]. To account for climatic conditions, plants used in this study were grown in conditions simulating their usual environments in terms of temperature and relative humidity (**Table 1**). Other challenges will be discussed in more details.

Solubility of plant extracts

Crude extracts from extracting solvents of varying polarities dissolve better in acetone as compared to the commonly used DMSO 10% solution. For instance, 17 out of 24 extracts showed better solubilities in acetone during the preparation of stock solutions (**Table 4**). However, the MIC values of extracts dissolved in acetone were generally not different from those dissolved in DMSO (**Tables 2 and 3**).

Since most of antibacterial plant-derived compounds are of low to intermediate polarities [14, 44–46], acetone is well suited for uses in extracting and dissolving of crude extracts from plant matrices. This is as well due to its good miscibility with water and non-toxicity to bacteria at higher concentrations (25% v/v) [20, 21, 47]. On the other hand, the use of DMSO offers better compounds' stability in solution, as well as lower vapor pressure. These features are crucial when prolonged storage or testing times are needed [48].

Insufficient solubility of the many extracts in 10% DMSO prompted our undertaking to use acetone in order to avoid missing out compounds with antibacterial activity. LC-MS analysis showed qualitatively similar compositions of the working solutions prepared from the two solvents. Further, a better solubility of an extract in a particular solvent was related to the presence of higher quantities of corresponding phytochemicals in the resulting working solution (**Figs 1–3b**). However, some extracts indicated additional compounds in acetone dissolved extracts' solutions, and most likely did not miss any compounds present in 10% DMSO dissolved solutions.

Since the solvent used for extraction as well as the diluent have an influence on the composition of the resulting test/working solution, a careful selection of the two is necessary. Based on quantitative benefits, our findings are suggestive of favoring acetone as a diluent when handling extracts obtained from less polar solvents and using 10% DMSO for extracts from more polar organic solvents.

Plant materials, sampling, and phytochemicals composition

Reporting of essential details about plants used in the screening for antibacterial activities is inadequate in a big proportion of published research articles. This is demonstrated by the missing information on the season and location of collection, as well as the maturity state of plants at collection in a big number of referred studies (**Fig 4a**). This challenge is aggravated by the observed low reporting of both qualitative and quantitative phytochemical profiles of the studied extracts. Since plants contain different types and quantities of phytochemicals in different seasons and at different maturity stages, stating of these details is crucial [9, 11, 42, 43]. The lack of this information results in less objective plant collection, and contributes to low reproducibility.

However, the amount and types of phytochemicals in plants are likely to vary even during different hours of a day [49]. The availability of details of season and plant's stage of maturity at collection may therefore not address this challenge in full. Factors such as the amount of sunlight and water received, soil type, and predators or pathogens induction of phytochemicals production may also largely vary in different geographical locations even during a similar season [9–12]. This is examplified by the negative tests for sterols and caffeic acid in methanol leaves extract of *V. album* and acetone leaves extract of *O. europaea* respectively, as opposed to the corresponding previous reports. The absence of sterols in the *V. album* extract may have contributed to the observed discrepancies in antibacterial activities. However, this cannot be stated with certainty because the initial study did associate the observed antibacterial activities to any of the phytochemicals found in the extract. On the other hand, caffeic acid was shown to have inhibitory activity against *E. coli* and *K. pneumoniae* among other bacteria. The differences in antibacterial activities observed in *O. europaea* leaves extracts can therefore be related to the missing sole or synergistic role of caffeic acid [22, 24].

Taken together, it is of great necessity to indicate the season and state of maturity alongside location(s) of plant collection. This will enable others to make all possible adjustments towards conforming to the previously reported conditions. Moreover, doing qualitative and/or quantitative fingerprint profiling of tested extracts using techniques like Thin Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC), or Liquid Chromatography-Mass Spectrometry (LC-MS) among others, should be considered necessary. Using fingerprint profiles enables an objective comparison on the extents at which the extracts to be studied are similar to those used previously.

Testing for antibacterial activities

There is a limited use of reference bacteria isolates in carrying out antimicrobial susceptibility testing of crude extracts from plants. Moreover, uncomprehensive reporting of methodological aspects and the use of varying references for methods of antimicrobial susceptibility testing are common.

Our evaluation revealed that 9 out of 20 referred articles either indicated the use of clinical isolates or did not give any reference(s) of the studied bacteria (**Fig 4b**). The use of clinical isolates largely limits the reproducibility of obtained results by researchers elsewhere. Even upon successfully reproducing other factors, the genetic and phenotypic variations among clinical isolates of a particular bacterium may hinder the objective screening of plant extracts for antibacterial activity [14]. Lower susceptibilities to plant extracts have been observed in tests that involved the use of clinical isolates of bacteria or those with known resistance phenotypes [50–52].

Furthermore, we observed missing details on the solvent(s) used in the preparation of extracts' test solutions as well as on the applied positive and/or negative controls used (**Fig 4a**). The non-disclosure of solvents used in preparing test samples disguises a proper choice of solvent(s) in the follow-up studies. Moreover, the use of solvents toxic to bacteria such as methanol and ethanol, or using recommended solvents above optimal concentrations precipitates the reporting of false-positive results [13, 14, 53]. The lack of information on the used negative controls aggravates the magnitude of this challenge.

Additionally, the approaches in the testing methods may be widely varying due to the observed diversity of references of methods for antimicrobial susceptibility testing. Among the 20 articles we referred to, 16 cited other published research papers as a reference for the used AST method (**Fig 4b**). Since each cited article might have done slight modifications of a previously reported method(s), this is also likely to affect the reproducibility [13, 14].

To warrant good reproducibility, it is therefore necessary to ensure the use of reference bacterial strains, especially during initial screening of plant extracts for antibacterial activities. This will enable others to select objectively the type of bacterial strains to use during the follow-up studies. Additionally, a thorough reporting of key aspects of the AST experiments and the use of standard methods from bodies like CLSI and EUCAST is a commendable approach in safeguarding the reproducibility of reported results [13, 18, 19].

Antibacterial activity of related plant species

Most interestingly, we found more extracts with higher antibacterial activity in the species related to those primarily identified in our literature review. This indicates that, screening of related species for antibacterial activities is a good approach in the search for antibacterial hit compounds from plants. As shown in **Table 3**, antibacterial activities were observed in the extracts of *G. spicata* and *P. officinalis*. While the MIC values exhibited by *G. spicata* extracts were at least 6.5 higher than those previously reported in *G. smeathmannii*; that of *P. officinalis* extract against *K. pneumoniae* (128 µg/mL) was about two times lower than that previously reported in *P. broteroi* (250 µg/mL). Additionally, the antibacterial activity of the acetone extract of *P. officinalis* against *E. coli* (MIC = 256 µg/mL), was not seen previously.

Expanding the search perimeter by screening of species related to those with previously reported activities is therefore noteworthy [14, 54]. This approach is generally better than the common practice of selecting plants based on their traditional uses in the treatment of bacterial infections. This is underscored by the reports that antibacterial activities observed among randomly collected plants did not significantly differ from those being collected on the bases of their usage by traditional healers [14].

Conclusion

The global rise of antimicrobial resistance demands for diversified approaches in the search for novel antibacterial agents. Plants, among other natural sources, host a great potential in contributing to the discovery of new antibiotics. As it might be common among other research groups, we have observed a very low reproducibility of previous findings on antibacterial activities of plant extracts. We also noted inconsistencies and a wide variation in the amount of provided information regarding experimental procedures, plants, and bacterial strains used. Although poor reproducibility depreciates the usefulness of the initial efforts and discourages follow up works, plants remain to be a potential source of novel antibacterial agents. This necessitates putting in place adequate and collective measures in facing the reproducibility challenge.

Acknowledgments

The authors would like to acknowledge the technical assistance of Liane Dreher and Emmanuel Tola Adeniyi.

Competing interests: The authors have declared that no competing interests exist.

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4.3. Isolation and Characterization of Galloylglucoses Effective against Multidrug-Resistant Strains of *Escherichia coli* and *Klebsiella pneumoniae*

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Abstract

The search for new antibiotics against multidrug-resistant (MDR), Gram-negative bacteria is crucial with respect to filling the antibiotics development pipeline, which is subject to a critical shortage of novel molecules. Screening of natural products is a promising approach for identifying antimicrobial compounds hosting a higher degree of novelty. Here, we report the isolation and characterization of four galloylglucoses active against different MDR strains of *Escherichia coli* and *Klebsiella pneumoniae*. A crude acetone extract was prepared from *Paeonia officinalis* Linnaeus leaves, and bioautography-guided isolation of active compounds from the extract was performed by liquid–liquid extraction, as well as open column, flash, and preparative chromatographic methods. Isolated active compounds were characterized and elucidated by a combination of spectroscopic and spectrometric techniques. *In vitro* antimicrobial susceptibility testing was carried out on *E. coli* and *K. pneumoniae* using 2 reference strains and 13 strains hosting a wide range of MDR phenotypes. Furthermore, *in vivo* antibacterial activities were assessed using *Galleria mellonella* larvae, and compounds 1,2,3,4,6-penta-O-galloyl-β-d-glucose, 3-O-digalloyl-1,2,4,6-tetra-O-galloyl-β-d-glucose, 6-O-digalloyl-1,2,3,4-tetra-O-galloyl-β-d-glucose, and 3,6-bis-O-digalloyl-1,2,4-tri-O-galloyl-β-d-glucose were isolated and characterized. They showed minimum inhibitory concentration (MIC) values in the range of 2–256 µg/mL across tested bacterial strains. These findings have added to the number of known galloylglucoses from *P. officinalis* and highlight their potential against MDR Gram-negative bacteria.

Keywords: antimicrobial resistance; *Escherichia coli*; *Klebsiella pneumoniae*; Enterobacteriaceae; Paeonia; gallotannins; isolation; structural elucidation

1. Introduction

Due to the ongoing global threat of antimicrobial resistance, the search for novel antimicrobial agents is crucial. Approaches aimed at the discovery and development of novel antibiotics are key with respect to continuously filling the antibiotics development pipeline. Many approaches have been applied in the search for new antibiotics, including modification of existing antibiotics, combination therapies, the use of resistance modifiers, as well as searching for novel antibiotics from scratch or from natural sources [1,2].

Antimicrobial resistance among Gram-negative bacteria poses a serious public health threat, as such infections are generally difficult to treat. *Escherichia coli* and *Klebsiella pneumoniae*, among other Gram-negative bacteria, are categorized by the World Health Organization (WHO) as high-priority pathogens against which the development of new therapies is vital. This is mainly due to their increasing resistance to carbapenems, which has narrowed the available treatment options. The use of the drug of last resort (colistin) is also challenged by the rise of plasmid-mediated transfer of the mcr-1 colistin resistance gene [3,4].

To significantly tackle the antimicrobial resistance (AMR) crisis, antibiotics with novel chemical structures and novel targets that act by new modes of action and lack cross resistance to existing antibiotics are urgently needed. However, most candidates currently in the pipeline fall short of these noble traits, which necessitates continual efforts in the search for ideal candidates [2].

Natural sources, especially from the Fungi and Monera kingdoms, have long been a reliable source of a number of available antibiotics. Due to their structural novelty and complexity, nature-derived compounds have formed successful classes of antibiotics, enabling the realization of new targets and modes of action [5]. Nevertheless, the role played by the driving factors for the development and spread of AMR has rendered most natural compounds less useful. Plants remain an attractive source for the discovery of new antibiotics due to their potential to host compounds with a higher degree of structural novelty, among other features of ideal new antibiotics [6].

Paeonia officinalis Linnaeus. (Paeoniaceae) is a small, non-bushy flowering plant naturally distributed in North America, Asia, and southern Europe [7]. Preparations from different parts of the plant are traditionally used to treat a broad range of diseases, including liver diseases, epilepsy, infections, pain, gastritis, amenorrhea, dysmenorrhea, as well as treatment of wounds, among other applications [7]. Flavonoids, tannins, glucosides, triterpenoids, phenols, and stilbenes are among the compounds previously isolated from this genus. Numerous galloylglucoses, among other tannins, were previously isolated from the leaves, fruits, petals, and roots of several peonies [7]. The available toxicological data favor plants from the genus Paeonia as largely safe. However, compared to other species from the genus, the

availability of data from *P. officinalis* on isolated compounds and their biological activities is scarce [8,9].

A number of tannins were previously isolated from different parts of *P. officinalis* and are typically classified into hydrolysable, condensed, and complex tannins. Tannins play important roles in plant growth, reproduction, and natural defense systems. In addition to providing a chemical barrier against the penetration and colonization of plant tissues by microorganisms, they help to deter predation by herbivores and insects [10,11,12]. Galloylglucoses, as hydrolysable tannins, are biosynthesized in plants following a strictly position-specific series of galloylation of glucose [10,13]. In addition to antibacterial activities, galloylglucoses are reported to exhibit nematicidal, insecticidal, antioxidant, anti-inflammatory, antidiabetic, cardioprotective, neuroprotective, anticancer, antiplatelet, anticoagulant, and immune-modulatory effects, among other biological activities [12,13,14,15,16].

Our previous efforts in the search for antibacterial compounds from numerous plants revealed antibacterial activity of crude acetone leaf extract of *P. officinalis* against *E. coli* and *K. pneumoniae* [17]. Here, we report the isolation, purification, and characterization of four galloylglucoses from the leaves of *P. officinalis* and their antibacterial activities against 2 reference and 13 multidrug-resistant (MDR) strains of *E. coli* and *K. pneumoniae*.

2. Materials and Methods

2.1. Chemical Reagents and Antibiotics

Acetone, n-hexane, toluene, ethyl acetate, dichloromethane, methanol, acetonitrile, silica gel 60 GF254 plates, and silica gel 60 powder were purchased from Sigma-Aldrich Chemie (Schnelldorf, Germany). Mueller–Hinton broth (MHB), Lysogeny broth (LB), and agar were purchased from Carl Roth (Karlsruhe, Germany). Gentamicin sulfate was purchased from AppliChem (Darmstadt, Germany). Phosphate-buffered saline (PBS) pH 7.4 was purchased from Fischer Scientific (Schwerte, Germany) and Millipore water was prepared by the Milli-Q® direct laboratory water purification system (Merk, Darmstadt, Germany).

2.2. Study Organisms

Bacterial reference strains of *Escherichia coli* (ATCC 25922) and *Klebsiella pneumoniae* (ATCC 10031) were purchased from the American Type Culture Collection (ATCC-LGC, Wesel, Germany). MDR Strains with the following identification numbers and resistance phenotypes in the parentheses were obtained from the Institute of Hygiene and Microbiology of the University of Wuerzburg (Wuerzburg, Germany): *E. coli*: NRZG 176 (OXA-48), Stich E 866 (VIM-1), UR481/1/2/13 (ESBL), NRZG 222 (KPC-3), RV3/A2/12 (VIM-1/4, TEM-1), and NRZG 14,408 (KPC-2, mcr-1); and *K. pneumoniae*: NRZG 246 (OXA 48), Stich E 895 (TEM/SHV/CTX-M), UR 3397/1/15 (NDM-1), Stich 787 (DHA-1 (AmpC)), NRZG 103 (KPC-2), NRZG 002 (OXA-48), and Stich E 785 (SHV-4). Moreover, *Galleria*

mellonella larvae were purchased from Feeders & More GmbH (Au in der Hallertau, Germany).

2.3. Plant Material Collection, Preparation, and Extraction

Leaves of flowering *Paeonia officinalis* L. plants were collected from the Botanical Garden of the University of Wuerzburg, Germany in August 2019. Plant identification was carried out by a botanist (Dr. Gerd Vogg) and was assigned internal ascension number 2013-11-S-10. The collected leaves were then transported to the laboratory in aerated paper bags, where they were chopped into small pieces and dried under shade and open air for two weeks. Dried leaves were thereafter reduced into a coarse powder using an electric blender (Braun, Kronberg in Taunus, Germany).
Extraction was performed under cold maceration using 44 g of powdered dry leaves and acetone at a solvent with a feed ratio of 10 mL/g for 72 h under constant magnetic stirring. The contents were then filtered, and the solvent was evaporated in vacuo at 40 °C. The recovered crude extract (4.58 g, 10.4% w/w) was stored at −15 °C.

2.4. Isolation and Purification

Bioassay-guided isolation was performed after the identification of the antibacterial activities of the crude extract on the reference bacterial strains. A contact bioautography technique was used to guide the identification of spots with antibacterial activity on developed thin-layer chromatography (TLC) plates.

2.4.1. Contact Bioautography

A mobile phase containing n-hexane: ethyl acetate: methanol (7.5:2:0.5 v/v/v) resulted in the largest number of distinct spots on a precoated silica gel 60 GF254 plate. To develop a TLC profile, 20 µL of a 10 mg/mL crude extract solution in acetone was spotted at two separate positions 1 cm from the bottom of an 8 cm × 2.5 cm TLC plate. The plates were developed using the above mobile phase and allowed to dry for 6 h in a sterile hood. Another control plate was developed in a similar way, and the position of each spot was marked under ultraviolet (UV) light at 254 and 366 nm. The control plate served as a template for the identification of the position, size, and intensity of the bioactive spots. A plate spotted with 20 µL of acetone with similar subsequent treatments served as a negative control [18].

A volume of 100 µL of bacterial suspensions with 10^8 colony-forming units (CFU)/mL prepared from fresh cultures was inoculated and uniformly spread on Mueller–Hinton agar plates. The inoculated plates (one for each bacterium and one for control) were lid-covered and left for 30 min at room temperature. The developed TLC described above was thereafter placed on the surface of the inoculated agar plate (the silica-coated surface facing the agar) and gently pressed to ensure uniform contact. The position of the TLC plate on the agar plate was traced/marked from the agar side of the agar plate's exterior. The entire set was kept at 4 °C for 30 min to facilitate the diffusion of the compounds into the agar. The TLC plate was thereafter carefully removed under sterile conditions, and the agar plates were incubated at 37 °C for 24

h. The spots containing compounds with antibacterial activity were identified by matching the zones of inhibition to the corresponding position on the control TLC plate [18].

2.4.2. Liquid–Liquid Extraction

Guided by the results of contact bioautography, liquid–liquid extraction was carried out to simplify the crude extract's composition. The extraction was performed as per a previously described procedure, with slight modifications [19]. A total of 4 g of the crude extract was dissolved into 200 mL of 90% methanol and extracted with three 100 mL portions of petroleum ether using a separating funnel. Both layers were dried in vacuo at 40 °C, and the methanolic extract was resuspended in 200 mL of water and extracted with three 100 mL portions of chloroform. Combined chloroform layers were dried in vacuo at 40 °C, whereas the water layer was freeze-dried (Martin Christ Gefriertrocknungsanlagen, Osterode am Harz, Germany) for 24 h to recover 1.92 g (48%) of a dark-brownish powder. Dried subfractions from petroleum ether, chloroform, and water, were tested for antibacterial activity using disc diffusion assays on Mueller–Hinton agar. The optimum mobile phase composition of dichloromethane: toluene: methanol (20:35:45% v/v/v) was thereafter used to develop the TLC plates for bioautography assay of the recovered dried water fraction. The results from bioautography and the developed TLC profile guided the subsequent isolation step by open-column chromatography.

2.4.3. Open-Column and Flash Chromatography

The dried water subfraction (1.92 g) from liquid–liquid extraction was subjected to open-column subfractionation using silica gel 60 and dichloromethane: toluene: methanol (20:35:45% v/v/v) as a starting mobile phase system, followed by methanol: water (95:5% v/v). The fractions containing the spot with the active compound(s) (corresponding to a spot with an Rf value of 0.54) were pooled and dried in vacuo at 40 °C.

Further refining of the resulting subfraction was performed by flash chromatography (Interchim PuriFlash 430 Flash Chromatography System, France) using a reversed-phase column (Chromabond® Flash RS 40 C18 ec, Macherey-Nagel, Dueren, Germany) eluted with n-hexane: ethyl acetate: water (25: 65:10% v/v/v) under isocratic conditions. The classic reversed-phase chromatography (methanol or acetonitrile-water) systems could not yield optimal results. Fractions containing a spot corresponding to the active compounds at Rf = 0.72 on reversed-phase, pre-coated silica gel C18 TLC plates (ALUGRAM® RP-18W/UV254, Macherey-Nagel, Dueren, Germany) developed with the above mobile phase were pooled and dried in vacuo at 40 °C followed by freeze drying for 24 h to recover 656.6 mg (32.4%) of light-brownish amorphous powder.

2.4.4. Preparative Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC)

Reversed-phase HPLC analysis of the refined water subfraction revealed the presence of multiple components, which necessitated further separation under preparative HPLC (Agilent 1100 Series Preparative HPLC, Santa clara, CA, US). Isolation of subfractions from the above fraction was carried out under conditions indicated in **Table S1**.

Purified subfractions/compounds were dried in vacuo at 40 °C, followed by freeze-drying for 24 h. The recovered compounds were stored at −15 °C until they were needed for further experiments. Bioactivities of each recovered subfraction (compound) were tested using either broth microdilution assay or disc diffusion assay in cases where only small amounts were recovered.

2.5. Characterization and Structural Elucidation

2.5.1. Infrared Spectral Measurements

Infrared spectra of the active purified compounds were acquired using a JASCO FT/IR-4700 Fourier transform infrared (FT-IR) spectrometer equipped with an attenuated total reflectance (ATR) accessory (JASCO Labor und Datentechnik GmbH, Pfungstadt, Germany).

2.5.2. High-Resolution Mass Spectrometry

Exact masses of the active purified compounds were obtained with an Exactive™ Plus Orbitrap high-resolution mass spectrometer (HR-ESI-MS) (ThermoFischer Scientific, Waltham, MA, US) under electrospray ionization in both positive and negative modes. Moreover, full mass spectra, simulated spectra, and calculated masses for the proposed molecular formulae were also acquired/processed.

The identity of the measured compounds was partly confirmed by the screening of relevant databases (MassBank Europe, PubChem, ChemSpider, and SciFinder) and available literature for compounds with similar exact masses and proposed molecular formulae.

2.5.3. Nuclear Magnetic Resonance

One-dimensional (^{1}H, ^{13}C, and distortionless enhancement by polarization transfer-135 (DEPT-135)) and two-dimensional (COrrelation SpectroscopY (COSY), heteronuclear single-quantum correlation (HSQC), and heteronuclear multiple-bond correlation (HMBC)) 600 MHz NMR measurements of the active purified compounds were carried out on a Bruker Avance III HD 600 MHz NMR spectrometer (Bruker, Billerica, MA, US). All HMBC measurements were done at the long-range (2–3 bonds) J(CH) coupling constant of 8.3 Hz. Based on differences in their solubilities, the compounds were dissolved in different proportions of acetone-d6 in D_2O. Compounds NMA2 (21 mg/mL), NMB4 (8.3 mg/mL), NMB6 (25 mg/mL), and NMC3 (15.4 mg/mL) were dissolved in 91, 75, 100 and 96.2% of acetone-d6 in D_2O (v/v), respectively [20,21,22,23,24,25].

2.6. Antibacterial Activity Testing

2.6.1. Disc Diffusion Assays

A disc diffusion assay was used to evaluate antibacterial activities of fractions isolated in small quantities before deciding on their further isolation or structural elucidation. All assays were conducted as per the guidelines of the European Committee for Antimicrobial Susceptibility Testing (EUCAST), with slight modifications. Briefly, Mueller–Hinton agar plates were inoculated with 100 µL of 10^8 CFU/mL bacterial suspensions and left under sterile conditions for 30 min at room temperature. Then, 6 mm diameter test discs were loaded with an amount equivalent to 256 µg of the test substance solution dissolved in acetone. The discs were left to dry for 1h in a biosafety hood before being transferred to the inoculated agar plates mentioned above. Discs loaded with acetone alone were used as negative controls. Zones of inhibition were read after 24 h of incubation at 37 °C [26].

2.6.2. Broth Microdilution Assays for Determination of Minimum Inhibitory Concentration (MIC)

Preparation of Bacterial Suspensions

To prepare overnight cultures, one isolated colony of each bacterium was picked up using a sterile tip and used to inoculate 2 mL of lysogeny broth (LB) medium in sterile test tubes. The tubes were then incubated overnight (12–14 h) at 37 °C under constant shaking (200 rpm). Fresh cultures were thereafter prepared by transferring 200 µL of the overnight cultures into 20 mL of LB medium in sterile flasks and further incubated for 5–6 h under the same conditions as above. Volumes of fresh cultures needed to prepare final test bacterial suspensions were determined using the optical density (OD_{600}) (Eppendorf BioPhotometer Plus, Eppendorf, Hamburg, Germany) of the respective fresh culture and the Newman's correlation curve. All test bacterial suspensions were prepared at concentrations of 10^6 CFU/mL [27].

Loading of 96-Well Plates

MICs of the crude extract, fractions, and purified compounds were determined by the broth microdilution method as per EUCAST guidelines. Stock solutions of the crude extract and petroleum ether, chloroform and water subfractions were prepared at 10 mg/mL, whereas those of purified compounds were prepared at 2 mg/mL in acetone. Through dilution with MHB media, working solutions at concentrations of 2048 µg/mL and 512 µg/mL were made from the above stock solutions. The concentration of acetone in the working solution was 25.6% v/v.

To ensure a uniform concentration of acetone across all wells, 100 µL of 25.6% v/v acetone in MHB medium was prefilled in wells on columns 3 to 11 of the 96-well plate in triplicate. This was followed by loading 200 µL of the working solutions into the wells on column 2 of the corresponding prefilled triplicate rows. The working solution was serially diluted using a multichannel pipette by drawing 100 µL of the working solution and mixing it well with the above prefilled 100 µL acetone-MHB mixture in

the successive columns. The procedure was repeated until the 11th column was reached, at which point the final 100 µL was discarded.

To the above wells, 100 µL of bacterial suspension in MHB medium at 10^6 CFU/mL were added, attaining test concentration ranges of 2–1024 µg/mL for the crude extract and 0.5–256 µg/mL for subfractions/compounds. Furthermore, a final concentration of 12.8% v/v acetone in MHB medium was achieved across all test wells. The wells on the outer ring of the plate were filled with 200 µL of MHB medium, which reduced the evaporation of acetone from the treatment and control wells within. Gentamicin sulfate was used as a positive control in the range of 0.25–128 µg/mL µg/mL, whereas a 12.8% v/v of acetone in MHB medium mixture was used as a negative control. The loaded and inoculated plates were thereafter incubated at 37 °C for 18 h. All experiments were performed in triplicate in one assay and repeated three times on separate days. MIC values were determined by visual observation for the absence of pinpointed bacterial growths at the bottom of treatment wells. MIC values were recorded as the highest of the values obtained from all individual replicas [27,28].

2.6.3. Minimum Bactericidal Concentration (MBC) Testing

Minimum bactericidal concentration (MBC) values were determined as per the EUCAST guidelines. Briefly, after 18 h of incubation for determination of MIC values as described above, 20 µL was drawn from the test wells without bacterial growth (MIC and above) and applied to the MHB agar plates in triplicate. The plates were then incubated for a further 24 h at 37 °C. The MBC was determined, as the wells with the lowest concentration with no bacterial growth (colonies) were observed at the end of the incubation time.

2.7. *In vivo* Antibacterial Assay Using *Galleria mellonella* Larvae

In vivo antibacterial assays were performed using Galleria mellonella larvae. To avoid maturation and pupation, all larvae were used within 4 days after their delivery. Bacterial suspensions of the reference strains (*E. coli* (ATCC 25922) and *K. pneumoniae* (ATCC 10031)) to infect the larvae were prepared from overnight and fresh cultures using similar procedures as those described above. Moreover, to remove residual bacterial toxins, the fresh cultures were centrifuged under mild conditions (3500 rcf for 5 min at 4 °C), and the supernatant was discarded, followed by resuspension of the bacteria in PBS. The resulting suspension was recentrifuged under the same conditions, and the bacteria were resuspended in PBS after careful removal of the supernatant. The optical density (OD600) of the resulting bacterial suspension in PBS was measured, and using Newman's correlation curves, the desired concentrations of bacterial suspensions in PBS were prepared for inoculation of the *G. mellonella* larvae.

The optimal bacterial concentrations to be used for infection of the larvae before treatments were identified after testing the doses between 0.05×10^8–3.0×10^8 CFU/mL for *E. coli* (ATCC 25922) and *K. pneumoniae* (ATCC 10031). The selected

optimal concentration was that which did not kill more than 50% of the untreated infected larvae within 12 h of incubation while ensuring that 80% or more of the untreated larvae were killed within 24 h of incubation.

Larvae were infected by injecting 20 µL of the bacterial suspension on the second last appendage on the left-hand side of each larva using a BD MicroFine + Demi 0.3 mL, 0.30 mm (30 G) × 8 mm insulin syringe (BD, Wokingham, UK). A total of 20 larvae were used per test group. Each group was placed on plastic Petri dish plates with a small amount of wooden chips. The infected larvae were thereafter incubated at 37 °C for 30 min before they were treated with the test compounds and controls [28].

Larvae were treated with the test compounds and controls by injecting 20 µL of each test compound in 12.8% acetone in PBS on the same appendage as described above. Compounds were tested at fixed concentrations of 64 or 128 µg/mL, which were generally two times their *in vitro* MIC values. The larvae were then incubated at 37 °C, and dead larvae were counted every 6 h for the first 48 h and then every 12 h until 96 h after treatment. Dead larvae were identified by the virtue of a complete lack of response to touch stimuli and were removed from the plates [28]. The negative control group was treated with 20 µL of 12.8% acetone in PBS, whereas the positive control was given the same volume of gentamicin 64 µL/mL solution in PBS. Furthermore, one group without any treatment was included.

2.8. Evaluation of the Amino Acid Composition of the Expressed Resistance Enzymes

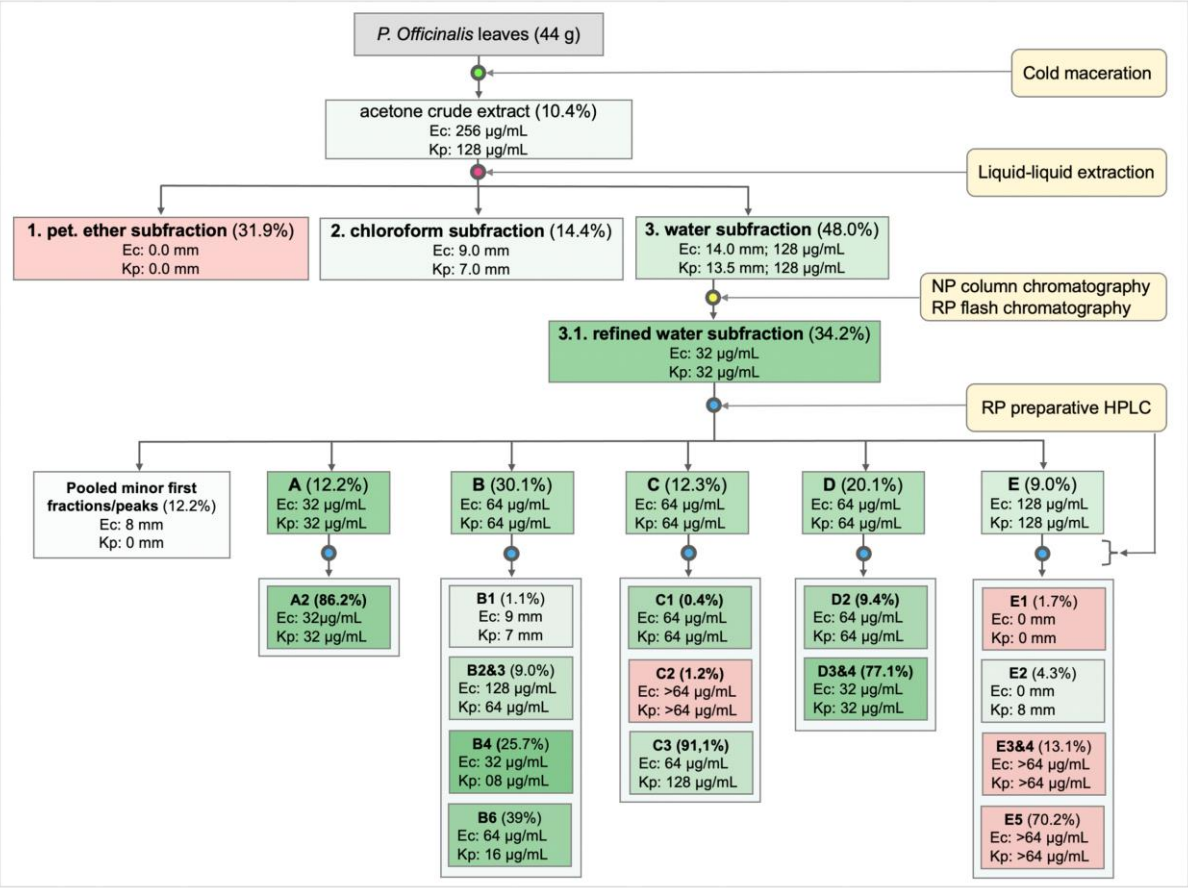
The literature broadly indicates stronger interactions of galloylglucoses with proteins with higher contents of aromatic amino acids, as well as their electrostatic adsorption onto the surfaces of positively charged moieties [29,30,31,32,33,34]. On these grounds, we evaluated the relationships between the observed MIC values and the contents of aromatic amino acid, as well as net charges in the resistance enzymes in the studied MDR bacteria. Sequences of the amino acids in each resistance enzyme were obtained from the Protein Data Bank (PDB) and UniProt databases [35,36], and their relative contents of aromatic amino acids and net charges were determined using an online protein analysis tool (ExPASy) and Origin® software [37]. To determine possible relationship patterns, the above parameters were scatter-plotted against the MIC values observed among studied MDR strains hosting the indicated resistance enzymes. The analysis considered only cases in which the MIC values within our highest tested concentration of 256 µg/mL were obtained.

3. Results

3.1. Extraction, Isolation, and Purification of Antibacterial Compounds

Crude acetone leaf extract from *P. officinalis* dried leaves was obtained with a 10.4% w/w yield after 72 h of cold maceration. The extract showed moderate antibacterial activities (MIC = 128–258 µg/mL) against *E. coli* (ATCC 25922) and *K. pneumoniae*

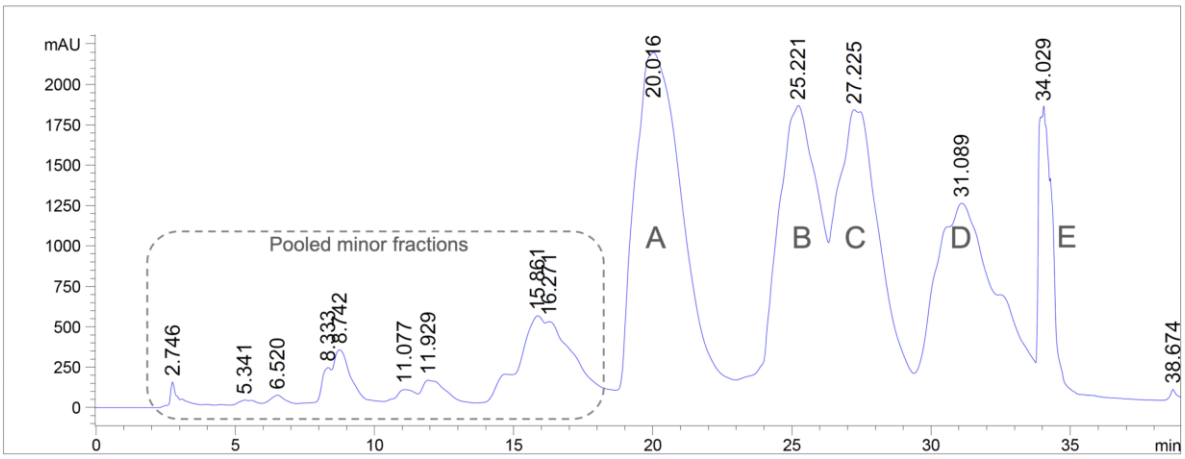
(ATCC 130031) (**Scheme 1**). Upon TLC profiling of the extract (n-hexane: ethyl acetate: methanol (7.5:2:0.5 v/v/v)), multiple spots (compounds) were visible across the entire run distance. Contact bioautography on the TLC plate developed under the above conditions exhibited a zone of inhibition at the position of sample application (Rf = 0). These findings suggested a high polarity of the antibacterial compound(s) present in the crude extract, which informed the decision to conduct liquid–liquid extraction to simplify the extract. As shown in **Scheme 1**, the aqueous fraction from liquid–liquid extraction was found to host the antibacterial activity at MIC = 128 µg/mL against both bacteria, whereas no or little activity was noted in the petroleum ether and chloroform fractions (**Scheme 1**).



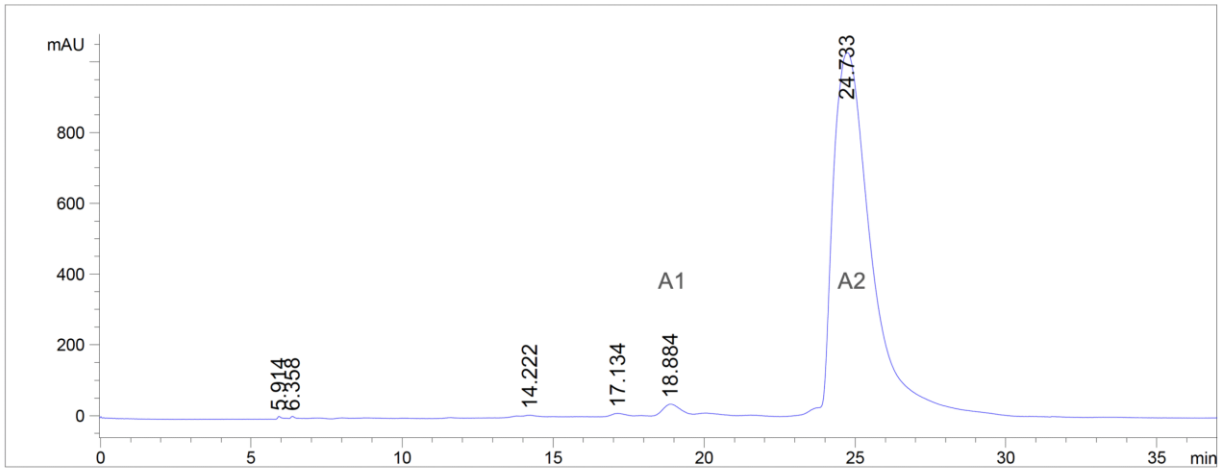
Scheme 1. The overall scheme of extraction, bioassay-guided isolation and purification of antibacterial compounds from the *P. officinalis* leaves.

Subfractionation of the aqueous subfraction by means of silica gel open-column and RP flash chromatography achieved a subfraction 3.1, showing a single spot on a reversed-phase TLC plate (Rf = 0.72; n-hexane: ethyl acetate: water, 5:13:2 v/v/v) at a yield of 34.2% w/w (**Scheme 1**). Despite a fourfold increase in the antibacterial activity of the purified water fraction (MIC = 32 µg/mL), HPLC analysis of subfraction 3.1 showed multiple peaks under the UV chromatogram (RT = 6.0–7.8 min), denoting the presence of multiple closely related compounds. Further analysis of subfraction 3.1 under preparative RP-HPLC conditions (acetonitrile: water, 20–30%, 25 min.

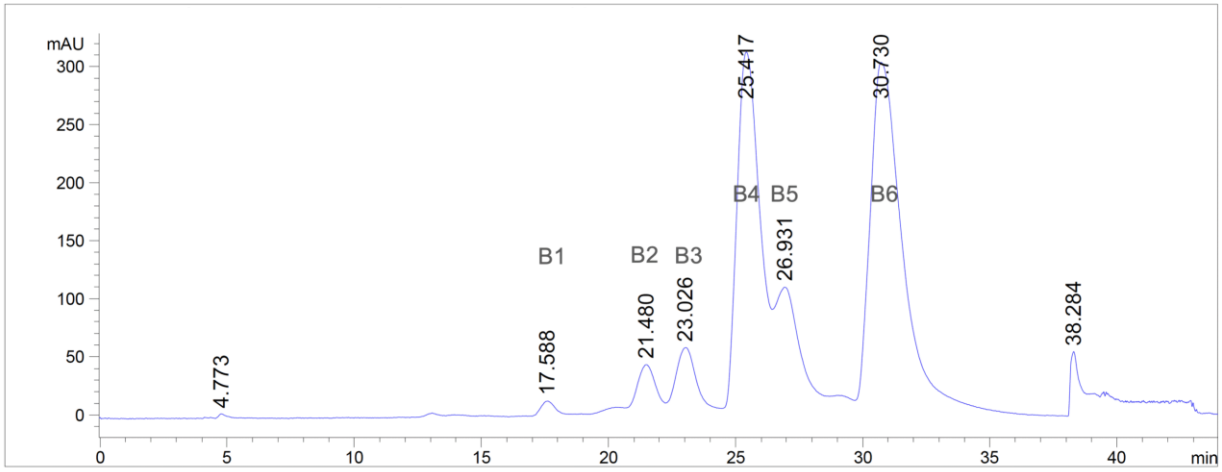
gradient) revealed at least 15 UV peaks (254 nm) of broadly varying sizes and retention times (**Figure 1a**). The subfractions corresponding to the observed peaks were collected, as indicated in **Figure 1a**, all first minor fractions were pooled in one fraction, and the five major subfractions (A–E) were individually collected (**Figure 1a–e** and **Figures S1** and **S2**).



(**a**)



(**b**)



(**c**)

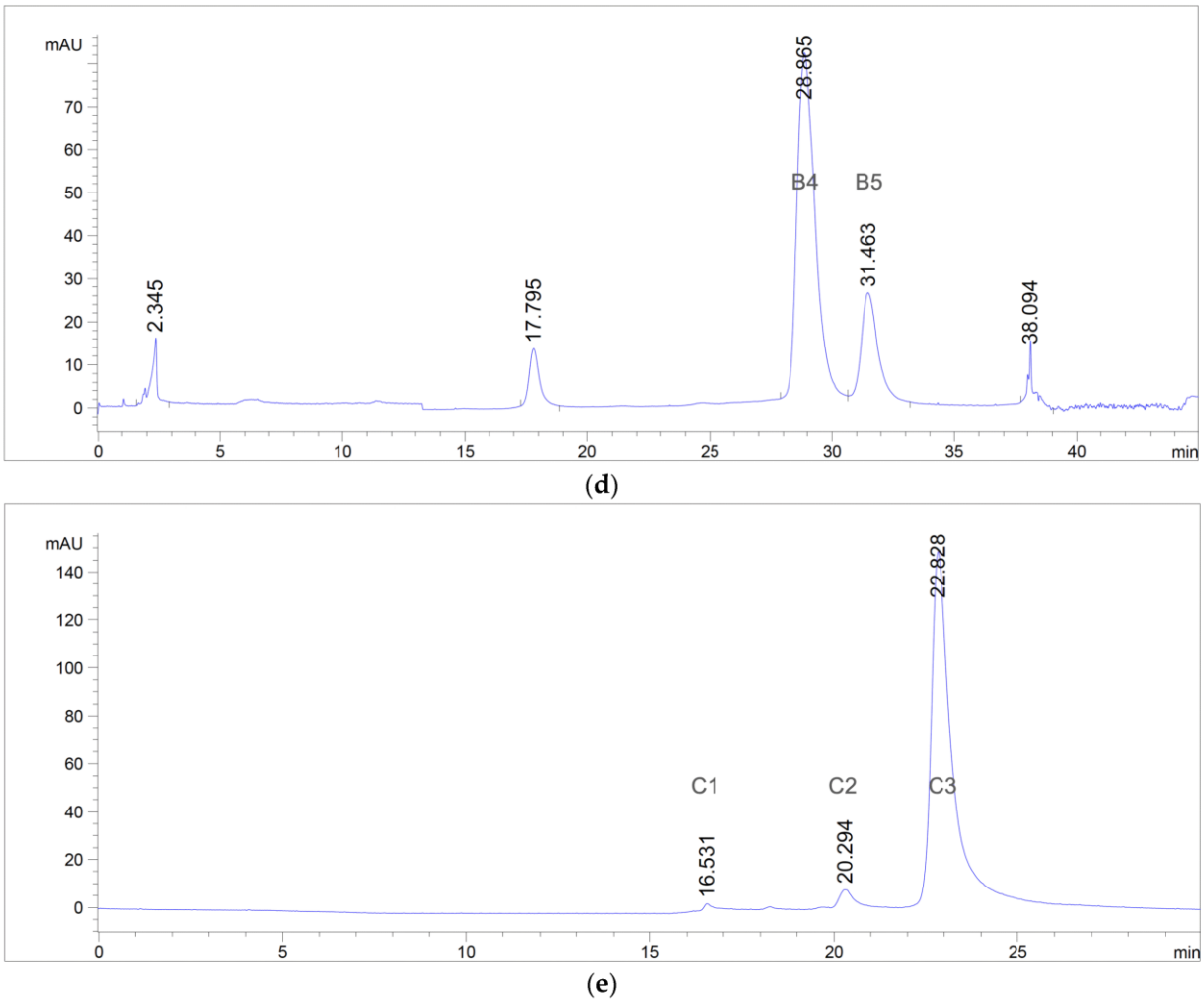


Figure 1. (**a**) UV chromatogram of all fractions under preparative RP-HPLC isolation conditions. The first minor fractions were pooled, whereas subfractions A–E were individually collected, followed by further attempts at subfractionation. (**b**) UV chromatogram upon further purification of subfraction A by preparative RP-HPLC. Subfraction A2 was obtained as a pure compound, whereas subfraction A1 was recovered in trace amounts. (**c**) UV chromatogram upon further purification of subfraction B by preparative RP-HPLC. Subfractions B4 and B5 were collected together, and subfraction B6 was collected individually, whereas subfractions B1–B3 were obtained in trace amounts. (**d**) UV chromatogram upon further purification of a mixture of subfraction B4 and B5 by preparative RP-HPLC. Subfraction B4 was obtained as a pure compound, whereas subfraction B5 was obtained in trace amounts. (**e**) UV chromatogram upon further purification of subfraction C by preparative RP-HPLC. Subfraction C3 was obtained as a pure compound, whereas subfractions C1 and C2 were obtained in trace amounts.

Antibacterial activities in the range of 32–128 µg/mL were observed towards the reference *E. coli* and *K. pneumoniae* strains in the subfractions corresponding to all the major peaks (A–E), in which fractions A and E exhibited the highest and lowest activities, respectively (**Scheme 1**). As shown in **Scheme 1**, the purified and semipurified compounds from subfractions A–E exhibited varying levels of

antibacterial activities against reference and MDR strains of *E. coli* and *K. pneumoniae*. The active compounds corresponding to UV peaks/subfractions A2, B4, B6, and C3 were obtained in substantial quantities, and purity levels and were selected for structural elucidation and other biological studies. Further purification approaches to separate the compounds in subfraction D 3 and 4 under subfraction D were unsuccessful.

3.2. Characterization and Structural Elucidations of Selected Isolated Compounds

The infrared spectra of compounds corresponding to peaks A2, B4, B6, and C3 were similar and largely superimposable. Characteristic absorptions across the four compounds corresponded to aromatic O–H stretching (3353–3383 cm^{-1}), aryl-substituted ester C=O stretching (1698–1704 cm^{-1}), aromatic C=C stretching (1609–1610 cm^{-1} and 1446–1448 cm^{-1}), alcoholic ester C–O stretching (1313–1316 cm^{-1}), phenolic C–O stretching (1191–1195 cm^{-1}), and ester C–O stretching (1026–1029 cm^{-1}) (**Figures S3–S6**).

These data, together with the corresponding HR-ESI-MS, 1D, and 2D NMR spectra and comparison with data from spectral databases and the literature, enabled the identification of the compounds, as indicated below.

The compound corresponding to UV peak A2 in **Figure 1b** was obtained as an amorphous white powder and was assigned the molecular formula of $C_{41}H_{32}O_{26}$ by its HR-ESI-MS *m/z* of 963.1054 $[M+Na]^+$ (calculated for 963.1074). The compound was identified as 1,2,3,4,6-Penta-O-galloyl-β-d-glucose (PGG) and was coded as **NMA2** (**Figure 2** and **Figures S3, S7**, and **S11–S18**; **Table 1**) [23].

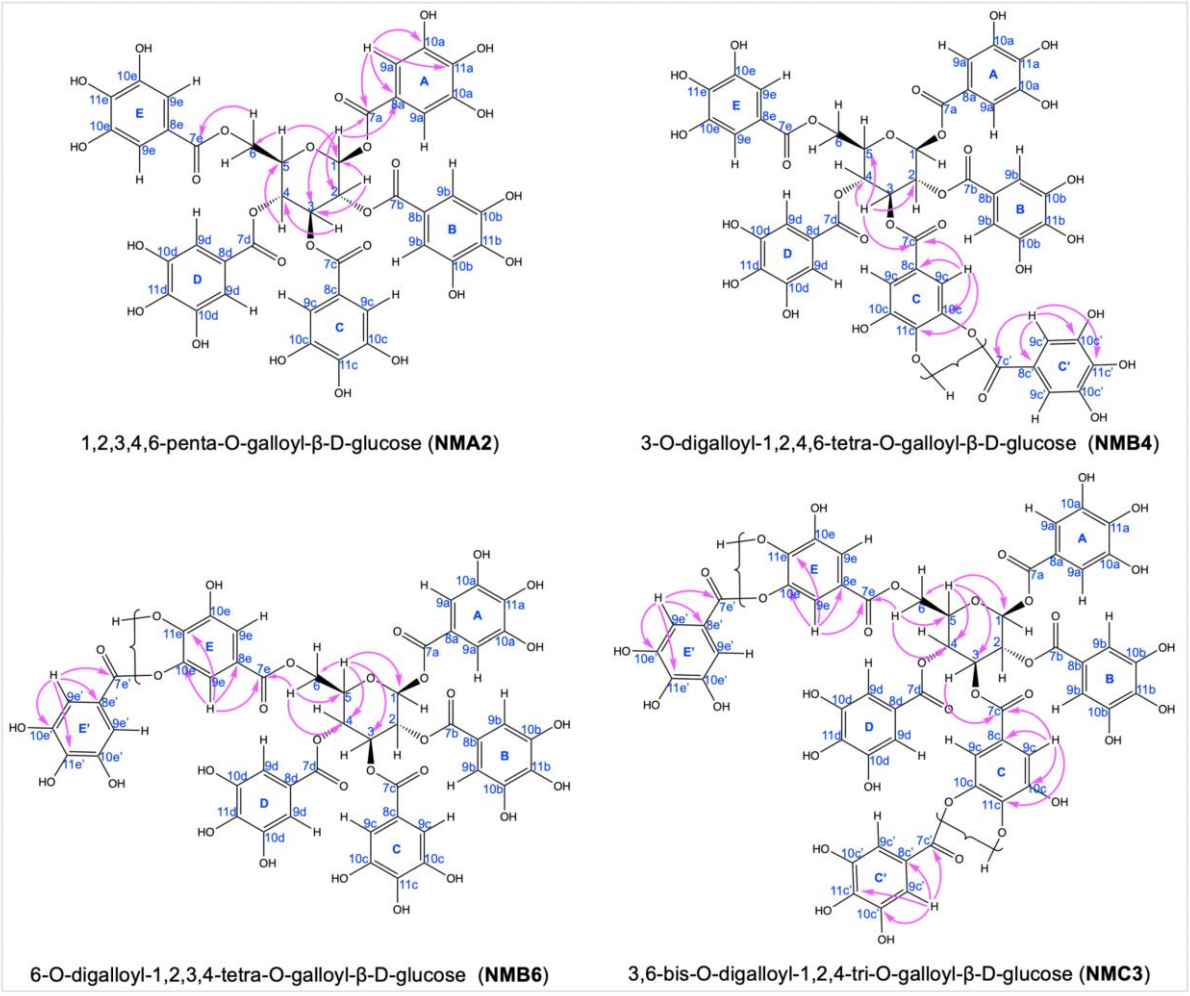


Figure 2. Structures of isolated compounds with annotations of selected HMBC correlations.

The compound corresponding to UV peak B4 in **Figure 1d** was obtained as an amorphous white powder and was assigned a molecular formula of $C_{48}H_{36}O_{30}$ by its HR-ESI-MS *m/z* of 1115.1166 $[M+Na]^+$ (calculated for 1115.1184). The compound was identified as 3-O-digalloyl-1,2,4,6-tetra-O-galloyl-β-d-glucose and was coded as NMB4 (**Figure 2** and **Figures S4**, **S8**, and **S19–S26**; **Table 1**) [22].

The compound corresponding to UV peak B6 in **Figure 1c** was obtained as an amorphous white powder and was assigned a molecular formula of $C_{48}H_{36}O_{30}$ by its HR-ESI-MS *m/z* of 1115.1167 $[M+Na]^+$ (calculated for 1115.1184). The compound was identified as 6-O-digalloyl-1,2,3,4-tetra-O-galloyl-β-d-glucose and was coded as NMB6 (**Figure 2** and **Figures S5**, **S9**, and **S27–S34**; **Table 2**) [24].

The compound corresponding to UV peak C3 was obtained as an amorphous white powder and was assigned a molecular formula of $C_{55}H_{40}O_{34}$ by its HR-ESI-MS *m/z* of 1267.1268 $[M+Na]^+$ (calculated for 1267.1293). The compound was identified as 3,6-bis-O-digalloyl-1,2,4-tri-O-galloyl-β-d-glucose and was coded as NMC3 (**Figure 2** and **Figures S6**, **S10**, and **S35–S42**; **Table 2**) [25].**Table 1**. ^{1}H and ^{13}C chemical

shifts and HMBC correlations for compounds NMA2 and NMB4 (600 MHz). Chemical shifts of the same compounds previously reported in the literature are shown in square brackets.

Group		1,2,3,4,6-Penta-O-Galloyl-β-D-Glucose (NMA2) [23]			3-O-Digalloyl-1,2,4,6-Tetra-O-Galloyl-β-D-Glucose (NMB4) [22]		
		δC	δH, Multip., (J in Hz)	HMBC	δC	δH, Multip., (J in Hz)	HMBC
Glucose	1	93.27 [93.8]	6.26, d (8.2) [6.26]	2, 3, 7a, 8a	93.11 [93.77]	6.21–6.25, m [6.14]	3, 7a
	2	71.70 [70.8]	5.62, dd (9.8, 8.3) [5.61]	1, 3, 7b, 8b	71.46 [72.14]	5.57–5.62 [a], m [5.81]	1, 3, 4, 7b
	3	73.26 [74.1]	6.00, t (9.6) [5.65]	1, 2, 4, 5, 7c, 8c	73.44 [74.06]	5.94–6.02, m [5.53]	2, 4, 7c
	4	69.34 [68.4]	5.66, t (9.6) [5.90]	3, 5, 6, 7d, 8d	69.13 [69.75]	5.62–5.68 [a], m [5.48]	3, 6, 7d
	5	73.76 [72.2]	4.55, oddd [4.42]	1, 3, 4, 6	73.44 [74.35]	4.52–4.4 [a], m [4.30]	1, 3, 4, 6
	6	62.94 [62.2]	4.29, dd (12.3, 4.6) 4.58, odd [4.39]	4, 5, 7e, 8e	62.80 [63.08]	4.25 [a], m 4.54–4.56 [a], m [4.28; 4.42]	4, 5, 7e
Galloyl A	7a	165.40 [166.2]			165.20 [166.88]		
	8a	119.09 [119.7]			118.74 [119.68]		
	9a	110.14 [110.3]	7.06, s [7.06]	7a, 8a, 10a, 11a	110.05 [110.43]	7.01–7.02 [a], m [6.94]	7a, 8a, 10a, 11a
	10a	146.14 [146.2]			145.85 [146.44]		
	11a	140.18 [140.0]			140.11 [140.72]		
Galloyl B	7b	166.32 [166.9]			166.21 [167.89]		
	8b	119.70 [120.2]			119.23 [120.31]		
	9b	109.98 [110.3]	6.99, s [6.94]	7b, 8b, 10b, 11b	109.88 [110.37]	6.92–6.96, m [6.80]	7b, 8b, 10b, 11b
	10b	146.00 [146.4]			145.69 [146.32]		
	11b	139.72 [140.1]			139.65 [140.25]		
Galloyl C	7c	166.48 [167.9]			165.77 [166.98]		
	8c	119.80 [121.1]			118.95-119.08 [b] [120.16]		
	9c	109.96 [110.7]	6.96, s [7.14]	7c, 8c, 10c, 11c	114.45 [b]; 117.21 [b]	7.15, d (3.87) [b]; 7.27, d (2.03) [b]	7c, 8c, 10c, 11c
					109.35 [c]	6.99–7.00 [ac], m	

Group		1,2,3,4,6-Penta-O-Galloyl-β-D-Glucose (NMA2) [23]			3-O-Digalloyl-1,2,4,6-Tetra-O-Galloyl-β-D-Glucose (NMB4) [22]		
		δC	δH, Multip., (J in Hz)	HMBC	δC	δH, Multip., (J in Hz)	HMBC
					[110.00]	[6.88]	
	10c	145.90 [146.5]			145.69–145.83 [b] [146.42]		
	11c	139.55 [140.8]			139.39–139.49 [b] [139.96]		
Galloyl D	**7d**	166.14 [167]			166.01 [167.25]		
	8d	119.65 [120.2]			119.23 [120.19]		
	9d	110.06 [110.4]	7.03, s [7.01]	7d, 8d, 10d, 11d	109.35 [110.00]	6.99–7.00 [a], m [6.85]	7d, 8d, 10d, 11d
	10d	146.02 [146.4]			140.11 [140.31]		
	11d	139.75 [140.3]			145.86 [146.50]		
Galloyl E	**7e**	166.82 [167.0]			166.87 [169.00]		
	8e	120.72 [120.2]			120.37 [121.39]		
	9e	109.91 [110.4]	7.12, s [6.98]	7e, 8e, 10e, 11e	109.80 [110.30]	7.07, d (6.21) [6.95]	7e, 8e, 10e, 11e
	10e	145.98 [146.4]			145.69 [146.23]		
	11e	139.23 [140.3]			139.14 [139.70]		
Galloyl C'	**7c'**				164.58–165.20 [166.18]		
	8c'				119.69–119.76 [121.00]		
	9c'				110.28–110.33 [110.58]	7.14, d (12.18) [7.01]	7c', 8c', 10c', 11c'
	10c'				145.69–145.83 [146.38]		
	11c'				139.49–139.65 [140.07]		

[a] = overlapped signal; [b] = signals on the galloyl proximal group due to the attachment of the distal galloyl group in a meta position; c = signals on the galloyl proximal group due to the attachment of the distal galloyl group in a para position; HMBC correlations are from protons with respect to the indicated carbons. Assignments were confirmed by DEPT-135, COSY, and HSQC.

Table 2. ^{1}H and ^{13}C chemical shifts and HMBC correlations for compounds NMB6 and NMC3 (600 MHz). Chemical shifts of the same compounds previously reported in the literature are shown in square brackets.

Group		6-O-Digalloyl-1,2,3,4-Tetra-O-Galloyl-β-D-Glucose (NMB6) [24]			3,6-Bis-O-Digalloyl-1,2,4-Tri-O-Galloyl-β-D-Glucose (NMC3) [25]		
		δC	δH, Multip.,(J in Hz)	HMBC	δC	δH, Multip.	HMBC
Glucose	1	93.40 [93.87]	6.31–6.34, m [6.23]	3, 5, 7a, 8a	93.25–93.33 [93.3]	6.26–6.32, m	2, 3, 5, 7a
	2	71.77–71.80 [72.25]	5.59–5.62, om [5.58]	1, 3, 4, 7b, 8b	71.67 [71.7]	5.63–5.66 [a], m	1, 3, 4, 7b
	3	73.30–73.32 [74.09]	5.98–6.03, m [5.91]	1, 2, 4, 7c, 8c	73.65–73.86 [73.9]	6.01–6.07, m	1, 2, 4, 5, 7c
	4	69.22–69.43 [70.02]	5.63–5.68, om [5.58]	2, 3, 5, 6, 7d, 8d	69.25–69.47 [69.3]	5.66–5.72 [a], m	3, 5, 6, 7d
	5	73.91–73.95 [74.38]	4.55–4.60, om [4.52]	1, 3, 4, 6	73.65–73.86 [73.9]	4.56–4.64 [a], m	1, 3, 4, 6
	6	63.12–63.24 [63.60]	4.45–4.54, om [4.43]	4, 5, 7e	63.25–63.43 [63.1]	4.35–4.43, m 4.52–4.56 [a], m	4, 5, 7e
Galloyl A	7a	164.97–165.05 [166.22]			165.26–165.44 [164.9]		
	8a	119.93–119.95 [119.78]			119.20–119.84		
	9a	110.39 [110.66]	7.10, d (8.42) [7.04]	7a, 8a, 10a, 11a	110.12–110.18	7.05–7.07 [a], m	7a, 8a, 10a, 11a
	10a	146.12 [146.59]			146.05–146.13		
	11a	139.84 [140.80]			139.46–140.08		
Galloyl B	7b	165.74–165.75 [167.01]			166.25–166.31 [165.0]		
	8b	120.53 [120.50]			119.72–119.84		
	9b	110.17 [110.50]	7.00, d (6.23) [6.97]	7b, 8b, 10b, 11b	110.03	6.99–7.00, m	7b, 8b, 10b, 11b
	10b	145.90 [146.47]			145.98–146.00		
	11b	139.33 [140.39]			139.61–139.63		
Galloyl C	7c	165.93–165.98 [167.34]			165.80 [b] [165.9]		
	8c	120.64–120.67 [120.40]			119.72 [b]; 120.66 [b]		
	9c	110.05–110.11 [110.41]	6.96, d (3.63) [6.89]	7c, 8c, 10c, 11c	114.68 [b]; 117.43 [b]	7.31–7.34 [b], m 7.19–7.21 [b], m	7c, 8c, 10c, 11c

Group		6-O-Digalloyl-1,2,3,4-Tetra-O-Galloyl-β-D-Glucose (NMB6) [24]			3,6-Bis-O-Digalloyl-1,2,4-Tri-O-Galloyl-β-D-Glucose (NMC3) [25]		
		δC	δH, Multip.,(J in Hz)	HMBC	δC	δH, Multip.	HMBC
					109.55 [c]	7.03, d (7.11)[c]	
	10c	145.89 [146.32]			144.00 [b]; 144.35 [b]		
	11c	139.18–139.20 [140.28]			139.63 [b]; 139.73 [b]		
Galloyl D	**7d**	165.74–165.75 [167.07]			166.16–166.21 [165.6]		
	8d	120.55 [120.28]			119.21–119.79		
	9d	110.29 [110.44]	7.05, d (6.43) [6.94]	7d, 8d, 10d, 11d	110.12–110.18	7.04–7.05 [c], m	7d, 8d, 10d, 11d
	10d	145.97 [146.42]			145.98–146.07		
	11d	139.35 [140.34]			139.48–139.61		
Galloyl E	**7e**	165.93–165.98 [167.23]			166.16 [b] [165.8]		
	8e	121.30 [121.13]			119.72 [b]; 120.66		
	9e	114.77 [b]; 117.71 [b] [115.09; 117.60]	7.40, d (1.99) [b]; 7.51, d (1.99) [b]	7e, 8e, 10e, 11e	114.68 [b];117.34 [b]	7.47–7.48 [b], m 7.31–7.34 [b], m	7e, 8e, 10e, 11e
		110.05 [c] [-]	7.25, s [c] [7.29]		109.55 [c]	7.17–7.21 [c], m	
	10e	143.86–146.96 [144.62; 147.55]			144.00 [b]; 144.35 [b]		
	11e	139.86–139.95 [140.39]			139.73–139.79 [b]		
Galloyl E'	**7e'**	164.97–165.98 [166.70]			164.54–165.26		
	8e'	120.88–128.58 [120.54]			120.07–120.33		
	9e'	110.71–110.78 [110.91]	7.28, d (7.41) [7.23]	7e', 8e', 10e', 11e'	110.42–110.46	7.23–7.24, m	7e', 8e', 10e', 11e'
	10e'	146.16–151.44 [146.64]			146.03–146.11		
	11e'	132.67–139.43 [140.54]			139.48–139.67		
Galloyl C'	**7c'**				164.55–165.14		
	8c'				120.07–120.18		
	9c'				110.38–110.42	7.17–7.19, m	7c', 8c',

Group		6-O-Digalloyl-1,2,3,4-Tetra-O-Galloyl-β-D-Glucose (NMB6) [24]			3,6-Bis-O-Digalloyl-1,2,4-Tri-O-Galloyl-β-D-Glucose (NMC3) [25]		
		δC	δH, Multip.,(J in Hz)	HMBC	δC	δH, Multip.	HMBC
							10c', 11c'
	10c'				145.99–146.05		
	11c'				139.38–139.67		

[a] = overlapped signal; [b] = signals on the galloyl proximal group due to the attachment of the distal galloyl group in a meta position; c = signals on the galloyl proximal group due to the attachment of the distal galloyl group in a para position. HMBC correlations are from protons with respect to the indicated carbons. Assignments were confirmed by DEPT-135, COSY, and HSQC.

The isotopic distributions observed in HR-ESI-MS for all four compounds were consistent with the assigned molecular formula, as it was also revealed by the spectra simulated from the respective molecular formula (**Figures S3–S6**).

3.3. Antibacterial Activity Testing

Table 3 shows the MIC values obtained from broth microdilution susceptibility testing of compounds NMA2, NMB4, NMB6, and NMC3 against both the reference strains of *E. coli* and *K. pneumoniae* and those showing multidrug resistance to the indicated antibiotics. The observed MIC values of the tested compounds were noted to vary depending on the type of compound, as well as the prevailing resistance phenotypes of the studied bacteria. Upon MBC testing, bacterial growth colonies on agar plates were observed in wells containing compound concentrations greater than four times the MIC values; therefore, the compounds were regarded as bacteriostatic.

Table 3. MICs of compounds NMA2, NMB4, NMB6, and NMC3 against reference and MDR strains of *E. coli* and *K. pneumoniae*.

	Bacteria and resistance phenotypes and MICs (µg/mL)														
	E. coli							***K. pneumoniae***							
Antibiotic/ Compound	**ATCC 25922**	OXA-48	VIM 1	ESBL	KPC-3	Vim1 /4 TEM-1	KPC-2 Mcr-1	**ATCC 10031**	OXA-48	TEM-/ SHV-/ CTX-M-pos	NDM -1	DHA-1 (AmpC)	KPC-2	OXA-48	SHV-4
Cefotaxime		S	R	R	R	R	R		R	R	R	R	R	R	R*
Ceftazidime		S	R	R	R	R	R		R	R	R	R	R	R	R
Ertapenem		R	S	S	R	R	R		R	S	R	R	R	R	S
Imipenem		S	S	S	R	R	R		R	S	R	R*	R	R*	S
Meropenem		S	S	S	R*	R	R		R	S	R	R*	R	R*	S
Ciprofloxacin		R	S	R	S	R	R		R	R	R	R	R	R	S
Piperacillin-Taz		R	R	R	R	R	R		R	I	R	R	R	R	R
NMA2	32	>256	>256	>256	4	256	>256	32	32	128	256	>256	2	8	256
NMB4	32	256	>256	>256	4	>256	>256	08	16	64	256	>256	4	4	256
NMB6	64	>256	256	32	4	128	>256	16	32	64	256	2	4	8	256
NMC3	64	>256	>256	16	8	>256	>256	128	16	>256	256	16	2	8	256
Gentamicin	<0.25	1	4	1	1	2	128	<0.25	128	64	>128	>128	2	<0.5	8

S = susceptible, standard dosing regimen; I = susceptible, increased exposure; R = resistant; R * = MIC of the antibiotic is within the sensitive range *in vitro* but must be considered resistant *in vivo* due to the expression of a beta-lactamase/carbapenemase. Standard abbreviations for the expressed resistance enzymes and genes are used to identify the resistance types in each strain.

3.4. *In vivo* Antibacterial Assays

At an inoculation dose of 1.5 × 10^8 CFU/mL, the 24 h untreated G. mellonella larvae survival probabilities were 80% for *K. pneumoniae* (ATCC 10031) and 90% for *E. coli* (ATCC 25922)-infected larvae. This dose was therefore used to inoculate larvae in the subsequent tests for *in vivo* antibacterial activities of compounds NMA2, NMB4, NMB6, and NMC3.

A higher probability of survival was observed among larvae treated with the test compounds as compared to the negative controls after 96 h of incubation. Whereas the probability of survival among the *E. coli*-infected larvae was in the range of 20–40% (negative control = 5%), the larvae infected with *K. pneumoniae* showed 40–65% survival probabilities (negative control = 10%). The probabilities of survival of the larvae treated with gentamicin as a positive control were 20% and 45% among the *E. coli* and *K. pneumoniae*-infected larvae, respectively (**Figure 3** and **Figure 4**).

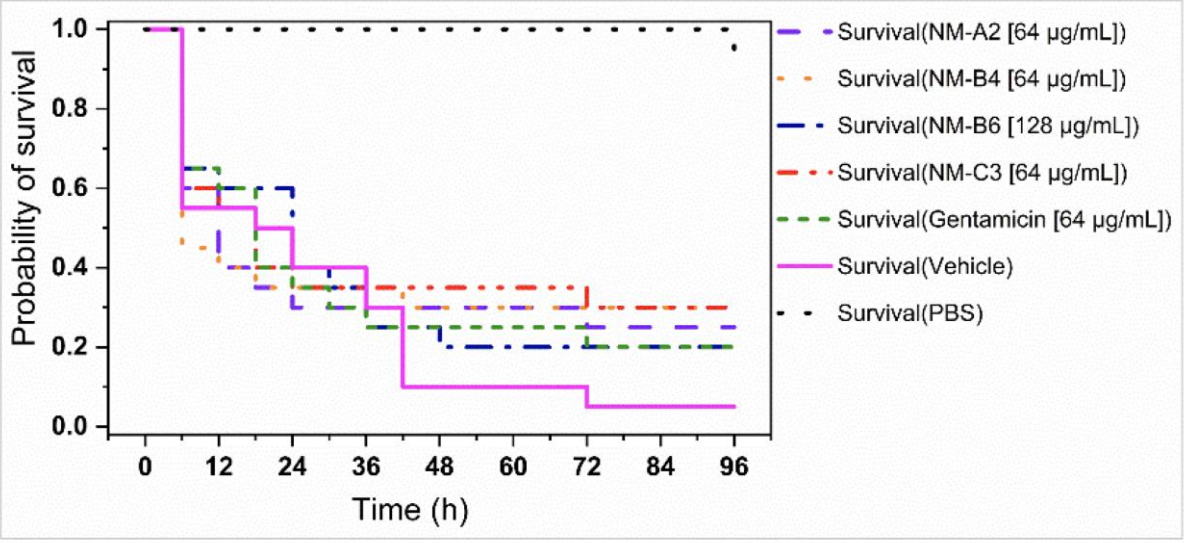


Figure 3. Kaplan–Meier survival plot showing the probability of survival of *G. mellonella* larvae infected with *E. coli* (ATCC 25922) and treated with the isolated compounds, as well as positive and negative controls.

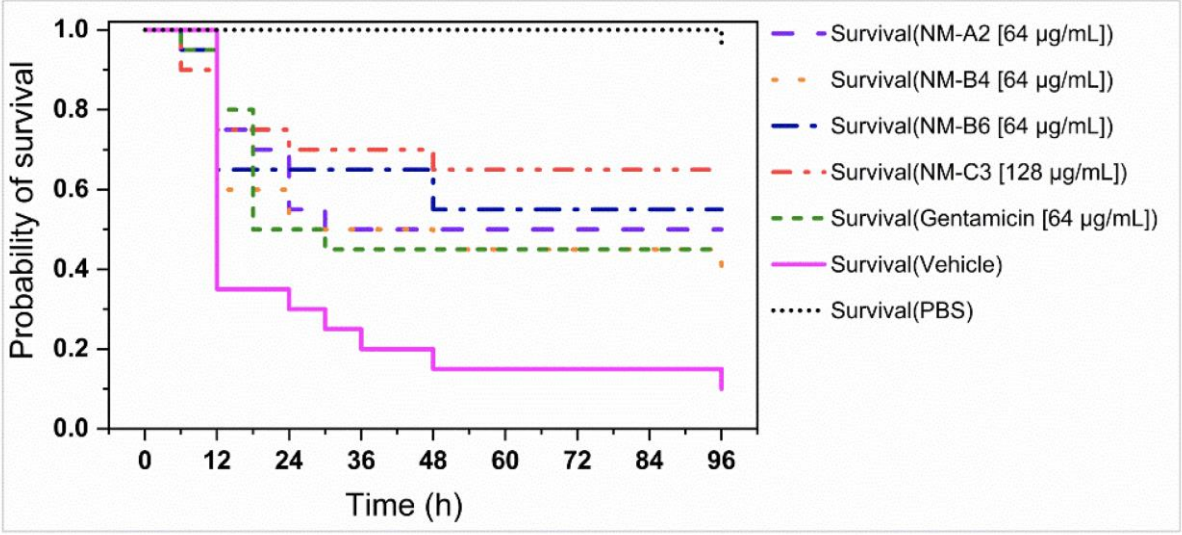


Figure 4. Kaplan–Meier survival plot showing the probability of survival of G. mellonella larvae infected with *K. pneumoniae* (ATCC 10031) and treated with the isolated compounds, as well as positive and negative controls.

3.5. Relationships between the MIC Values and the Nature of Expressed Resistance Enzymes by the Tested MDR Strains

Beta-lactamases formed a majority of the enzymes indicated to be expressed by the tested MDR strains of *E. coli* and *K. pneumoniae*. The relative contents of aromatic amino acids among the resistance enzymes expressed by the tested MDR bacteria were found to be in the range of 7.02–15.90% w/w. Moreover, the calculated net charges within these enzymes ranged from −15 to 4 (**Table 4**).

Table 4. Classes, percentages of aromatic amino acids, and net charges of the resistance enzymes indicated to be expressed by the studied MDR strains of *E. coli* and *K. pneumoniae*.

Protein code	Enzyme's class	PDB code	% Aromatic AA (w/w)	Net charge
OXA-48	Beta-lactamase	7KHQ	15,90	0
VIM-1	Metallo-beta-lactamase	5N5I	9,20	-15
KPC-3	Beta-lactamase	6QWD	11,67	0
TEM-1	Beta-lactamase	1M40	7,45	-7
KPC-2	Beta-lactamase	3DW0	12,57	1
MCR-1	Phosphoethanolamine transferase	5GRR	14,43	-12
CTX-M 9	Beta-lactamase	1YLJ	7,09	1
SHV-1	Beta-lactamase	2ZD8	7,02	-1
NDM-1	Metallo-beta-lactamase	4EXY	9,54	-6
DHA-1 or AmpC	Beta-lactamase	Q84AE1 (uniprot)	14,22	4

We observed a pattern of relationships between the magnitude of the observed MIC values and the content of the aromatic amino acids, as well as the net charge of the resistance enzymes expressed by the tested MDR strains. MIC values of 32 µg/mL or lower were observed among the bacteria expressing resistance enzymes with

more than 11% w/w of aromatic amino acids or those with a net-zero or positive charge (**Table 4**, **Figure 5**).

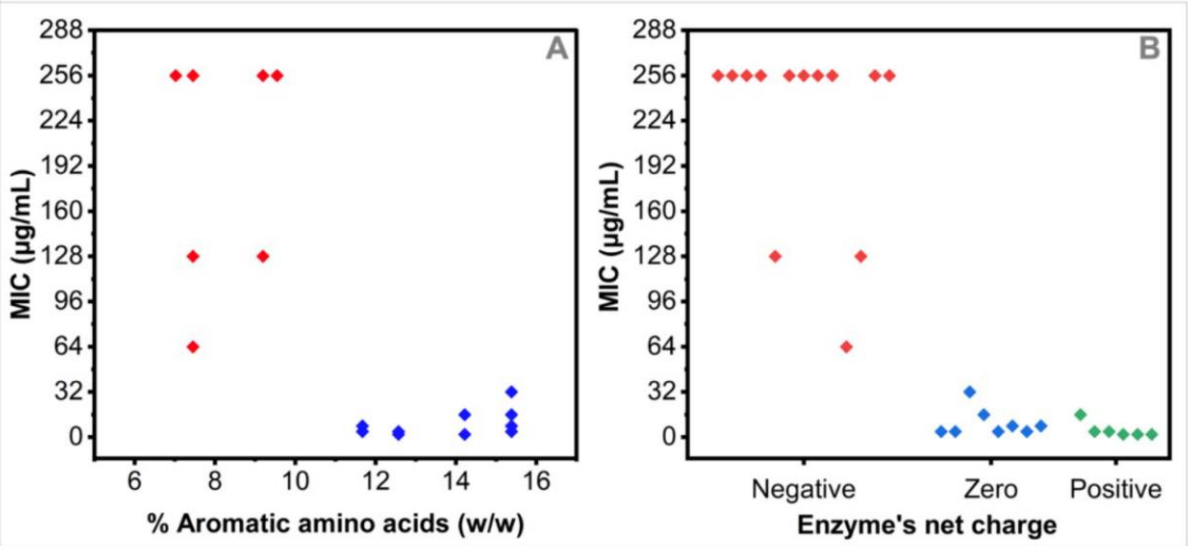


Figure 5. Scatter plots showing the relationships between the observed MIC values and the percentage aromatic amino acid content (**A**), as well as the net charge (**B**) of the resistance enzymes expressed by the studied MDR strains of *E. coli* and *K. pneumoniae*.

These observations prompted a hypothesis with respect to the possible role of the expressed enzymes in influencing the ultimate susceptibility of the respective bacteria strains to the tested compounds. **Figure 6** shows the possible interplay between bacteria, enzymes of varying nature, and the studied compounds. Enzymes expressed with higher proportions of aromatic amino acids and/or zero or positive net charge might cause increased proximity, higher surrounding concentrations, and enhanced interactions of the test compounds with the outer bacterial cell membrane (**Figure 6A**). Collectively, these factors could result in higher susceptibilities of the respective bacteria to the tested compounds. On the contrary, enzymes with contrasting features might bring about lower concentrations of the test compounds around the bacterial cells, leading to lower susceptibilities in such cases (**Figure 6B**).

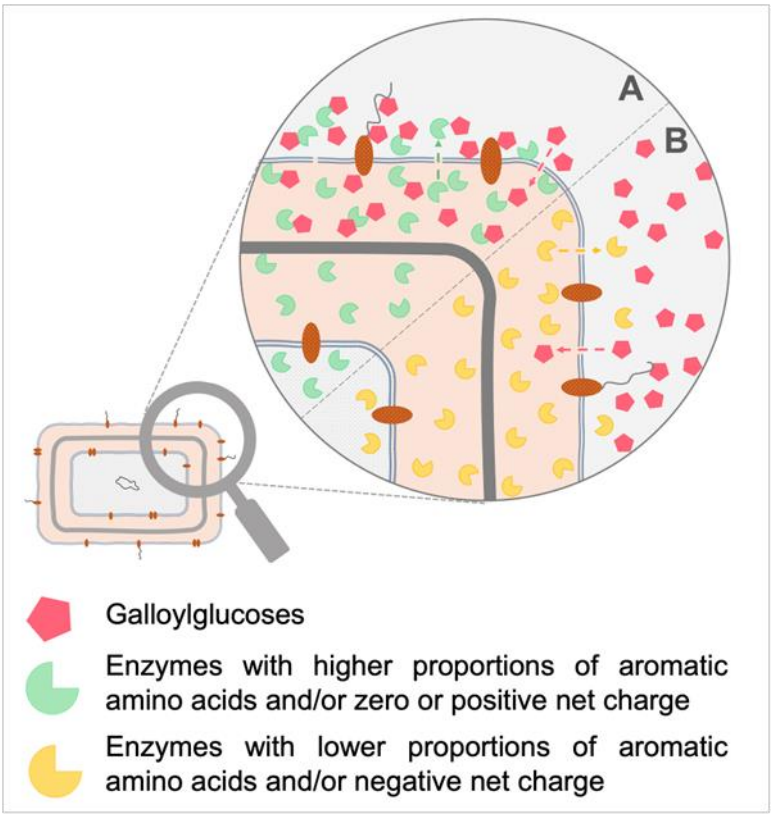


Figure 6. Hypothesized roles of the nature of expressed resistance enzymes in mediating the interactions between bacterial cells and galloylglucoses. The resulting differences in the galloylglucose concentration around the bacterial cells might contribute to the observed differences in susceptibilities.

4. Discussion

Extraction, isolation, and purification of bioactive compounds from crude plant extracts is an acceptably challenging task. This is mostly due to the complexity of a majority of crude plant mixtures, posing difficulty in the hunt for compounds exhibiting the activities of interest. The choice of acetone as an extractant was guided by previously reported antibacterial activities of *Paeonia broteoli* leaf extracts obtained from multiple solvents with varying polarities [17,38]. Other findings have shown the type and molecular weight of galloylglucoses recovered during extraction to be highly dependent on the nature of the extractant used. Acetone and ethyl acetate are the most suitable solvents for the extraction of low-molecular-weight galloylglucoses, among other tannins. Conversely, methanol and other organic solvent–water mixtures were found to mostly recover higher-molecular-weight galloylglucoses [10]. Although the nature of the antibacterial compounds was not known at the time of extraction, avoiding the use of acids to modify the extracting solvent and the isolation mobile phases was crucial because acidic conditions would have encouraged the hydrolysis of galloylglucoses in the course of their extraction and isolation [39].

Through contact bioautography, the identification of fractions/spots containing compounds with antibacterial activity was prominently simplified (**Scheme 1**). The outcomes of bioautographic screening considerably influenced subsequent focus and

choice of other isolation techniques to be employed. Whereas three main bioautography techniques are known, the type of bacteria under study can influence the outcomes of each technique [18]. Here, both *E. coli* (ATCC 25922) and *K. pneumoniae* (ATCC10031) performed better with contact bioautography as compared to direct TLC and immersion/agar overlay bioautography techniques.

The presence of multiple subfractions (A–E) with antibacterial activities from semipurified aqueous subfraction 3.1 signaled the possible existence of numerous structurally similar compounds in this subfraction (**Figure 1a**). This was underscored by the findings of at least two UV peaks (compounds) within each of subfractions A–E (**Scheme 1**; **Figure 1b–e**; **Figures S1** and **S2**). As a consequence, those mixtures required the use of isolation techniques and methods with a higher-resolution power and, in most cases, longer isolation times. The use of separate methods customized for the isolation of compounds within each subfraction (A–E) was crucial for the final step, in which compounds with suitable levels of purity were isolated.

Priority for further subfractionation was given to compounds isolated under peaks A2, B4, B6, and C3, in which suitable activity levels and complete isolations in appropriate quantities were attained (**Scheme 1**, **Figure 1b–e**). Moreover, the efforts to purify subfractions D3 and D4 were not successful (**Scheme 1**, **Figure S1**), and subfractions E1–E5 were not further characterized due to low or lack of antibacterial activities (**Scheme 1**, **Figure S2**).

The presence of many structural isomers of galloylglucoses in the studied extract challenged the isolation of the compounds exhibiting antibacterial activities. The same challenge was previously implicated in studies involving a similar type of compound [21]. Owing to the limited number of studies conducted using purified galloylglucoses of known structures, reports on methods for their isolation and purification are valuable.

All of the isolated compounds were previously isolated and characterized from several other plant species. To the best of our knowledge, the isolation of compounds NMB4, NMB6, and NMC3 from *P. officinalis*, as well as their *in vitro* and *in vivo* antibacterial activities against reference and MDR strains of *E. coli* and *K. pneumoniae*, are reported here for the first time.

The spectrometric and spectroscopic data presented herein are similar to those previously reported for the same compounds isolated from other plant species [13,20,21,22,40]. Among others, the resonances typical of the glucose moiety are those corresponding to the anomeric carbon (C-1) appearing at δ = 93.11–93.40 ppm across all compounds (**Table 1** and **Table 2**). The chemical shifts are indicative of the presence of beta-d-glucopyranose anomers, in contrast to the alpha anomers (δ~90 ppm) [41]. Additionally, the beta anomers of galloylglucoses are most commonly isolated from nature, whereas the occurrence and isolation of alpha anomers is very rare [12,42]. Moreover, the characteristic splitting of the ^{1}H NMR shifts corresponding

to methylene C-6 of the glucose core was noted at δ = 4.29–4.58 ppm in pentagalloylglucose (NMA2) as a split duplet of duplets. Signals in a similar ppm range were also evident in the remaining compounds. The existence of a methylene group at this position was confirmed by the ^{13}C DEPT-135 spectra, showing δ = 62.80–63.43 across all compounds (**Table 1** and **Table 2**; **Figures S11–S14**, **S19–S22**, **S27–S30**, and **S35–S38**).

The galloyl units surrounding the glucose core were characterized by the resonances of the protons at position 9 of the galloyl groups (δ = 6.92–7.51), among other signals. Signals resulting from these protons appeared as five distinct singlets in compound NMA2 and were more complex among the hexa- and heptagalloylglucoses (**Table 1** and **Table 2**; **Figures S11**, **S19**, **S27**, and **S35**). The resonances conforming to the carbonyl carbons on the ester groups of the galloyl units occurred at δ = 165–166.87 ppm across all compounds (**Table 1** and **Table 2**). Although galloyl units are typically esterified with a glucose polyol to yield galloylglucoses such as those reported here, other possible polyols include fructose, saccharose, xylulose, glucitol, and hamamelose, as well as shikimic and quinic acids [10,39].

The ascertainment of the exact position of attachment of each galloyl unit (A–E) on carbons C 1–4 and C–6 of the glucose cores was a key undertaking. This was achieved by aligning the three-bond correlations (HMBC) between each of the protons on the glucose core and the carbonyl carbons at positions 7a–7e on one hand, as well as between those carbonyl carbons and the aromatic protons at positions 9a–9e on the galloyl units on the other hand (**Table 1** and **Table 2**; **Figure 2** and **Figures S17**, **S18**, **S25**, **S26**, **S33**, **S34**, **S41**, and **S42**). Following this step, the determination of the galloyl units carrying an additional/distal galloyl unit (digalloyl units) was feasible.

The attachment of a (distal) galloyl unit on a particular galloyl group proximal to the glucose core by a depsidic bond resulted in the downfield shift of the ^{13}C and ^{1}H signals originating from the respective proximal galloyl unit (**Table 1** and **Table 2**). The resulting most downfield ^{1}H signals on the aromatic region corresponding to those at position 9 of the proximal unit of the digalloyl moieties were therefore earmarked. This was supported by the convergence of the HMBC correlations of such protons and those of the protons in the respective position on the glucose core to the same carbonyl carbon (position-7) of the ester bond in between (**Table 1** and **Table 2**; **Figure 2**).

Despite the three-bond HMBC correlations between the protons at positions 9c’ (NMB4), 9e′ (NMB6), as well as those at positions 9c′ and 9e′ (NMC3) and the carbonyl carbon on the adjacent depsidic bonds, the lack of protons in proximity on the proximal galloyl unit hindered the determination of connectivity using HMBC alone (**Figure 2**). Nevertheless, due to the deshielding effect of the proximal galloyl unit on the distal galloyl unit, the shifts resulting from protons at position 9 of the

distal galloyl units appeared at the second most downfield positions in the aromatic region of the spectra and correlated with the carbonyl carbon in the depsidic bond rather than that in the underlying ester bond (**Table 1** and **Table 2**; **Figures S11**, **S19**, **S27**, and **S35**) [43]

Other authors have frequently indicated the use of the downfield shifting of the ^{13}C NMR resonances of the respective carbons on the glucose core in comparison to those of the pentagalloylglucose to justify the position of the distal galloyl groups on hexa-, hepta-, octa-, and other polygalloylglucoses [20,21,41]. Similarly, in compound NMB4, the downfield shift of the C-3 signal led to its overlap with the C-5 signal at δ = 73.44 ppm. The same phenomenon was noted among the signals due to C-6 in NMB6 and C-3 plus C-6 in NMC3 (**Table 1** and **Table 2**) [21]. Using a similar approach, Nishizawa and Yamagishi implied that the C-3 and C-6 positions of the glucose core are predominant for attachments of depsidic galloyl groups [21].

Previous studies have indicated that in solutions, the distal galloyl groups tend to migrate between the ortho and para positions of the proximal galloyl unit and coexist in an equilibrium mixture of the two isomers [20]. Furthermore, it was noted that this migration induces the shifting of the ^{1}H and ^{13}C NMR resonances in other positions of the respective compounds, resulting in multiplets and an increased overlapping pattern of the signals (**Table 1** and **Table 2**; **Figure 2**) [21]. In addition, the stated migration causes the observed differences in resonances of the protons attached at position 9 of the proximal galloyl units. Therefore, the carbon (position 9) next to the meta carbon appears to be more downfield-shifted (^{13}C δ~117 ppm) when the distal galloyl group has migrated to the meta position, whereas the corresponding carbon in a similar position resonates at δ~114 ppm. Furthermore, the shifts of the protons in these positions appeared to follow the same pattern. On the other hand, the migration of the distal galloyl unit to the para position resulted in unified and more upfield-shifted (^{13}C δ = 109–110 ppm) signals corresponding to position 9 of the proximal galloyl unit (**Table 1** and **Table 2**). Due to the observed migratory nature of the distal galloyl groups in compounds NMB4, NMB6, and NMC3 in solution, it was not possible to ascertain their exact position(s) (meta, para, or a mixture of both) in these compounds based on NMR data alone.

The isolated galloylglucoses showed bacteriostatic activities against *E. coli* and *K. pneumoniae* strains of different resistance phenotypes. Generally, some or all compounds exhibited higher activity against MDR strains expressing KPC-2, KPC-3, OXA-48, and DHA-1 enzymes. Conversely, moderate or lower activity levels were observed among the strains expressing VIM-1, VIM-4, TEM-1, SHV, MCR-1, and NDM-1, either alone or together with other enzymes. The MIC values observed for *E. coli* and *K. pneumoniae* strains with ESBL and DHA-1 phenotypes, respectively, were many folds lower among the compounds NMB6 and NMC3.

The observed differences in the susceptibilities of the studied MDR bacteria to galloylglucoses are unlikely to be based on enzyme–substrate interactions due to the

broad structural differences between galloylglucoses and the usual substrates (e.g., beta-lactam antibiotics) of the resistance enzymes expressed by the studied MDR bacteria. The influence of other resistance-enzyme-related factors might have therefore contributed to the observed variation in susceptibilities.

Antimicrobial activities of galloylglucoses have been reported in various species of bacteria, fungi, and viruses [10,15,42]. Similar to our findings, previously reported antibacterial activities were mainly bacteriostatic against both Gram-negative and Gram-positive bacteria, and the Gram-negative bacteria were less susceptible [10,44,45]. Moreover, similar antibacterial activities of pentagalloylglucose (MIC or minimum regrowth concentration (MRC) = 32–256 µg/mL) against different reference and MDR strains of *E. coli* and *K. pnemouniae* were previously reported [46,47,48]. Additionally, galloylglucoses are reported to inhibit extracellular bacterial enzymes, toxins, adhesins, surface transport proteins, and biofilm formation [10,11,14]. These activities signify the potential of galloylglucoses against different mechanisms of pathogenicity and antibacterial resistance. No antibacterial activities of compounds NMB4, NMB6, and NMC3 were previously reported. However, compound NMB4 was reported to inhibit the enzyme alpha-glucosidase, the influx of Ca^{2+} in skin and respiratory cells, lipid formation in adiposities, and Alzheimer's amyloid beta-peptide aggregation [22,49,50,51]. Further, compound NMC3 was reported to block cell-membrane-based Ca^{2+}-dependent-chloride currents, induce formation of interferon, and exhibit antitumor activity [29,52].

Very low solubility of the isolated compounds was observed in solvents systems commonly used for broth microdilution assays. Complete dissolution of the compounds could not be attained using up to 2.5% DMSO in water or MHB media. This prompted efforts to explore other solubilization approaches to enable a more objective screening of the compounds' antibacterial potentials. This was achieved by preparing stock solutions by first dissolving the compounds in acetone, followed by working solutions, which contained 25.6% v/v of acetone in MHB. Ensuring a uniform concentration of acetone across all test wells (12.8% v/v) and filling the outermost wells with MHB media minimized acetone evaporation during the incubation time. Previous studies showed non-toxicity to bacteria at concentrations of up to 25% v/v of acetone in the test media [53,54].

The low water solubility of galloylglucoses hinders objective investigation of their antibacterial potentials *in vitro* and *in vivo*. Many studies have opted for disc diffusion assays, in which galloylglucoses are dissolved in an organic solvent before loading the discs. This approach achieves proper solubilization but is subject to less objective results, as the diffusion of compounds into water-based agar media is apparently low [16,44,55]. Furthermore, the solubility of galloylglucoses is highly influenced by the extent of their galloylation; those with more than four galloyl groups show lower water solubility profiles as compared to those with a lesser degree of galloylation [31]. This decrease in hydrophilicity is related to an increased degree of intramolecular hydrogen bonding and intermolecular stacking attained with a higher number of

galloyl groups [39]. The degree of galloylation might therefore be important with respect to finetuning the balance between compound solubility in test media and the degree of lipophilicity ideal for their interaction with bacteria cells. This is emphasized by the occurrence of optimal antibacterial activities among galloylglucoses with 6–7 galloyl groups [11].

All compounds resulted in *G. mellonella* larvae survival rates similar to or higher than those of the positive control (gentamicin); compound NMC3 ensured the best survival rates of larvae against both *E. coli* and *K. pneumoniae*. Furthermore, all compounds yielded better survival rates among the larvae infected with *K. pneumoniae* than with *E. coli*, which was consistent with the *in vitro* profiles.

The availability of data on *in vivo* antibacterial activities of galloylglucoses in higher animals is limited by their low oral bioaccessibility and bioavailability levels [10,39]. Improved *in vivo* anticancer and antiallergy activities were observed when galloylglucoses were administered via intraperitoneal or intravenous routes [13,32]. Conversely, other researchers have questioned the *in vivo* activities of galloylglucoses based on their likelihood of interacting with numerous proteins, limiting the attainment of effective concentrations [56]. Moreover, galloylglucoses are substrates of a range of hydrolytic and oxidative enzymes produced by gut microbiota in higher animals [10,39]. Most of the resulting metabolites can be absorbed and are highly linked to the observed systemic activities after oral administration [39].

The strains expressing enzymes KPC-2, KPC-3, OXA-48, DHA-1, and CTX-M, which have 11.7–15.4% w/w aromatic amino acid content and 0–4 net charges, were more susceptible (MIC = 2–64 µg/mL) to at least two of the galloylglucoses (**Figure 5**; **Table 3**). On the other hand, strains expressing VIM-1, TEM-1, SHV-1, and NDM-1 with aromatic amino acid contents of 7.0–9.5% w/w only and net charges of −1 to −15 were generally less susceptible (MIC = 64– > 256 µg/mL) to all galloylglucoses. Furthermore, the E. coli strains with phenotypes for both KPC-2 (net charge = +1) and MCR-1 (net charge = −12) were the least susceptible to each of the galloylglucoses (MIC > 256 µg/mL) (**Figure 5**; **Table 3**). These findings suggest a relationship between the nature of the resistance enzymes expressed by the MDR bacteria and their susceptibility to galloylglucoses.

However, the MIC values observed in the strain of *E. coli* expressing resistance enzyme OXA-48 (net charge = 0, aromatic AAs content = 15.4% w/w) were remarkably higher (256- > 256 µg/mL) than those in the *K. pneumoniae* strain expressing the same enzyme (4–32 µg/mL) (**Table 3**). Thus, we postulate that other factors, such as the presence of unidentified resistance enzyme(s) with opposing features, favored the observed lower susceptibility of the *E. coli* strain.

The observed antibacterial activities of galloylglucoses are related to their previously reported ability to interact with proteins, carbohydrates, lipids, and metal ions [10,11]. The compounds characteristically bind to different macromolecules through

hydrophobic interactions, as well as via hydrogen, covalent, and ionic or electrostatic bonds [10,15,39]. Proteins with higher proportions of aromatic amino acids were reported to show stronger hydrophobic interactions with galloylglucoses [29,30,31,33]. The compounds are also capable of electrostatically adsorbing to surfaces of macromolecules or elements carrying opposite charges [15,29,32,34].

These behaviors might explain the observed variations in MICs of the investigated compounds among MDR strains expressing enzymes with different contents of aromatic amino acids and net charges. The nature of resistance enzymes might influence the ultimate concentration of galloylglucoses around bacterial cells. To this end, enzymes richer in aromatic amino acids or with zero or positive net charges attract and interact more with galloylglucoses. The presence of those enzymes and their interactions with galloylglucoses might result in higher concentrations of galloylglucoses in the vicinity of bacterial cells (**Figure 6A**). Therefore, the compounds can attack the bacterial cells more intensely via a number of previously described modes of action. In contrast, the presence of enzymes with lower content of aromatic amino acids and/or net negative charges can accomplish the opposite effect [15,30,31,32,33,34]. In this case, lower concentrations of galloylglucoses around the bacterial cells make the respective bacteria less susceptible (**Figure 6B**).

5. Conclusions

Screening and isolation of antibacterial compounds from nature remains an important and challenging approach to the discovery and development of novel antibiotics. This study highlights a range of useful approaches to first-time extraction, isolation, purification, and characterization of three of the four galloylglucoses from the leaves of *P. officinalis*. Importantly, the challenge posed by the common coexistence of closely related galloylglucoses was mostly addressed by a combination of bioautography-guided extractive and chromatographic techniques.

The observed moderate-to-high bacteriostatic activities of the isolated compound against reference and MDR strains of *E. coli* and *K. pneumoniae* underline the previous reports on antimicrobial activities of galloylglucoses. Furthermore, the relative content of aromatic amino acids and net charges of the expressed resistance enzymes were noted to influence bacterial susceptibilities to the studied galloylglucoses. Moreover, diverse modes of action targeting different macromolecules on bacterial surfaces, as well as enzymes, toxins, and nutrients in the surrounding media, were previously indicated.

Despite limitations with respect to their absorption, metabolism, and lower target selectivity, galloylglucoses can potentially be applied in the agriculture and food industries, as well as in the management of septic wounds and other topical microbial infections. Through these and other possible avenues, galloylglucoses can substantially contribute to supplementing, reducing, or replacing the use of

contemporary antibiotics in order to mitigate the development of antimicrobial resistance.

Acknowledgments

The authors extend their sincere thanks to Christine Heinz, Liane Pogorelaja, Liane Dreher, and Eslam Ebrahim for their technical assistance; Christoph Mahler and Merle Arrowsmith for their kind support with HR-MS measurements; Gerd Vogg for identification and provision of the plant materials; and Christoph Schoen for the generous provision of the MDR bacterial strains.

Conflicts of Interest

The authors declare no conflict of interest. The sponsors had no role in the design, execution, interpretation, or writing of the manuscript.

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Supporting information

Table of Contents

Table S1: Summary of preparative HPLC parameters for isolation and purification of subfractions/compounds from the purified water fraction.

Fraction to be Refined	**Stationary phase**	**Mobile phase composition as %v/v of acetonitrile in water and other chromatographic conditions. (UV detection wavelength = 254 nm)**	**Recovered subfractions/ compounds**
Purified water fraction	ZORBAX XDB-C18, 21.2 x 150 mm, 5 µm (Agilent, US)	20 – 20% (0 – 3 min), 20 – 45% (3 – 32 min), 45 – 45% (32 – 33 min), 45 – 95% (33 – 35 min), 95 – 95% (35 – 36 min), 95 – 20% (36 – 38 min). Sample conc. = 250 mg/ mL, injection volume = 50 µL, Flow rate = 4 ml/min.	pooled first minor fractions, and major fractions A, B, C, D, E
A		20 – 20% (0 – 29 min), 20 – 50% (29 – 32 min), 50 – 50% (32 – 34 min), 50 – 20% (34 – 36 min), 20 – 20% (36 – 40 min). Sample conc. = 100 mg/ mL, injection volume = 100 µL, Flow rate = 4 ml/min.	A1, A2
B		0 – 20% (0 – 32 min), 20 – 75% (32 – 35 min), 75 – 75% (35 – 38 min), 75 – 20% (38 – 41 min), 20 – 20% (41 – 44 min). Sample conc. = 22.5 mg/ mL, injection volume = 8 µL, Flow rate = 6 ml/min.	B1, B2, B3, B4&5, B6
C		5 – 5 % (0 – 5 min), 5 – 26% (5 – 7 min), 26 – 26% (7 – 27 min), 26 – 5% (27 – 29 min), 5 – 5% (29 – 31 min). Sample conc. = 100 mg/ mL, injection volume = 20 µL, Flow rate = 4 ml/min.	C1, C2, C3
D		31 – 31% (0 – 32 min) Sample conc. = 100 mg/ mL, injection volume = 20 µL, Flow rate = 2 ml/min.	D1, D2, D3&4
E		31 – 31% (0 – 35 min) Sample conc. = 100 mg/ mL, injection volume = 35	E1, E2, E3&4, E5

		µL, Flow rate = 2 ml/min.	
B4&5	ZORBAX Eclipse XDB-18, Semi-Preparative 9.4 x 250 mm, 5 µ (Agilent, US)	0 – 17.5% (0 – 34 min), 17.5 – 75% (34 – 37 min), 75 – 75% (37 – 41 min), 75 – 17.5% (41 – 43 min), 17.5 – 17.5 % (29 – 31 min). Sample conc. = 13.5 mg/ mL, injection volume = 50 µL, Flow rate = 4 ml/min.	B4, B5

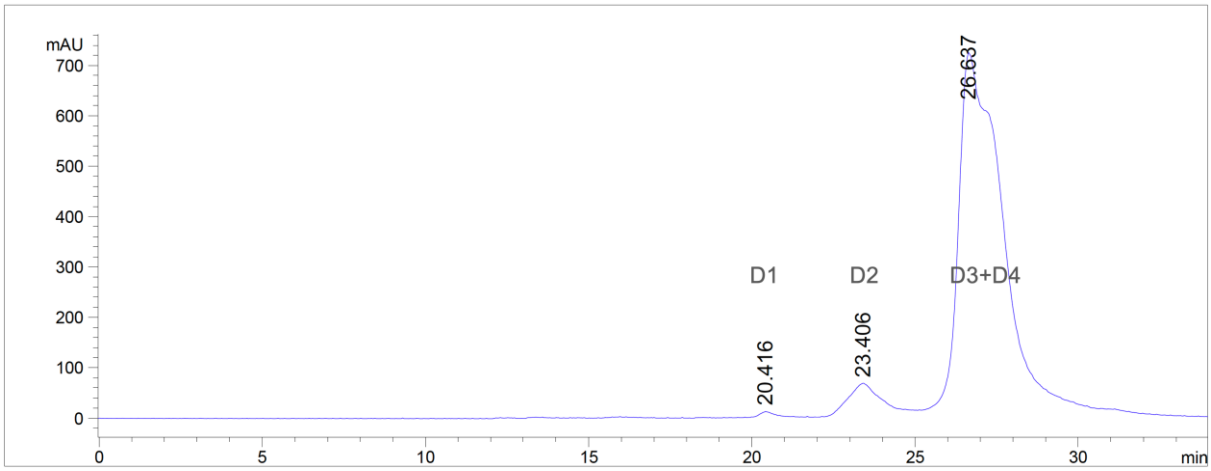

Figure S1: UV chromatogram subfraction D

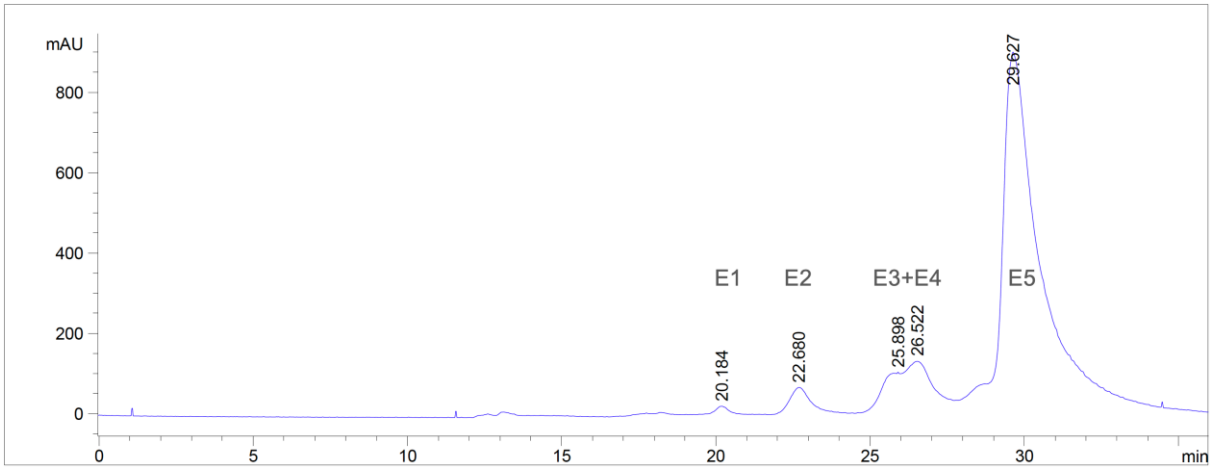

Figure S2: UV chromatogram of subfraction E

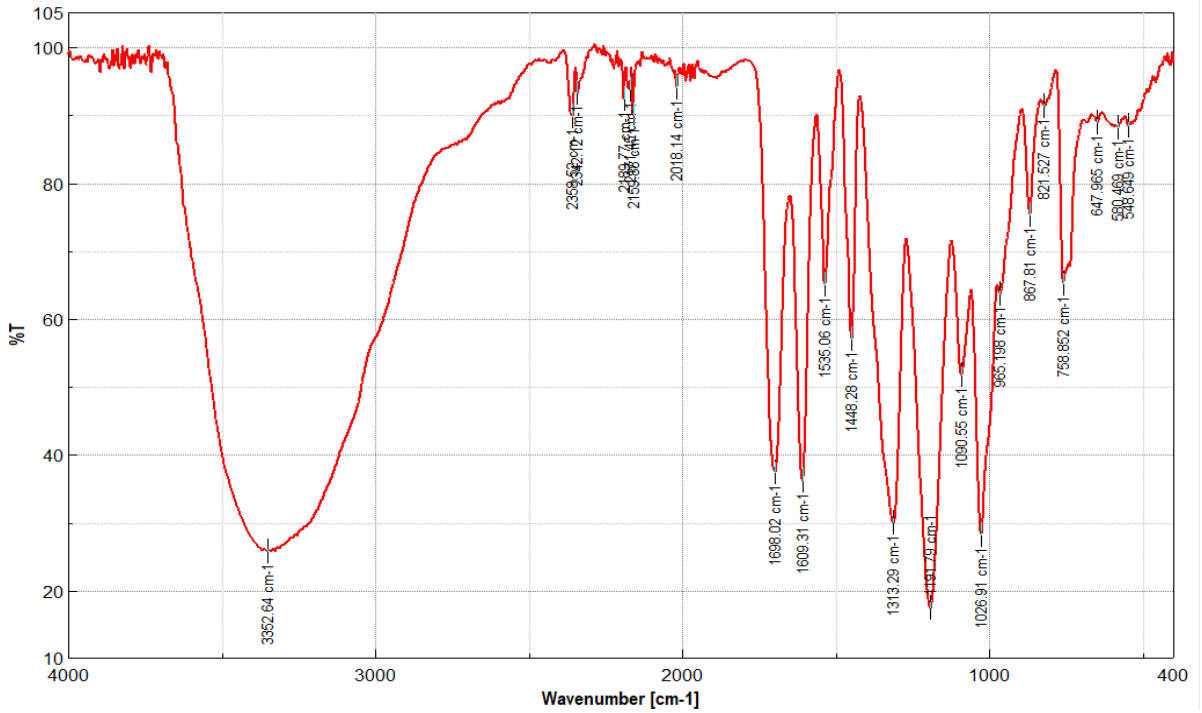

Figure S3: Infrared spectrum of 1,2,3,4,6-penta-O-galloyl-β-D-glucose (NMA2)

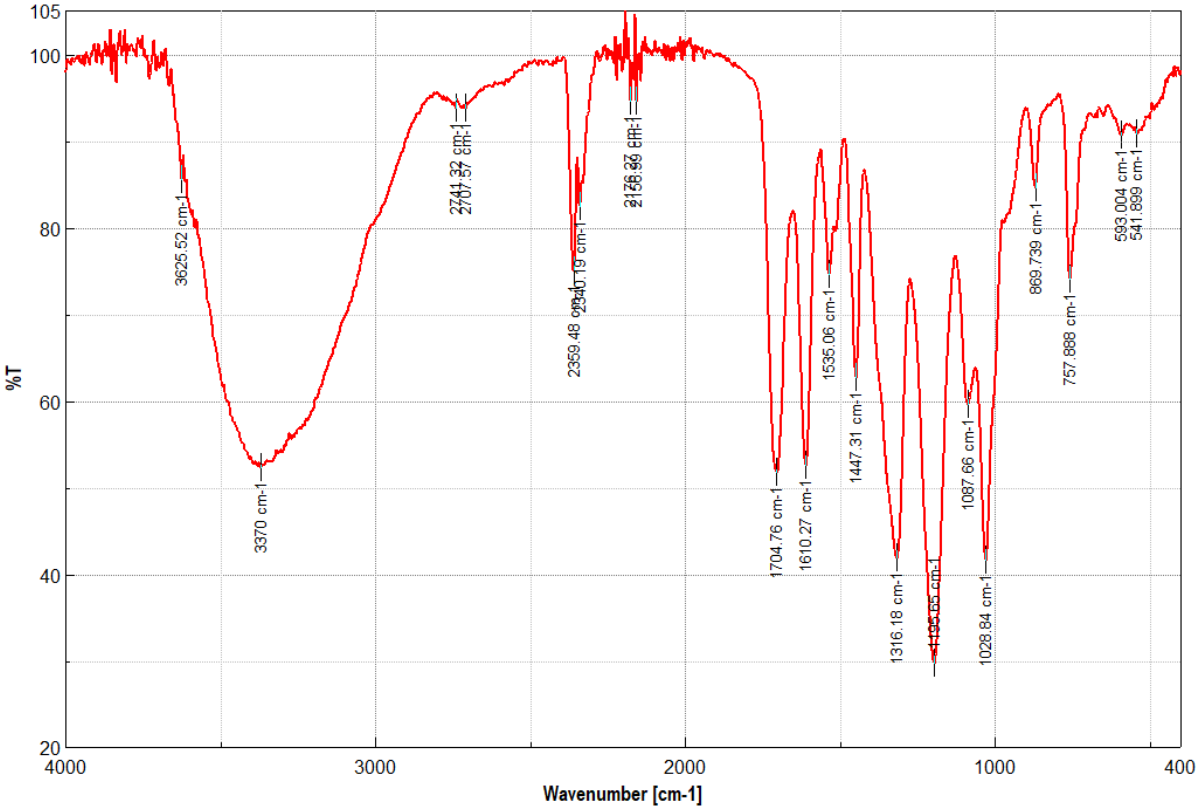

Figure S4: Infrared spectrum of 3-O-digalloyl-1,2,4,6-tetra-O-galloyl-β-D-glucose (NMB4)

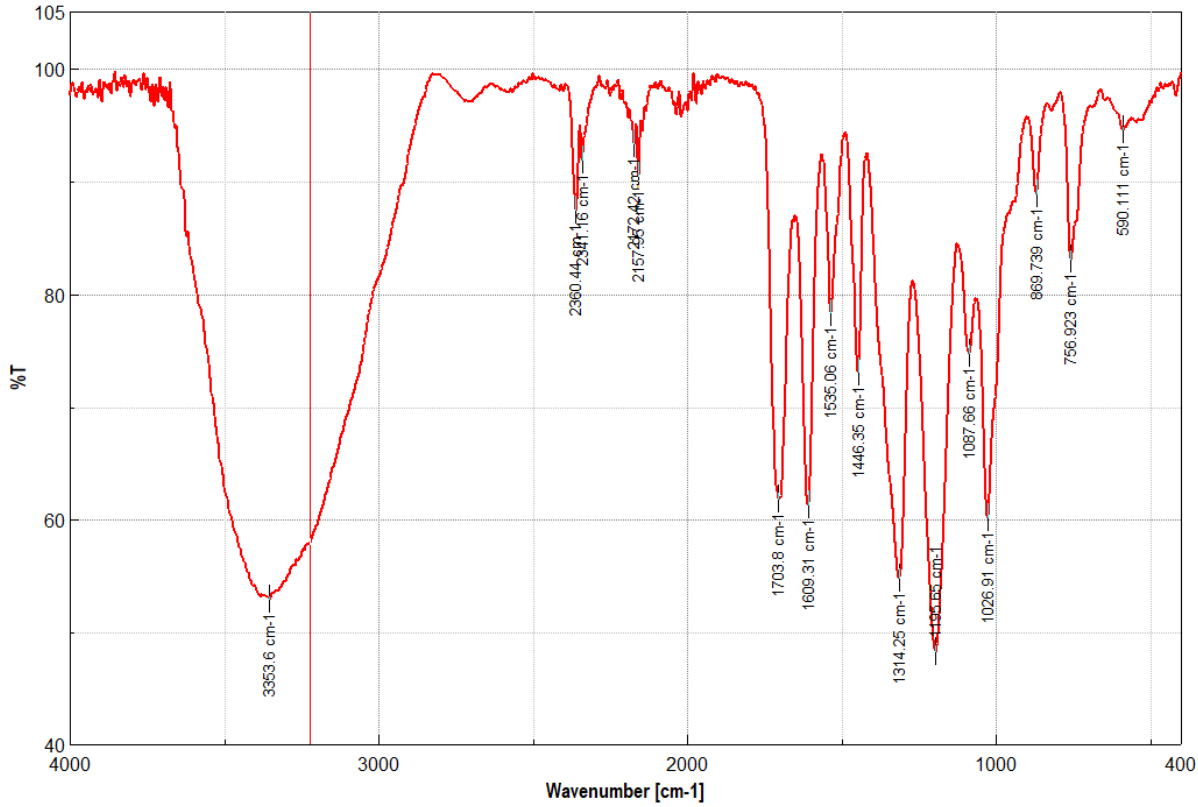

Figure S5: Infrared spectrum of 6-O-digalloyl-1,2,3,4-tetra-O-galloyl-β-D-glucose (NMB6)

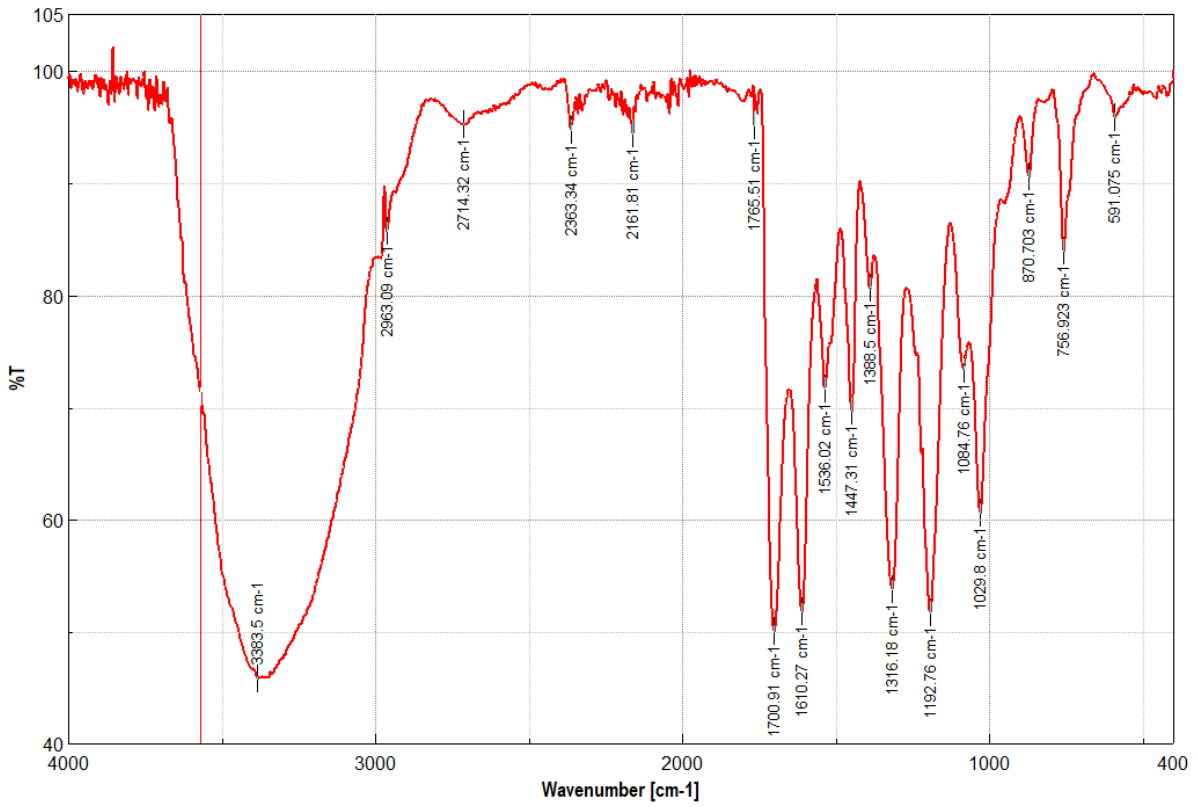

Figure S6: Infrared spectrum of 3,6-bis-O-digalloyl-1,2,4-tri-O-galloyl-β-D-glucose (NMC3)

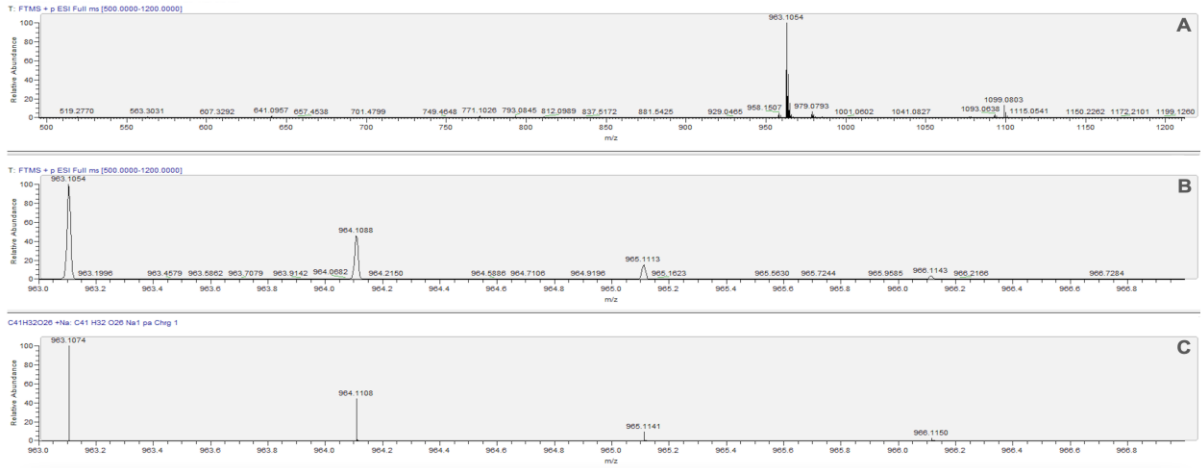

Figure S7: HR-ESI-MS spectra of 1,2,3,4,6-penta-O-galloyl-β-D-glucose (NMA2) showing the full mass spectrum (A), zoom in of the major peak region (B) and simulated spectrum for the shown molecular formula (C).

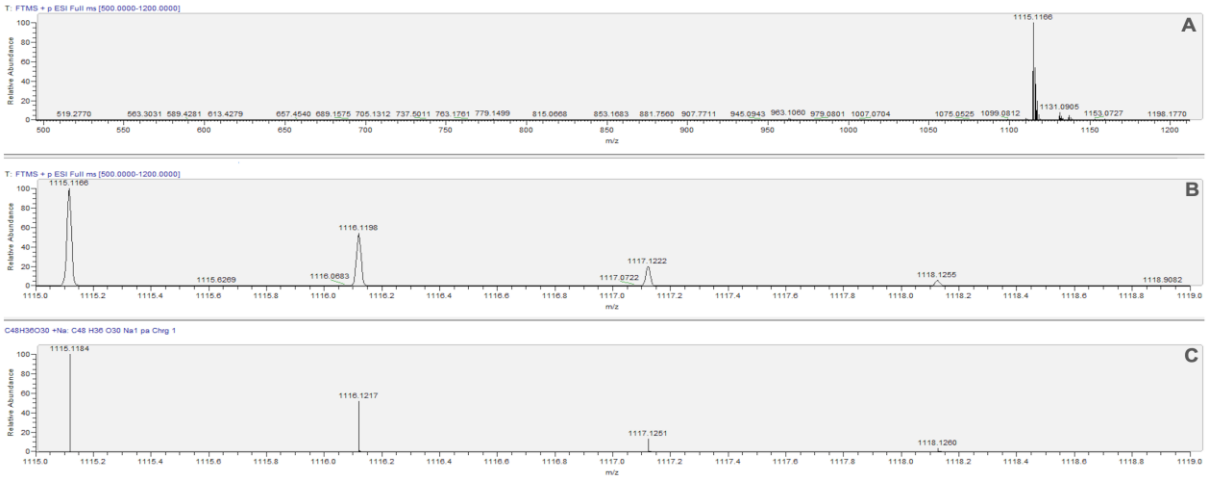

Figure S8: HR-ESI-MS spectra of 3-O-digalloyl-1,2,4,6-tetra-O-galloyl-β-D-glucose (NMB4) showing the full mass spectrum (A), zoom in of the major peak region (B) and simulated spectrum for the shown molecular formula (C).

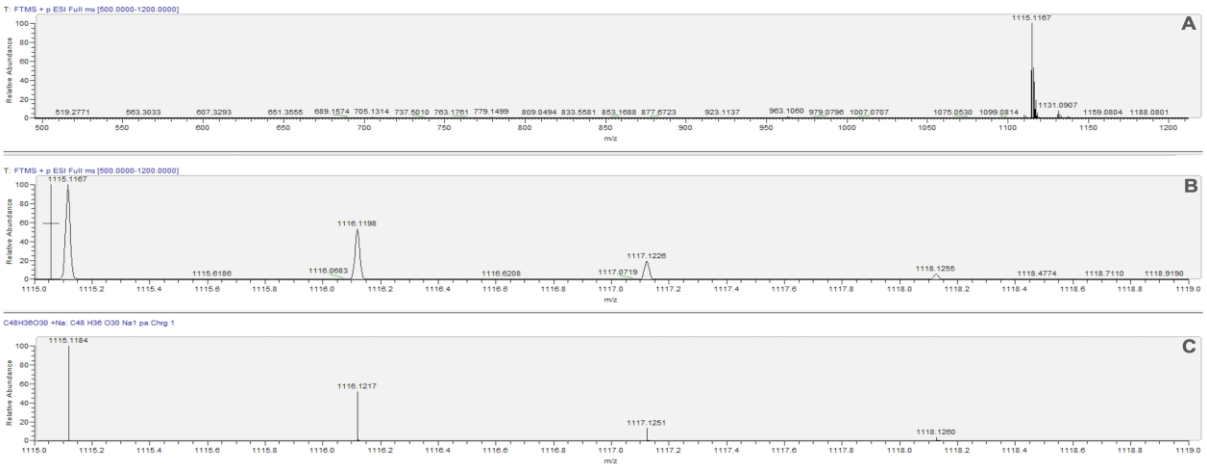

Figure S9: HR-ESI-MS spectra of 6-O-digalloyl-1,2,3,4-tetra-O-galloyl-β-D-glucose (NMB6) showing the full mass spectrum (A), zoom in of the major peak region (B) and simulated spectrum for the shown molecular formula (C).

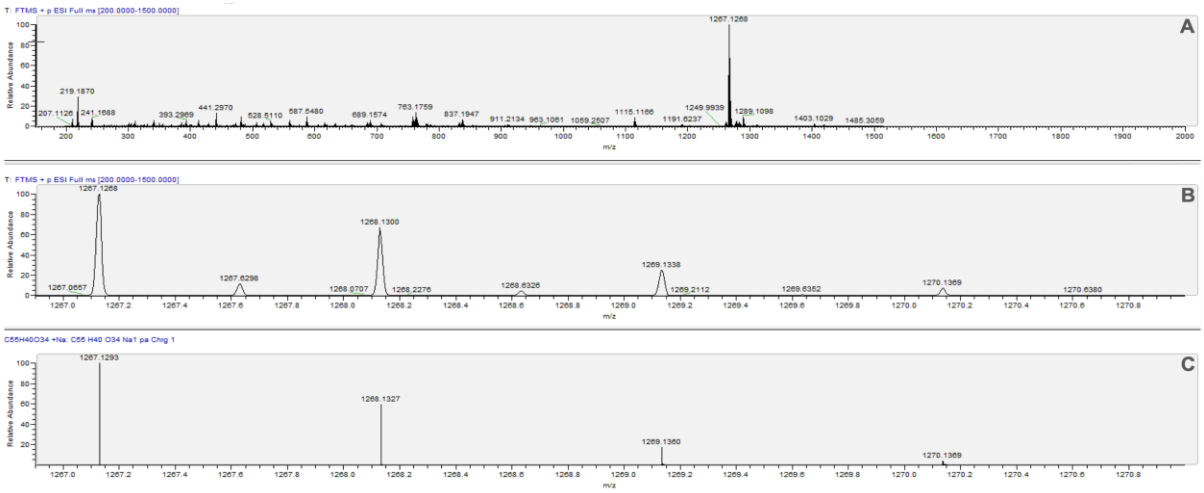

Figure S10: HR-ESI-MS spectra of 3,6-bis-O-digalloyl-1,2,4-tri-O-galloyl-β-D-glucose(NMC3) showing the full mass spectrum (A), zoom in of the major peak region (B) and simulated spectrum for the shown molecular formula (C).

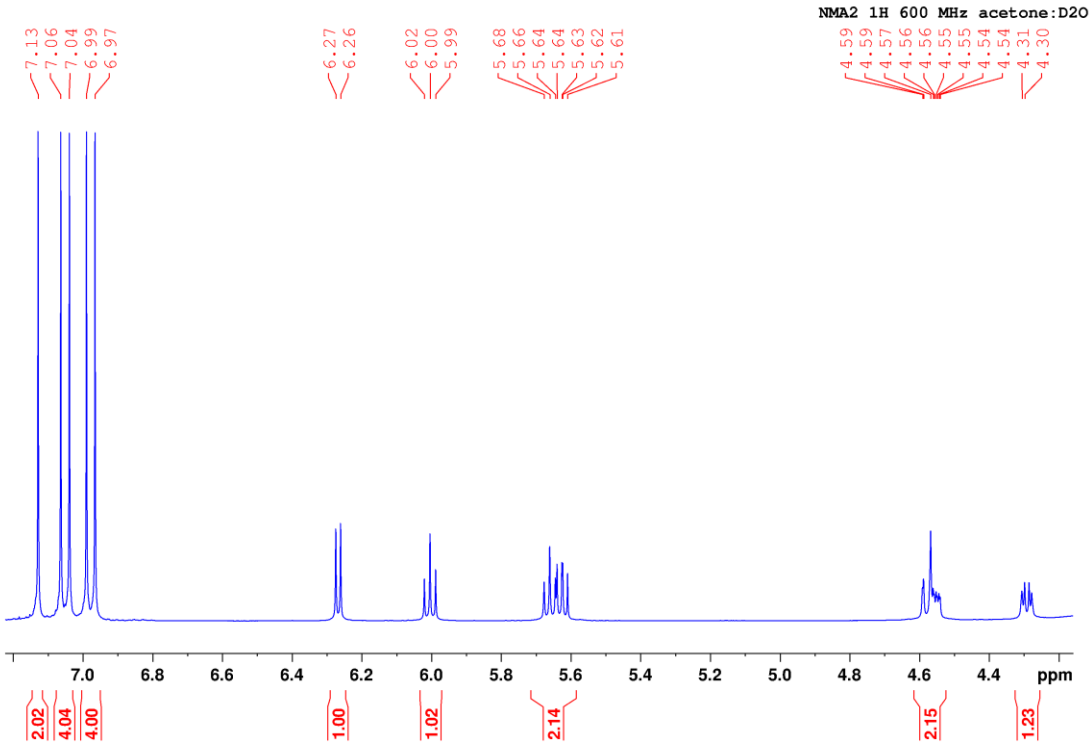

Figure S11: ^{1}H spectrum of 1,2,3,4,6-penta-O-galloyl-β-D-glucose (NMA2)

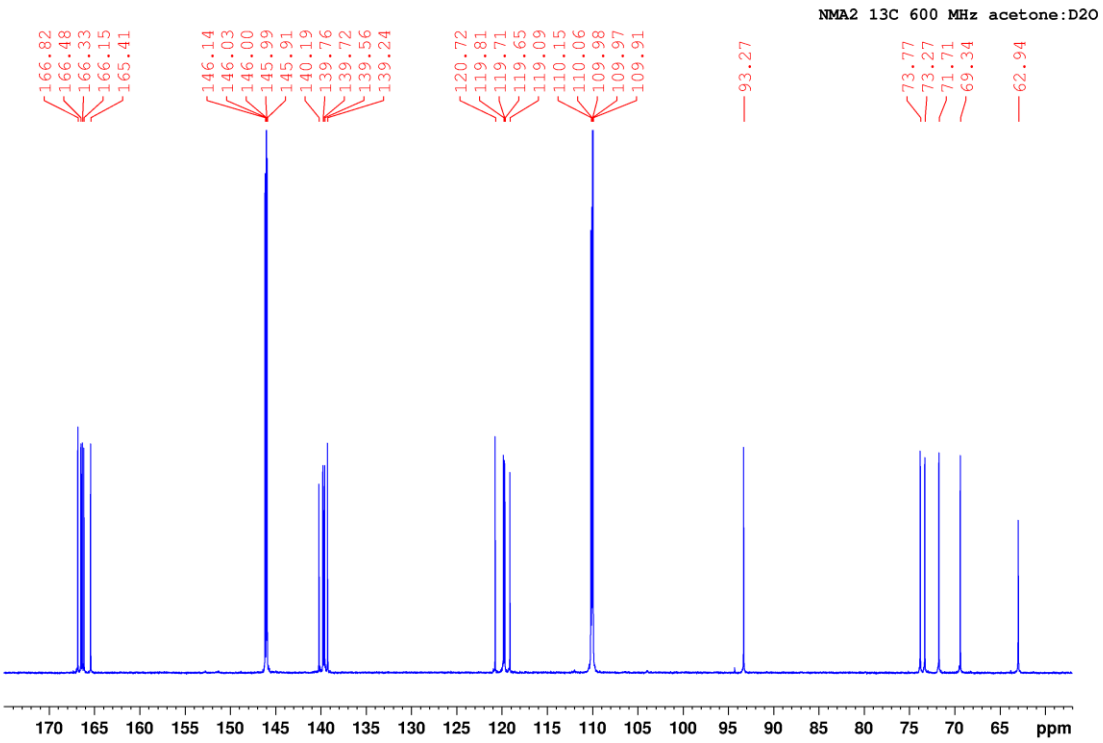

Figure S12: ^{13}C spectrum of 1,2,3,4,6-penta-O-galloyl-β-D-glucose (NMA2)

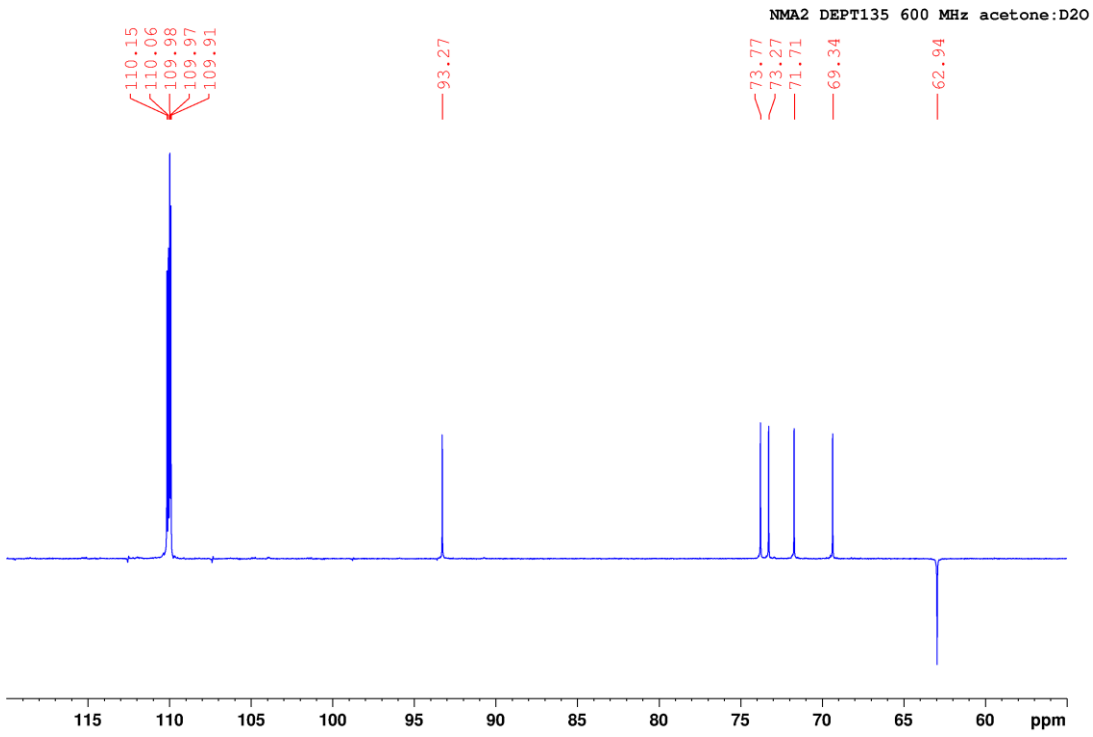

Figure S13: DEPT- 135 spectrum of 1,2,3,4,6-penta-O-galloyl-β-D-glucose (NMA2)

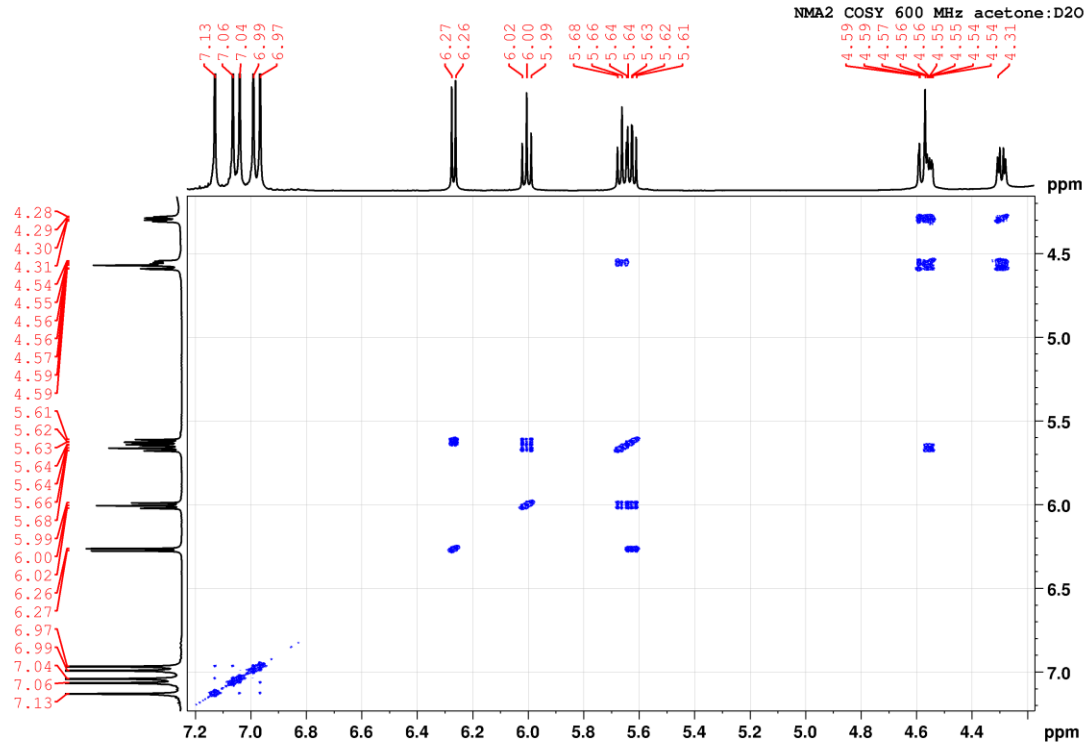

Figure S14: COSY spectrum of 1,2,3,4,6-penta-O-galloyl-β-D-glucose (NMA2)

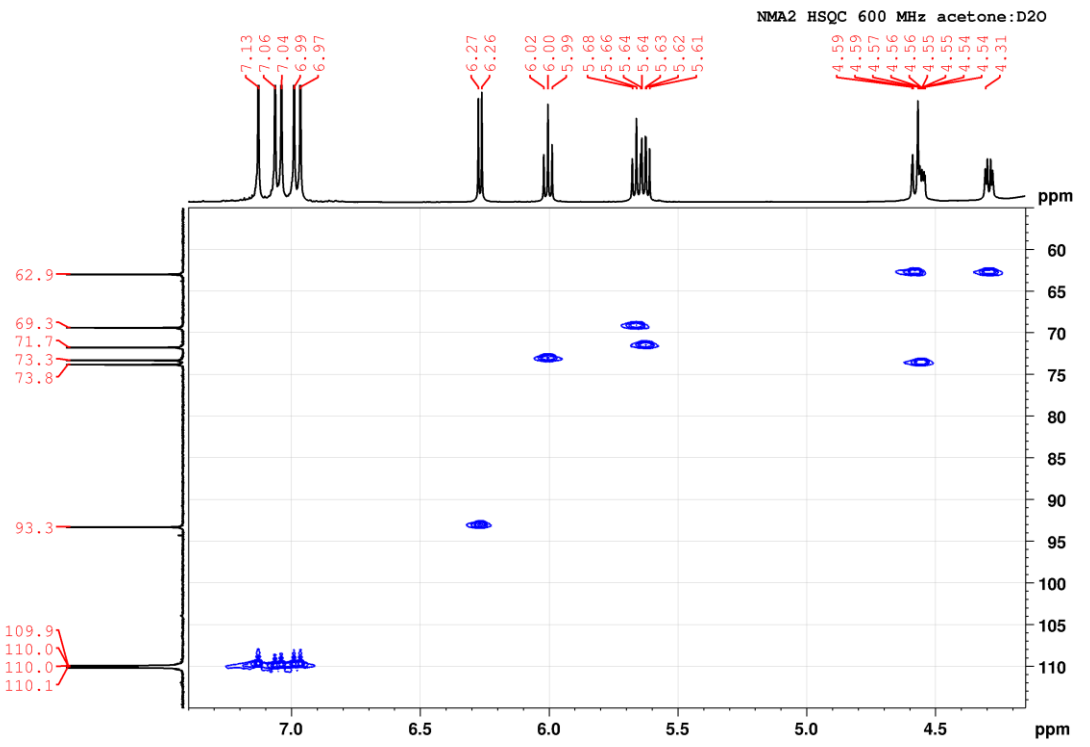

Figure S15: HSQC spectrum of 1,2,3,4,6-penta-O-galloyl-β-D-glucose (NMA2)

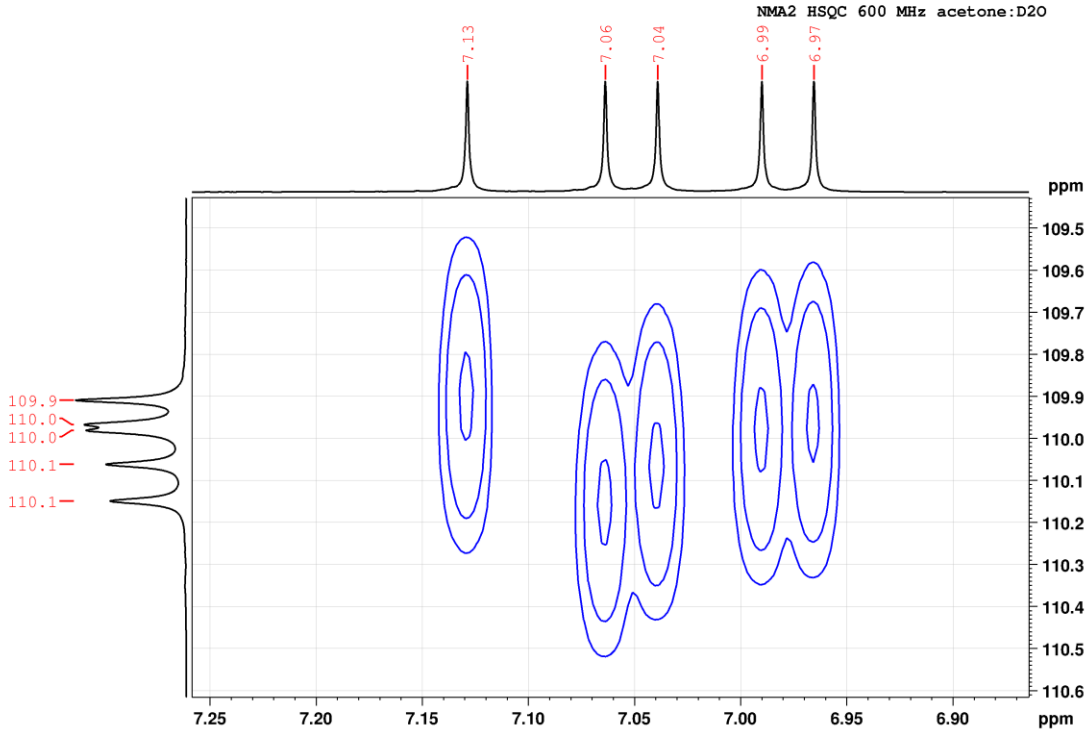

Figure S16: Zoom in of the aromatic aromatic region of HSQC spectrum of 1,2,3,4,6-penta-O-galloyl-β-D-glucose (NMA2)

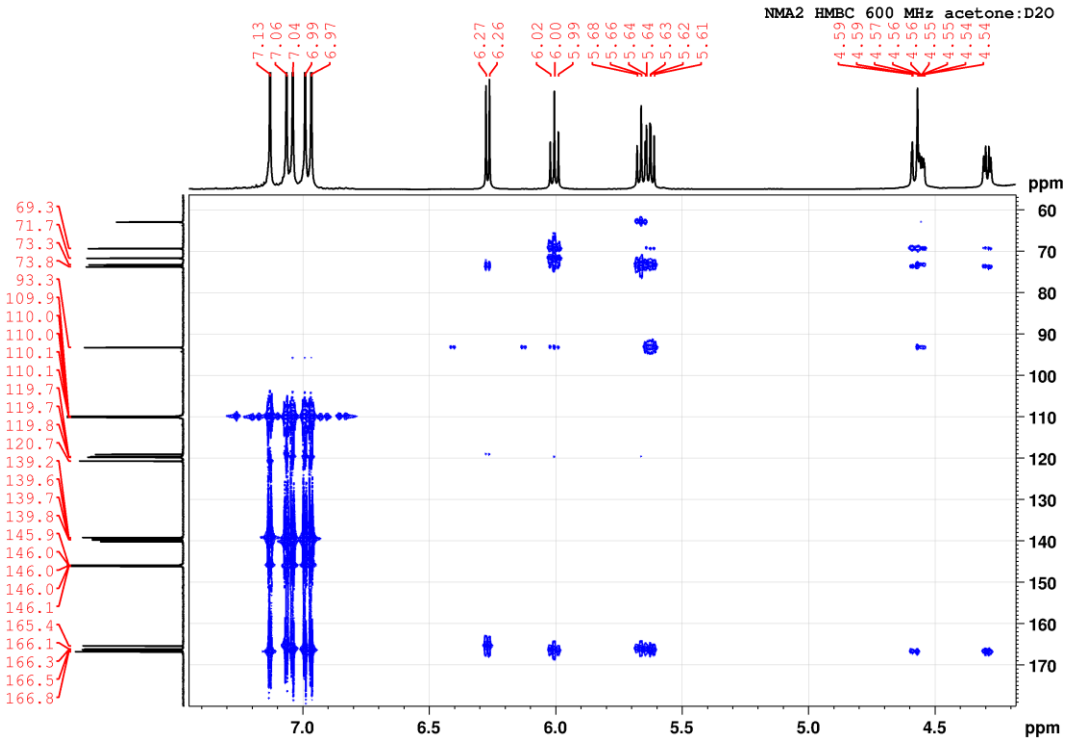

Figure S17: HMBC spectrum of 1,2,3,4,6-penta-O-galloyl-β-D-glucose (NMA2)

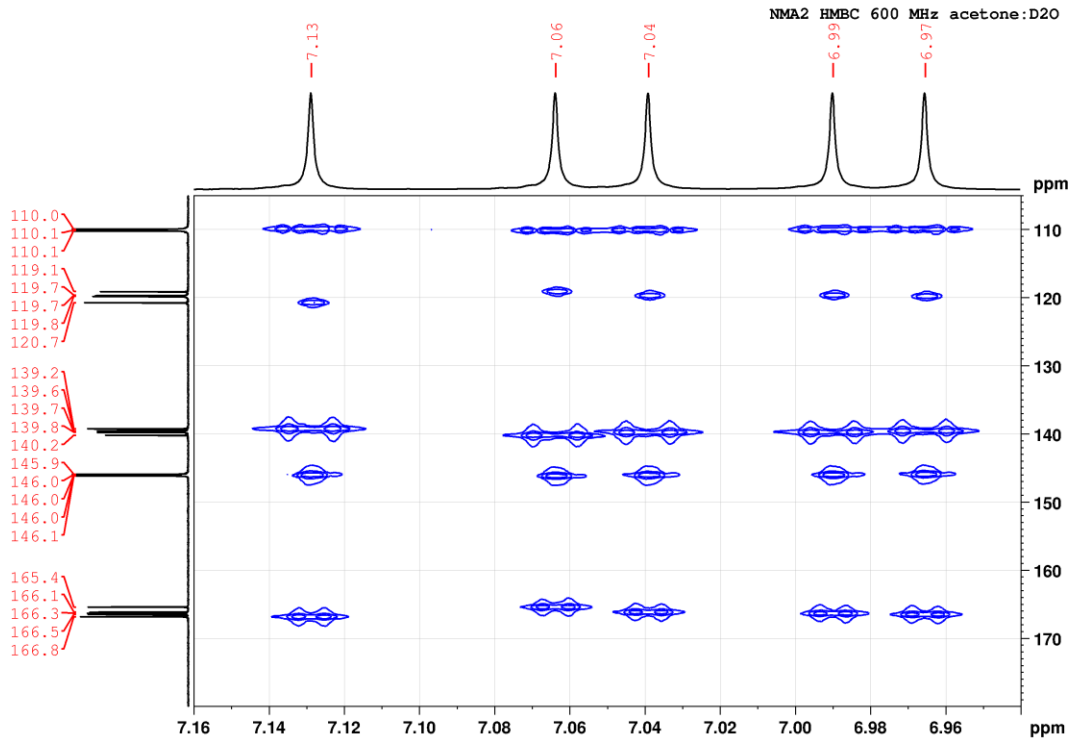

Figure S18: Zoom in of the aromatic region of HMBC spectrum of 1,2,3,4,6-penta-Ogalloyl-β-D-glucose (NMA2)

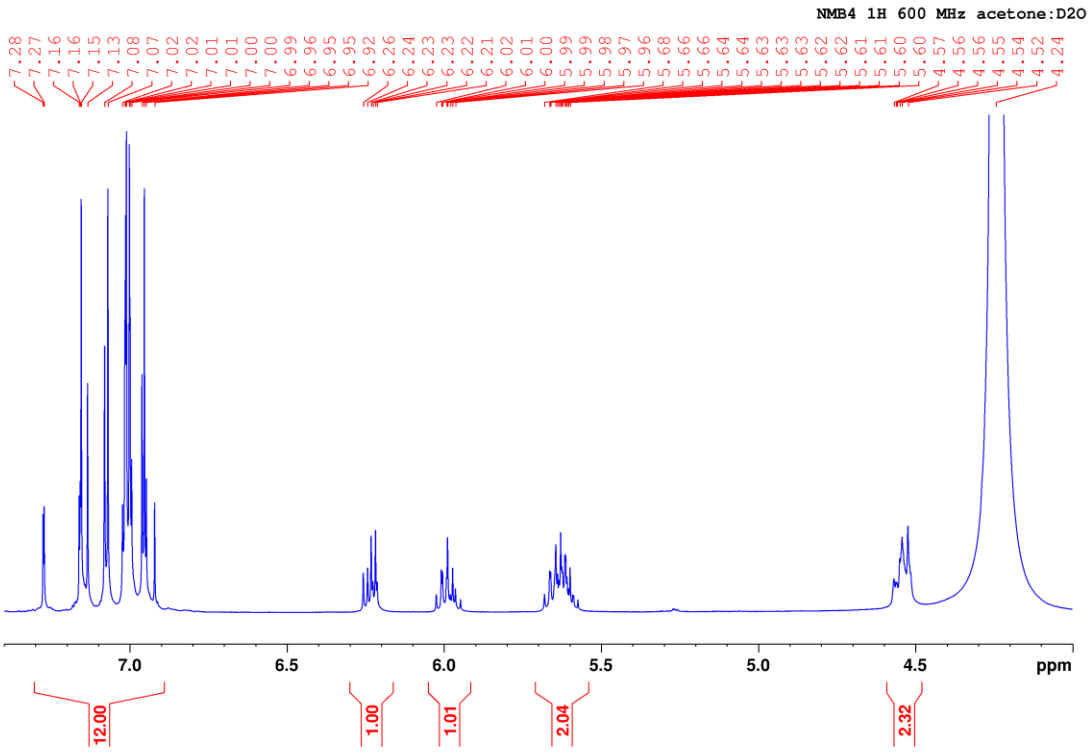

Figure S19: ^{1}H spectrum of 3-O-digalloyl-1,2,4,6-tetra-O-galloyl-β-D-glucose (NMB4)

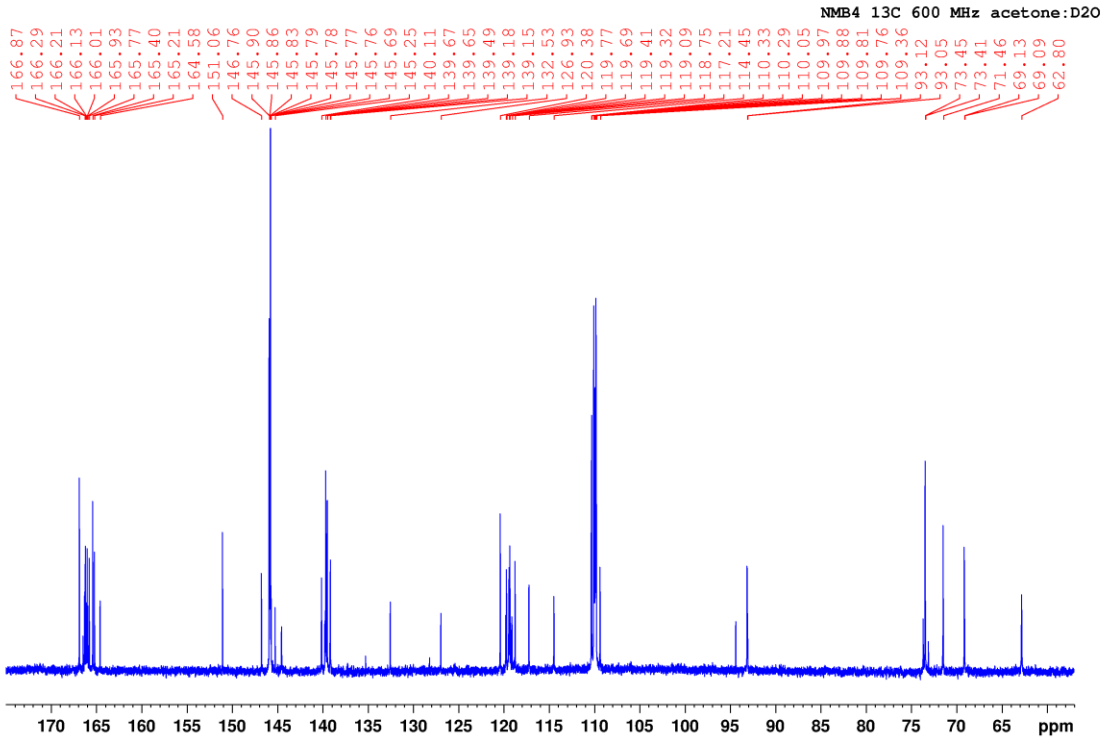

Figure S20: ^{13}C spectrum of 3-O-digalloyl-1,2,4,6-tetra-O-galloyl-β-D-glucose (NMB4)

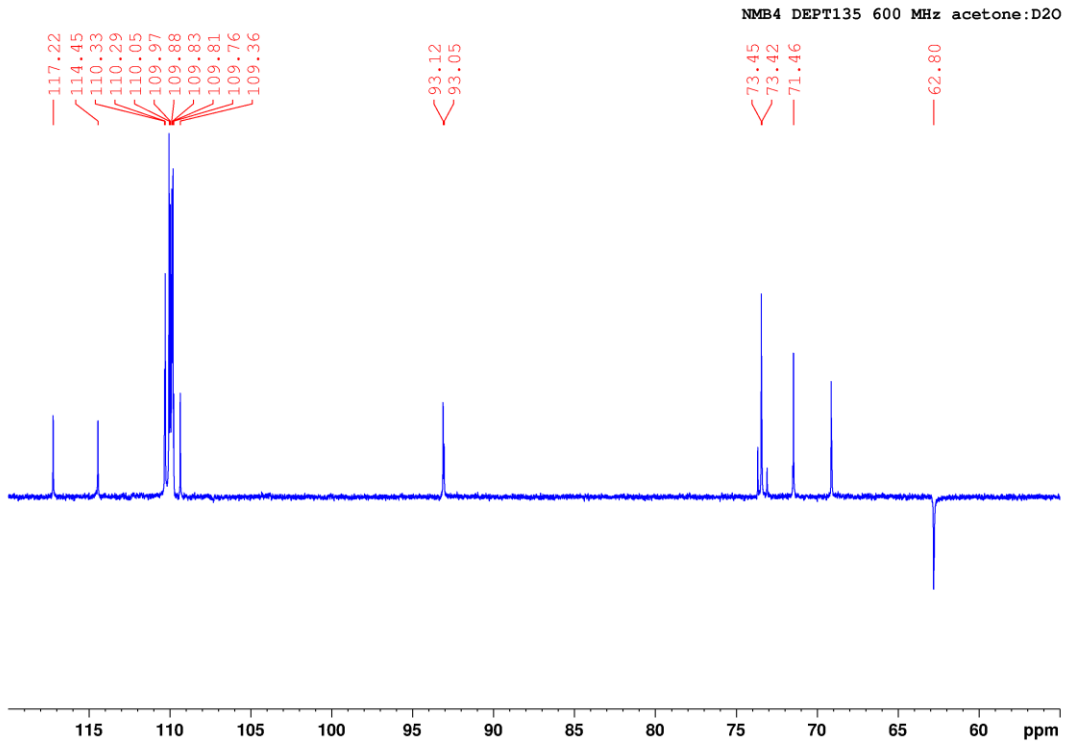

Figure S21: DEPT-135 of 3-O-digalloyl-1,2,4,6-tetra-O-galloyl-β-D-glucose (NMB4)

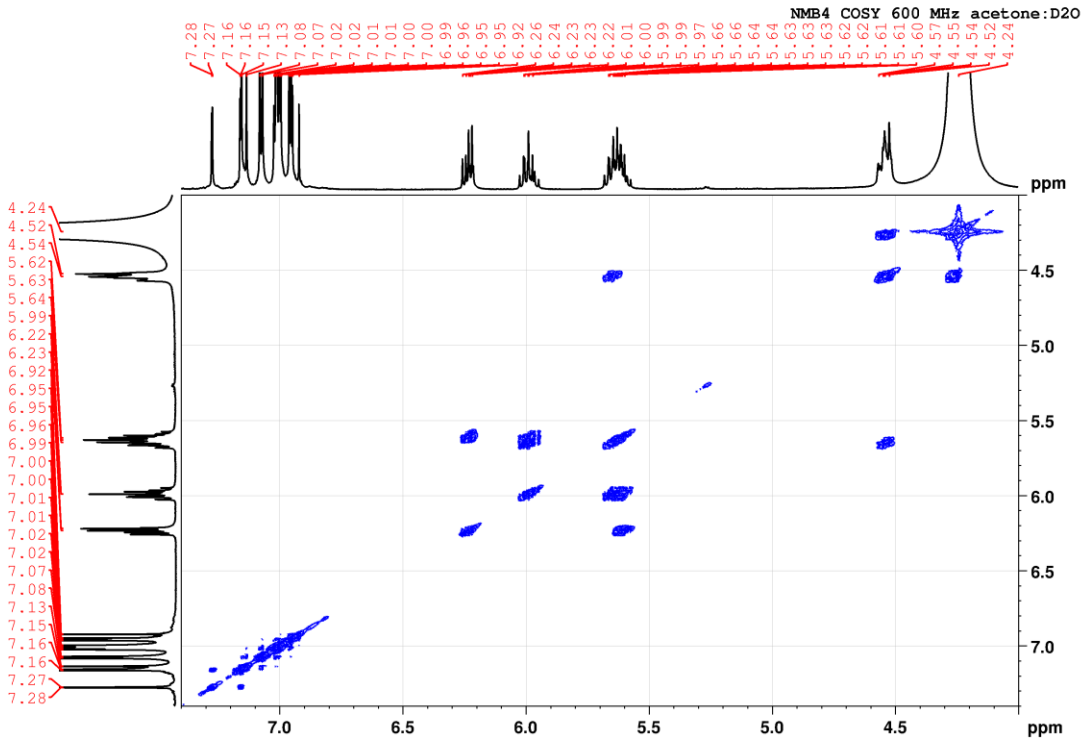

Figure S22: COSY Spectrum of 3-O-digalloyl-1,2,4,6-tetra-O-galloyl-β-D-glucose (NMB4)

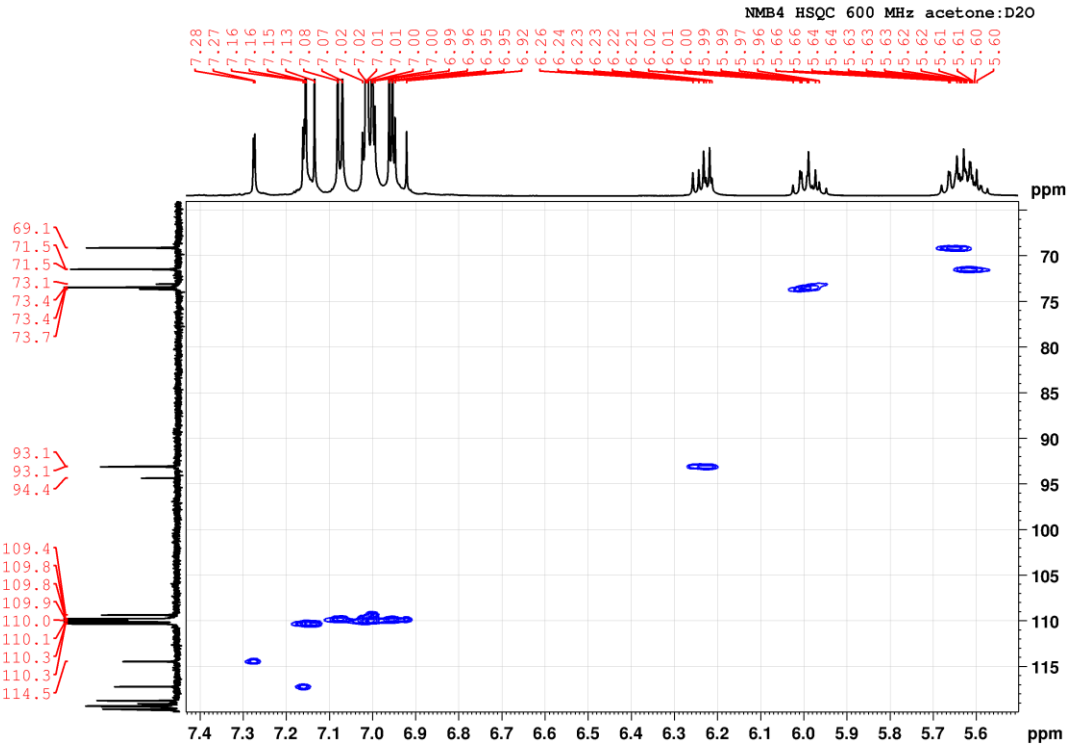

Figure S23: HSQC spectrum of 3-O-digalloyl-1,2,4,6-tetra-O-galloyl-β-D-glucose (NMB4)

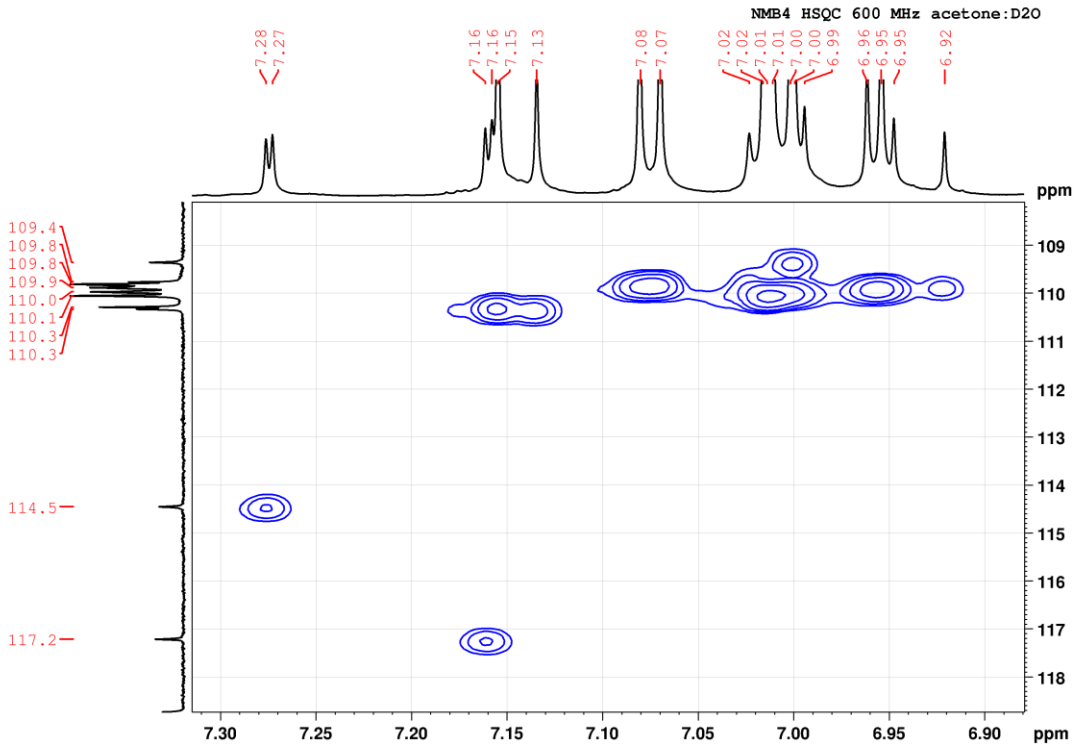

Figure S24: Zoom in of the aromatic region of HSQC spectrum of 3-O-digalloyl-1,2,4,6-tetra-O-galloyl-β-D-glucose (NMB4)

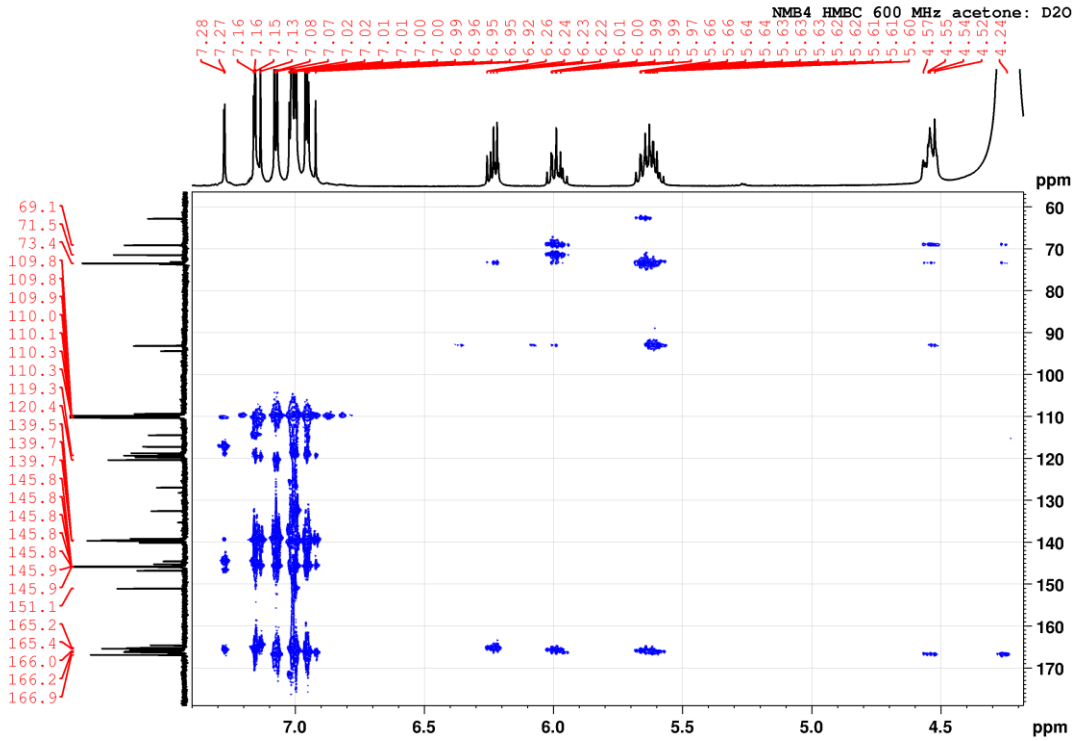

Figure S25: HMBC spectrum of 3-O-digalloyl-1,2,4,6-tetra-O-galloyl-β-D-glucose (NMB4)

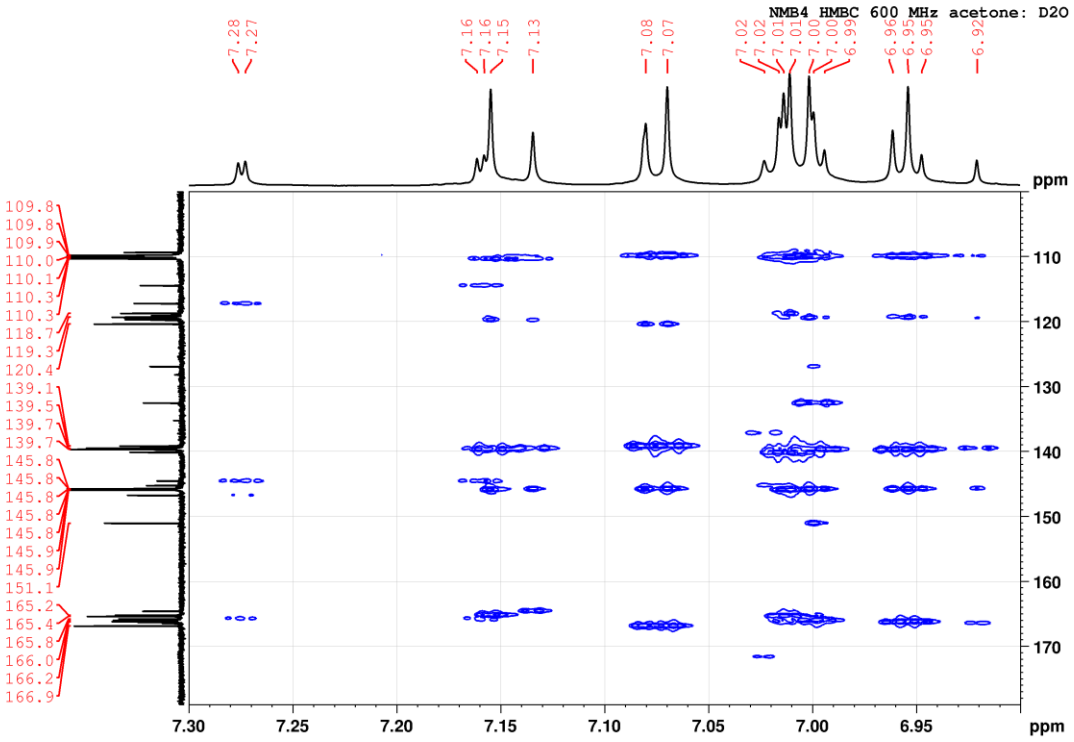

Figure S26: Zoom in of the aromatic region of HMBC spectrum of 3-O-digalloyl-1,2,4,6-tetra-O-galloyl-β-D-glucose (NMB4)

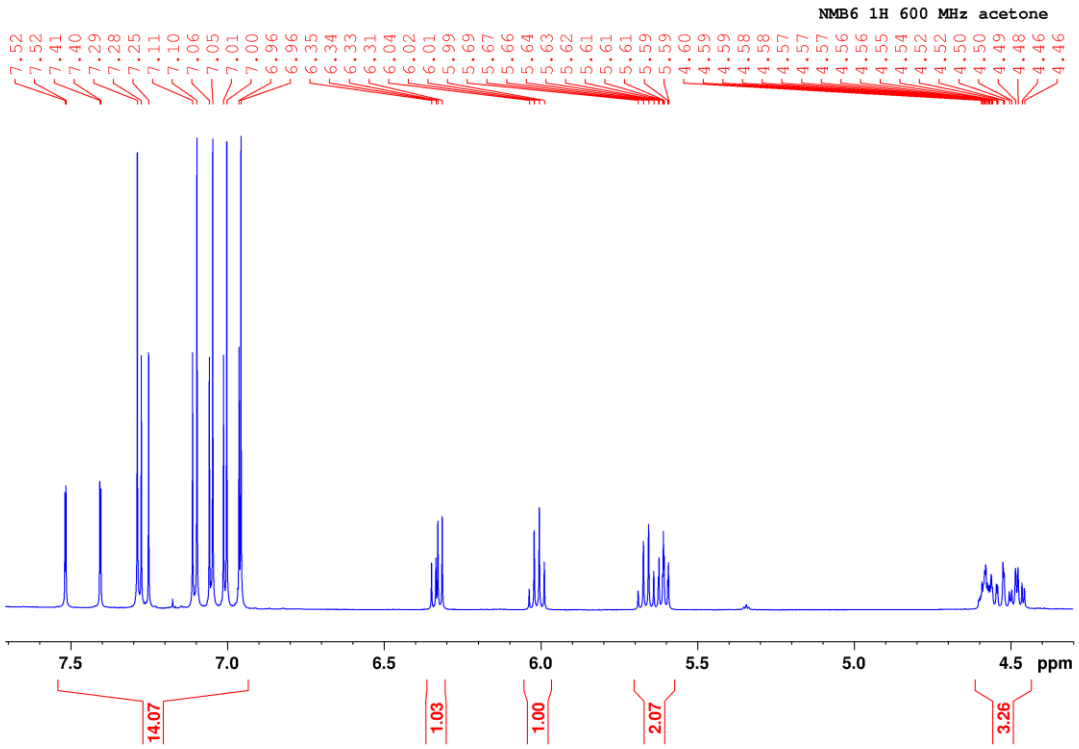

Figure S27: 1H spectrum of 6-O-digalloyl-1,2,3,4-tetra-O-galloyl-β-D-glucose (NMB6)

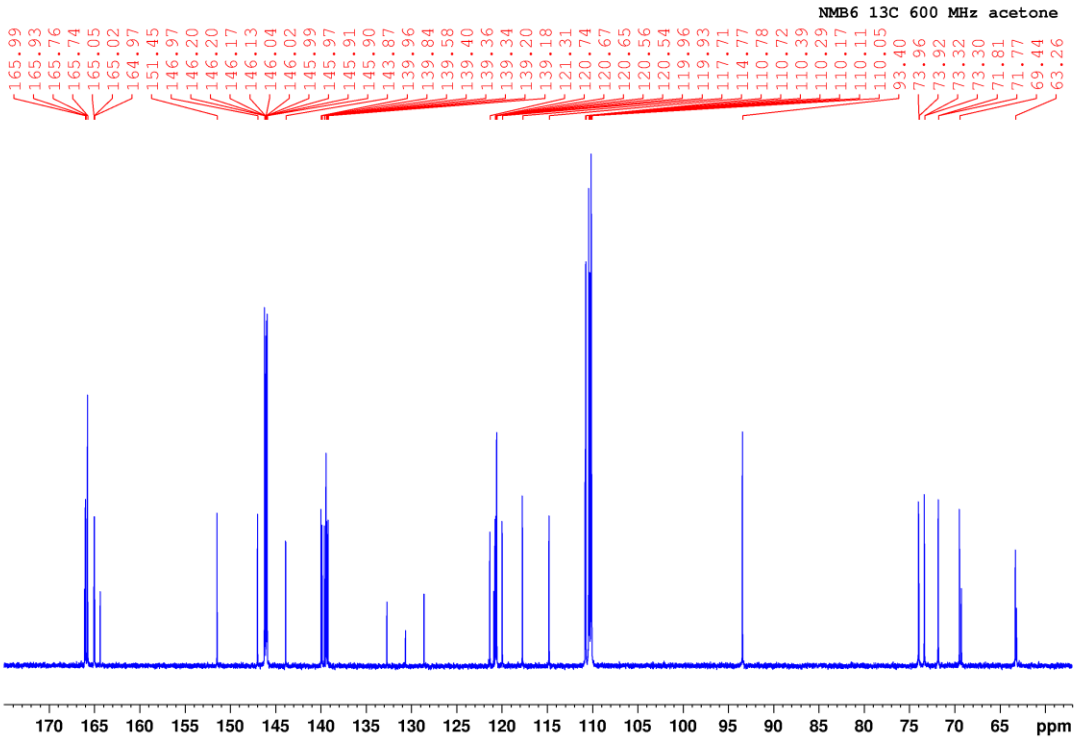

Figure S28: ^{13}C spectrum of 6-O-digalloyl-1,2,3,4-tetra-O-galloyl-β-D-glucose (NMB6)

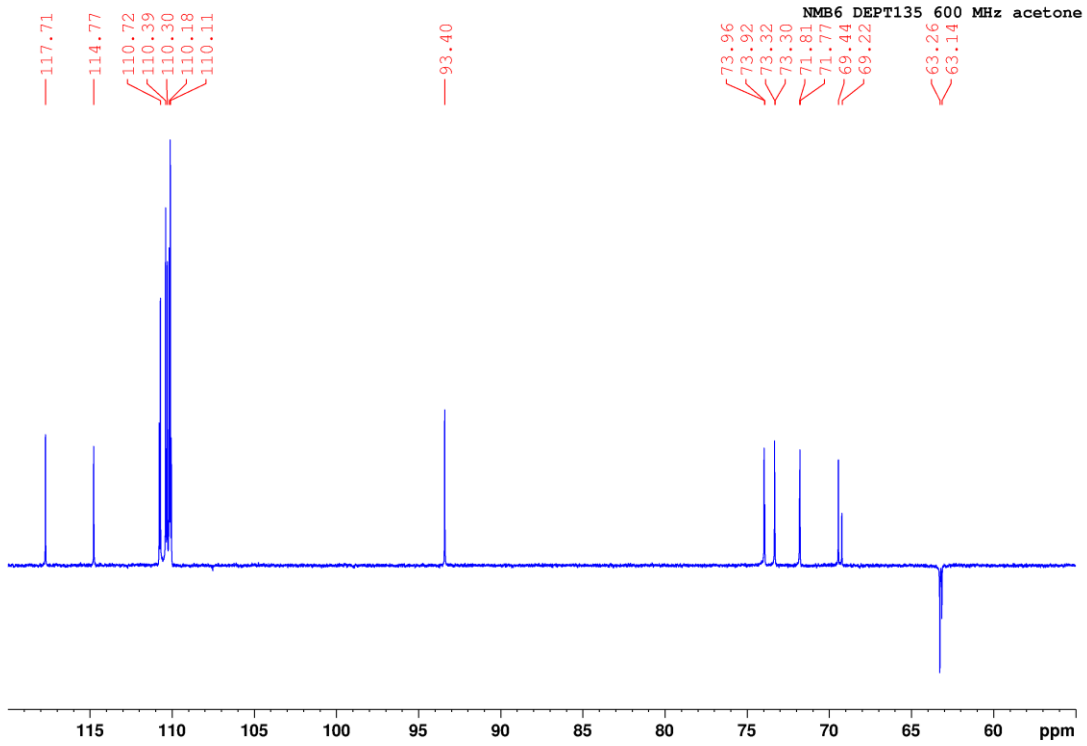

Figure S29: DEPT135 spectrum of 6-O-digalloyl-1,2,3,4-tetra-O-galloyl-β-D-glucose (NMB6)

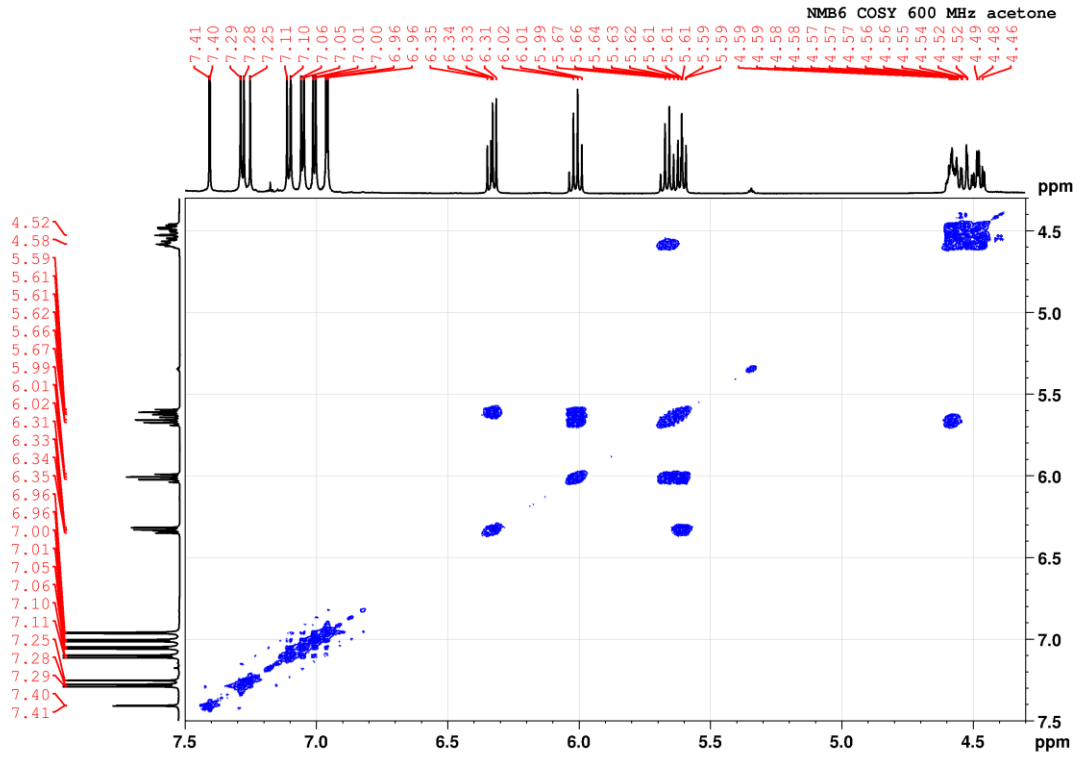

Figure S30: COSY spectrum of 6-O-digalloyl-1,2,3,4-tetra-O-galloyl-β-D-glucose (NMB6)

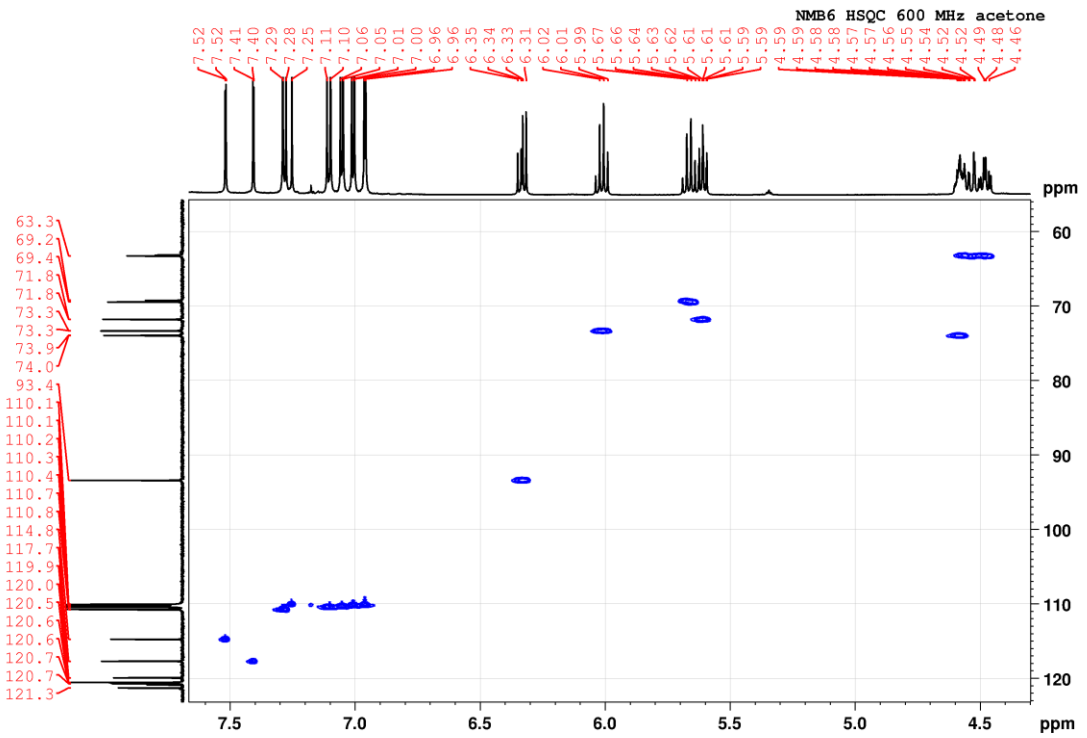

Figure S31: HSQC spectrum of 6-O-digalloyl-1,2,3,4-tetra-O-galloyl-β-D-glucose (NMB6)

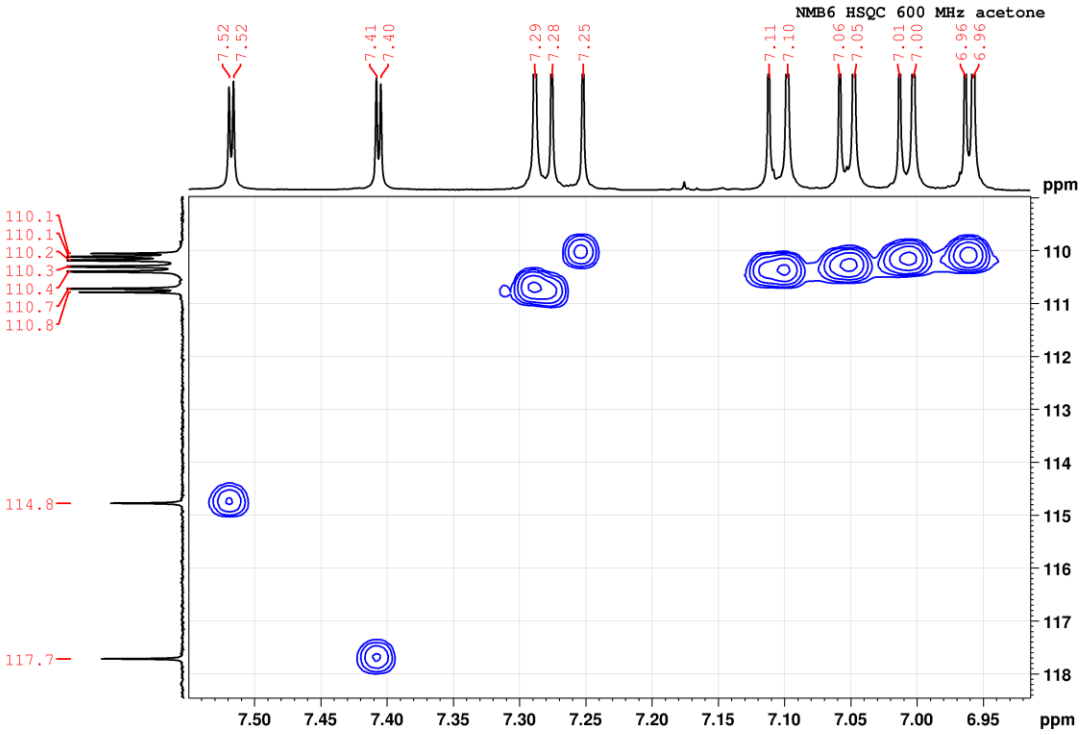

Figure S32: Zoom in of the aromatic region of HSQC spectrum of 6-O-digalloyl-1,2,3,4-tetra-O-galloyl-β-D-glucose (NMB6)

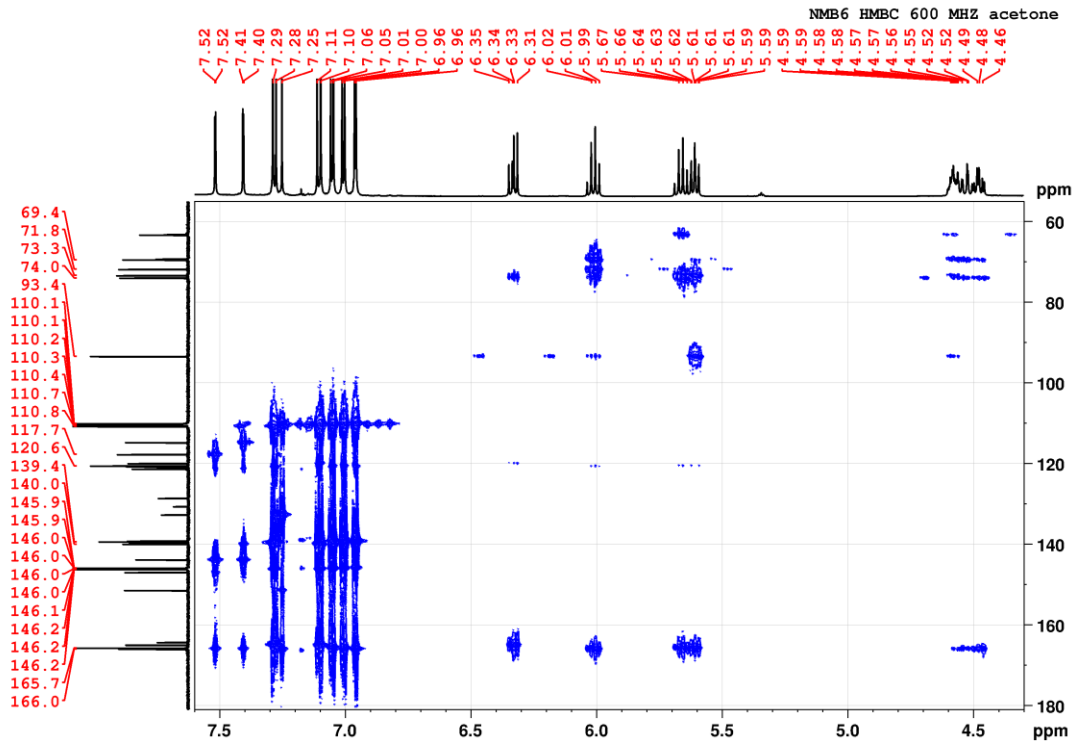

Figure S33: HMBC spectrum of 6-O-digalloyl-1,2,3,4-tetra-O-galloyl-β-D-glucose (NMB6)

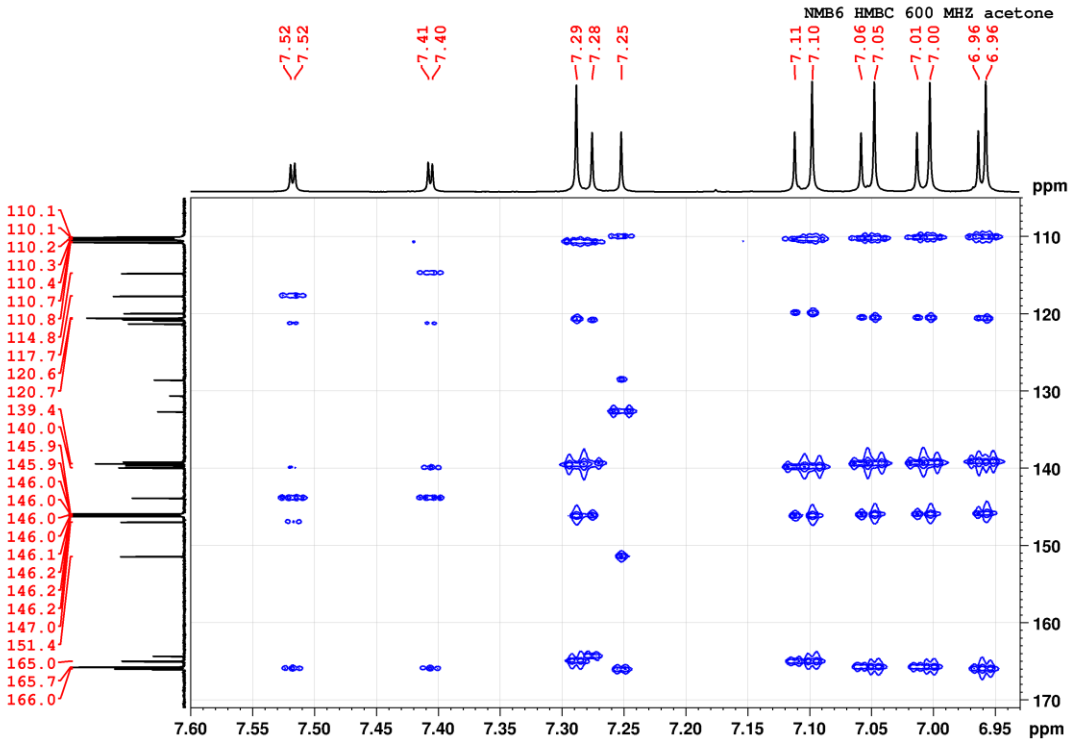

Figure S34: Zoom in of the aromatic region of HMBC spectrum of 6-O-digalloyl-1,2,3,4-tetra-O-galloyl-β-D-glucose (NMB6)

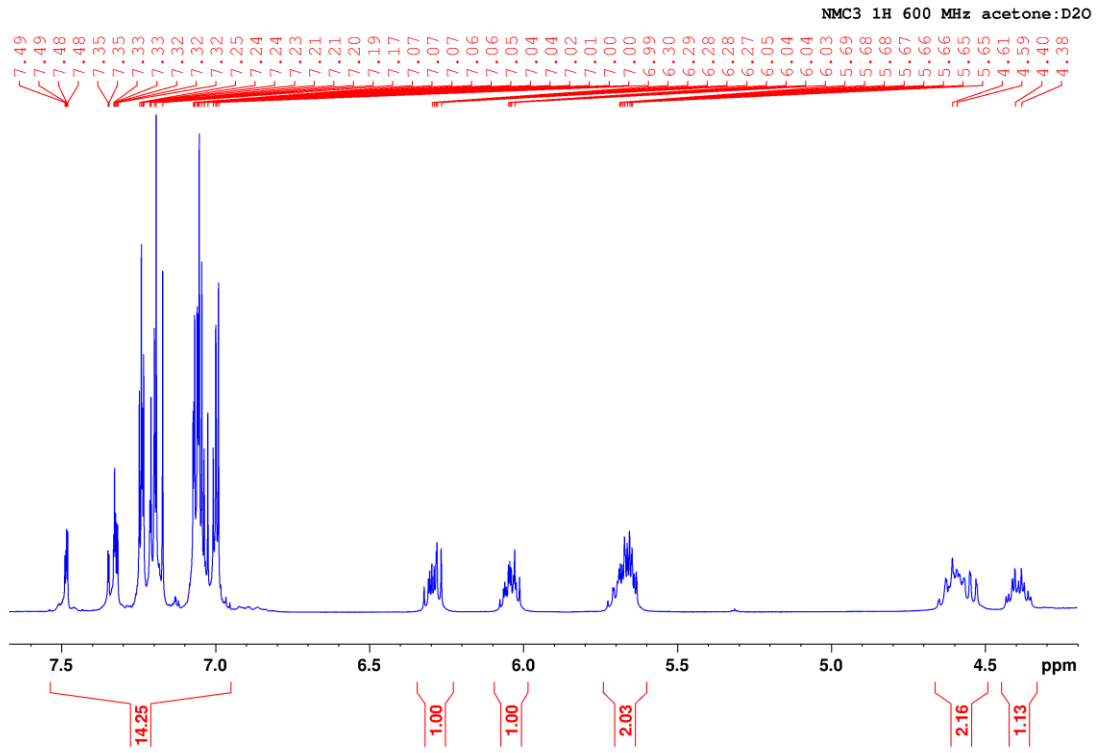

Figure S35: ^{1}H spectrum of 3,6-bis-O-digalloyl-1,2,4-tri-O-galloyl-β-D-glucose (NMC3)

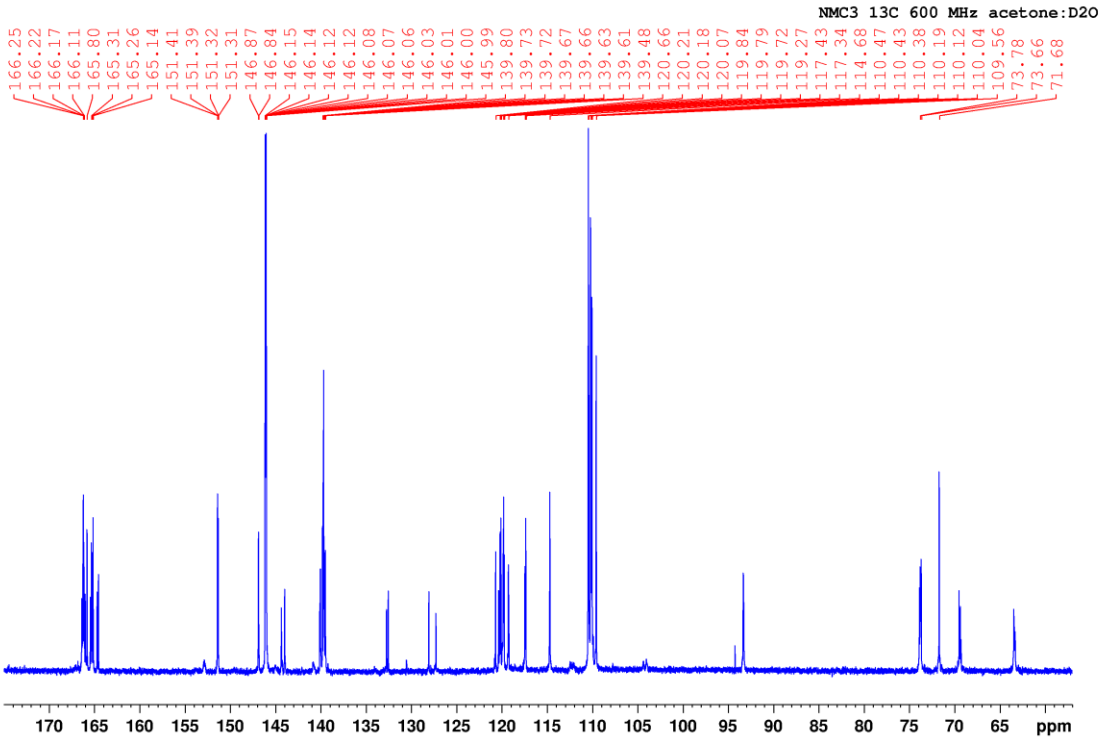

Figure S36: ^{13}C spectrum of 3,6-bis-O-digalloyl-1,2,4-tri-O-galloyl-β-D-glucose (NMC3)

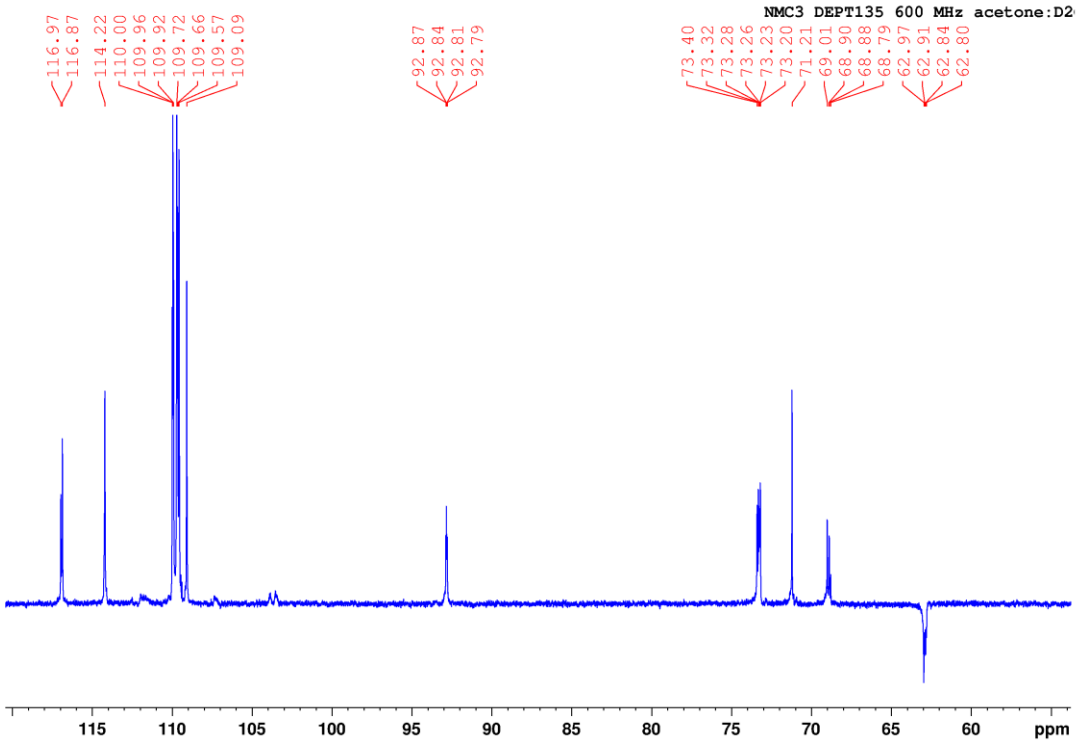

Figure S37: DEPT-135 spectrum of 3,6-bis-O-digalloyl-1,2,4-tri-O-galloyl-β-Dglucose (NMC3)

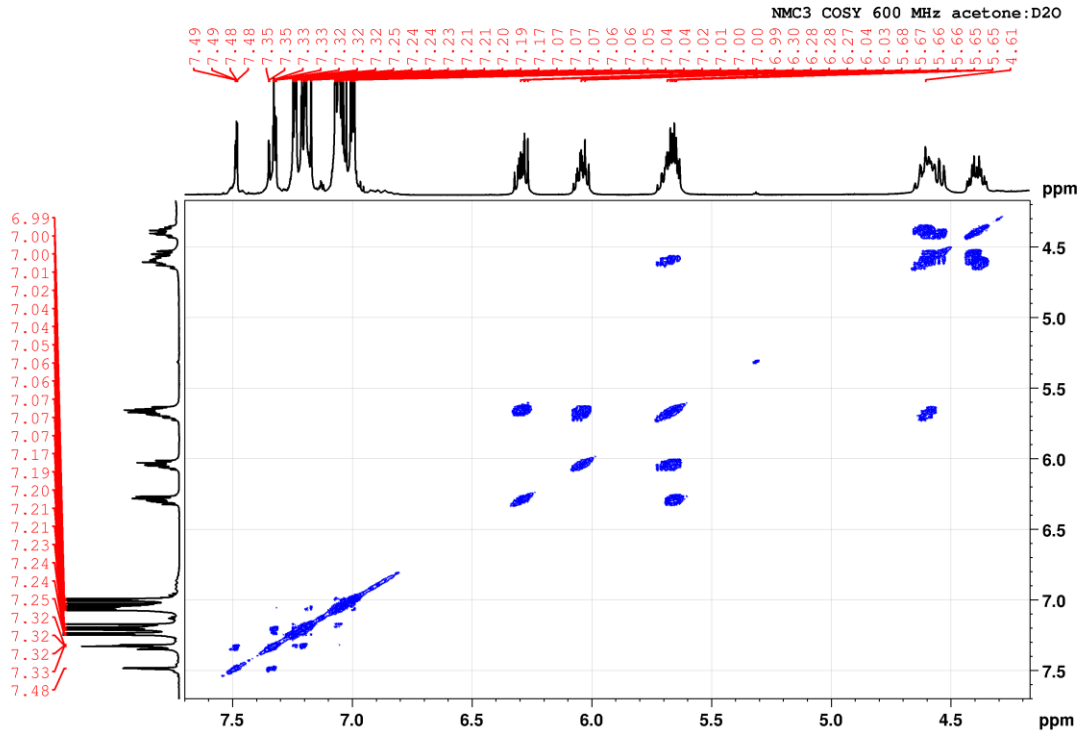

Figure S38: COSY spectrum of 3,6-bis-O-digalloyl-1,2,4-tri-O-galloyl-β-D-glucose (NMC3)

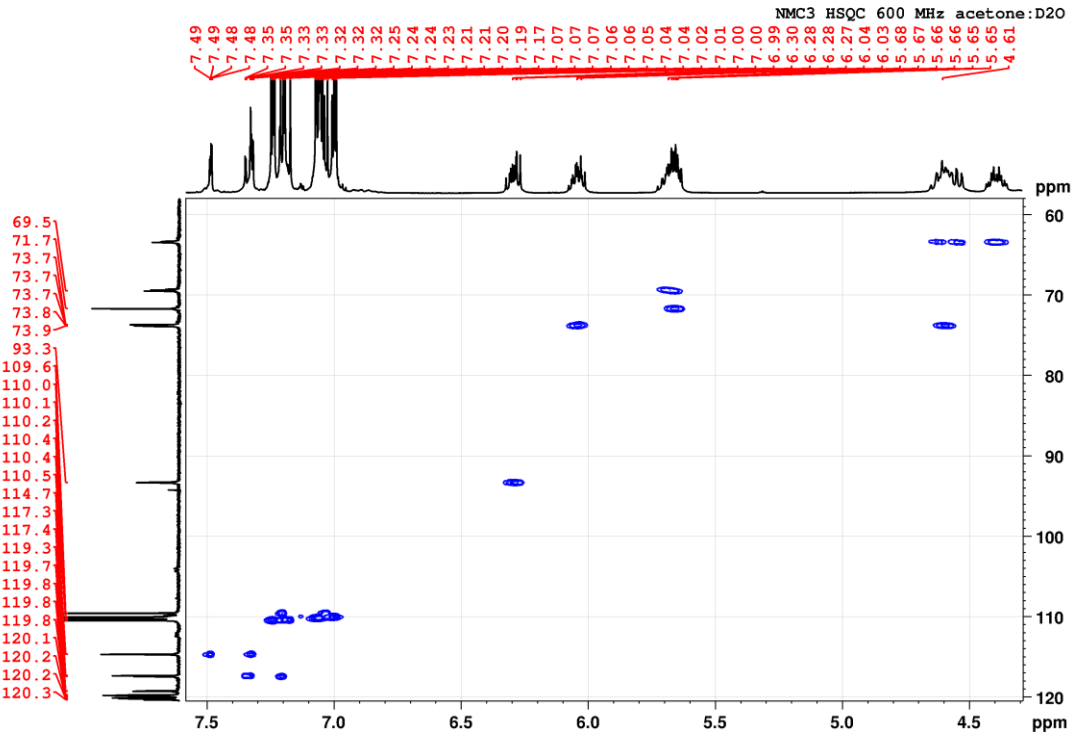

Figure S39: HSQC spectrum of 3,6-bis-O-digalloyl-1,2,4-tri-O-galloyl-β-D-glucose (NMC3)

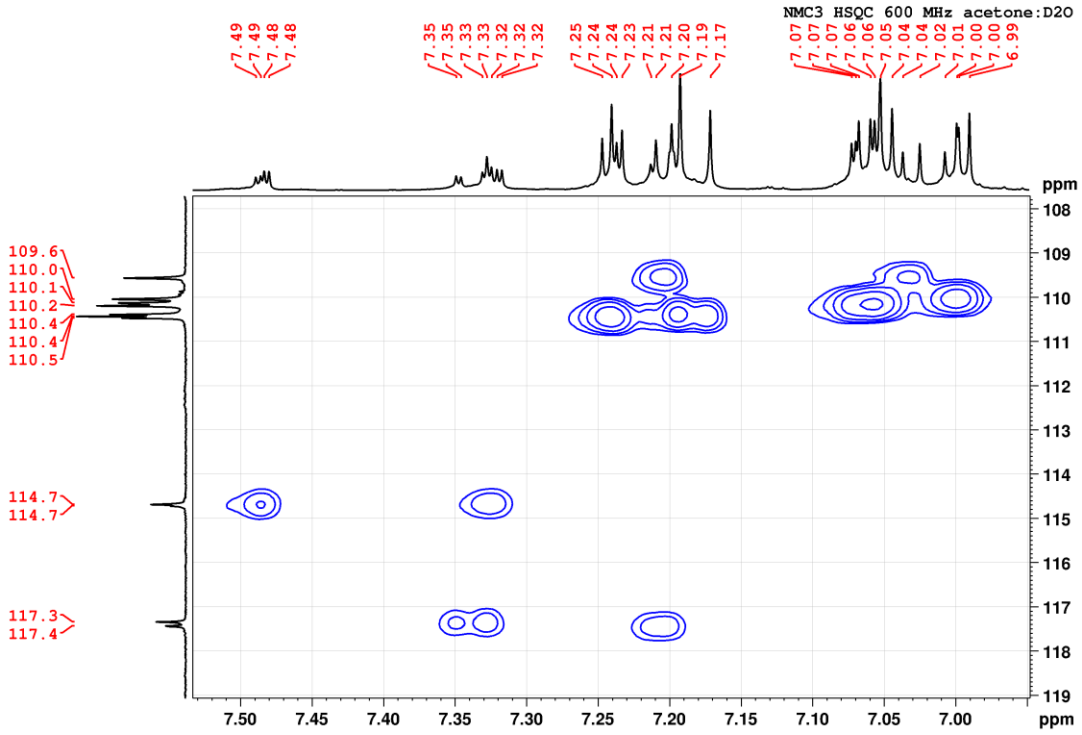

Figure S40: Zoom in of the aromatic region of the HSQC spectrum of 6-O-digalloyl-1,2,3,4-tetra-O-galloyl-β-D-glucose (NMB6)

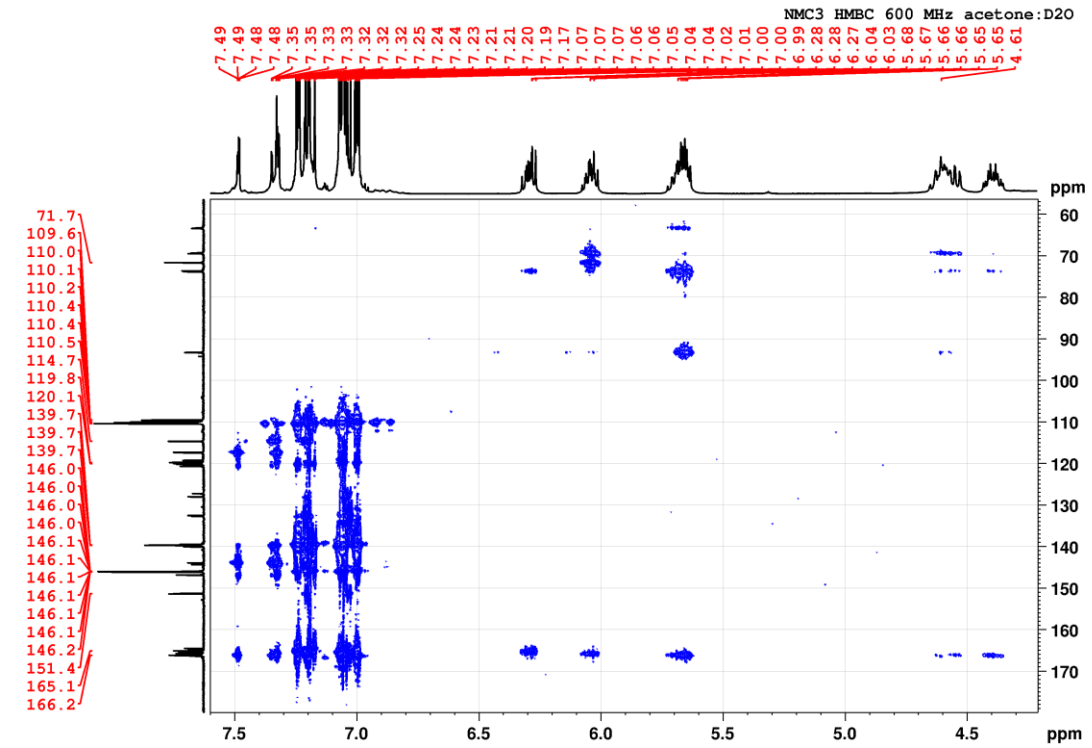

Figure S41: HMBC spectrum of 3,6-bis-O-digalloyl-1,2,4-tri-O-galloyl-β-D-glucose (NMC3)

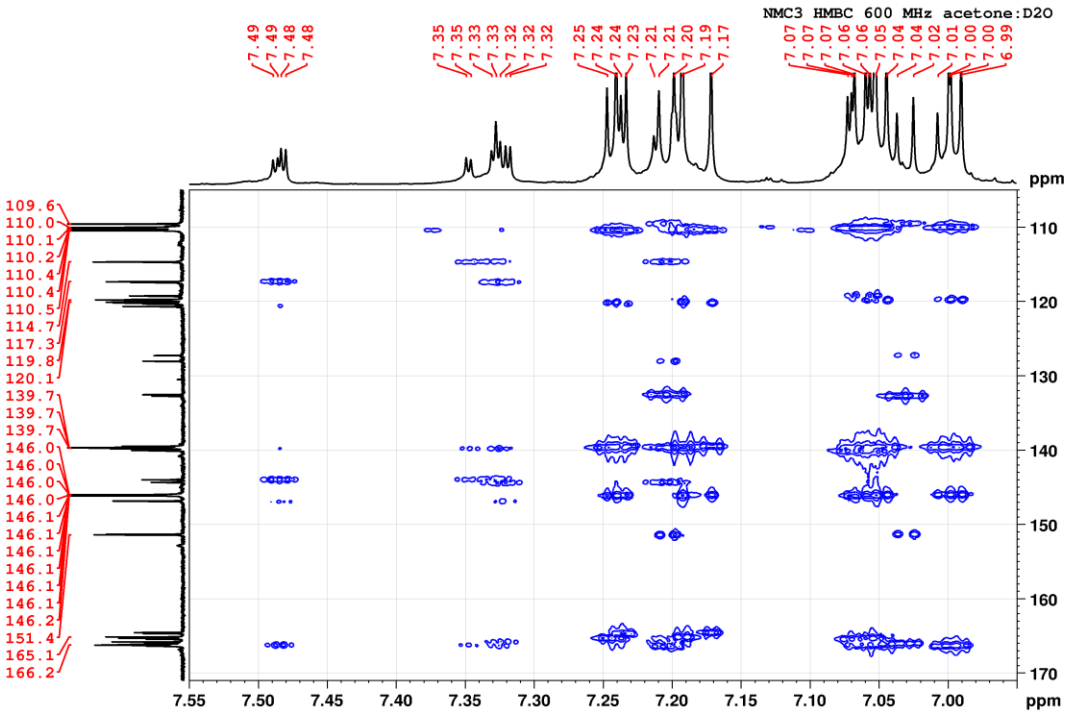

Figure S42: Zoom in of the aromatic region of HMBC spectrum of 3,6-bis-Odigalloyl-1,2,4-tri-O-galloyl-β-D-glucose (NMC3)

4.4. Nature-inspired synthesis of antibacterial glucovanillin derivatives

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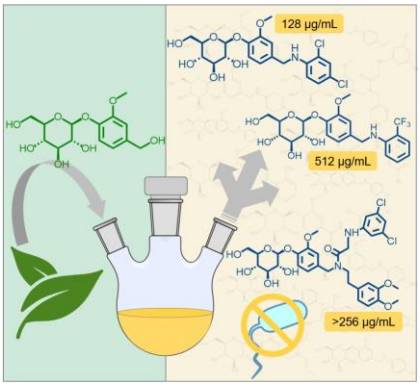
Manuscript submitted to Fitoterapia



Abstract

The ongoing threat of Antimicrobial Resistance (AMR) complicated by the rise of Multidrug-Resistant (MDR) pathogens calls for increased efforts in the search for novel treatment options. While deriving inspiration from antibacterial natural compounds, this study aimed at using synthetic approaches to generate a series of glucovanillin derivatives and explore their antibacterial potentials. Among the synthesized derivatives, optimum antibacterial activities were exhibited by those containing 2,4- and 3,5-dichlorophenylamino group coupled to a glucovanillin moiety (compounds **6h** and **8d** respectively). In those compounds, the Minimum Inhibitory Concentrations (MIC) of 128 – 256 µg/mL were observed against reference and MDR strains of *Klebsiella pneumoniae*, Methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecium* (VRE). Moreover, these findings emphasize the claims from previous reports on the essence of smaller molecular size, the presence of protonatable amino groups and halogens in potential antibacterial agents. The observed moderate and broad-spectrum activities of the stated derivatives point to their suitability as potential leads towards further efforts to improve their antibacterial activities.

Keywords: Antibacterial, *Enterobacteriaceae*, MDR, MRSA, VRE.

1. INTRODUCTION

The ever-increasing rates of Antimicrobial Resistance (AMR) continue to pose a great threat to public health globally. Among others, the infections caused by Gram-negative bacteria need an urgent attention as they are more difficult to treat, and the rise of Multidrug-Resistant (MDR) bacteria has left very limited treatment options [1, 2]. The World Health Organization (WHO) categorizes carbapenem-resistant and third-generation cephalosporin-resistant Enterobacteriaceae among the critical priority pathogens against which new antibiotics are urgently needed [3]. This challenge is intensified by the increasing trends of resistance against the current antibiotic of the last resort for those pathogens, colistin [3, 4]. Furthermore, the slow entry rates of novel antibacterial agents into the antibiotic development pipeline have long led to an almost empty pipeline [5].

In the face of these challenges, the necessity for constant efforts in the search for novel antibacterial agents is outstanding. Favourably, diverse approaches in the discovery and development of antibacterial agents are well established. Among them, the screening of natural products from plants, fungi, bacteria etc. for compounds with antibacterial activities has increasingly reported a good number of potential compounds [6-8]. Natural compounds are particularly attractive due to their higher likelihood of hosting novel bioactive scaffolds beyond those from synthetic or computational approaches. Concerning antibacterial compounds, this attribute is essential towards the discovery of agents with novel targets and modes of action, hence lowering chances for their cross-resistance with the existing antibiotics [6-8].

Moreover, the chemical synthesis of natural compounds or their modifications stands as a valuable tool towards increased access and diversity of such compounds for different purposes. Here, compounds/scaffolds from natural compounds can potentially inspire synthetic approaches targeting increased potency, better pharmacokinetic and physicochemical profiles, as well as reduced toxicities [9-12].

The development of feasible synthetic approaches to prepare natural compounds and their derivatives is therefore essential in optimizing the combined benefits from the ideal qualities of nature-derived compounds and their synthetic derivatives. The development of agents against Gram-negative bacteria is challenged by the roles played by several morphological features. These include the presence of outer and inner cell membranes, selective porins, as well as single- or multi-component efflux transporters [13, 14]. Apart from limiting the entry of antibacterial agents into the Gram-negative bacteria by the cell membranes and porin channels, the forced efflux of agents, which manage to enter the bacterial cells, hinder the exhibition of their targeted effect(s) [13, 14]. To account for such limitations, the development of small molecules with low globularity and lower flexibility, having amphiphilic characters, and which possess protonatable amino group(s) are among the highly recommended strategies [13]. In line with those approaches, this study, aimed at identifying and profiling a potential natural compound, followed by its synthesis, derivatization, and

assessment of antibacterial activities. Generally, the exploration of a wider chemical space around the selected natural product was hypothesized to possibly reveal other compounds with antibacterial potentials.

2. RESULTS AND DISCUSSION

2.1. Selection of the natural hit compound

From the review of literature, the compound vanilloloside (compound **4**) was selected by virtue of having reported MIC values of 16 – 32 µg/mL against the Gram-negative bacteria *Escherichia coli*, *Klebsiella pneumoniae, Salmonella typhimurium* and *Pseudomonas aeruginosa* [15]. Moreover, vanilloloside was previously isolated from honeybee venom and several plant species, whereby it was associated with wound healing, neuroprotection, anti-inflammatory, antibacterial, antimutagenic, and anticancer activities, among others [16-22].

A total of 57 compounds were retrieved from the PubChem® search for compounds similar to vanilloloside with reported biological activities (Tanimoto coefficient ≥ 0.9). It was noted that the compounds exhibit an array of biological activities including those related to anti-infective potentials (**Figure 1**).

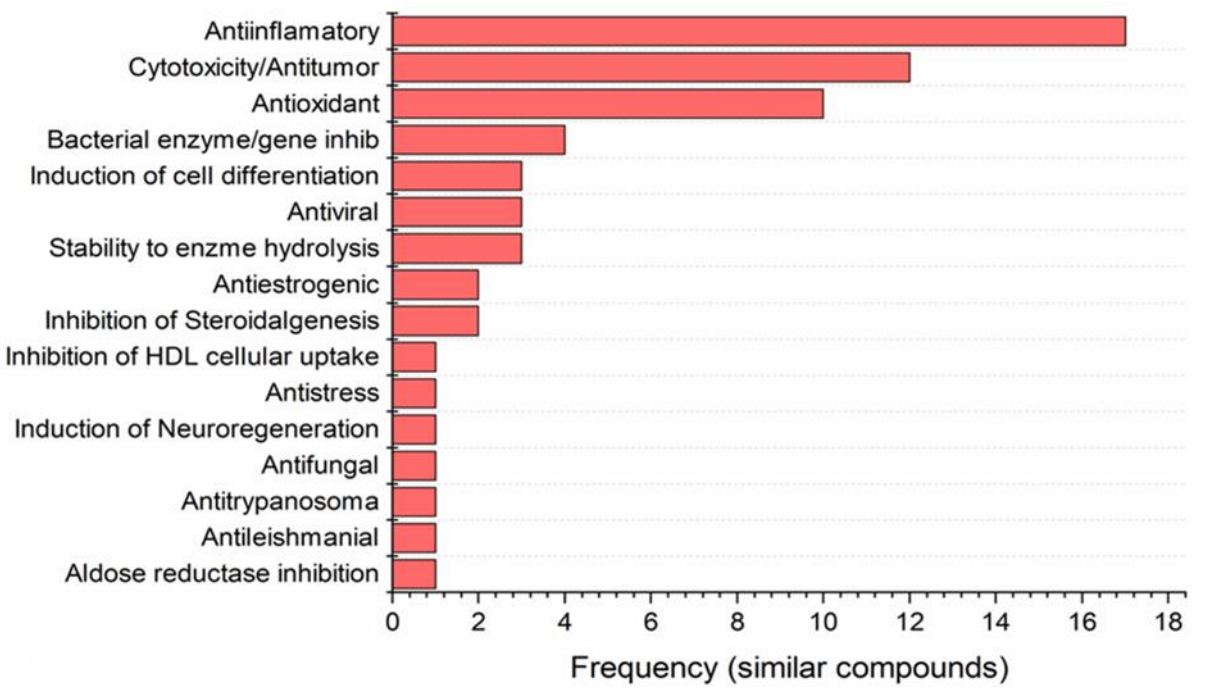


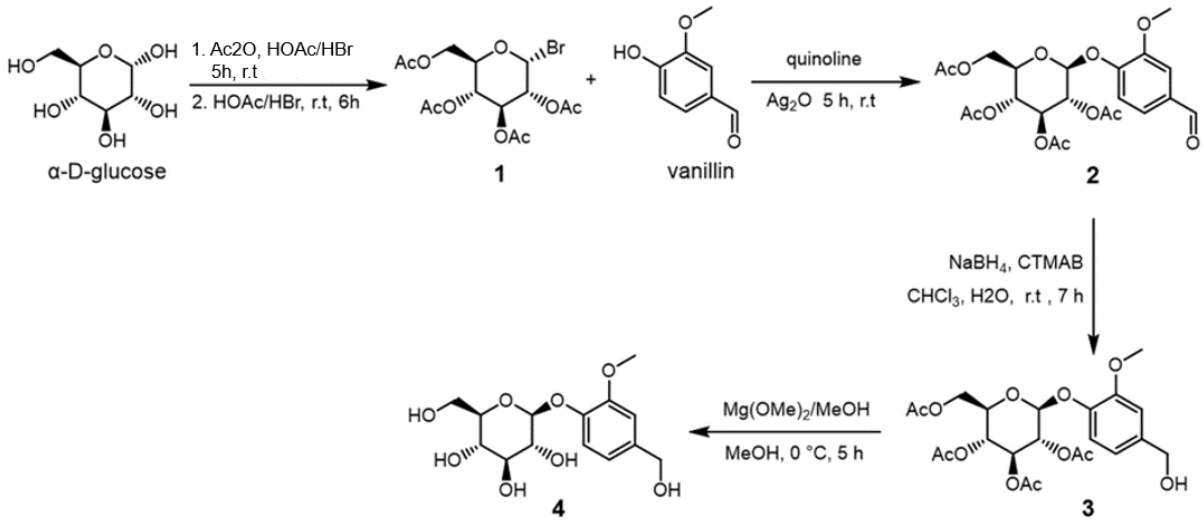
Figure 1: Frequency distribution of biological activities among 57 compounds holding similarity to vanilloloside as determined by the Tanimoto coefficient of ≥ 0.9.

Furthermore, a number of biological activities such as antimicrobial, wound healing, anti-inflammatory, anti-tumor and antioxidant were frequently reported from vanillin and its natural or synthetic derivatives [23-26]. Additionally, different roles of sugar residues were established on different families of compounds exhibiting antibacterial activities. In addition to modifying pharmacokinetic profiles (absorption, distribution, metabolism, and excretion), sugar residues play important roles as cell surface

recognition and anchoring units, increasing target specificity, binding efficacy, active transportation, water solubility, as well as lowering toxicity, hence potentiating the overall activities of the respective aglycones [27, 28]. These findings inspired the undertaking of further steps to explore possible antibacterial potentials of vanilloloside and its synthetic derivatives.

2.2. Chemistry

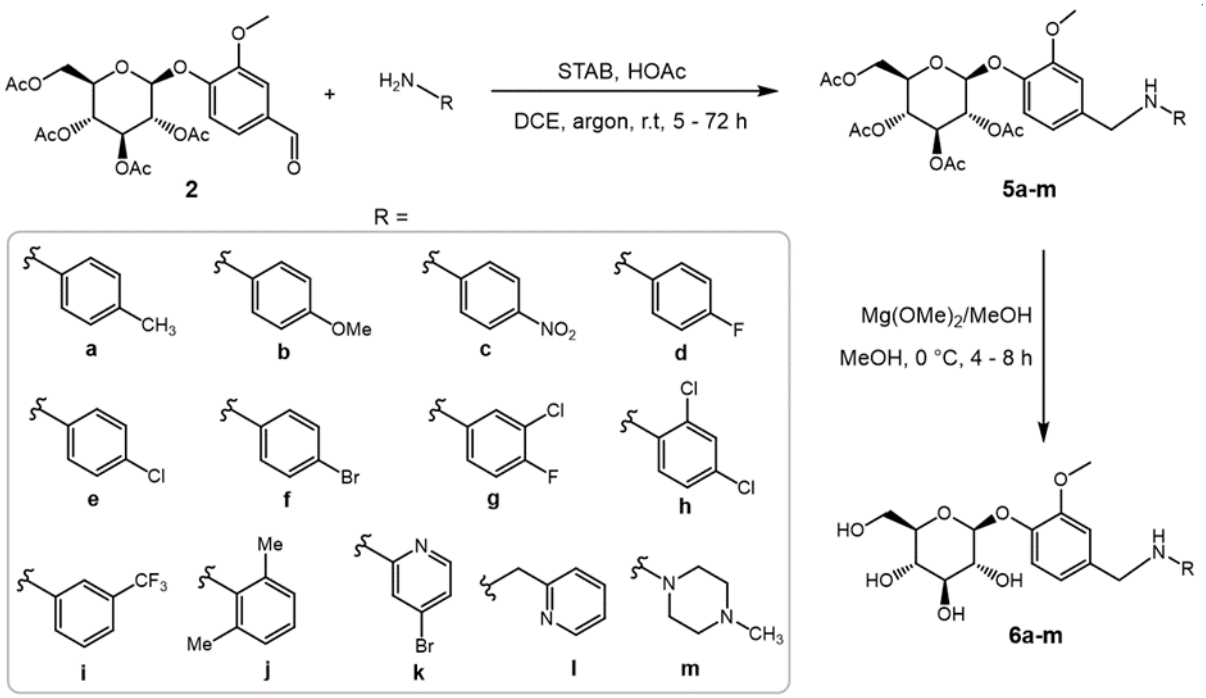
Following the synthesis of acetobromoglucose (**1**), vanilloloside (**4**) could be synthesized following a 3 steps route previously described by Avetyan *et al*., with some modifications (**Scheme 1**) [29]. Attempts to glycosylate vanillin using reagents typical of Koenigs–Knorr reaction e.g., K_2CO_3, Ag_2CO_3, Cs_2CO_3, TEA in solvents like acetone, acetonitrile and THF showed very slow to no conversion. The best yields were therefore attained using Ag_2O as a promoter and quinoline as a basic solvent [29, 30]. Following the Walden inversion at position-2 of the glucose moiety, the β-D-glucose enantiomeric form was expected in the obtained product (**2**). The subsequent reduction of compound **2** by $NaBH_4$ under cetyltrimethylammonium bromide (CTMAB) as a phase transfer catalyst yielded compound **3** at good yields [29]. Further, the choice of $Mg(OMe)_2$ as a de-O-acetylating agent was aimed at avoiding the substantial formation artefacts observed when other bases such as sodium methoxide are employed [31]. To avoid products' degradation under strongly basic conditions, it was crucial to perform neutralization of the reaction mixture before its concentration *in vacuo*.



Scheme 1: Synthesis route for vanilloloside (**4**)

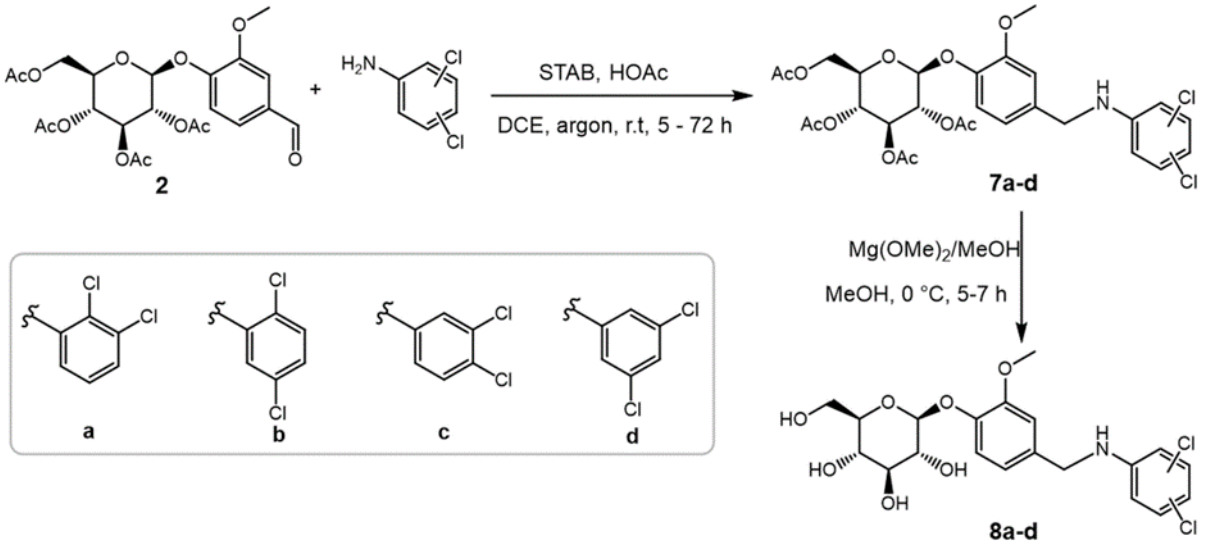
The versatility of the formyl group on compound **2** was further used to enable the exploration of a broader chemical space with possible biological activities. This way, a total of 13 amino derivatives were prepared via a direct reductive amination of the formyl group with the corresponding primary amines (**Scheme 2**) [32]. The incorporation of the amino group was inspired by reports on the antibacterial properties of vanillin-derived amines, and its amide, capsaicin [33-35]. Depending on the nature of the involved amine, compounds **6a-m** were obtained in good yields (41

– 98%) following simple purification steps. The use of 1,2-dichloroethane as a solvent and sodium triacetoxyborohydride (STAB) as a reducing agent, in the presence of acetic acid, ensured faster completion of the reactions, at high yields and fewer side products [32]. However, this approach had limited feasibility in cases where the basicity of the aromatic primary amine was lowered by some electron-withdrawing groups on the aromatic moiety. (e.g., 2, 6- dibromo-/dichloroanilines, difluoroanilines, amino-hydroxy pyridines etc.). Additionally, the attempts to carry out the glycosylation step after reductive amination of vanillin with the respective amine yielded complex and difficult-to-purify mixtures, hence this route was avoided.



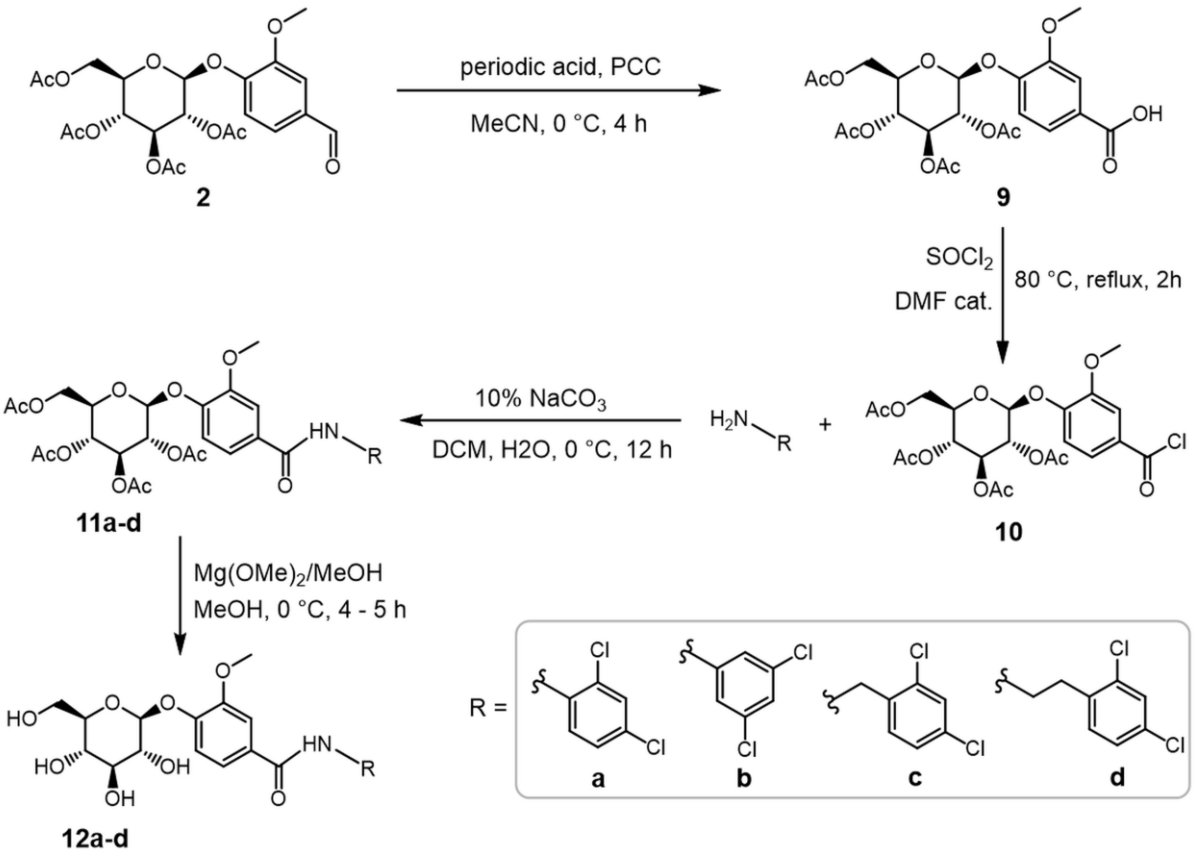
Scheme 2: Synthesis of amino derivatives of β-D-glucovanillin via a direct reductive amination with subsequent de-O-acetylation

In a similar way, the exploration of other dichloroaniline derivatives was achieved (**Scheme 3**). As described above, the attempted preparation of 2,6-dichloroaniline observed extremely slow progress, whereas no product was formed with 2,4,5 trichloroaniline. Notably, compounds, **7a** and **7b** prepared from 2,3- and 2,5-dichloroanilines were obtained at lower yields (28% and 30% resp.) compared to those from the 3,4- (92%) and 3, 5- (74%) dichloroanilines (**7c** and **7d**), even after prolonged reaction durations.



Scheme 3: Synthesis of dichloroaniline derivatives via a direct reductive amination with subsequent de-O-acetylation

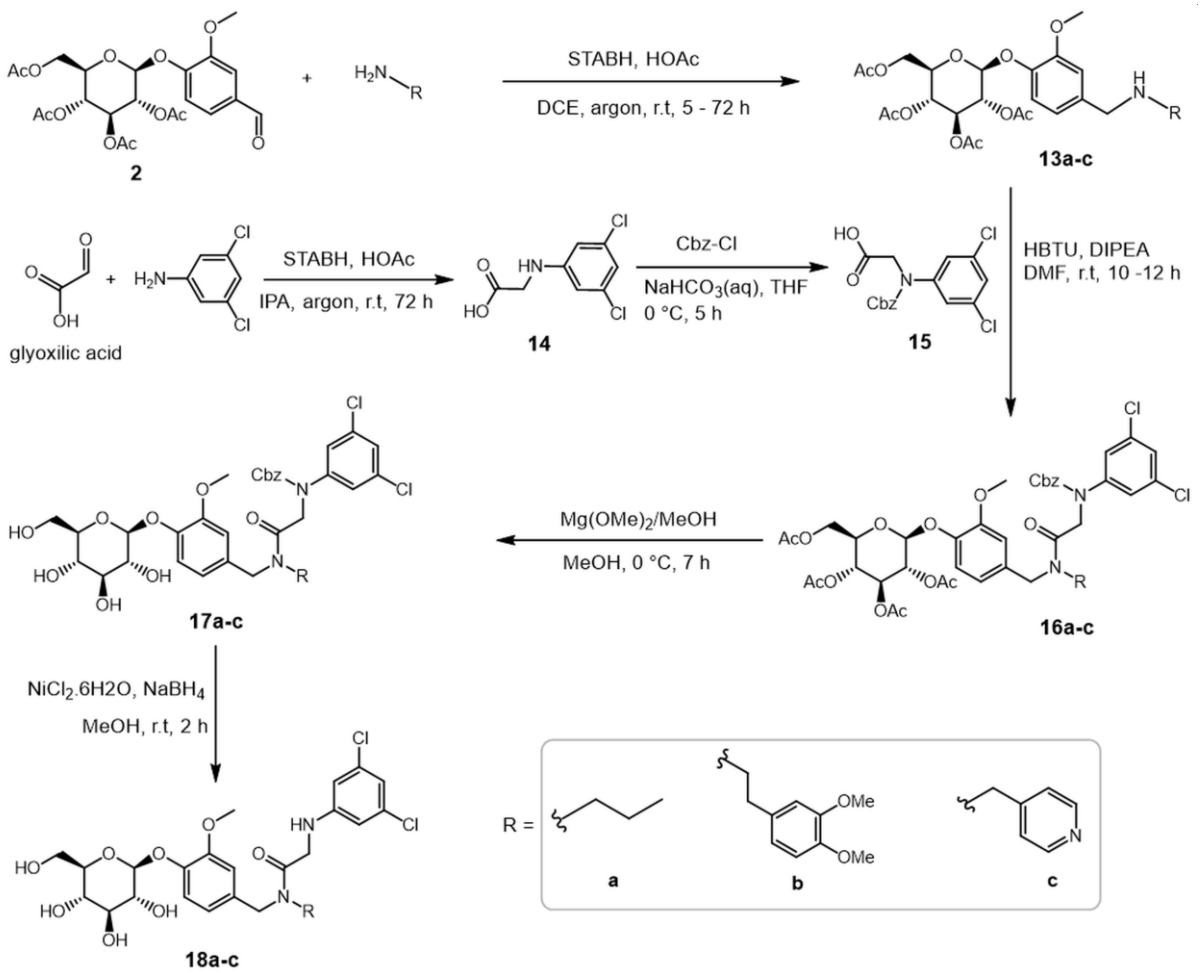
To enable the preparation of selected amide derivatives, the intermediate acid chloride (**10**) was first obtained from the oxidation formyl group of compound **2** with a subsequent refluxing of the resulting carboxylic acid (**9**) with $SOCl_2$ under DMF catalysis (**Scheme 4**) [36, 37]. Although the oxidation of compound **2** under pyridinium chlorochromate (PCC) was straightforward, the repeated addition of PCC at half of the overall reaction time was crucial in the reaction's completion. The syntheses of the amides **11a-d** at 59 – 92% yields were done under the Schotten-Baumann conditions. The final de-O-acetylation yielded the target compounds **12a-d** (**Scheme 4**).



Scheme 4: Synthesis of selected amide derivatives

The capsaicin-like moiety, in which the amide's carbonyl group is distal to the vanillyl moiety was successfully incorporated in compounds **18a-c** via the initial formation of the respective secondary amines **13a-c** (**Scheme 5**). Particularly, these modifications aimed at evaluating changes in observed antibacterial potentials of compound **8d** upon the incorporation of the above moiety with extensively reported antibacterial potentials [35, 38]. Moreover, compound **8d** was chosen based on its better (observed) aqueous solubility compared to **6h**.

Attempts to convert the formyl group on compound **2** into a primary amine under ammonium formate/Mg [39], ammonium acetate/$NaBH_3CN$ [40], or ammonium hydroxide/$NaBH_4$/$ZnCl_2$ [41] conditions were not successful. Also, similar efforts via the reduction of the respective oxime under H_2/Pd, ammonium formate/Mg [39], $LiAlH_4$ [42], as well as reduction of the respective nitrile under or $NaBH_4$ or $LiAlH_4$ [43, 44] did afford the expected acetogluco-vanillylamine. To bypass this challenge, the preparation of three secondary amine derivatives of acetogluco-vanillylamine (**13a-c**) followed by their amidation was pursued. Choices on the secondary amine to be prepared were based on the quest to explore diverse chemical scaffolds on this position for possible antibacterial potentials [45-48].



Scheme 5: Synthesis of derivatives with the reintroduction of dichlorophenyl amino moiety, inversion of the amide group to mimic a capsaicin-like amide, and formation of tertiary amides

Moreover, the re-introduction of the aniline moiety was achieved using a glyoxylic acid-derived linker coupled to 3,5-dichloroaniline by direct reductive amination to obtain compound **14** [32]. The protection of the secondary amine of compound **14** using benzyl chloroformate (Cbz) was necessary to avoid possible self-reactions in the subsequent coupling reaction. The use of Cbz was ideal as its removal was devoid of strongly acidic or basic conditions which might hydrolyze the acetal on the glucose moiety (in compounds **16a-c**) or prematurely de-O-acetylate it, respectively. The above undertakings led to the successful coupling of compounds **13a-c** and **15** using HBTU and DIPEA conditions to give compounds **16a-c** at yields of 73 – 83% [49]. Notably, the alternative route involving the amidation glyoxylic acid with compounds **13a-c** and subsequent reductive amination of the residual formyl group afforded very low yields during the first step, hence avoided.

The target compounds **18a-c** were obtained via Cbz-deprotection of de-O-acetylated compounds **17a-c** using nickel boride generated in situ from $NaBH_4$ and $NiCl_2.6H_2O$ in methanol [50]. Deprotection under the standard conditions (H_2, Pd/C) resulted in a mixture of untargeted products including the one in which both Cbz and 3,5-phenyl

groups were removed [51]. Nevertheless, the deprotection by nickel boride resulted in low yields (19 – 37%). Additionally, compounds **18b&c** were obtained at relatively low HPLC purities of 84 – 85% even after purification under reversed phase flash chromatographic conditions (**Table S2**).

2.3. Structure-activity relationships

For the sake of comparison, **Table 1** summarizes the antibacterial activities of the new compounds and known antibiotics against susceptible and Multidrug-resistant bacterial strains. In addition to the non-resistant *E. coli* (ATCC 25922) *and K. pneumoniae* (ATCC 10031) strains, Methicillin-resistant *Staphylococcus aureus* (MRSA) and Vancomycin-resistant *Enterococcus faecium* (VRE), 6 strains of *K. pneumoniae* showing resistance towards many antibiotics were studied.

Despite previously reported antibacterial activities (MIC = 16–32 µg/mL) of vanilloloside (**4**), no activity was observed at up to 256 µg/mL when it was tested against *E. coli* (ATCC 25952) and *K. pneumoniae* (ATCC 10031) strains with no known resistances to antibiotics (**Scheme 1, Table 1**) [15]. The observed discrepancies were presumed to be related to possible differences in the tested bacterial strains or experimental approaches, of which the actual previously studied strains were not disclosed [52].

Table 1: Minimum inhibitory concentrations (µg/mL) of studied compounds against susceptible and MDR bacterial strains. S = susceptible, standard dosing regimen; I = susceptible, increased exposure; R = resistant; R * = MIC of the antibiotic is within the sensitive range *in vitro* but must be considered resistant *in vivo* due to the expression of a beta-lactamase/carbapenemase. Standard abbreviations for the expressed resistance enzymes and genes are used to identify the resistance types in each strain

	Bacteria, their resistance phenotypes, and MICs (µg/mL)									
	E. coli	*K. pneumoniae*							*S. aureus*	*E. faecium*
Compound/ Antibiotic	*ATCC 25922*	*ATCC 10031*	DHA-1 (Amp C)	KPC-2	SHV-4	OXA-48	TEM-/ SHV-/ CTX-M-pos	NDM-1	MRSA	VRE
Cefotaxime			R	R	R*	R	R	R		
Ceftazidime			R	R	S	R	R	R		
Ertapenem			R	R	S	R	S	R		
Imipenem			R*	R	S	R*	S	R		
Meropenem			R*	R	S	R*	S	R		
Ciprofloxacin			R	R	S	R	I	R		
Piperacillin-Taz			R	R	R*	R	R	R		
Methicillin									R	
Vancomycin										R
4	>125	>128								
6(a-d, j-m)	>512	>512								
6(e, f)	>256	>256								
6(g, i)	>512	**512**								
6h	>256	**128**	**256**	**256**	>256	>256	>256	**256**	**256**	**256**
8 (a-c)	>256	**256**								
8d	>256	**128**	>256	**256**	>256	>256	>256	>256	>256	>256
12(a-d)	>256	>256								
18(a-c)	>256	>256								
Gentamicin	<0.25	<0.25	>128	2	8	0.5	64	>128		
Vancomycin									4	>512

However, moderate to low antibacterial activities were observed from the exploration of other derivatives (**Scheme 2, Table 1**). In that, the replacement of the hydroxyl group on the vanilloloside's aglycone with dihalophenyl amino- and trifluoromethylphenyl amino- groups yielded moderate to low (MIC 128 – 512 µg/mL) activities against *K. pneumoniae*. Furthermore, lower activities against *K. pneumoniae* (MIC 256 µg/mL) were obtained from the 2,4-, 2,5- and 3,4-dichlorophenyl amino derivatives (**8(a-c)**), whereas the 3,5-dichlorophenyl amino derivative (**8d**) had activity similar to that observed in the initial 2,4-dichlorophenyl amino derivative (**6h**) (MIC = 128 µg/mL).

These findings resonate with previous claims on the antibacterial potentials of halogenated natural and synthetic compounds [10, 53, 54]. In those cases, halogenated derivatives were reported to exhibit broad-spectrum activities against bacteria and fungi [10, 53, 54]. Further, the presence of halogen groups was stated to enhance biological activities while improving toxicity profiles through enhanced selectivity [55, 56]. Decreasing hydrophobicity, increasing membrane penetrability, and enhanced self-assembly of halogen-containing compounds are among the most highlighted roles of halogens within bioactive compounds [56]. Such activities are partly linked to halogens' ability to act as hydrogen bond acceptors as well as to form halogen bonds [56]. Additionally, an increase in the amphiphilic character of the molecules brought by coupling with a dichlorophenyl moiety might also play a role in improving their potential against Gram-negative bacteria [13]. However, their *ortho-para* (**6h**) and *meta-meta* (**8d**) positioning relative to the amino group were notably

crucial for the exhibition of antibacterial activities, as other positions resulted in a two-fold decrease in activity (**8a-c, Table 1**)

Moreover, the replacement of the amino-methyl group between the vanillin and the dichlorophenyl moieties with an amide functionality (**12a&b**) led to the loss of activities among compounds **6h** and **8d**. (**Scheme 4, Table 1**). No activity was observed upon insertion of methylene (**12c**) or ethylene (**12d**) groups between the amide and the 2,4-dichlorophenyl groups. These observations underscored the significance of the secondary amine group in compounds **6h** and **8d** in an exhibition of antibacterial activities. The loss of activity upon separation of the amino and dichlorophenyl moiety with an ethylene group (data not shown), underlined the necessity for its placement next to the dichlorophenyl unit in those compounds. Similarly, the reintroduction of the dichlorophenyl amino group, inversion of the amide group and insertion of diverse groups to yield corresponding tertiary amides (**18a-c**), resulted in the loss of antibacterial activities (**Scheme 5, Table 1**). Taken together, the observed loss in activities can be brought about by factors similar to those reported by Richter et al. [13], whereby, the accumulation of compounds within the bacterial cells was more associated with smaller molecules which contain a protonatable amine while having a rigid structure and low globularity.

Furthermore, compounds **6h** and **8d** inhibited the growth (MIC 256 µg/mL) of 3 MDR strains of *K. pneumoniae* expressing Amp C, KPC-2 and NDM-1 enzymes and showing resistance towards multiple antibiotics. Additionally, compound **6h** showed the same level of activity against MRSA and Vancomycin-resistant *E. faecium* (**Table 1**). These findings highlight the potential of these compounds against MDR bacterial strains and their broad-spectrum nature against both Gram-negative and Gram-positive bacteria. Further efforts to improve the observed potential are therefore encouraged. Among other aspects, such efforts should focus on increasing their globularity, reducing their flexibility, and introducing primary amine moieties [13].

3. CONCLUSIONS

Nature-inspired synthesis of antibacterial compounds is a promising way to maximize the benefits of chemical diversity, novelty, and biological potential of nature-derived compounds. While well-studied natural hits might not be attractive for further studies, less explored hits are likely to be associated with limited reproducibility of their scarcely available data. This study has highlighted the usefulness of a surrogate approach to explore the biological profiles of natural hits with scarce biological data by collectively evaluating the profiles of closely similar compounds. Moreover, highly feasible synthetic routes towards the preparation of various glucovanillin derivatives at good yields and purity levels were presented. This way, it was possible to explore a wider chemical space around glucovanillin using both natural and synthetic scaffolds in hunting for derivatives exhibiting antibacterial potentials against selected bacteria. Altogether, the moderate activities of compounds **6h** and **8d** against both

susceptible and MDR Gram-negative and Gram-negative bacteria encourages further efforts to improve such potentials and other related profiles.

4. EXPERIMENTAL

4.1. Selection of a natural hit compound

Vanilloloside (a glucovanillin derivative) was selected from the library of compounds created from screening the literature for plant-isolated compounds with MIC ≤ 100 µg/mL against *E. coli* and *K. pneumoniae* (unpublished data). The profile of previously reported biological activities was thereafter established, which was accompanied by the search for vanilloloside-similar compounds on PubChem®. The search installed filters to retrieve only compounds with reported biological activities and with a Tanimoto similarity coefficient of ≥ 0.9. Evaluation of reported activity profiles of vanilloloside and its related compounds informed the ultimate decision towards its synthesis and derivatization.

4.2. General information and instrumentation

Chemicals, media and antibiotics: All chemicals used for synthesis were purchased from Sigma-Aldrich (Schnelldorf, Germany), Acros Organics (Heel, Belgium), and VWR International (Darmstadt, Germany), and were used without further purification. Deionized water prepared using a Milli-Q ® system (Merk, Darmstadt, Germany) was used in all chromatographic applications. Mueller–Hinton broth (MHB), Lysogeny broth (LB), and agar were purchased from Carl Roth (Karlsruhe, Germany). Gentamicin sulfate and vancomycin were purchased from AppliChem (Darmstadt, Germany). Moreover, precoated Thin-Layer Chromatography (TLC) plates (silica gel 60 F254) were purchased from Merck (Darmstadt, Germany), and precoated Reversed-phase (RP) TLC plates (ALUGRAM® RP-18W/UV254) sourced from Macherey-Nagel (Dueren, Germany). Furthermore, for flash chromatography, Puriflash® F0025, silica gel 50 µm columns were purchased from Interchim (Los Angeles CA, United States) and the reversed-phase columns (CHROMABOND® Flash RS 25 C18ec) were bought from Macherey-Nagel GmbH Co. KG (Dueren, Germany).

Chromatography and Mass Spectrometry: open column chromatography was carried out using glass columns loaded with silica gel of 230 – 400 mesh particle size with a pore size of 60 Å. When needed, deactivation of the silica gel was done by treating silica gel powder with ammonia 25% solution at a ratio of 100 g/ 7.5 mL in a tightly closed container over 30 minutes. Moreover, flash chromatography was conducted on a Puriflash® 430 system (Interchi, Montlucon, France). Appropriate solvent systems for both techniques were developed based on the nature of the target compounds and prevailing impurities (**Table S2**). **HPLC-MS:** measurements were carried out using an LCMS-2020 (Shimadzu, Japan) equipped with SPD-20A UV/Vis and ESI-MS detectors. The chromatographic conditions entailed a reversed-phase column (RP C18; 4 µm, 150 × 4.6 mm; Agilent Technology) and elution was done using a mixture of solvent A (water + 0.1% FA) and solvent B (methanol + 0.1%

FA) under the gradient elution conditions of 5–100% B (0–12 min), 100% B (12–17 min), and 100–5% B (17–18 min) (Method 1). **HR-MS:** measurements were performed using an Exactive™ Plus Orbitrap high-resolution mass spectrometer (HR-ESI-MS) (ThermoFischer Scientific, Waltham, MA, US) under electrospray ionization in a negative mode.

Nuclear Magnetic Resonance (NMR): ^{1}H (400.132 MHz) and ^{13}C (100.613 MHz) NMR spectra were recorded on a Bruker AV 400 instrument (Bruker Biospin, Ettlingen, Germany). As internal standards, signals of deuterated solvents were used (CDCl3: ^{1}H 7.24 ppm, ^{13}C 77.23 ppm, DMSO-d_6: ^{1}H 2.50 ppm, ^{13}C 39.52, acetone-d_6: ^{1}H 2.05 ppm, ^{13}C 28.84 ppm, methanol-d4: ^{1}H 3.31 ppm, ^{13}C 48.00 ppm). The abbreviations: (s) singlet, (d) doublet, (t) triplet, (q) quartet, (dd) doublet of doublets, (ddd) doublet of doublets of doublets, (m) multiplet, (sext) sextet, were used during analyses and interpretations of the spectra. Moreover, the coupling constants J are given in Hz.

Infrared (IR): spectra were acquired on a JASCO FT/IR-4700 Fourier transform infrared (FT-IR) spectrometer equipped with an attenuated total reflectance (ATR) accessory (JASCO Labor und Datentechnik GmbH, Pfungstadt, Germany). The abbreviations: (w) weak, (m) medium, (s) strong, (vs) very strong, (br) broad, were used during analyses and interpretations of the spectra.

Melting points: were determined on a Mettler Toledo MP70 melting point system (Mettler-Toledo AG Analytical, Schwerzenbach, Switzerland) and they were not corrected.

General procedures: Completion of the reactions was monitored TLC or LC-MS When needed, deactivation of TLC plates was done by inserting the plates into an ammonia vapour chamber for 5 minutes. Visualization of the spots was done under UV light at 254 nm. Moreover, H_2SO_4: MeOH 1:1 v/v, 2,4-dinitrophenyl hydrazine, and ninhydrin spray reagents were used for visualization of glucosides, carbonyl and amino groups respectively.

4.3. Synthesis procedures

*Synthesis of (2R,3R,4S,5R,6R)-2-(acetoxymethyl)-6-bromotetrahydro-2H-pyran-3,4,5-triyl triacetate (acetobromoglucose) (**1**)*

α-D-glucose (20.0 g, 111 mmol) was dissolved in acetic anhydride (105 mL, 1.1 mol) at room temperature. A 33% w/v solution of HBr in glacial acetic acid (28.8 mL, 167 mmol) was added to the above solution and stirred further for 5 h. Another portion of HBr/glacial acetic acid solution (143.8 mL, 777 mmol) was thereafter added and the reaction was continued for another 6 h [57]. Reaction completion was monitored using H_2SO_4 in MeOH (1:1 v/v) as a spray reagent with oven heating at 120 °C for 10 minutes. The reaction mixture was concentrated *in vacuo* with subsequent co-evaporation with toluene to recover a brownish syrup. Crystallization was achieved by dissolving the syrup into 100 mL of diisopropyl ether and 48h of refrigeration. The

obtained crystals were washed with pet. ether, dried under suction to obtain 39 g of **1-** Yield 85%, light brown solid, mp 84 – 86 °C. IR (ATR, ṽ [cm^{-1}]): 2961,1738, 1212, 1164, 668. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.58 (d, 1H, J = 4.0), 5.53 (t, 1H, J = 9.7), 5.14 (t, 1H, J = 9.7), 4.81 (dd, 1H, J = 9.7 Hz, 4.0 Hz), 4.25 - 4.32 (m, 2H), 4.11 (dd, 1H, J = 10.6 Hz, 1.8 Hz), 2.01-2.08 (m, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.7, 169.6, 86.7, 72.3, 70.8, 70.3, 67.4, 61.1, 20.8, 20.7. ESI-MS: $[M+H]^+$ = 410.85 *m/z* ($C_{14}H_{19}BrO_9$), HPLC purity: 89% (Method 1), Rf = 0.73 (silica gel, pet. ether/EtOAc 1:1 v/v). (Compound **1** should be stored at -15 °C in a sealed container under an argon atmosphere).

*(2R,3S,4S,5R,6S)-2-(acetoxymethyl)-6-(4-formyl-2-methoxyphenoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**2**)*

Compound **1** (1,84 g, 4.49 mmol) was dissolved in quinoline (15 mL) r.t., Ag_2O (2.1 g, 8.97 mmol) was added and the mixture was stirred for 1 h. Thereafter, 4-hydroxy-3-methoxybenzaldehyde (vanillin) (750.9 mg, 4.94 mmol) was added in small portions and the reaction was continued for a further 11 h [58, 59]. The mixture was filtered through celite, the celite cake was washed with $CHCl_3$, 3% aqueous HCl (100 mL) was added to the filtrate and the mixture was extracted with $CHCl_3$. Combined organic phases were re-washed with 3% aqueous HCl, sat. aqueous $NaHCO_3$ solution, brine, dried over anh. Na_2SO_4, filtered, and the solvent was removed *in vacuo*. The residue was purified by column chromatography on silica gel (silica gel pet. ether/EtOAc 1:1 v/v, Rf = 0.37) to yield 2.06 g of **2**. Yield 95%, off-white solid, mp 144 – 145 °C. IR (ATR, ṽ [cm^{-1}]): 3014 (w, Ar–H), 2942 (w, C–H), 1752 (s, ester C═O), 1209 (vs, ether C–O). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.86 (s, 1H), 7.43 – 7.35 (m, 2H), 7.18 (d, *J* = 8.0 Hz, 1H), 5.33 – 5.24 (m, 2H), 5.20 – 5.10 (m, 1H), 5.11 – 5.03 (m, 1H), 4.25 (dd, *J* = 12.3, 5.2 Hz, 1H), 4.16 (dd, *J* = 12.3, 2.5 Hz, 1H), 3.89 – 3.78 (m, 4H), 2.04 (s, 6H), 2.01 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 191.1, 170.7, 170.4, 169.6, 169.4, 151.3, 151.2, 133.0, 125.5, 118.4, 111.0, 99.9, 72.6, 72.5, 71.2, 68.5, 62.1, 56.3, 20.9, 20.8, 20.8, 20.8. ESI-MS: *m/z* = 505.05 ($[M+Na]^+$, $C_{22}H_{26}O_{12}$), HPLC purity: 99% (Method 1),

(2R,3S,4S,5R,6S)-2-(acetoxymethyl)-6-(4-(hydroxymethyl)-2-methoxyphenoxy) tetrahydro-2H-pyran-3,4,5-triyl triacetate **(3)**

Compound **2** (160 mg, 331.65 µmol) was dissolved in $CHCl_3$ (10 mL) of chloroform at r.t. with subsequent addition of distilled water (10 mL). To the above mixture were added $NaBH_4$ (12.55 mg, 331.65 µmol) and cetyltrimethylammonium bromide (CTMAB) (6.04 mg, 16.58 µmol) as a phase transfer catalyst. Another portion of $NaBH_4$ (6.28 mg, 165.83 µmol) was added to the reaction mixture after 5 h of stirring at r.t. and the reaction was continued for a further 2 h. Upon reaction completion (TLC control), 50 mL of distilled water was added, and the mixture was extracted with $CHCl_3$. The combined organic layers were washed with brine, dried over Na_2SO_4, filtered, and the solvent was removed *in vacuo*. The residue was purified by column chromatography (silica gel, pet. ether/EtOAc 1:9 v/v, Rf = 0.46) to obtain 143 mg of **3**. Yield 89%, white solid, mp 154 °C. IR (ATR, ṽ [cm^{-1}]): 3548 (br, O–H), 2935 (w, C–H), 1752 (s, ester C═O), 1209 (vs, ether C–O), 1066 (vs, alc. C–O). ^{1}H NMR (400

MHz, $CDCl_3$) δ 7.07 (d, 1H, J = 8.1 Hz), 6.93 (d, 1H, J = 1.5 Hz), 6.82 (dd, 1H, J = 8.1, 1.5 Hz), 5.24 – 5.26 (m, 2H), 5.11-5.16 (m, 1H), 4.91 – 4.92 (m, 1H), 4.62 (s, 2H), 4.25 (dd, 1H, J = 12.2, 5.0 Hz), 4.14 (dd, 1H, J = 12.2, 2.3 Hz), 3.81 (s, 3H), 3.71 – 3.75 (m, 1H), 2.05 (s, 6H), 2.01 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.6, 120.5, 119.3, 111.7, 101.1, 72.8, 72.2, 71.4, 68.6, 65.2, 62.1, 56.2, 20.9, 20.8. ESI-MS: $[M+K]^+$ = 523.05 *m/z* ($C_{22}H_{28}O_{12}$), HPLC purity: 85% (Method 1).

*General procedure (**A**) for the synthesis of compounds **4, 6a-m, 8a-d, 12a-d, and 17a-c***

The appropriate acetylated compound (1 eq) was dissolved in 19 mL dry MeOH at 0 °C followed by an addition 1mL of a 6 – 10 %w/v solution of $Mg(OMe)_2$ in MeOH and stirred for 4 – 8 h at 0 °C. Thereafter, 1.25 M ethanolic HCl was added to arrive at a pH of 7 – 8, the solvent was removed *in vacuo* and the residue was purified by column or flash chromatography to yield the desired product (**Table S2**).

*(2R,3S,4S,5R,6S)-2-(hydroxymethyl)-6-(4-(hydroxymethyl)-2-methoxyphenoxy)tetrahydro-2H-pyran-3,4,5-triol (Vanilloloside) (**4**)*

According to general procedure **A**, compound **3** (134 mg, 276.60 µmol) was treated with an approx. 0.5 %w/v solution of $Mg(OMe)_2$ in dry MeOH (20 mL) for 4h to yield 80 mg of **4**. Yield 91%, white solid, mp 119 - 120 °C. IR (ATR, ṽ [cm^{-1}]): 3423 (br, O–H), 3016 (w, Ar–H), 2916 (w, C–H), 1265 (m, ether C–O), 1074 (s, alc. C–O). ^{1}H NMR (400 MHz, DMSO-D6) δ 7.02 (d, *J* = 8.3 Hz, 1H), 6.94 (d, *J* = 1.6 Hz, 1H), 6.85 – 6.72 (m, 1H), 5.17 (d, *J* = 4.8 Hz, 1H), 5.09 – 5.04 (m, 2H), 4.97 (d, *J* = 5.2 Hz, 1H), 4.86 (d, *J* = 7.2 Hz, 1H), 4.49 (t, *J* = 5.7 Hz, 1H), 4.42 (d, *J* = 5.7 Hz, 2H), 3.75 (s, 3H), 3.68 – 3.64 (m, 1H), 3.48 – 3.43 (m, 1H), 3.29 – 3.21 (m, 3H), 3.19 – 3.13 (m, 1H). ^{13}C NMR (101 MHz, DMSO-D6) δ 148.8, 145.3, 136.4, 118.6, 115.3, 111.1, 100.3, 77.0, 76.9, 73.3, 69.7, 62.7, 60.7, 55.6. ESI-MS: $[M+Na]^+$ = 338.90 *m/z* ($C_{14}H_{20}O_8$), HPLC purity: 92% (Method 1), Rf = 0.71 (silica gel, EtOAc/MeOH 7:3 v/v).

*General procedure (**B**) for the synthesis of compounds **5a-m**, **7a-d**, **13a-c** and **14***

The appropriate aldehydes (1 eq), amines (1.2 – 1.5 eq), triacetoxyborohydride (STAB) (3 - 4 eq) and acetic acid (3 - 4 eq) were dissolved into 20 – 25 mL of 1,2-dichloroethane (DCE) or isopropyl alcohol (IPA) at r.t. under argon atmosphere and stirred for 5 – 72 h. The reaction was quenched by the addition of water and the reaction mixture was extracted with CH_2Cl_2. The combined organic layers were washed with brine, dried over Na_2SO_4, filtered, and the solvent was removed *in vacuo*. The residue was purified by column chromatography to obtain the desired product (Supporting information, **Table S1**).

*(2R,3S,4S,5R,6S)-2-(hydroxymethyl)-6-(2-methoxy-4-((p-tolylamino)methyl)phenoxy)tetrahydro-2H-pyran-3,4,5-triol (**6a**)*

According to general procedure **A**, compound **5a** (230 mg, 400.98 µmol) was treated with approx. 0.5 %w/v solution of $Mg(OMe)_2$ in dry MeOH (20 mL) for 4 h to yield 151 mg of 6a. Yield 93%, brown solid, mp 59 – 60 °C. IR (ATR, ṽ [cm^{-1}]): 3370 (br, O–H),

3019 (w, Ar–H), 2919 (w, C–H), 1218 (s, ether C–O), 1070 (vs, alc. C–O). ^{1}H NMR (400 MHz, Acetone-d_6) δ 7.10 (d, *J* = 8.2 Hz, 1H), 7.05 (d, *J* = 1.8 Hz, 1H), 6.89 (d, *J* = 8.2 Hz, 3H), 6.58 – 6.54 (m, 2H), 4.87 (d, *J* = 7.3 Hz, 1H), 4.42 – 4.24 (m, 5H), 3.90 – 3.82 (m, 2H), 3.80 (s, 3H), 3.70 – 3.68 (m, 2H), 3.57 – 3.44 (m, 5H), 2.15 (s, 3H). ^{13}C NMR (101 MHz, Acetone-d_6) δ 150.8, 147.6, 146.7, 136.1, 130.2, 126.0, 120.4, 118.1, 113.7, 112.9, 102.8, 77.9, 77.7, 74.8, 71.3, 62.7, 56.5, 48.3, 30.4, 30.2, 30.0, 29.8, 29.7, 3.46, 29.3, 20.4. ESI-MS: $[M+H]^+$ = 406.60 *m/z* ($C_{21}H_{27}NO_7$), HPLC purity: 94% (Method 1). Rf = 0.32 (silica gel, EtOAc/MeOH 9:1 v/v),

*(2R,3S,4S,5R,6S)-2-(hydroxymethyl)-6-(2-methoxy-4-(((4-methoxyphenyl)amino)methyl)phenoxy)tetrahydro-2H-pyran-3,4,5-triol (**6b**)*

According to general procedure **A**, compound **5b** (245 mg, 415.54 µmol) was treated with approx. 0.5 %w/v solution of $Mg(OMe)_2$ in dry MeOH (20 mL) for 5 h to yield 169 mg of **6b**. Yield 97%, brown semi-solid, IR (ATR, ṽ [cm^{-1}]): 3338 (br, O–H), 3019 (w, Ar–H), 2915 (w, C–H), 1221 (m, ether C–O), 1068 (vs, alc. C–O). ^{1}H NMR (400 MHz, Acetone-d_6) δ 7.09 (d, *J* = 8.3 Hz, 1H), 7.04 (d, *J* = 1.4 Hz, 1H), 6.89 (dd, *J* = 8.3, 1.3 Hz, 1H), 6.70 – 6.67 (m, 2H), 6.61 – 6.57 (m, 2H), 5.49 (s, 1H), 5.16 – 4.88 (m, 5H), 4.40 – 4.33 (m, 1H), 4.18 (s, 2H), 3.79 – 3.74 (m, 4H), 3.64 (s, 3H), 3.60 – 3.44 (m, 4H). ^{13}C NMR (101 MHz, Acetone-d_6) δ 151.8, 149.9, 146.4, 143.8, 135.1, 122.7, 120.1, 116.5, 115.0, 114.9, 114.2, 112.7, 101.6, 77.8, 74.2, 70.7, 61.7, 56.2, 55.6, 48.1. ESI-MS: $[M+H]^+$ = 422.00 *m/z* ($C_{21}H_{27}NO_8$), HPLC purity: 93% (Method 1), Rf = 0.60 (silica gel, EtOAc/MeOH 9:1 v/v).

*(2R,3S,4S,5R,6S)-2-(hydroxymethyl)-6-(2-methoxy-4-(((4-nitrophenyl)amino)methyl)phenoxy)tetrahydro-2H-pyran-3,4,5-triol (**6c**)*

According to general procedure **A**, compound **5c** (100 mg, 165.41 µmol) was treated with approx. 0.5 %w/v solution of $Mg(OMe)_2$ in dry MeOH (20 mL) for 6 h to yield 70 mg of **6c**. Yield 97%, yellow solid, mp 101 °C. IR (ATR, ṽ [cm^{-1}]): 3348 (br, O–H), 3011 (w, Ar–H), 2850 (w, C–H), 1512 (m, nitro N–O), 1221 (s, ether C–O), 1072 (vs, alc. C–O). ^{1}H NMR (400 MHz, Acetone-d_6) δ 7.99 (d, *J* = 9.3 Hz, 2H), 7.54 (t, *J* = 5.4 Hz, 1H), 7.14 (d, *J* = 8.3 Hz, 1H), 7.05 (d, *J* = 1.5 Hz, 1H), 6.91 (dd, *J* = 8.3, 1.7 Hz, 1H), 6.74 (d, *J* = 9.3 Hz, 2H), 4.93 – 4.86 (m, 4H), 4.39 (d, *J* = 5.7 Hz, 2H), 4.26 (t, *J* = 5.7 Hz, 1H), 3.81 – 3.76 (m, 4H), 3.62 – 3.56 (m, 1H), 3.42 – 3.55 (m, 4H). ^{13}C NMR (101 MHz, Acetone-d_6) δ 155.2, 150.3, 147.0, 137.3, 133.2, 126.6, 120.3, 116.9, 113.0, 101.8, 77.9, 74.3, 70.9, 61.9, 56.4, 46.8. ESI-MS: $[M+Na]^+$ = 459.05 *m/z*, ($C_{20}H_{24}N_2O_9$), HPLC purity: 98% (Method 1), Rf = 0.54 (silica gel, EtOAc/MeOH 9:1 v/v).

*(2R,3S,4S,5R,6S)-2-(4-(((4-fluorophenyl)amino)methyl)-2-methoxyphenoxy)-6-(hydroxymethyl)tetrahydro-2H-pyran-3,4,5-triol (**6d**)*

According to general procedure **A**, compound **5d** (160 mg, 227.03 µmol) was treated with approx. 0.5 %w/v solution of $Mg(OMe)_2$ in dry MeOH (20 mL) for 5 h to yield 110 mg of **6d**. Yield 97%, pale yellow solid, mp 99 – 100 °C. IR (ATR, ṽ [cm^{-1}]): 3547 (m, N–H), 3357 (br, O–H), 2916 (w, C–H), 1220 (m, ether C–O), 1073 (vs, alc. C–O). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.03 – 6.99 (m, 2H), 6.90 – 6.83 (m, 3H), 6.56 (dd, *J* = 8.6, 4.5 Hz, 2H), 6.04 (t, *J* = 5.4 Hz, 1H), 5.16 (d, *J* = 3.7 Hz, 1H), 5.04 (d, *J* = 1.6 Hz,

1H), 4.98 (d, *J* = 4.9 Hz, 1H), 4.85 (d, *J* = 6.6 Hz, 1H), 4.49 (t, *J* = 5.4 Hz, 1H), 4.15 (d, *J* = 5.4 Hz, 2H), 3.74 – 3.64 (m, 4H), 3.47 – 3.41 (m, 1H), 3.29 – 3.15 (m, 4H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 155.4, 153.1, 148.9, 145.4, 145.4, 133.7, 119.3, 115.3, 115.3, 115.0, 113.1, 113.0, 111.9, 100.2, 79.3, 77.0, 76.9, 73.2, 69.7, 60.7, 55.7, 46.8. ESI-MS: $[M+Na]^+$ = 432.00 *m/z* ($C_{20}H_{24}FNO_7$), HPLC purity: 98% (Method 1), Rf = 0.28 (silica gel, EtOAc/MeOH 9:1 v/v),

*(2R,3S,4S,5R,6S)-2-(4-(((4-chlorophenyl)amino)methyl)-2-methoxyphenoxy)-6-(hydroxymethyl)tetrahydro-2H-pyran-3,4,5-triol (**6e**)*

According to general procedure **A**, compound **5e** (145 mg, 244.10 µmol) was treated with approx. 0.5 %w/v solution of $Mg(OMe)_2$ in dry MeOH (20 mL) for 4 h to yield 80 mg of **6e**. Yield 77%, off-white solid, mp 149 °C. IR (ATR, ṽ [cm^{-1}]): 3375 (br, O–H), 3002 (w, Ar–H), 2935 (w, C–H), 1233 (m, ether C–O), 1072 (vs, alc. C–O). ^{1}H NMR (400 MHz, DMSO- d_6) δ 7.06 – 6.98 (m, 4H), 6.83 (d, *J* = 8.0 Hz, 1H), 6.57 (d, *J* = 8.6 Hz, 2H), 6.33 (t, *J* = 5.4 Hz, 1H), 5.17 (d, *J* = 2.2 Hz, 1H), 5.05 – 4.98 (m, 2H), 4.85 (d, *J* = 6.6 Hz, 1H), 4.49 (t, *J* = 4.9 Hz, 1H), 4.16 (d, *J* = 5.4 Hz, 2H), 3.74 – 3.65 (m, 4H), 3.46 – 3.41 (m, 1H), 3.28 – 3.16 (m, 4H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 148.9, 147.6, 145.4, 133.4, 128.5, 119.2, 119.0, 115.3, 113.7, 111.9, 100.2, 77., 76.9, 73.2, 69.7, 60.7, 55.7, 46.3. HPLC purity: 99% (Method 1), ESI-MS: $[M+Na]^+$ = 448.00 *m/z* ($C_{20}H_{24}FNO_7$), Rf = 0.55 (silica gel, EtOAc/MeOH 9:1 v/v).

*(2R,3S,4S,5R,6S)-2-(4-(((4-bromophenyl)amino)methyl)-2-methoxyphenoxy)-6-(hydroxymethyl)tetrahydro-2H-pyran-3,4,5-trio (**6f**)*

According to general procedure **A**, compound **5f** (162 mg, 400.98 µmol) was treated with approx. 0.5 %w/v solution of $Mg(OMe)_2$ in dry MeOH (20 mL) for 8 h to yield 89 mg of **6f**. Yield 75%, white solid, mp 178 °C. IR (ATR, ṽ [cm^{-1}]): 3397 (br, O–H), 3370, 3019 (w, Ar–H), 2922 (w, C–H), 1228 (m, ether C–O), 1077 (vs, alc. C–O). ^{1}H NMR (400 MHz, DMSO- d_6) δ 7.16 (d, *J* = 8.7 Hz, 2H), 7.03 – 6.97 (m, 2H), 6.82 (d, *J* = 8.1 Hz, 1H), 6.53 (d, *J* = 8.7 Hz, 2H), 6.36 (t, *J* = 5.7 Hz, 1H), 5.17 – 5.00 (m, 3H), 4.85 (d, *J* = 6.9 Hz, 1H), 4.49 (t, *J* = 5.1 Hz, 1H), 4.16 (d, *J* = 5.6 Hz, 2H), 3.74 (s, 3H), 3.67 – 3.64 (m, 1H), 3.47 – 3.41 (m, 1H), 3.28 – 3.15 (m, 4H).^{13}C NMR (101 MHz, DMSO-d_6) δ 148.9, 148.0, 145.4, 133.3, 131.3, 119.2, 115.3, 114.3, 111.8, 106.3, 100.2, 77.0, 76.9, 73.2, 69.7, 60.7, 55.7, 46.2. ESI-MS: $M+Na]^+$ = 493.90 *m/z* ($C_{20}H_{24}BrNO_7$), HPLC purity: 100% (Method 1), Rf = 0.65 (silica gel, EtOAc/MeOH 9:1 v/v).

*(2R,3S,4S,5R,6S)-2-(4-(((3-chloro-4-fluorophenyl)amino)methyl)-2-methoxyphenoxy)-6-(hydroxymethyl)tetrahydro-2H-pyran-3,4,5-triol (**6g**)*

According to general procedure **A**, compound **5g** (180 mg, 294.12 µmol) was treated with approx. 0.5 %w/v solution of $Mg(OMe)_2$ in dry MeOH (20 mL) for 5 h to yield 124 mg of **6g**. Yield 95%, white solid, mp 171 – 172 °C. IR (ATR, ṽ [cm^{-1}]): 3416 (br, O–H), 3041 (w, Ar–H), 2898 (w, C–H), 1222 (s, ether C–O), 1067 (vs, alc. C–O). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.09 – 6.99 (m, 3H), 6.84 (d, *J* = 8.2 Hz, 1H), 6.67 (dd, *J* = 6.2, 2.7 Hz, 1H), 6.55 – 6.51 (m, 1H), 6.34 (t, *J* = 5.8 Hz, 1H), 5.17 (d, *J* = 3.1 Hz, 1H), 5.04 (s, 1H), 4.98 (d, *J* = 4.6 Hz, 1H), 4.86 (d, *J* = 7.0 Hz, 1H), 4.48 (t, *J* = 5.2

Hz, 1H), 4.16 (d, *J* = 5.7 Hz, 2H), 3.74 (s, 3H), 3.66 (dd, *J* = 11.6, 3.1 Hz, 1H), 3.47 – 3.41 (m, 1H), 3.29 – 3.15 (m, 4H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 150.2, 148.9, 147.8, 146.3, 145.5, 133.1, 119.3, 119.2, 116.9, 116.7, 115.3, 112.5, 111.9, 100.2, 77.0, 76.9, 73.2, 69.7, 60.7, 55.7, 46.4. HR-MS: $[M+Cl]^-$ calc 478.0841, found 478.0838 *m/z* ($C_{20}H_{23}ClFNO_7$), HPLC purity: 99% (Method 1), Rf = 0.30 (silica gel, EtOAc/MeOH 9:1 v/v).

*(2R,3S,4S,5R,6S)-2-(4-(((2,4-dichlorophenyl)amino)methyl)-2-methoxyphenoxy)-6-(hydroxymethyl)tetrahydro-2H-pyran-3,4,5-triol (**6h**)*

According to general procedure **A**, compound **5h** (84 mg, 133.66 µmol) was treated with approx. 0.5 %w/v solution of $Mg(OMe)_2$ in dry MeOH (20 mL) for 4 h to yield 50 mg of **6h**. Yield 81%, off-white solid, mp 153 – 154 °C. IR (ATR, ṽ [cm^{-1}]): 3379 (br, O–H), 3021 (w, Ar–H), 2923 (w, C–H), 1228 (s, ether C–O), 1071 (vs, alc. C–O). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.34 (dd, *J* = 2.3, 1.1 Hz, 1H), 7.08 (dd, *J* = 8.8, 1.1 Hz, 1H), 7.03 – 6.94 (m, 2H), 6.79 (d, *J* = 8.3 Hz, 1H), 6.56 (d, *J* = 8.8 Hz, 1H), 6.21 (t, *J* = 6.0 Hz, 1H), 5.15 (d, *J* = 3.9 Hz, 1H), 5.02 (s, 1H), 4.96 (d, *J* = 5.0 Hz, 1H), 4.83 (d, *J* = 6.7 Hz, 1H), 4.47 (t, *J* = 5.6 Hz, 1H), 4.32 (d, *J* = 6 Hz, 2H), 3.73 (s, 3H), 3.64 (dd, *J* = 11.4, 5.0 Hz, 1H), 3.46 – 3.39 (m, 1H), 3.25 – 3.10 (m, 4H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 148.9, 145.5, 143.1, 132.9, 128.2, 127.7, 118.9, 118.8, 118.3, 115.3, 112.7, 111.5, 100.2, 77.0, 73.2, 69.7, 60.6, 55.7, 45.7. HR-MS: $[M+Cl]^-$ calc 494.0546, found 494.0546 *m/z* ($C_{20}H_{23}Cl_2NO_7$), HPLC purity: 99% (Method 1), Rf = 0.45 (silica gel, EtOAc/MeOH 9:1 v/v).

*(2R,3S,4S,5R,6S)-2-(hydroxymethyl)-6-(2-methoxy-4-(((3-(trifluoromethyl)phenyl)amino)methyl)phenoxy)tetrahydro-2H-pyran-3,4,5-triol (**6i**)*

According to general procedure **A**, compound **5i** (184 mg, 293.2 µmol) was treated with approx. 0.5 %w/v solution of $Mg(OMe)_2$ in dry MeOH (20 mL) for 8 h to yield 128 mg of **6i**. Yield 95%, off-white solid, mp 119 – 120 °C. IR (ATR, ṽ [cm^{-1}]): 3361 (br, O–H), 3016 (w, Ar–H), 2925 (w, C–H), 1227 (s, ether C–O), 1064 (vs, alc. C–O). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.24 (t, *J* = 7.9 Hz, 1H), 7.05 – 7.00 (m, 2H), 6.87 – 6.78 (m, 4H), 6.63 (t, *J* = 5.7 Hz, 1H), 5.17 (d, *J* = 4.6 Hz, 1H), 5.04 (d, *J* = 3.8 Hz, 1H), 4.97 (d, *J* = 5.1 Hz, 1H), 4.86 (d, *J* = 7.0 Hz, 1H), 4.48 (t, *J* = 5.7 Hz, 1H), 4.23 (d, *J* = 5.7 Hz, 2H), 3.74 (s, 3H), 3.67 – 3.64 (m, 1H), 3.47 – 3.41 (m, 1H), 3.29 – 3.15 (m, 4H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 149.2, 148.9, 145.5, 133.02 129.8, 129.8, 129.5, 119.3, 115.6, 115.3, 112.0, 111.6, 108.2, 100.2, 77.0, 76.9, 73.2, 69.7, 60.7, 55.7, 46.0. HR-MS: $[M+Cl]^-$ calc 494.1199, found 494.1199 m/z ($C_{21}H_{24}F_3NO_7$). HPLC purity: 99% (Method 1), Rf = 0.68 (silica gel, EtOAc/MeOH 9:1 v/v).

*(2R,3S,4S,5R,6S)-2-(4-(((2,6-dimethylphenyl)amino)methyl)-2-methoxyphenoxy)-6-(hydroxymethyl)tetrahydro-2H-pyran-3,4,5-triol (**6j**)*

According to general procedure **A**, compound **5j** (142 mg, 241.65 µmol) was treated with approx. 0.5 %w/v solution of $Mg(OMe)_2$ in dry MeOH (20 mL) for 5 h to yield 92 mg of **6j**. Yield 91%, light brown solid, mp 148 – 148 °C. IR (ATR, ṽ [cm^{-1}]): 3381 (br, O–H), 3029 (w, Ar–H), 2926 (w, C–H), 1231 (s, ether C–O), 1072 (vs, alc. C–O). ^{1}H NMR (400 MHz, DMSO) δ 6.98 (d, *J* = 8.3 Hz, 1H), 6.93 – 6.89 (m, 3H), 6.79 (dd, *J* =

8.3, 1.6 Hz, 1H), 6.70 (t, *J* = 7.4 Hz, 1H), 5.16 (d, *J* = 4.4 Hz, 1H), 5.05 (s, 1H), 4.98 (d, *J* = 4.8 Hz, 1H), 4.85 (d, *J* = 7.1 Hz, 1H), 4.49 (t, *J* = 5.7 Hz, 1H), 4.02 (s, 2H), 3.73 – 3.64 (m, 4H), 3.51 (s, 1H), 3.48 – 3.42 (m, 1H), 3.29 – 3.15 (m, 4H), 2.20 (s, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 149.2, 148.9, 145.5, 133.0, 129.8, 129.8, 129.5, 119.3, 115.6, 115.3, 112.0, 111.6, 108.2, 100.2, 77.0, 76.9, 73.2, 69.7, 60.7, 55.7, 46.0. ESI-MS: $[M+H]^+$ = 420.25 *m/z* ($C_{22}H_{29}NO_7$), HPLC purity: 96% (Method 1), Rf = 0.56 (silica gel, EtOAc/MeOH 9:1 v/v).

*(2R,3S,4S,5R,6S)-2-(4-(((4-bromopyridin-2-yl)amino)methyl)-2-methoxyphenoxy)-6-(hydroxymethyl)tetrahydro-2H-pyran-3,4,5-triol (**6k**)*

According to general procedure **A**, compound **5a** (72 mg, 414.56 µmol) was treated with approx. 0.5 %w/v solution of $Mg(OMe)_2$ in dry MeOH (20 mL) for 4 h to yield 43 mg of **6k**. Yield 81%, white solid, mp (n.d., decomposes). IR (ATR, ṽ [cm^{-1}]): 3346 (br, O–H), 3016 (w, Ar–H), 2969 (w, C–H), 1227 (s, ether C–O), 1069 (vs, alc. C–O). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.85 (d, *J* = 5.4 Hz, 1H), 7.22 (t, *J* = 5.8 Hz, 1H), 7.03 – 6.94 (m, 2H), 6.81 – 6.78 (m, 1H), 6.72 (s, 1H), 6.67 (dd, *J* = 5.4, 1.5 Hz, 1H), 5.17 – 4.84 (m, 5H), 4.38 (d, *J* = 5.7 Hz, 2H), 3.74 – 3.64 (m, 4H), 3.47 – 3.42 (m, 1H), 3.25 – 3.15 (m, 4H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 159.6, 157.7, 149.0, 148.8, 145.4, 134.0, 133.6, 131.68, 119.4, 115.4, 114.5, 112.1, 111.1, 110.2, 100.2, 77.0, 76.96 73.2, 69.78, 60.6, 59.6, 55.79 43.9. ESI-MS: $[M+H]^+$ *m/z* = 472.90, ($C_{19}H_{23}BrN_2O_7$), HPLC purity 98% (Method 1), Rf = 0.26 (silica gel, EtOAc/MeOH 9:1 v/v).

*(2R,3S,4S,5R,6S)-2-(hydroxymethyl)-6-(2-methoxy-4-(((pyridin-2-ylmethyl)amino)methyl)phenoxy)tetrahydro-2H-pyran-3,4,5-triol (**6l**)*

According to general procedure **A**, compound **5a** (160 mg, 278.46 µmol) was treated with approx. 0.5 %w/v solution of $Mg(OMe)_2$ in dry MeOH (20 mL) for 5 h to yield 94 mg of **6l**. Yield 83%, white solid, mp 78 – 80 °C. IR (ATR, ṽ [cm^{-1}]): 3455 (m, N–H), 3289 (br, O–H), 3016 (w, Ar–H), 2969 (w, C–H), 1228 (s, ether C–O), 1071 (vs, alc. C–O). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.52 – 8.43 (m, 2H), 7.75 (d, *J* = 7.6 Hz, 1H), 7.34 (dd, *J* = 7.3, 4.9 Hz, 1H), 7.03 – 6.98 (m, 2H), 6.81 (d, *J* = 8.1 Hz, 1H), 5.18 – 4.52 (m, 5H), 3.75 (s, 3H), 3.37 – 3.61 (m, 5H), 3.47 – 3.43 (m, 1H), 3.26 – 3.16 (m, 4H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 149.4, 148.9, 147.8, 145.3, 136.2, 135.7, 134.3, 123.3, 119.9, 115.1, 112.4, 100.2, 77.0, 76.9, 73.3, 69.7, 60.7, 59.8, 55.6, 51.9, 49.4. ESI-MS: ($[M+H]^+$ = 407.25 *m/z* ($C_{20}H_{26}N_2O_7$), HPLC purity: 85% (Method 1), Rf = 0.13 (RP-18, MeCN/water 3:2 v/v + TEA) (Flash chromatography method S1).

*(2R,3S,4S,5R,6S)-2-(hydroxymethyl)-6-(2-methoxy-4-(((4-methylpiperazin-1-yl)amino)methyl)phenoxy)tetrahydro-2H-pyran-3,4,5-triol (**6m**)*

According to general procedure **A**, compound **5m** (83 mg, 142.71 µmol) was treated with approx. 0.5 %w/v solution of $Mg(OMe)_2$ in dry MeOH (20 mL) for 5 h to yield 46 mg of **6m**. Yield 78%, white solid, mp 123 °C. IR (ATR, ṽ [cm^{-1}]): 3435 (m, N–H), 3358 (br, O–H), 3026 (w, Ar–H), 2969 (w, C–H), 2920 (w, C–H), 1227 (s, ether C–O), 1071 (vs, alc. C–O). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.57 (s, 1H), 7.22 (s, 1H), 7.07

– 7.01 (m, 2H), 5.22 – 4.91 (m, 4H), 4.52 (s, 1H), 3.77 (s, 3H), 3.67 (d, *J* = 11.4 Hz, 1H), 3.48 – 3.14 (m, 11H), 3.07 (s, 4H), 2.22 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 149.1, 146.6, 135.4, 130.4, 119.3, 115.1, 108.6, 99.9, 77.0, 76.9, 73.2, 69.6, 60.6, 55.5, 54.0, 50.8, 45.5. ESI-MS: $[M+H]^+$ = 412.10 *m/z* ($C_{19}H_{31}N_3O_7$), HPLC purity: 99% (Method 1), Rf = 0.21 (RP-18, MeCN/H_2O 3:2 v/v + TEA) (Flash chromatography method S2).

*(2R,3S,4S,5R,6S)-2-(4-(((2,3-dichlorophenyl)amino)methyl)-2-methoxyphenoxy)-6-(hydroxymethyl)tetrahydro-2H-pyran-3,4,5-triol (**8a**)*

According to general procedure **A**, compound **7a** (62 mg, 98.66 µmol) was treated with approx. 0.5 %w/v solution of $Mg(OMe)_2$ in dry MeOH (20 mL) for 5 h to yield 42 mg of **8a**. Yield 91%, white solid, mp 186 °C. IR (ATR, ṽ [cm^{-1}]): 3376 (br, O–H), 3002 (w, Ar–H), 2931 (w, C–H), 1233 (s, ether C–O), 1072 (vs, alc. C–O). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.05 – 6.99 (m, 3H), 6.81 (dd, *J* = 8.3, 1.7 Hz, 1H), 6.74 (dd, *J* = 7.9, 1.2 Hz, 1H), 6.55 (dd, *J* = 8.3, 1.1 Hz, 1H), 6.38 (t, *J* = 6.1 Hz, 1H), 5.15 (d, *J* = 4.7 Hz, 1H), 5.04 (s, 1H), 4.97 (d, *J* = 5.1 Hz, 1H), 4.84 (d, *J* = 7.3 Hz, 1H), 4.48 (t, *J* = 5.7 Hz, 1H), 4.34 (d, *J* = 6.0 Hz, 2H), 3.73 (s, 3H), 3.67 – 3.63 (m, 1H), 3.46 – 3.40 (m, 1H), 3.28 – 3.12 (m, 4H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 148.9, 145.7, 145.5, 132.9, 131.5, 128.2, 118.8, 116.6, 115.5, 115.3, 111.6, 110.1, 100.2, 77.0, 76.9, 73.2, 69.7, 60.6, 55.7, 45.9. HR-MS: $[M+Cl]^-$ calc 494.0545 m/z, found 494.0546 m/z ($C_{20}H_{23}Cl_2NO_7$), HPLC purity: >99% (Method 1), Rf = 0.30 (silica, EtOAc/MeOH 9:1 v/v).

*(2R,3S,4S,5R,6S)-2-(4-(((2,5-dichlorophenyl)amino)methyl)-2-methoxyphenoxy)-6-(hydroxymethyl)tetrahydro-2H-pyran-3,4,5-triol (**8b**)*

According to general procedure **A**, compound **5a** (63 mg, 100.25 µmol) was treated with approx. 0.5 %w/v solution of $Mg(OMe)_2$ in dry MeOH (20 mL) for 7 h to yield 36 mg of **8b**. Yield 78%, white solid, mp 162 – 163 °C. IR (ATR, ṽ [cm^{-1}]): 3401 (m, N–H), 3272 (br, O–H), 3081 (w, Ar–H), 2921 (w, C–H), 1269 (s, ether C–O), 1069 (vs, alc. C–O). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.25 (d, *J* = 8.2 Hz, 1H), 7.04 – 7.01 (m, 2H), 6.81 (dd, *J* = 8.2, 1.6 Hz, 1H), 6.58 – 6.55 (m, 3H), 6.34 (t, *J* = 6.1 Hz, 1H), 5.16 (d, *J* = 4.5 Hz, 1H), 5.04 (s, 1H), 4.98 (d, *J* = 5.0 Hz, 1H), 4.86 (d, *J* = 7.3 Hz, 1H), 4.49 (t, *J* = 5.7 Hz, 1H), 4.33 (d, *J* = 6.0 Hz, 2H), 3.74 (s, 3H), 3.67 – 3.63 (m, 1H), 3.47 – 3.41 (m, 1H), 3.29 – 3.14 (m, 4H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 148.9, 145.5, 145.1, 132.6, 132.4, 130.1, 118.8, 116.5, 115.8, 115.3, 111.7, 111.0, 100.1, 77.0, 76.9, 73.2, 69.6, 60.6, 55.7, 45.5. HR-MS: $[M+Cl]^-$ calc 494.0546 m/z, found 494.0545 m/z ($C_{20}H_{23}Cl_2NO_7$), HPLC purity: 99% (Method 1), Rf = 0.58 (silica gel, EtOAc/MeOH 9:1 v/v).

*(2R,3S,4S,5R,6S)-2-(4-(((3,4-dichlorophenyl)amino)methyl)-2-methoxyphenoxy)-6-(hydroxymethyl)tetrahydro-2H-pyran-3,4,5-triol (**8c**)*

According to general procedure **A**, compound **7c** (208 mg, 330.97 µmol) was treated with approx. 0.5 %w/v solution of $Mg(OMe)_2$ in dry MeOH (20 mL) for 6 h to yield 128 mg of **8c**. Yield 84%, white solid, mp 174 °C. IR (ATR, ṽ [cm^{-1}]): 3409 (m, N–H), 3360 (br, O–H), 3071(w, Ar–H), 2912 (w, C–H), 1275 (s, ether C–O), 1063 (vs, alc. C–O).

^{1}H NMR (400 MHz, DMSO-d_6) δ 7.22 (dd, *J* = 8.8, 2.4 Hz, 1H), 7.04 – 6.98 (m, 2H), 6.83 (dd, *J* = 8.3, 1.8 Hz, 1H), 6.75 (d, *J* = 2.7 Hz, 1H), 6.64 – 6.55 (m, 2H), 5.17 – 5.16 (m, 1H), 5.04 – 4.97 (m, 2H), 4.87 – 4.85 (m, 1H), 4.50 – 4.47 (m, 1H), 4.18 (d, *J* = 5.7 Hz, 2H), 3.74 (s, 3H), 3.68 – 3.63 (m, 1H), 3.47 – 3.41 (m, 1H), 3.29 – 3.15 (m, 4H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 149.0, 148.8, 145.5, 132.8, 131.1, 130.4, 119.3, 116.3, 115.4, 113.0, 112.8, 111.9, 100.2, 77.0, 76.9, 73.2, 69.7, 60.7, 55.7, 46.0. HR-MS: $[M+Cl]^-$ calc 494.0546 m/z, found 494.0546 m/z, ($C_{20}H_{23}Cl_2NO_7$), HPLC purity: 99% (Method 1), Rf = 0.31 (silica gel, EtOAc/MeOH 9:1 v/v).

*(2R,3S,4S,5R,6S)-2-(4-(((3,5-dichlorophenyl)amino)methyl)-2-methoxyphenoxy)-6-(hydroxymethyl)tetrahydro-2H-pyran-3,4,5-triol (**8d**)*

According to general procedure **A**, compound **7d** (171 mg, 272.10 µmol) was treated with approx. 0.5 %w/v solution of $Mg(OMe)_2$ in dry MeOH (20 mL) for 5 h to yield 116 mg of **8d**. Yield 92%, brown solid, mp 175 – 176 °C. IR (ATR, ṽ [cm^{-1}]): 3423 (m, N–H), 3358 (br, O–H), 3096 (w, Ar–H), 2980 (w, C–H), 1222 (s, ether C–O), 1071 (vs, alc. C–O).^{1}H NMR (400 MHz, DMSO-d_6) δ 7.04 (d, *J* = 8.3 Hz, 1H), 6.98 (d, *J* = 1.6 Hz, 1H), 6.84 – 6.78 (m, 2H), 6.58 (s, 3H), 5.17 (d, *J* = 4.7 Hz, 1H), 5.04 (d, *J* = 2.9 Hz, 1H), 4.98 (d, *J* = 5.1 Hz, 1H), 4.87 (d, *J* = 7.2 Hz, 1H), 4.49 (t, *J* = 5.7 Hz, 1H), 4.19 (d, *J* = 5.7 Hz, 2H), 3.75 (s, 3H), 3.68 – 3.64 (m, 1H), 3.48 – 3.42 (m, 1H), 3.30 – 3.13 (m, 4H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 150.9, 149.0, 145.6, 134.3, 132.5, 119.3, 115.4, 114.3, 112.0, 110.4, 100.2, 77.0, 76.9, 73.23, 69.7, 60.7, 55.7, 45.8. HR-MS: $[M+Cl]^-$ calc 494.0546 m/z, found 494.0545 m/z ($C_{20}H_{23}Cl_2NO_7$), HPLC purity: 99% (Method 1), Rf = 0.38 (silica gel, EtOAc/MeOH 9:1 v/v).

*3-methoxy-4-(((2R,3S,4S,5R,6S)-3,4,5-triacetoxy-6-(acetoxymethyl) tetrahydro-2H-pyran-2-yl)oxy)benzoic acid (**9**)*

Periodic acid (756 mg, 3.32 mmol) was dissolved in MeCN (100 mL) and stirred at r.t. for 20 min. The solution was cooled to 0 °C and compound **2** (800 mg, 1.66 mmol) and pyridinium chlorochromate (PCC) (7.15 mg, 33.17 µmol) dissolved in MeCN (5 mL) were added and stirred for 2h. The same portion of PCC was added and the reaction was continued for another 2h [36]. The mixture was diluted with EtOAc (100 mL) washed with brine/water 1:1 v/v solution, followed by sat. $NaHSO_3$ aq. solution, and dried over anhydrous Na_2SO_4 and filtered. The solvent was removed *in vacuo* and the residue was purified by column chromatography (silica gel, pet. ether/EtOAc 2:3 v/v + 0.1%FA, Rf = 0.40) to yield 601 mg of **9**. Yield 97%, white solid, mp 181 – 182 °C. IR (ATR, ṽ [cm^{-1}]): 3352 (br, O–H), 3081(w, Ar–H), 2951 (w, C–H), 1748 (s, ester C═O), 1212 (s, ether C–O). ^{1}H NMR (400 MHz, Acetone-d_6) δ 7.66 – 7.63 (m, 2H), 7.31 (d, *J* = 8.3 Hz, 1H), 5.46 – 5.43 (m, 1H), 5.40 (dd, *J* = 9.6, 1.2 Hz, 1H), 5.28 – 5.23 (m, 1H), 5.18 – 5.13 (m, 1H), 4.34 – 4.29 (m, 1H), 4.23 – 4.19 (m, 2H), 3.91 (s, 3H), 2.05 – 2.04 (m, 9H), 2.00 – 1.99 (m, 3H). ^{13}C NMR (101 MHz, Acetone-d_6) δ 170.67, 170.32, 170.06, 169.65, 167.09, 151.12, 150.90, 126.92, 123.86, 118.20, 114.60, 100.10, 73.24, 72.80, 71.93, 69.30, 62.73, 56.59, 20.62, 20.59, 20.54. ESI-MS: $[M+Na]^+$ = 521.00 *m/z* ($C_{22}H_{26}O_{13}$), HPLC purity: 98% (Method 1).

*(2R,3S,4S,5R,6S)-2-(acetoxymethyl)-6-(4-(chlorocarbonyl)-2-methoxyphenoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**10**)*

Compound **9** (800 mg, 1.61 mmol) was dissolved in $SOCl_2$ (10 mL, excess) and 1 drop of abs. DMF was added the solution was heated at 80 °C for 2 h under reflux. The excess thionyl chloride was removed *in vacuo* to yield 821 mg of **10**, which was used without further purification [37]. Yield = 99%, brown solid, mp n.d (decomposes). IR (ATR, ṽ [cm^{-1}]): 3017 (w, Ar–H), 2947 (w, C–H), 1747 (s, C═O), 1215 (s, ether C–O), 1079 (vs, alc. C–O). ^{1}H NMR (400 MHz, Acetone-d_6) δ 7.80 (dd, *J* = 8.6, 2.2 Hz, 1H), 7.64 (d, *J* = 2.2 Hz, 1H), 7.41 (d, *J* = 8.6 Hz, 1H), 5.57 (d, *J* = 7.9 Hz, 1H), 5.40 (t, *J* = 9.4 Hz, 1H), 5.26 (dd, *J* = 9.7, 7.9 Hz, 1H), 5.16 (t, *J* = 9.4 Hz, 1H), 4.32 – 4.18 (m, 3H), 3.93 (s, 1H), 2.03 – 2.02 (m, 9H), 1.98 (s, 3H). ^{13}C NMR (101 MHz, Acetone-d_6) δ 170.7, 170.3, 170.0, 169.6, 167.4, 153.4, 151.1, 128.6, 126.9, 117.7, 115.30, 99.5, 73.1, 73.0, 71.8, 69.1, 62.6, 56.8, 20.6, 20.5. HPLC purity (n.d, reacts with mobile phase), Rf = n.d (reacts with silica gel).

*General procedure (**C**) for the synthesis of compounds **11a-d***

To a solution of an appropriate amine (1.1 eq) in CH_2Cl_2 (50 mL) at 0 °C, aqueous Na_2CO_3 (10% w/v) (50 mL) and compound **10** (1 eq) were added and the mixture was vigorously stirred for 12 h while warming up to r.t. [60]. Phases were separated and the aqueous layer was additionally extracted with CH_2Cl_2. Combined CH_2Cl_2 layers were dried over Na_2SO_4, filtered, and the solvent was removed *in vacuo*. The residue was purified by column chromatography to yield a desired product (Supporting information, **Table S1**).

*N-(2,4-dichlorophenyl)-3-methoxy-4-(((2R,3S,4S,5R,6S)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)benzamide (**12a**)*

According to general procedure **A**, compound **11a** (80 mg, 124.53 µmol) was treated with approx. 0.5 %w/v solution of $Mg(OMe)_2$ in dry MeOH (20 mL) for 4 h to yield 51 mg of **12a**. Yield 86%, white solid, mp 180 – 181 °C. IR (ATR, ṽ [cm^{-1}]): 3366 (br, O–H), 3073 (w, Ar–H), 2961 (w, C–H), 2924 (w, C–H), 1649 (m, amide C═O), 1258 (s, ether C–O), 1072 (vs, alc. C–O). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.93 (s, 1H), 7.69 (d, *J* = 2.4 Hz, 1H), 7.66 – 7.60 (m, 3H), 7.46 (dd, *J* = 8.6, 2.4 Hz, 1H), 7.24 – 7.22 (m, 1H), 5.27 (d, *J* = 4.6 Hz, 1H), 5.07 – 5.06 (m, 2H), 4.99 (d, *J* = 5.1 Hz, 1H), 4.49 (t, *J* = 5.7 Hz, 1H), 3.86 (s, 3H), 3.74 – 3.70 (m, 1H), 3.52 – 3.47 (m, 1H), 3.41 – 3.33 (m, 3H), 3.24 – 3.19 (m, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 167.4, 164.8, 152.4, 149.7, 148.7, 143.2, 134.6, 130.5, 130.3, 129.4, 129.0, 127.5, 127.1, 120.9, 114.5, 111.8, 99.7, 77.2, 77.0, 73.2, 69.7, 60.8, 55.7. ESI-MS: $[M+H]^+$ = 473.90 *m/z* ($C_{20}H_{21}Cl_2NO_8$), HPLC purity: >99% (Method 1), Rf = 0.40 (silica, EtOAc/MeOH 9:1 v/v).

*N-(3,5-dichlorophenyl)-3-methoxy-4-(((2R,3S,4S,5R,6S)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)benzamide (**12b**)*

According to general procedure **A**, compound **11b** (86 mg, 133.87 µmol) was treated with approx. 0.5 %w/v solution of $Mg(OMe)_2$ in dry MeOH (20 mL) for 4 h to yield 54 mg of **12b**. Yield 85%, white solid, mp 230 – 322 °C. IR (ATR, ṽ [cm^{-1}]): 3318 (br, O–

H), 3078 (w, Ar–H), 2926 (w, C–H), 2871 (w, C–H), 1647 (m, amide C═O), 1271 (s, ether C–O), 1070 (vs, alc. C–O). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.38 (s, 1H), 7.87 (d, *J* = 1.8 Hz, 2H), 7.56 – 7.53 (m, 2H), 7.29 (t, *J* = 1.8 Hz, 1H), 7.20 (d, *J* = 8.4 Hz, 1H), 5.37 (d, *J* = 4.7 Hz, 1H), 5.14 (d, *J* = 3.7 Hz, 1H), 5.09 (d, *J* = 5.3 Hz, 1H), 5.04 (d, *J* = 7.3 Hz, 1H), 4.62 (t, *J* = 5.7 Hz, 1H), 3.84 (s, 3H), 3.70 – 3.66 (m, 2H), 3.39 – 3.29 (m, 4H), 3.20 – 3.14 (m, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 156.0, 140.0, 139.1, 132.1, 124.5, 117.8, 113.3, 111.6, 108.9, 104.9, 102.3, 90.0, 67.6, 67.27, 63.6, 60.2, 51.2, 46.4. ESI-MS: $[M+Na]^+$ = 495.90 *m/z* ($C_{20}H_{21}Cl_2NO_8$), HPLC purity: 99% (Method 1), Rf = 0.38 (silica gel, EtOAc/MeOH 9:1 v/v).

*N-(2,4-dichlorobenzyl)-3-methoxy-4-(((2R,3S,4S,5R,6S)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)benzamide (**12c**)*

According to general procedure **A**, compound **11c** (47 mg, 71.60 µmol) was treated with approx. 0.5 %w/v solution of $Mg(OMe)_2$ in dry MeOH (20 mL) for 5 h to yield 13 mg of **12c**. Yield 37%, off-white solid, mp 242 – 243 °C. IR (ATR, ṽ [cm^{-1}]): 3364 (br, O–H), 3048 (w, Ar–H), 2980 (w, C–H), 2887 (w, C–H), 1617 (m, amide C═O), 1269 (s, ether C–O), 1073 (vs, alc. C–O). ^{1}H NMR (400 MHz, MeOD) δ 7.54 (d, *J* = 2.0 Hz, 1H), 7.50 (dd, *J* = 8.5, 2.0 Hz, 1H), 7.47 (d, *J* = 2.0 Hz, 1H), 7.38 (d, *J* = 8.3 Hz, 1H), 7.32 (dd, *J* = 8.3, 2.0 Hz, 1H), 7.23 (d, *J* = 8.5 Hz, 1H), 5.03 (d, *J* = 7.3 Hz, 1H), 4.62 – 4.60 (m, 7H), 3.92 – 3.87 (m, 4H), 3.71 (dd, *J* = 12.2, 5.4 Hz, 1H), 3.58 – 3.41 (m, 4H). ^{13}C NMR (101 MHz, MeOD) δ 148.9, 134.8, 133.7, 129.9, 127.0, 115.2, 111.3, 100.6, 76.8, 76.2, 73.3, 69.8, 55.5, 40.9. ESI-MS: $[M+H]^+$ = 487.90 *m/z* ($C_{21}H_{23}Cl_2NO_8$), HPLC purity: 99% (Method 1), Rf = 0.19 (silica gel, EtOAc/MeOH 9:1 v/v).

*N-(2,4-dichlorophenethyl)-3-methoxy-4-(((2R,3S,4S,5R,6S)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)benzamide (**12d**)*

According to general procedure **A**, compound **12 d** (50 mg, 74.57 µmol) was treated with approx. 0.5 %w/v solution of $Mg(OMe)_2$ in dry MeOH (20 mL) for 5 h to yield 27 mg of **12d**. Yield 72%, white solid, mp 222 – 223 °C. IR (ATR, ṽ [cm^{-1}]): 3282 (br, O–H), 3074 (w, Ar–H), 2930 (w, C–H), 2863 (w, C–H), 1632 (m, amide C═O), 1273 (m, ether C–O), 1071 (vs, alc. C–O). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.46 (t, *J* = 5.5 Hz, 1H), 7.58 (d, *J* = 0.6 Hz, 1H), 7.42 – 7.35 (m, 4H), 7.11 (d, *J* = 8.6 Hz, 1H), 5.26 (d, *J* = 4.8 Hz, 1H), 5.08 (d, *J* = 3.9 Hz, 1H), 5.01 (dd, *J* = 10.0, 6.2 Hz, 2H), 4.53 (t, *J* = 5.7 Hz, 1H), 3.80 (s, 3H), 3.69 – 3.65 (m, 1H), 3.51 – 3.42 (m, 3H), 3.31 – 3.26 (m, 3H), 3.20 – 3.14 (m, 1H), 2.95 (t, *J* = 7.0 Hz, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 165.7, 148.8, 148.4, 136.2, 134.1, 132.5, 131.7, 128.6, 127.9, 127.3, 126.5, 120.0, 114.2, 111.3, 99.6, 77.1, 76.9, 73.1, 69.6, 60.6, 55.7, 32.4. ESI-MS: $[M+H]^+$ = 502.05 *m/z* $C_{22}H_{25}Cl_2NO_8$), HPLC purity: 99% (Method 1), Rf = 0.16 (silica gel, EtOAc/MeOH 9:1 v/v).

*N-((benzyloxy)carbonyl)-N-(3,5-dichlorophenyl)glycine (**15**)*

Compound **14** (1.5 g, 6.82 mmol) was dissolved in THF (20 mL) at r.t. and sat. $NaHCO_3$ aq. solution (20 mL) was added. The mixture was cooled to 0°C and stirred for a further 30 min. Benzyl chloroformate (2.4 g, 13.63 mmol) dissolved THF (60 mL)

was slowly added to the reaction mixture (over 2h) using a dropping funnel and stirred for further 3h [61]. The reaction was quenched by the addition of water (100 mL) and acidification to pH 2 – 3 using 4 M HCl. The mixture was extracted with CH_2Cl_2, combined organic layers were washed with brine, dried over anh. Na_2SO_4, filtered and the solvent was removed *in vacuo*. The residue was purified by flash chromatography (Method S4) to yield 1.4 g (58%) of **15**. (Supporting information, **Table S1**).

*General procedure (**D**) for the synthesis of compounds **16a-c***

Compound **15** (1.2 eq), 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (1.2 eq), and N, N-Diisopropylethylamine (DIPEA) (1.2 eq) in DMF (5 mL) at r.t. stirred for 2 h. Appropriate compound (**13a-c**) (1.2 equiv.) was dissolved DMF (5 mL) and added dropwise to the above solution, and the reaction mixture was stirred for a further 8 – 10 h under LC-MS control [49]. Afterwards, water (100 mL) was added to the reaction mixture and stirred for 15 minutes. The aqueous layer was then extracted with CH_2Cl_2, combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, filtered, and the solvent was removed *in vacuo*. The residual DMF was removed by repeated co-evaporated with n-heptane and the residue was purified by flash chromatography (Methods S5 – S7) to yield a desired product (Supporting information, **Table S1**).

*General procedure (**E**) for the synthesis of compounds **18a-c***

Appropriate compound (**17a-c**) (1 equiv.) was dissolved in dry MeOH (10 mL) r.t, and $NiCl_2.6H_2O$ (5 eq) was added. $NaBH_4$ (15 eq) was added to the solution in small portions and the mixture was stirred for 1 h. Thereafter, other portions of $NiCl_2.6H_2O$ (2.5 eq.) and $NaBH_4$ (7.5 eq.) were added and the reaction was continued for further 30 minutes [50]. The suspension was filtered through celite two times, and the solvent of the filtrate was removed *in vacuo*. The residue was re-suspended in water and extracted with CH_2Cl_2. The combined organic phases were washed with water and brine, dried over anhydrous Na_2SO_4, filtered, and the solvent was removed *in vacuo*. The residue was purified under flash chromatography (Methods S8 – S10) to obtain compounds a desired product.

*2-((3,5-dichlorophenyl)amino)-N-(3-methoxy-4-(((2R,3S,4S,5R,6S)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)benzyl)-N-propylacetamide (**18a**)*

According to general procedure **E**, compound **17a** (140 mg, 201.85 µmol) was dissolved in dry MeOH (10 mL) and treated with $NiCl_2.6H_2O$ (239.89 mg, 1.01 mmol), and $NaBH_4$ (114.55 mg, 3.03 mmol). The mixture was stirred at r.t. for 1.5 h. The crude product was purified by flash chromatography (Method S8) to yield 33 mg of **18a.** Yield 37%, white solid, mp 63 – 65 °C. IR (ATR, $\tilde{\nu}$ [cm^{-1}]): 3358 (br, O–H), 3076 (w, Ar–H), 2980 (w, C–H), 2970 (w, C–H), 1646, 1223 (s, ether C–O), 1071 (vs, alc. C–O). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39 – 7.26 (m, 1H), 7.22 – 7.16 (m, 2H), 7.01 – 6.91 (m, 1H), 6.76 – 6.56 (m, 2H), 6.37 (d, *J* = 28.0 Hz, 1H), 5.19 – 4.76 (m, 4H), 4.47 – 4.32 (m, 3H), 3.81 – 3.64 (m, 9H), 3.37 – 3.24 (m, 2H), 3.13 – 3.04 (m, 1H), 1.58 – 1.48 (m, 2H), 0.83 – 0.79 (m, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 168.7, 149.6,

149.1, 135.6, 128.7, 117.3, 111.3, 73.4, 56.2, 32.2, 30.0, 21.7, 11.5. ESI-MS: $[M+H]^+$ = 559.15 *m/z* ($C_{25}H_{32}Cl_2N_2O_8$), HPLC purity: 93% (Method 1), Rf = 0.72 (RP-18, MeCN/H_2O 1:1 v/v + TEA).

*2-((3,5-dichlorophenyl)amino)-N-(3,4-dimethoxyphenethyl)-N-(3-methoxy-4-(((2R,3S,4S,5R,6S)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)benzyl)acetamide (**18b**)*

According to general procedure **E**, compound **17b** (180 mg, 220.67 µmol) was dissolved in dry MeOH (10 mL) and treated with $NiCl_2.6H_2O$ (262.25 mg, 1.10 mmol), and $NaBH_4$ (125.23 mg, 3.31 mmol). The mixture was stirred at r.t. for 1.5 h. The crude product was purified by flash chromatography (Method S9) to yield 49 mg of **18b**. Yield 33%, white solid, mp 95 – 96 °C. IR (ATR, ṽ [cm^{-1}]): 3374 (br, O–H), 3078 (w, Ar–H), 2918 (w, C–H), 2850 (w, C–H), 1645 (m, amide C═O), 1227 (s, ether C–O), 1070 (vs, alc. C–O). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.32 – 7.15 (m, 3H), 7.05 – 6.93 (m, 1H), 6.77 – 6.55 (m, 5H), 6.30 (d, *J* = 36.0 Hz, 1H), 5.14 – 4.77 (m, 4H), 4.48 (s, 1H), 4.18 (t, *J* = 54.7 Hz, 1H), 3.84 – 3.56 (m, 13H), 3.42 – 3.35 (m, 3H), 2.77 – 2.73 (m, 2H), 2.25 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 162.8, 157.4, 148.5, 146.1, 135.5, 130.5, 128.7, 121.0, 112.3, 111.6, 111.2, 56.1, 32.1, 29.9, 29.6, 26.9, 22.9, 14.3. ESI-MS: $[M+H]^+$ = 681.10 *m/z* ($C_{32}H_{38}Cl_2N_2O_{10}$), HPLC purity: 84% (Method 1), Rf = 0.69 (RP-18, MeCN/ H_2O 1:1 v/v + TEA).

*2-((3,5-dichlorophenyl)amino)-N-(3-methoxy-4-(((2R,3S,4S,5R,6S)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)benzyl)-N-(pyridin-4-ylmethyl)acetamide (**18c**)*

According to general procedure **E**, compound **17c** (110 mg, 148.13 µmol) was dissolved in dry MeOH (10 mL) and treated with $NiCl_2.6H_2O$ (176 mg, 740.64 µmol), and $NaBH_4$ (84 mg, 2.22 mmol). The mixture was stirred at r.t. for 1.5 h. The crude product was purified by flash chromatography (Method S10) to yield 13 mg of **18c**. Yield 19%, off-white semisolid, IR (ATR, ṽ [cm^{-1}]): 3345 (br, O–H), 3071 (w, Ar–H), 2922 (w, C–H), 2846 (w, C–H), 1650 (m, amide C═O), 1220 (m, ether C–O), 1070 (vs, alc. C–O). ^{1}H NMR (400 MHz, Acetone-d_6) δ 8.49 (d, *J* = 26.9 Hz, 2H), 7.36 – 7.34 (m, 1H), 7.27 – 7.23 (m, 2H), 7.11 (dd, *J* = 25.9, 8.3 Hz, 1H), 6.96 – 6.92 (m, 1H), 6.80 – 6.77 (m, 1H), 6.72 (d, *J* = 1.48 Hz, 1H), 6.68 (d, *J* = 1.48 Hz, 1H), 6.61 (d, *J* = 6.1 Hz, 1H), 4.89 (t, *J* = 7.4 Hz, 1H), 4.78 – 4.59 (m, 5H), 4.26 (s, 1H), 4.12 (s, 1H), 3.87 – 3.83 (m, 1H), 3.78 (d, *J* = 6.1 Hz, 3H), 3.67 (dd, *J* = 11.8, 4.6 Hz, 1H), 3.44 – 3.41 (m, 4H), 3.26 – 3.22 (m, 3H). ^{13}C NMR (101 MHz, Acetone-d_6) δ 168.6, 154.6, 153.6, 150.5, 142.2, 136.0, 116.6, 112.0, 102.6, 86.5, 78.0, 74.7, 71.2, 62.6, 56.6, 53.2. ESI-MS: $[M+H]^+$ = 608.05 *m/z* ($C_{28}H_{31}Cl_2N_3O_8$), HPLC purity: 85% (Method 1), Rf = 0.7 (RP-18, MeCN/ H_2O 1:1 v/v + TEA).

4.4. Antimicrobial susceptibility testing

Fresh bacterial cultures were prepared from their respective overnight cultures in LB medium under incubation at 37°C. Test bacterial suspensions at 10^6 CFU/mL were prepared via dilution of the respective fresh cultures using Newman's correlation

curve after the determination of their optical densities (OD600) (Eppendorf BioPhotometer Plus, Eppendorf, Hamburg, Germany) [62].

Determination of the compound Minimum Inhibitory Concentrations (MIC) was done using broth microdilution assays as per the guidelines prescribed by the European Committee for Antimicrobial Susceptibility Testing (EUCAST) [62]. Briefly, stock solutions of purified compounds were prepared at 10 mg/mL in DMSO or acetone depending on the respective compound's solubility (**Table S3**). Working solutions were prepared by dilution of the stock solutions to concentrations of 512 or 1024 µg/mL using an MHB medium. The concentrations of DMSO in working solutions were 5.12 – 10.24%, whereas those of acetone were 25.6 %v/v. In cases where acetone was used, its concentration across wells was achieved by prefilling the wells on columns 3 to 11 of the 96-wells plate with 100 µL of acetone/MHB medium 25.6 %v/v. For samples dissolved in DMSO, prefilling of the corresponding wells was done using the same volume of MHB medium.

Following the loading of 200 µL of the compounds' working solutions into wells on column 2 of the plates in triplicate, serial dilution was performed using a multichannel pipette. During the procedure, 100 µL of the working solution was drawn from wells on column 2 and mixed well with the prefilled 100 µL of the respective media in the successive columns. Following the repeated procedure to the 11^{th} column, the final 100 µL was discarded.

Finally, 100 µL of bacterial suspensions in MHB (10^6 CFU/mL) were added in each test well to arrive at the test concentrations of 0.5–256 µg/mL or 1–512 µg/mL of the respective compounds (**Table S3**). The final concentration of acetone was 12.8% v/v, whereas the maximum tested concentration of DMSO was 2.56–5.12%. All outside wells of the 96-well plates were filled with 200 µL of MHB medium. All tests employed gentamicin sulfate (0.25–128 µg/mL) as a positive control. No bacterial growth inhibition was observed at the highest tested concentrations of acetone and DMSO stated above.

All experiments were carried out in triplicate for one assay and assays for compounds showing activities were repeated three times on separate days. The loaded plates were incubated at 37°C for 18 h. Ascertainment of the MIC values was based on visual observation for the absence of pinpointed bacterial growth at the bottom of the treatment wells. In that, the MIC values were taken as the highest of the values obtained from all individual replicas [9, 28].

ACKNOWLEDGEMENTS

The authors would like to extend their sincere appreciation to Florian Geyer, Joshua Weinmann, Marie Hovah, Cristian Lombo, Mohammed Marzouk, Liling Tryasmono, Liane Pogorelaja, Liane Dreher, Eslam Ibrahim and Curd Schollmayer for their technical assistance; Christoph Mahler and Merle Arrowsmith for assistance with HR-MS measurements; Prof. Dr. Christoph Schoen for the generous provision of the MDR bacterial strains, and the German Academic Exchange Service (DAAD) for the financial support.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest

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Supporting information

Table of contents

Table S1: Synthesis procedures, yields, properties, and spectra data for intermediate compounds

Cpd	Particulars
5a	*(2R,3R,4S,5R,6S)-2-(acetoxymethyl)-6-(2-methoxy-4-((p-tolylamino)methyl)phenoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**5a**).* According to general procedure **B**, compound **2** (248 mg, 513.27 µmol) was treated with *p*-toluidine (66 mg, 615.92 µmol), triacetoxyborohydride (STAB) (326 mg, 1.54 mmol) and acetic acid (88 µL, 1.54 mmol) in 20 mL of 1,2-dichloroethane (DCE) and stirred for overnight under argon. The crude product was purified by column chromatography to yield 292 mg of **5a**. Yield 94%, brown semisolid, mp 94 – 96 °C. IR (ATR, ṽ [cm^{-1}]): 3387 (w, N–H), 3016 (w, Ar–H), 2968 (w, C–H), 2947 (w, C–H), 1737 (vs, ester C═O), 1215 (s, ether C–O), 1083 (vs, alc. C–O). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.05 (d, J = 8.2 Hz, 1H), 6.96 (d, J = 8.2 Hz, 2H), 6.91 (d, J = 1.8 Hz, 1H), 6.84 (dd, J = 8.2, 1.8 Hz, 1H), 6.56 – 6.50 (m, 2H), 5.29 – 5.21 (m, 2H), 5.17 – 5.09 (m, 1H), 4.94 – 4.89 (m, 1H), 4.26 (dd, J = 12.3, 5.1 Hz, 1H), 4.22 (s, 2H), 4.13 (dd, J = 12.2, 2.5 Hz, 1H), 3.76 (d, J = 8.0 Hz, 3H), 3.75 – 3.71 (m, 1H), 2.05 – 2.01 (m, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.8, 170.5, 169.6, 169.6, 151.0, 146.0, 145.3, 136.6, 129.9, 127.2, 120.4, 119.8, 113.3, 112.1, 101.1, 72.8, 72.1, 71.4, 68.6, 62.1, 56.2, 48.7, 20.9, 20.8, 20.8, 20.8, 20.6. ESI-MS: $[M+H]^+$ = 574.40 *m/z* ($C_{29}H_{35}NO_{11}$), HPLC purity: 95% (Method 1), Rf = 0.40 (silica gel, Pet. ether/EtOAc 1:1 v/v).
5b	*(2R,3R,4S,5R,6S)-2-(acetoxymethyl)-6-(2-methoxy-4-(((4-methoxyphenyl)amino)methyl)phenoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**5b**)* According to general procedure **B**, compound **2** (240 mg, 497.47 µmol) was treated with 4-methoxyaniline (74 mg, 596.97 µmol),

	triacetoxyborohydride (STAB) (316 mg,1.49 mmol) and acetic acid (85 µL,1.49 mmol) in 25 mL of 1,2-dichloroethane (DCE) and stirred overnight under argon. The crude product was purified by column chromatography to yield 262 mg of **5b**. Yield 89%, brown semisolid, mp 109 – 111 °C. IR (ATR, ṽ [cm^{-1}]): 3384 (w, N–H), 3013 (w, Ar–H), 3002 (w, Ar–H), 2969 (w, C–H), 2943 (w, C–H), 1736 (vs, ester C═O), 1216 (s, ether C–O), 1082 (vs, alc. C–O). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.05 (d, J = 8.1 Hz, 1H), 6.91 (d, J = 1.8 Hz, 1H), 6.84 (dd, J = 8.1, 1.8 Hz, 1H), 6.78 – 6.75 (m, 1H), 6.75 – 6.73 (m, 1H), 6.61 – 6.58 (m, 1H), 6.58 – 6.56 (m, 1H), 5.28 – 5.23 (m, 2H), 5.17 – 5.11 (m, 1H), 4.92 (dd, J = 5.3, 2.5 Hz, 1H), 4.26 (dd, J = 12.2, 5.3 Hz, 1H), 4.20 (s, 2H), 4.14 (dd, J = 12.2, 2.5 Hz, 1H), 3.79 – 3.70 (m, 7H), 2.05 (s, 6H), 2.01 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.8, 170.5, 169.6, 169.6, 152.6, 151.0, 145.4, 136.6, 120.5, 119.9, 115.1, 114.5, 112.2, 101.1, 72.9, 72.2, 71.5, 68.7, 62.2, 56.3, 56.0, 49.4, 20.9, 20.9, 20.9, 20.8. ESI-MS: $[M+H]^+$ = 590.50 *m/z* ($C_{29}H_{35}NO_{12}$), HPLC purity: 74% (Method 1), Rf = 0.35 (silica gel, Pet. ether/EtOAc 1:1 v/v).
5c	*(2R,3R,4S,5R,6S)-2-(acetoxymethyl)-6-(2-methoxy-4-(((4-nitrophenyl)amino)methyl)phenoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**5c**)* According to general procedure **B**, compound **2** (243 mg, 504.0 µmol) was treated with 4-nitroaniline (84 mg, 604.8 µmol), triacetoxyborohydride (STAB) (320 mg, 1.51 mmol) and acetic acid (86 µL, 1.51 mmol) in 25 mL of 1,2-dichloroethane (DCE) and stirred for overnight under argon. The crude product was purified by column chromatography to yield 132 mg of **5c**. Yield 46%, yellow solid, mp 154 °C. IR (ATR, ṽ [cm^{-1}]): 3364 (w, N–H), 3024 (w, Ar–H), 2969 (w, C–H), 2945 (w, C–H), 1738 (vs, ester C═O), 1216 (s, ether C–O), 1065 (vs, alc. C–O). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.06 – 8.02 (m, 2H), 7.07 (d, J = 8.1 Hz, 1H), 6.84 – 6.80 (m, 2H), 6.55 – 6.52 (m, 2H), 5.28 – 5.21 (m, 2H), 5.15 – 5.11 (m, 1H), 4.93 (dd, J = 4.9, 2.5 Hz, 1H), 4.34 (s, 2H), 4.24 (dd, J = 12.2, 4.9 Hz, 1H), 4.14 (dd, J = 12.2, 2.5 Hz, 1H), 3.77 (s, 3H), 3.75 – 3.71 (m, 1H), 2.05 – 2.00 (m, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.8, 170.5, 169.6, 169.6, 153.2, 151.3, 145.8, 138.6, 134.2, 126.6, 120.6, 119.6, 112.0, 111.6, 100.9, 72.7, 72.2, 71.4, 68.6, 62.1, 56.3, 47.6, 20.9, 20.8, 20.8, 20.8. ESI-MS: $[M+H]^+$ = 627.15 *m/z* ($C_{28}H_{32}N_2O_{13}$), HPLC purity: >99% (Method 1), Rf = 0.47 (silica gel, Pet. ether/EtOAc 1:1 v/v).
5d	*(2R,3R,4S,5R,6S)-2-(acetoxymethyl)-6-(4-(((4-fluorophenyl)amino)methyl)-2-methoxyphenoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**5d**)* According to general procedure **B**, compound **2** (200 mg, 414.56 µmol) was treated with 4-fluoroaniline (92 mg, 829.12 µmol), triacetoxyborohydride (STAB) (351 mg, 1.66 mmol) and acetic acid (95 µL, 1.66 mmol) in 20 mL of 1,2-dichloroethane (DCE) and stirred for 36 h under argon. The crude product was purified by column chromatography to yield 210 mg of **5d**. Yield 88%, brown solid, mp 120 – 121 °C. IR (ATR, ṽ

	[cm^{-1}]): 3389 (w, N–H), 3016 v, 2969 (w, C–H), 2943 (w, C–H), 1741 (vs, ester C═O), 1216 (s, ether C–O), 1072 (vs, alc. C–O). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.05 (d, *J* = 8.1 Hz, 1H), 6.89 – 6.81 (m, 4H), 6.55 – 6.50 (m, 2H), 5.28 – 5.21 (m, 2H), 5.17 – 5.11 (m, 1H), 4.93 – 4.91 (m, 1H), 4.25 (dd, *J* = 12.2, 5.0 Hz, 1H), 4.20 (s, 2H), 4.13 (dd, *J* = 12.2, 2.4 Hz, 1H), 3.77 (s, 3H), 3.76 – 3.71 (m, 1H), 2.05 – 2.01 (s, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.8, 170.5, 169.6, 169.6, 157.3, 155.0, 151.1, 145.4, 144.6, 136.2, 120.5, 119.8, 116.0, 115.8, 113.9, 113.9, 112.1, 101.1, 72.8, 72.2, 71.4, 68.6, 62.1, 56.2, 48.9, 20.9, 20.8, 20.8, 20.8. ESI-MS: $[M+H]^+$ = 578.05 *m/z* ($C28H_{32}FNO_{11}$), HPLC purity: 96% (Method 1), Rf = 0.48 (silica gel, Pet. ether/EtOAc 1:1 v/v).
5e	*(2R,3R,4S,5R,6S)-2-(acetoxymethyl)-6-(4-(((4-chlorophenyl)amino)methyl)-2-methoxyphenoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**5e**)* According to general procedure **B**, compound **2** (183 mg, 379.98 µmol) was treated with 4-chloroaniline (58 mg, 455.98 µmol), triacetoxyborohydride (STAB) (241 mg, 1.14 mmol) and acetic acid (65 µL, 1.14 mmol) in 20 mL of 1,2-dichloroethane (DCE) and stirred overnight under argon. The crude product was purified by column chromatography to yield 221 mg of **5e**. Yield 98% off-white solid, mp 139 – 140 °C. IR (ATR, ṽ [cm^{-1}]): 3389 (w, N–H), 3016 (w, Ar–H), 2969 (w, C–H), 2942 (w, C–H), 1740 (vs, ester C═O), 1216 (s, ether C–O), 1071 (vs, alc. C–O). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.09 – 7.04 (m, 3H), 6.87 (d, *J* = 1.8 Hz, 1H), 6.82 (dd, *J* = 8.2, 1.8 Hz, 1H), 6.53 – 6.49 (m, 2H), 5.28 – 5.21 (m, 2H), 5.15 – 5.10 (m, 1H), 4.92 – 4.90 (m, 1H), 4.25 (dd, *J* = 12.3, 5.1 Hz, 1H), 4.22 (s, 2H), 4.13 (dd, *J* = 12.3, 2.5 Hz, 1H), 3.77 (s, 3H), 3.75 – 3.71 (m, 1H), 2.04 – 2.00 (m, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.8, 170.5, 169.6, 169.6, 151.1, 146.7, 145.4, 135.8, 129.3, 122.5, 120.5, 119.7, 114.2, 112.0, 101.0, 72.8, 72.2, 71.4, 68.6, 62.1, 56.3, 48.2, 20.9, 20.8, 20.8, 20.8. ESI-MS: $[M+H]^+$ = 595.00 *m/z* ($C_{28}H_{32}ClNO_{11}$), HPLC purity: >99% (Method 1), Rf = 0.30 (silica gel, Pet. ether/EtOAc 1:1 v/v).
5f	*(2R,3R,4S,5R,6S)-2-(acetoxymethyl)-6-(4-(((4-bromophenyl)amino)methyl)-2-methoxyphenoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**5e**)* According to general procedure **B**, compound **2** (200 mg, 415 µmol) was treated with 4-bromoaniline (86 mg, 497.47 µmol), triacetoxyborohydride (STAB) (351 mg, 1.66 mmol) and acetic acid (95 µL, 1.66 mmol) in 20 mL of 1,2-dichloroethane (DCE) and stirred overnight under argon. The crude product was purified by column chromatography to yield 183 mg of 5f. Yield 70%, Off-white solid, mp 144 – 145 °C. IR (ATR, ṽ [cm^{-1}]): 3388, 3057, 2966 (w, C–H), 2938 (w, C–H), 1741 (vs, ester C═O), 1214 (s, ether C–O), 1069 (vs, alc. C–O). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.23 – 7.20 (m, 2H), 7.05 (d, *J* = 8.1 Hz, 1H), 6.87 (d, *J* = 1.8 Hz, 1H), 6.82 (dd, *J* = 8.1, 1.8 Hz, 1H), 6.49 – 6.45 (m, 2H), 5.26 – 5.24 (m, 2H), 5.16 – 5.11 (m, 1H),

	4.93 – 4.91 (m, 1H), 4.25 (dd, *J* = 12.2, 5.0 Hz, 1H), 4.22 (s, 2H), 4.14 (dd, *J* = 12.2, 2.5 Hz, 1H), 3.77 (s, 3H), 3.76 – 3.71 (m, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.6, 170.3, 169.4, 169.4, 150.9, 146.9, 145.3, 135.6, 132.0, 120.3, 119.5, 114.5, 111.8, 109.4, 100.9, 72.6, 72.0, 71.2, 68.5, 62.0, 56.1, 48.1, 20.7, 20.7, 20.6, 20.6. ESI-MS: $[M+H]^+$, = 639.98 *m/z* ($C_{28}H_{32}BrNO_{11}$), HPLC purity: >99% (Method 1), Rf = 0.40 (silica gel, Pet. ether/EtOAc 1:1 v/v).
5g	*(2R,3R,4S,5R,6S)-2-(acetoxymethyl)-6-(4-(((3-chloro-4-fluorophenyl)amino)methyl)-2-methoxyphenoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**5g**)* According to general procedure **B**, compound **2** (200 mg,414.56 µmol) was treated with 3-chloro-4-fluoro-aniline (91 mg, 621.84 µmol), triacetoxyborohydride (STAB) (351 mg, 1.66 mmol) and acetic acid (95 µL, 1.66 mmol) in 20 mL of 1,2-dichloroethane (DCE) and stirred for 36 h under argon. The crude product was purified by column chromatography to yield 231 mg of **5g**. Yield 91%, off-white solid, mp 122 – 1123 °C. IR (ATR, ṽ [cm^{-1}]): 3400 (w, N–H), 2955 (w, C–H), 2896 (w, C–H), 1756 (vs, ester C═O), 1211 (s, ether C–O), 1066 (vs, alc. C–O). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.06 (d, *J* = 8.1 Hz, 1H), 6.25 – 6.86 (m, 1H), 6.81 (dd, *J* = 8.1, 2.0 Hz, 1H), 6.59 (dd, *J* = 6.0, 2.9 Hz, 1H), 6.45 – 6.37 (m, 1H), 5.26 – 5.24 (m, 2H), 5.16 – 5.11 (m, 1H), 4.94 – 4.92 (m, 1H), 4.25 (dd, *J* = 12.2, 5.0 Hz, 1H), 4.19 (s, 2H), 4.13 (dd, *J* = 12.2, 2.5 Hz, 1H), 3.77 (s, 3H), 3.76 – 3.70 (m, 1H), 2.05 – 2.01(m, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.8, 170.5, 169.6, 169.6, 152.4, 151.1, 150.1, 145.5, 145.1, 135.5, 121.4, 121.2, 120.5, 119.7, 117.14, 116.9, 114.0, 112.4, 112.3, 112.0, 101.0, 72.8, 72.2, 71.4, 68.6, 62.1, 56.3, 48.6, 20.9, 20.8, 20.8, 20.8. ESI-MS: ($[M+H]^+$ = 588.60 *m/z* ($C_{28}H_{32}ClFNO_{11}$), HPLC purity: 83% (Method 1), Rf = 0.50 (silica gel, Pet. ether/EtOAc 1:1 v/v).
5h	*(2R,3R,4S,5R,6S)-2-(acetoxymethyl)-6-(4-(((2,4-dichlorophenyl)amino)methyl)-2-methoxyphenoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**5h**)* According to general procedure **B**, compound **2** (200 mg,414.56 µmol) was treated with 2,4-dichloroaniline (101 mg, 621.84 µmol), triacetoxyborohydride (STAB) (351 mg, 1.66 mmol) and acetic acid (95 µL, 1.66 mmol) in 20 mL of 1,2-dichloroethane (DCE) and stirred for 24 h under argon. The crude product was purified by column chromatography to yield 84 mg of **5h**. Yield 32%, off-white solid, mp 103 – 104 °C. IR (ATR, ṽ [cm^{-1}]): 3400 (w, N–H), 2980 (w, C–H), 2927 (w, C–H), 1742 (vs, ester C═O), 1206 (s, ether C–O), 1064 (vs, alc. C–O). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.25 (d, *J* = 2.4 Hz, 1H), 7.07 (d, *J* = 8.1 Hz, 1H), 7.03 (dd, *J* = 8.7, 2.4 Hz, 1H), 6.86 (d, *J* = 1.7 Hz, 2H), 6.82 (dd, *J* = 8.1, 1.7 Hz, 1H), 6.50 (d, *J* = 8.7 Hz, 1H), 5.26 – 5.25 (m, 1H), 5.17 – 5.12 (m, 1H), 4.94 – 4.92 (m, 1H), 4.30 (s, 2H), 4.26 (dd, *J* = 12.3, 5.0 Hz, 1H), 4.14 (dd, *J* = 12.3, 2.4 Hz, 1H), 3.78 (s, 3H), 3.76 – 3.72 (m, 1H), 2.06 – 2.01 (m, 12H).

	^{13}C NMR (101 MHz, $CDCl_3$) δ 170.8, 170.5, 169.6, 169.6, 151.2, 145.57, 142.7, 135.2, 129.0, 128.0, 121.8, 120.6, 119.7, 119.6, 112.4, 111.8, 101.0, 72.8, 72.2, 71.4, 68.7, 62.2, 56.3, 47.9, 20.9, 20.9, 20.8. ESI-MS: $[M+H]^+$ = 650.90 *m/z* ($C_{28}H_{31}Cl_2NO_{11}$), HPLC purity: >99% (Method 1), Rf = 0.40 (silica gel, Pet. ether/EtOAc 1:1 v/v).
5i	*(2R,3R,4S,5R,6S)-2-(acetoxymethyl)-6-(2-methoxy-4-(((3-(trifluoromethyl)phenyl)amino)methyl)phenoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**5i**)* According to general procedure **B**, compound **2** (200 mg, 414.56 µmol) was treated with 3-(trifluoromethyl)aniline (80 mg, 497.47 µmol), triacetoxyborohydride (STAB) (351 mg, 1.66 mmol) and acetic acid (95 µL, 1.66 mmol) in 20 mL of 1,2-dichloroethane (DCE) and stirred for 48 h under argon. The crude product was purified by column chromatography to yield 203 mg of **5i**. Yield 78%, off-white solid, mp 115 – 116 °C. IR (ATR, ṽ [cm^{-1}]): 3409 (w, N–H), 3046 (w, Ar–H), 2960 (w, C–H), 1757 (vs, ester C═O), 1221 (s, ether C–O), 1062 (vs, alc. C–O). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.23 (t, *J* = 7.7 Hz, 1H), 7.07 (d, *J* = 8.2 Hz, 1H), 6.93 (d, *J* = 7.7 Hz, 1H), 6.89 (d, *J* = 1.9 Hz, 1H), 6.84 (dd, *J* = 8.2, 1.9 Hz, 2H), 6.73 (dd, *J* = 8.2, 2.2 Hz, 1H), 5.27 – 5.24 (m, 2H), 5.16 – 5.12 (m, 1H), 4.94 – 4.92 (m, 1H), 4.27 (d, *J* = 2.4 Hz, 2H), 4.24 (d, *J* = 5.0 Hz, 1H), 4.14 (dd, *J* = 12.2, 2.4 Hz, 1H), 3.78 (s, 3H), 3.76 – 3.72 (m, 1H), 2.05 – 2.01 (m, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.8, 170.5, 169.6, 169.6, 156.2, 151.2, 148.3, 145.6, 135.5, 132.0, 131.6, 129.9, 125.8, 120.6, 119.9, 116.1, 114.4, 112.1, 109.4, 101.0, 72.8, 72.2, 71.4, 68.7, 62.2, 56.3, 48.2, 20.9, 20.9, 20.9, 20.8. ESI-MS: $[M+H]^+$, = 628.10 *m/z* ($C_{29}H_{32}F_3NO_{11}$), HPLC purity: >99% (Method 1), Rf = 0.38 (silica gel, Pet. ether/EtOAc 1:1 v/v).
5j	*(2R,3R,4S,5R,6S)-2-(acetoxymethyl)-6-(4-(((2,6-dimethylphenyl)amino)methyl)-2-methoxyphenoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**5j**)* According to general procedure **B**, compound **2** (200 mg, 414.56 µmol) was treated with 2,6-dimethylaniline (60 mg, 497.47 µmol), triacetoxyborohydride (STAB) (264 mg, 1.24 mmol) and acetic acid (75 µL,1.24 mmol) in 20 mL of 1,2-dichloroethane (DCE) and stirred overnight under argon. The crude product was purified by column chromatography to yield 142 mg of **5j**. Yield 58%, light brown semisolid, IR (ATR, ṽ [cm^{-1}]): 3372 (w, N–H), 2951 (w, C–H), 2927 (w, C–H), 1748 (vs, ester C═O), 1211 (s, ether C–O), 1064 (vs, alc. C–O). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.06 (d, *J* = 7.9 Hz, 1H), 7.00 (s, 1H), 6.98 (s, 1H), 6.85 – 6.71 (m, 3H), 5.27 – 5.25 (m, 2H), 5.17 – 5.12 (m, 1H), 4.93 – 4.91 (m, 1H), 4.26 (dd, *J* = 12.2, 5.0 Hz, 1H), 4.15 (dd, *J* = 12.2, 2.5 Hz, 1H), 4.05 (s, 2H), 3.76 – 3.72 (m, 4H), 2.24 (s, 6H), 2.06 (s, 6H), 2.02 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.8, 170.5, 169.6, 156.2, 150.9, 147.2, 145.5, 134.5, 130.0, 129.4, 129.1, 128.5, 122.5, 120.5, 120.3, 112.6, 101.2, 72.9, 72.2, 71.5, 68.7, 62.2, 56.2, 52.7, 20.9, 20.9, 20.8, 18.7, 17.8. ESI-MS: $[M+H]^+$ = 588.60 *m/z*

	($C_{30}H_{37}NO_{11}$), HPLC purity: 83% (Method 1) Rf = 0.47 (silica gel, Pet. ether/EtOAc 1:1 v/v).
5k	*(2R,3R,4S,5R,6S)-2-(acetoxymethyl)-6-(4-(((4-bromopyridin-2-yl)amino)methyl)-2-methoxyphenoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**5k**)* According to general procedure **B**, compound **2** (200 mg, 414.56 µmol) was treated with 4-bromopyridin-2-amine (143.45 mg, 829.12 µmol) and triacetoxyborohydride (STAB) (351 mg, 1.66 mmol) in 20 mL of 1,2-dichloroethane (DCE) and stirred for 24 h under argon. The crude product was purified by column chromatography to yield 82 mg of **5k**. Yield 83%, light brown semisolid, IR (ATR, ṽ [cm^{-1}]): 3398 (w, N–H), 2980 (w, C–H), 2969 (w, C–H), 2925, 1745 (vs, ester C═O), 1212 (s, ether C–O), 1065 (vs, alc. C–O). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.84 (d, *J* = 5.5 Hz, 1H), 7.06 (d, *J* = 8.1 Hz, 1H), 6.93 – 6.80 (m, 3H), 6.73 (dd, *J* = 5.5, 1.5 Hz, 1H), 6.53 (d, *J* = 1.5 Hz, 1H), 5.25 (dd, *J* = 6.4, 2.4 Hz, 2H), 5.16 – 5.11 (m, 1H), 4.93 – 4.91 (m, 1H), 4.38 (d, *J* = 3.0 Hz, 2H), 4.25 (dd, *J* = 12.2, 5.0 Hz, 1H), 4.13 (dd, *J* = 12.2, 2.4 Hz, 1H), 3.78 (s, 3H), 3.76 – 3.71 (m, 1H), 2.05 (s, 6H), 2.01 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.8, 170.5, 169.6, 159.4, 151.1, 148.4, 145.6, 135.2, 134.4, 120.6, 119.7, 116.7, 111.9, 109.7, 101.0, 72.8, 72.2, 71.4, 68.7, 62.2, 56.3, 46.2, 20.9, 20.9, 20.8. ESI-MS: $[M+H]^+$ = 639.3 *m/z* ($C_{27}H_{31}BrN_2O_{11}$), HPLC purity: >99% (Method 1), Rf = 0.47 (silica gel, Pet. ether/EtOAc 1:1 v/v).
5l	*(2R,3R,4S,5R,6S)-2-(acetoxymethyl)-6-(2-methoxy-4-(((pyridin-2-ylmethyl)amino)methyl)phenoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**5l**)* According to general procedure **B**, compound **2** (200 mg, 414.56 µmol) was treated with pyridine-4-ylmethanamine (200 mg, 414.56 µmol), and triacetoxyborohydride (STAB) (351.45 mg, 1.66 mmol) in 20 mL of 1,2-dichloroethane (DCE) and stirred for 24 h under argon. The crude product was purified by column chromatography to yield 201 mg of **5l**. Yield 84%, brown semisolid, mp 92 – 93 °C. IR (ATR, ṽ [cm^{-1}]): 3358 (w, N–H), 3016 (w, Ar–H), 2969 (w, C–H), 2944 (w, C–H), 1738 (vs, ester C═O), 1216 (s, ether C–O), 1069 (vs, alc. C–O). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.54 (s, 1H), 8.47 (d, *J* = 3.8 Hz, 1H), 7.66 – 7.65 (m, 1H), 7.25 – 7.21 (m, 1H), 7.03 (d, *J* = 8.1 Hz, 1H), 6.88 (d, *J* = 1.8 Hz, 1H), 6.78 (dd, *J* = 8.1, 1.8 Hz, 1H), 5.27 – 5.21 (m, 2H), 5.15 – 5.10 (m, 1H), 4.92 – 4.90 (m, 1H), 4.24 (dd, *J* = 12.2, 5.0 Hz, 1H), 4.13 (dd, *J* = 12.2, 2.5 Hz, 1H), 3.78 – 3.70 (m, 7H) 2.04 (s, 6H), 2.00 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.8, 170.5, 169.6, 169.6, 150.9, 149.9, 148.7, 145.3, 136.8, 136.1, 135.6, 123.6, 120.4, 120.3, 112.7, 101.1, 72.8, 72.1, 71.4, 68.6, 62.1, 56.2, 53.0, 50.6, 20.9, 20.8, 20.8, 20.8. ESI-MS: $[M+H]^+$ = 575.60 *m/z* ($C_{28}H_{34}N_2O_{11}$), HPLC purity: 97% (Method 1), Rf = 0.27 (silica gel, Pet. ether/EtOAc 1:1 v/v).
5m	*(2R,3R,4S,5R,6S)-2-(acetoxymethyl)-6-(2-methoxy-4-(((4-methylpiperazin-1-yl)amino)methyl)phenoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**5m**)*

	According to general procedure **B**, compound **2** (200 mg, 414.56 µmol) was treated with 4-methylpiperazin-1-amine (57 mg, 497.47µmol), triacetoxyborohydride (STAB) (264 mg,1.24 mmol) and acetic acid (75 µL, 1.24 mmol) in 20 mL of 1,2-dichloroethane (DCE) and stirred overnight under argon. The crude product was purified by column chromatography to yield 96 mg of **5m**. Yield 42%, brown solid, mp 113 – 114 °C. IR (ATR, ṽ [cm^{-1}]): 3454 (w, N–H), 2980 (w, C–H), 2970 (w, C–H), 1735 (vs, ester C═O), 1219 (s, ether C–O), 1081 (vs, alc. C–O). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.46 (s, 1H), 7.27 (d, *J* = 1.8 Hz, 1H), 7.05 (d, *J* = 8.2 Hz, 1H), 6.95 (dd, *J* = 8.2, 1.8 Hz, 1H), 5.29 – 5.23 (m, 2H), 5.16 – 5.11 (m, 1H), 4.96 – 4.81 (m, 1H), 4.25 (dd, *J* = 12.2, 5.1 Hz, 1H), 4.14 (dd, *J* = 12.2, 2.5 Hz, 1H), 3.83 (s, 3H), 3.76 – 3.72 (m, 1H), 3.22 – 3.19 (m, 4H), 2.64 – 2.62 (m, 4H), 2.36 (s, 3H), 2.05 (s, 6H), 2.01 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.8, 170.5, 169.6, 151.1, 146.5, 135.7, 133.3, 120.1, 120.0, 109.1, 101.0, 72.8, 72.2, 71.5, 68.7, 62.2, 56.2, 54.6, 51.1, 46.0, 20.9, 20.9, 20.8. ESI-MS: ($[M+H]^+$ = 580.50 *m/z*, $C_{27}H_{39}N_3O_{11}$), HPLC purity: >99% (Method 1), Rf = 0.50 (silica gel, EtOAc/MeOH 4:1 v/v + 0.1% ammonia 25%).
7a	*(2R,3R,4S,5R,6S)-2-(acetoxymethyl)-6-(4-(((2,3-dichlorophenyl)amino)methyl)-2-methoxyphenoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**7a**)* According to general procedure **B**, compound **2** (200 mg, 414.56 µmol) was treated with 2,3-dichloroaniline (101 mg, 621.84 µmol), triacetoxyborohydride (STAB) (351 mg, 1.66 mmol) and acetic acid (95 µL, 1.66 mmol) in 20 mL of 1,2-dichloroethane (DCE) and stirred for 72 h under argon. The crude product was purified by column chromatography to yield 72 mg of **7a**. Yield 28%, white solid, mp 158 – 160 °C. IR (ATR, ṽ [cm^{-1}]): 3412 (w, N–H), 2980 (w, C–H), 2971 (w, C–H), 1754 (vs, ester C═O), 1208 (s, ether C–O), 1063 (vs, alc. C–O). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.07 (d, *J* = 8.1 Hz, 1H), 6.99 (t, *J* = 8.1 Hz, 1H), 6.87 (d, *J* = 1.6 Hz, 1H), 6.83 (dd, *J* = 8.1, 1.6 Hz, 1H), 6.78 (dd, *J* = 8.1, 1.1 Hz, 1H), 6.50 – 6.48 (m, 1H), 5.29 – 5.22 (m, 2H), 5.16 – 5.12 (m, 1H), 4.94 – 4.92 (m, 1H), 4.32 (s, 2H), 4.26 (dd, *J* = 12.2, 5.0 Hz, 1H), 4.14 (dd, *J* = 12.2, 2.4 Hz, 1H), 3.78 (s, 3H), 3.76 – 3.72 (m, 1H), 2.05 – 2.04(m, 6H) 2.01 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.8, 170.5, 169.6, 169.6, 151.8, 151.2, 145.6, 145.5, 135.2, 133.1, 128.0, 120.6, 119.6, 118.6, 117.5, 111.8, 109.7, 101.0, 72.8, 72.2, 71.4, 68.7, 62.2, 56.3, 48.0, 20.9, 20.9, 20.8. ESI-MS: $[M+H]^+$ = 651.95 *m/z* ($C_{28}H_{31}Cl_2NO_{11}$), HPLC purity: >99% (Method 1), Rf = 0.3 (silica gel, Pet. ether/EtOAc 1:1 v/v).
7b	*(2R,3R,4S,5R,6S)-2-(acetoxymethyl)-6-(4-(((2,5-dichlorophenyl)amino)methyl)-2-methoxyphenoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**7b**)* According to general procedure **B**, compound **2** (200 mg, 414.56 µmol) was treated with 2,5-dichloroaniline (101 mg, 621.84 µmol),

	triacetoxyborohydride (STAB) (351 mg, 1.66 mmol) and acetic acid (95 µL, 1.66 mmol) in 20 mL of 1,2-dichloroethane (DCE) and stirred for 72 h under argon. The crude product was purified by column chromatography to yield 78 mg of **7b**. Yield 30%, white solid, mp 173 – 174 °C. IR (ATR, ṽ [cm^{-1}]): 3410 (w, N–H), 2980 (w, C–H), 1755 (vs, ester C═O), 1206 (s, ether C–O), 1067 (vs, alc. C–O). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.15 (d, *J* = 8.3 Hz, 1H), 7.08 (d, *J* = 8.3 Hz, 1H), 6.87 (d, *J* = 1.9 Hz, 1H), 6.83 (dd, *J* = 8.3, 1.9 Hz, 1H), 6.61 – 6.57 (m, 2H), 5.29 – 5.23 (m, 2H), 5.17 – 5.12 (m, 1H), 4.95 – 4.93 (m, 1H), 4.29 – 4.24 (m, 3H), 4.14 (dd, *J* = 12.2, 2.5 Hz, 1H), 3.79 (s, 3H), 3.77 – 3.73 (m, 1H), 2.06 – 2.05 (m, 6H), 2.01 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.8, 170.5, 169.6, 169.6, 151.2, 145.7, 144.8, 134.8, 133.8, 130.0, 120.7, 119.7, 117.6, 112.0, 111.6, 101.0, 72.8, 72.2, 71.5, 68.7, 62.2, 56.3, 47.8, 20.9, 20.9, 20.9, 20.8. ESI-MS: $[M+H]^+$ = 650.05 *m/z* ($C_{28}H_{31}Cl_2NO_{11}$), HPLC purity: >99% (Method 1), Rf = 0.52 (silica gel, Pet. ether/EtOAc 1:1 v/v).
7c	*(2R,3R,4S,5R,6S)-2-(acetoxymethyl)-6-(4-(((3,4-dichlorophenyl)amino)methyl)-2-methoxyphenoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**7c**)* According to general procedure **B**, compound **2** (200 mg, 414.56 µmol) was treated with 3,4-dichloroaniline (101 mg, 621.84 µmol), triacetoxyborohydride (STAB) (351 mg, 1.66 mmol) and acetic acid (95 µL, 1.66 mmol) in 20 mL of 1,2-dichloroethane (DCE) and stirred for 24 h under argon. The crude product was purified by column chromatography to yield 239 mg of **7c**. Yield 92%, light brown solid, mp 126 – 128 °C. IR (ATR, ṽ [cm^{-1}]): 3398 (w, N–H), 3010 (w, Ar–H), 2978 (w, C–H), 2958 (w, C–H), 1754 (vs, ester C═O), 1205 (s, ether C–O), 1052 (vs, alc. C–O). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.15 (d, *J* = 8.7 Hz, 1H), 7.06 (d, *J* = 8.2 Hz, 1H), 6.85 (d, *J* = 1.9 Hz, 1H), 6.81 (dd, *J* = 8.2, 2.0 Hz, 1H), 6.66 (d, *J* = 2.7 Hz, 1H), 6.42 (dd, *J* = 8.7, 2.7 Hz, 1H), 5.28 – 5.22 (m, 2H), 5.16 – 5.11 (m, 1H), 4.94 – 4.92 (m, 1H), 4.25 (dd, *J* = 12.2, 5.0 Hz, 1H), 4.21 (s, 2H), 4.14 (dd, *J* = 12.2, 2.5 Hz, 1H), 3.78 (s, 3H), 3.75 – 3.72 (m, 1H), 2.05 – 2.04 (m, 6H), 2.01 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.8, 170.5, 169.6, 169.6, 151.2, 147.6, 145.6, 135.2, 133.0, 130.8, 120.6, 120.4, 119.7, 114.2, 112.8, 112.0, 101.0, 72.8, 72.2, 71.4, 68.6, 62.1, 56.3, 48.1, 20.9, 20.9, 20.8, 20.8. ESI-MS: $[M+H]^+$, = 667.75 *m/z* ($C_{28}H_{31}Cl_2NO_{11}$), HPLC purity: 98% (Method 1), Rf = 0.35 (silica gel, Pet. ether/EtOAc 1:1 v/v),
7d	*(2R,3R,4S,5R,6S)-2-(acetoxymethyl)-6-(4-(((3,5-dichlorophenyl)amino)methyl)-2-methoxyphenoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**7d**)* According to general procedure **B**, compound **2** (200 mg, 414.56 µmol) was treated with 3,5-dichloroaniline (101 mg, 621.84 µmol), triacetoxyborohydride (STAB) (351 mg, 1.66 mmol) and acetic acid (95 µL, 1.66 mmol) in 20 mL of 1,2-dichloroethane (DCE) and stirred for 48 h under argon. The crude product was purified by column chromatography to

	yield 192 mg of **7d**. Yield 74%, off-white solid, mp 163 – 164 °C. IR (ATR, ṽ [cm^{-1}]): 3396 (w, N–H), 2974 (w, C–H), 2960 (w, C–H), 1756 (vs, ester C═O), 1213 (s, ether C–O), 1066 (vs, alc. C–O). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.07 (d, *J* = 8.1 Hz, 1H), 6.84 (d, *J* = 1.7 Hz, 1H), 6.80 (dd, *J* = 8.1, 1.9 Hz, 1H), 6.66 (t, *J* = 1.7 Hz, 1H), 6.45 (d, *J* = 1.7 Hz, 2H), 5.29 – 5.22 (m, 2H), 5.16 – 5.11 (m, 1H), 4.94 – 4.92 (m, 1H), 4.25 (dd, *J* = 12.3, 5.0 Hz, 1H), 4.21 (s, 2H), 4.14 (dd, *J* = 12.3, 2.5 Hz, 1H), 3.78 (s, 3H), 3.77 – 3.72 (m, 1H), 2.06 – 2.04 (m, 6H), 2.01 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.8, 170.5, 169.6, 169.6, 151.2, 149.7, 145.6, 135.7, 135.0, 120.6, 119.8, 117.6, 112.0, 111.3, 101.0, 72.8, 72.2, 71.4, 68.6, 62.2, 56.3, 47.9, 20.9, 20.9, 20.8, 20.8. ESI-MS: $[M+H]^+$ = 629.9 *m/z* ($C_{28}H_{31}Cl_2NO_{11}$), HPLC purity: 99% (Method 1), Rf = 0.30 (silica gel, Pet. ether/EtOAc 1:1 v/v),
11a	*(2R,3R,4S,5R,6S)-2-(acetoxymethyl)-6-(4-((2,4-dichlorophenyl)carbamoyl)-2-methoxyphenoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**11a**)* According to general procedure **C**, 2, 4-dichloroaniline (30 mg, 183.87 µmol) was treated with compound **10** (100 mg, 167.16 µmol) in a mixture of aqueous Na_2CO_3 (10% w/v) and CH_2Cl_2 and vigorously stirred for 12 h. The crude product was purified by column chromatography to yield 90 mg of **11a**. Yield 83%, white solid mp 159 – 160 °C. IR (ATR, ṽ [cm^{-1}]): 3419 (w, N–H), 3071 (w, Ar–H), 2980 (w, C–H), 1749 (vs, ester C═O), 1680 (vs, alc. C–O), 1213 (s, ether C–O), 1065 (vs, alc. C–O). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.48 (d, *J* = 8.9 Hz, 1H), 8.30 (s, 1H), 7.51 (d, *J* = 2.1 Hz, 1H), 7.41 (d, *J* = 2.4 Hz, 1H), 7.34 (dd, *J* = 8.4, 2.1 Hz, 1H), 7.29 (dd, *J* = 8.9, 2.4 Hz, 1H), 7.17 (d, *J* = 8.4 Hz, 1H), 5.32 – 5.26 (m, 2H), 5.21 – 5.13 (m, 1H), 5.08 – 5.03 (m, 1H), 4.26 (dd, *J* = 12.3, 4.9 Hz, 1H), 4.17 (dd, *J* = 12.3, 2.5 Hz, 1H), 3.89 (s, 3H), 3.82 – 3.78 (m, 1H), 2.06 (s, 6H), 2.04 – 2.01 (m, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.8, 170.5, 169.6, 169.5, 164.7, 151.2, 149.5, 133.7, 130.8, 129.5, 129.0, 128.3, 123.7, 122.3, 119.3, 119.2, 112.4, 100.3, 72.7, 72.4, 71.3, 68.5, 62.1, 56.5, 20.9, 20.8, 20.8. ESI-MS: $[M+Na]^+$ = 665.00 *m/z* ($C_{28}H_{29}Cl_2NO_{12}$), HPLC purity: >99% (Method 1), Rf = 0.38 (silica gel, $CHCl_3$/EtOAc 4:1 v/v).
11b	*(2R,3R,4S,5R,6S)-2-(acetoxymethyl)-6-(4-((3,5-dichlorophenyl)carbamoyl)-2-methoxyphenoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**11b**)* According to general procedure **C**, 3, 5-dichloroaniline (30 mg, 183.87 µmol) was treated with compound **10** (100 mg, 167.16 µmol) in a mixture of aqueous Na_2CO_3 (10% w/v) and CH_2Cl_2 and vigorously stirred for 12 h. The crude product was purified by column chromatography to yield 99 mg of **11b**. Yield 92%, light brown solid, mp 69 – 70 °C. IR (ATR, ṽ [cm^{-1}]): 3357 (w, N–H), 3079 (w, Ar–H), 2980 (w, C–H), 2970 (w, C–H), 1748 (vs, ester C═O), 1673 (vs, alc. C–O), 1210 (s, ether C–O), 1065 (vs, alc. C–O). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.99 (s, 1H), 7.61 – 7.60 (m, 2H), 7.45 (d, *J* = 2.0 Hz, 1H), 7.28 (dd, *J* = 8.4, 2.1 Hz, 1H), 7.14 – 7.11 (m, 2H), 5.30 – 5.24 (m, 2H), 5.18 – 5.10 (m, 1H), 5.06 – 5.01 (m, 1H), 4.23 (dd, *J* = 12.3, 4.8

	Hz, 1H), 4.16 (dd, *J* = 12.3, 2.6 Hz, 1H), 3.86 (s, 3H), 3.79 – 3.75 (m, 1H), 2.06 (s, 6H), 2.02 – 2.01 (m, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.8, 170.5, 169.6, 169.6, 165.2, 151.1, 149.3, 140.0, 135.5, 130.6, 124.7, 119.3, 119.2, 118.6, 112.4, 100.3, 72.6, 72.4, 71.3, 68.5, 62.0, 56.4, 20.9, 20.8, 20.8. ESI-MS: $[M+Na]^+$ = 665.15 *m/z* ($C_{28}H_{29}Cl_2NO_{12}$). HPLC purity: 98% (Method 1), Rf = 0.38, (silica gel, $CHCl_3$/EtOAc 4:1 v/v).
11c	*(2R,3R,4S,5R,6S)-2-(acetoxymethyl)-6-(4-((3,5-dichlorobenzyl)carbamoyl)-2-methoxyphenoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**11c**)* According to general procedure **C**, (2,4-dichlorophenyl)methanamine (29 mg, 166.85 µmol) was treated with compound **10** (80 mg, 151.68 µmol) in a mixture of aqueous Na_2CO_3 (10% w/v) and CH_2Cl_2 and vigorously stirred for 12 h. The crude product was purified by column chromatography to yield 59 mg of **11c**. Yield 59%, light brown semisolid, ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38 – 7.42 (m, 3H), 7.21 (dd, J = 8.2, 2.0 Hz, 1H), 7.17 (dd, J = 8.2, 2.0 Hz, 1H), 7.08 (d, J = 8.2 Hz, 1H), 6.52 (t, J = 5.6 Hz, 1H), 5.23 – 5.27 (m, 2H), 5.12 – 5.16 (m, 1H), 4.98 – 5.00 (m, 1H), 4.64 (d, J = 6.0 Hz, 2H), 4.23 (dd, J = 12.2, 4.9 Hz, 1H), 4.14 (dd, J = 12.2, 2.5 Hz, 1H), 3.84 (s, 3H), 3.73 – 3.78 (m, 1H), 2.01– 04 (m, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.5, 134.4, 131.5, 129.6, 127.6, 119.3, 119.0, 112.4, 100.4, 72.6, 72.3, 71.3, 68.5, 62.0, 56.4, 41.8, 20.8. ESI-MS: $[M+H]^+$ = 656.00 *m/z* ($C_{29}H_{31}Cl_2NO_{12}$), HPLC purity: 99% (Method 1), Rf = 0.44, (silica gel, $CHCl_3$/EtOAc 4:1 v/v).
11d	*(2R,3R,4S,5R,6S)-2-(acetoxymethyl)-6-(4-((3,5-dichlorophenethyl)carbamoyl)-2-methoxyphenoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**11d**)* According to general procedure **C**, 2-(2,4-dichlorophenyl)ethan-1-amine (27 mg, 141.58 µmol) was treated with compound **10** (77 mg, 128.71 µmol) in a mixture of aqueous Na_2CO_3 (10% w/v) and CH_2Cl_2 and vigorously stirred for 12 h. The crude product was purified by column chromatography to yield 63 mg of **11d**. Yield 74%, off-white semisolid, ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36-7.37 (m, 2H), 7.17 (d, J = 1.18 Hz, 2H), 7.07 – 7.08 (m, 2H), 6.12 (t, J = 5.7, 1H), 5.25 – 5.27 (m, 2H), 5.11 – 5.16 (m, 1H), 4.98 – 5.00 (m, 1H), 4.24 (dd, J = 12.2, 5.0 Hz, 1H), 4.14 (dd, J = 12.2, 2.5 Hz, 1H), 3.83 (s, 3H), 3.74 – 3.79 (m, 1H), 3.63 – 3.69 (m, 2H), 3.03 (t, J = 6.8 Hz, 2H), 2.01 – 2.04 (m, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.7, 170.4, 169.6, 169.5, 167.0, 150.9, 148.7, 135.4, 134.9, 133.3, 132.0, 131.0, 129.6, 127.5, 119.2, 118.8, 112.1, 100.4, 72.6, 72.3, 71.3, 68.5, 62.1, 60.5, 56.3, 39.9, 33.0, 20.9, 20.8, 20.7, 14.4. ESI-MS: $[M+H]^+$ = 656.00 *m/z* ($C_{30}H_{33}Cl_2NO_{12}$), HPLC purity: >99% (Method 1), Rf = 0.30 (silica gel, $CHCl_3$/EtOAc 4:1 v/v).
13a	*(2R,3R,4S,5R,6S)-2-(acetoxymethyl)-6-(2-methoxy-4-((propylamino)methyl)phenoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**13a**)* According to general procedure **B**, compound **2** (300 mg, 621.84 µmol)

	was treated with propan-1-amine (55 mg, 932.76 µmol), triacetoxyborohydride (STAB) (527mg, 2.49 mmol) and acetic acid (142 µL, 2.49 mmol) in 25 mL of 1,2-dichloroethane (DCE) and stirred overnight under argon. The crude product was purified by column chromatography to yield 259 mg of 13a. Yield 79%, pale-yellow semisolid, IR (ATR, ṽ [cm^{-1}]): 3346 (w, N–H), 3020 (w, Ar–H), 2961 (w, C–H), 2923 (w, C–H), 1751 (vs, ester C═O), 1215 (s, ether C–O), 1068 (vs, alc. C–O). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.99 (d, 1H, J = 8.1 Hz), 6.85 (d, J = 1.6 Hz,1H), 6.74 (dd, J = 8.1, 1.7 Hz, 1H), 5.17 – 5.24 (m, 2H), 5.06 – 5.11 (m, 1H), 4.86 – 4.88 (m, 1H), 4.21 (dd, J = 12.2, 5.0 Hz, 1H), 4.09 (dd, J = 12.2, 2.4 Hz, 1H), 3.75 (s, 3H), 3.68 – 3.72 (m, 1H), 3.67 (s, 2H), 2.53 (t, J = 7.2, 2H), 1.96 – 2.01 (m, 12H), 1.48 (sext, J = 7.2, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.6, 170.3, 169.5, 169.4, 150.7, 145.0, 137.4, 120.3, 120.1, 112.6, 101.0, 72.7, 72.0, 71.3, 68.5, 62.0, 56.1, 53.7, 51.4, 23.1, 20.8, 20.7, 20.6, 11.8. ESI-MS: $[M+H]^+$ = 526.60 *m/z* ($C_{25}H_{35}NO_{11}$), HPLC purity: 94% (Method 1), Rf = 0.50 (deactivated silica gel, Pet. ether/EtOAc 4:1 v/v).
13b	*(2R,3R,4S,5R,6S)-2-(acetoxymethyl)-6-(4-(((3,4-dimethoxyphenethyl)amino)methyl)-2-methoxyphenoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**13b**)* According to general procedure **B**, compound **2** (200 mg, 414.56 µmol) was treated with 2-(3,4-dimethoxyphenyl)ethan-1-amine (108 mL, 621.84 µmol), triacetoxyborohydride (STAB) (351 mg, 1.66 mmol) and acetic acid (95 µL, 1.66 mmol) in 20 mL of 1,2-dichloroethane (DCE) and stirred overnight under argon. The crude product was purified by column chromatography to yield 229 mg of **13b**. Yield 85%, pale-yellow oil, IR (ATR, ṽ [cm^{-1}]): 3378 (w, N–H), 2980 (w, C–H), 2970 (w, C–H), 1749 (vs, ester C═O), 1214 (s, ether C–O), 1065 (vs, alc. C–O). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.01 (d, *J* = 8.1 Hz, 1H), 6.84 (d, *J* = 1.8 Hz, 1H), 6.80 – 6.69 (m, 4H), 5.28 – 5.21 (m, 2H), 5.15 – 5.11 (m, 1H), 4.90 – 4.89 (m, 1H), 4.25 (dd, *J* = 12.3, 5.0 Hz, 1H), 4.13 (dd, *J* = 12.3, 2.5 Hz, 1H), 3.84 – 3.83 (m, 7H), 3.76 (s, 3H), 3.74 – 3.70 (m, 3H), 2.87 – 2.83 (m, 2H), 2.78 – 2.71 (m, 2H), 2.06 – 2.04 (m, 6H), 2.00 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.8, 170.5, 169.6, 169.6, 150.9, 149.1, 147.7, 145.2, 137.0, 132.6, 120.8, 120.5, 120.3, 112.7, 112.2, 111.5, 101.2, 72.8, 72.1, 71.4, 68.6, 62.1, 56.2, 56.1, 56.1, 53.7, 50.7, 35.9, 20.9, 20.9, 20.8, 20.8. ESI-MS: $[M+H]^+$ = 648.65 *m/z* ($C_{32}H_{41}NO_{13}$), HPLC purity: 82% (Method 1), Rf = 0.60 (deactivated silica gel, Pet. ether/EtOAc 4:1 v/v).
13c	*(2R,3R,4S,5R,6S)-2-(acetoxymethyl)-6-(2-methoxy-4-(((pyridin-4-ylmethyl)amino)methyl)phenoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**13c**)* According to general procedure **B**, compound **2** (200 mg, 414.56 µmol) was treated with pyridin-4-ylmethanamine (67 mg, 621.84 µmol) and triacetoxyborohydride (STAB) (351 mg, 1.66 mmol) and stirred for 36 h in 20 mL of 1,2-dichloroethane (DCE) under argon. The crude product was

	purified by column chromatography to yield 198 mg of **13c**. Yield 83%, brown semisolid, IR (ATR, ṽ [cm^{-1}]): 3375 (w, N–H), 2980 (w, C–H), 2970 (w, C–H), 1744 (vs, ester C═O), 1213 (s, ether C–O), 1064 (vs, alc. C–O). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.53 (d, *J* = 5.9 Hz, 2H), 7.27 (d, *J* = 6.0 Hz, 2H), 7.17 (dd, *J* = 4.5, 1.5 Hz, 1H), 7.05 (d, *J* = 8.1 Hz, 1H), 6.90 (d, *J* = 1.8 Hz, 1H), 6.80 (dd, *J* = 8.1, 1.8 Hz, 1H), 5.29 – 5.22 (m, 2H), 5.16 – 5.12 (m, 1H), 4.94 – 4.89 (m, 1H), 4.43 (d, *J* = 6.1 Hz, 1H), 4.25 (dd, *J* = 12.2, 5.0 Hz, 1H), 4.14 (dd, *J* = 12.2, 2.6 Hz, 1H), 3.80 (s, 4H), 3.76 – 3.71 (m, 3H), 2.06 – 2.05 (m, 6H), 2.02 – 2.00 (m, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.8, 170.5, 169.6, 169.6, 151.0, 150.3, 150.0, 145.4, 123.3, 120.5, 120.4, 112.8, 101.1, 72.8, 72.2, 71.4, 68.7, 62.2, 56.3, 53.0, 51.9, 20.9, 20.9, 20.9, 20.8. ESI-MS: $[M+H]^+$ = 575.65 *m/z* ($C_{28}H_{34}N_2O_{11}$), HPLC purity: 96 (Method 1), Rf = 0.60 (deactivated silica gel, Pet. ether/EtOAc 4:1 v/v).
14	*(3,5-dichlorophenyl)glycine (**14**)* According to general procedure **B**, glyoxylic acid (1.5 g, 15.8 mmol) was treated with 3,5-dichloroaniline (3.07 g, 18.96 mmol), triacetoxyborohydride (STAB) (13.4 mg, 63.21 mmol) and acetic acid (3.61mL, 63.21 mmol) in 25 mL of Isopropyl alcohol (IPA) and stirred for 72 h under argon. The crude product was purified by column chromatography to yield 3.29 g of **14**. Yield 95%, brown semisolid, IR (ATR, ṽ [cm^{-1}]): 3320 (br, O–H), 3074 (w, Ar–H), 2982 (w, C–H), 2943 (w, C–H), 1588 (vs, acid C═O). ^{1}H NMR (400 MHz, DMSO) δ 6.54 (s, 3H), 6.24 (s, 1H), 3.52 (s, 2H). ^{13}C NMR (101 MHz, DMSO) δ 172.4, 150.8, 134.2, 113.9, 110.2, 45.0. ESI-MS: $[M+H]^+$, = 219.00 *m/z* ($C_8H_7Cl_2NO_2$). HPLC purity: 99% (Method 1),
15	*N-((benzyloxy)carbonyl)-N-(3,5-dichlorophenyl)glycine (**15**)* A brown semisolid (Yield 58%), IR (ATR, ṽ [cm^{-1}]): 3477 (br, O–H), 3074, 3031, 2947 (w, C–H), 1704 (s, ester C═O), 1568 (s, acid C═O), 1218 (s, ether C–O). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36 – 7.30 (m, 8H), 5.18 (s, 2H), 4.28 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 155.2, 144.3, 136.1, 134.9, 128.7, 128.3, 127.8, 68.2, 45.7. ESI-MS: $[M+H]^+$, = 353.85 *m/z* ($C_{16}H_{13}Cl_2NO_4$). HPLC purity: 97% (Method 1), Rf = 0.60, (RP-18, MeCN/H_2O 1:1 v/v) (Method S3).
16a	*(2R,3R,4S,5R,6S)-2-(acetoxymethyl)-6-(4-((2-(((benzyloxy)carbonyl)(3,5-dichlorophenyl)amino)-N-propylacetamido)methyl)-2-methoxyphenoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**16a**)* According to general procedure **D**, compound **15** (129 mg, 365.33 µmol) was treated with compound **13a** (160 mg, 304.44 µmol), 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (139 mg, 365.33 µmol), and N, N-Diisopropylethylamine (DIPEA) (104 µL, 608.88 µmol) in DMF and stirred for 10 h. The crude product was purified by flash chromatography (Method S5) to yield 192 mg of **16a**. Yield 73%, light brown semisolid, IR (ATR, ṽ [cm^{-1}]): 3074 (w, Ar–H), 2980 (w, C–H), 2970 (w, C–H), 2887, 1750 (s, ester C═O), 1654 (vs, amide C═O), 1214 (s, ether C–O), 1063 (vs, alc. C–O). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.33 –

	7.25 (m, 7H), 7.22 – 7.15 (m, 1H), 7.01 (d, *J* = 8.1 Hz, 1H), 6.83 (d, *J* = 1.8 Hz, 1H), 6.67 (dd, *J* = 8.1, 1.8 Hz, 1H), 5.28 – 5.21 (m, 2H), 5.16 – 5.11 (m, 3H), 4.93 – 4.89 (m, 1H), 4.58 – 4.47 (m, 1H), 4.40 (s, 2H), 4.33 (s, 1H), 4.25 (dd, *J* = 12.2, 4.9 Hz, 1H), 4.13 (dd, *J* = 12.2, 2.2 Hz, 1H), 3.74 – 3.71 (m, 4H), 3.39 – 3.27 (m, 1H), 2.05 (s, 6H), 2.01 (s, 6H), 1.59 – 1.49 (m, 4H), 0.86 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.5, 169.6, 167.9, 167.9, 155.0, 151.1, 145.4, 136.3, 135.0, 128.7, 120.3, 101.1, 72.8, 72.2, 71.4, 62.1, 56.2, 52.2, 52.0, 49.0, 20.9, 20.9, 20.8, 11.5. ESI-MS: $[M+H]^+$ = 861.15 *m/z* ($C_{41}H_{46}Cl_2N_2O_{14}$), HPLC purity: 97% (Method 1), Rf = 0.55 (silica gel, Pet. ether/EtOAc 2:3 v/v).
16b	*(2R,3R,4S,5R,6S)-2-(acetoxymethyl)-6-(4-((2-(((benzyloxy)carbonyl)(3,5-dichlorophenyl)amino)-N-(3,4-dimethoxyphenethyl)acetamido)methyl)-2-methoxyphenoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**16b**)* According to general procedure **D**, compound **15** (144 mg, 407.61 µmol) was treated with compound **13b** (160 mg, 304.44 µmol), 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (159 mg, 407.61 µmol), and N, N-Diisopropylethylamine (DIPEA) (116 µL, 679.35 µmol) in DMF and stirred for 12 h. The crude product was purified by flash chromatography (Method S6) to yield 259 mg of **16b**. Yield 78%, off-white solid, mp 71 – 73 °C. IR (ATR, ṽ [cm^{-1}]): 3074 (w, Ar–H), 2980 (w, C–H), 2970 (w, C–H), 2926, 2366, 1751 (s, ester C═O), 1654 (vs, amide C═O), 1215 (s, ether C–O), 1064 (vs, alc. C–O). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.34 – 7.26 (m, 6H), 7.21 – 7.20 (m, 2H), 7.02 (d, *J* = 8.1 Hz, 1H), 6.84 (d, *J* = 1.6 Hz, 1H), 6.76 – 6.52 (m, 4H), 5.28 – 5.21 (m, 2H), 5.17 – 5.11 (m, 3H), 4.91 – 4.89 (m, 1H), 4.53 (q, *J* = 14.8 Hz, 1H), 4.33 (s, 1H), 4.27 – 4.22 (m, 2H), 4.13 (dd, *J* = 12.3, 2.4 Hz, 1H), 4.07 (s, 1H), 3.83 – 3.82 (m, 3H), 3.78 (m, 3H), 3.74 (s, 3H), 3.60 – 3.52 (m, 1H), 2.82 – 2.72 (m, 2H), 2.04 (s, 6H), 2.01 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.8, 170.5, 169.6, 155.0, 151.3, 149.4, 148.3, 136.1, 134.9, 134.1, 128.7, 128.3, 120.9, 120.7, 120.4, 112.2, 111.6, 110.6, 101.0, 72.8, 72.2, 71.3, 68.6, 62.1, 56.3, 56.1, 51.1, 33.7, 20.9, 20.8, 20.8. ESI-MS: $[M+H]^+$ = 983.15 *m/z* ($C_{48}H_{52}Cl_2N_2O_{16}$), HPLC purity: 96% (Method 1), Rf = 0.53 (silica gel, Pet. ether/EtOAc 2:3 v/v).
16c	*(2R,3R,4S,5R,6S)-2-(acetoxymethyl)-6-(4-((2-(((benzyloxy)carbonyl)(3,5-dichlorophenyl)amino)-N-(pyridin-4-ylmethyl)acetamido)methyl)-2-methoxyphenoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**16c**)* According to general procedure **D**, compound **15** (190 mg, 330.67 µmol) was treated with compound **13c** (140 mg, 396.81 µmol), 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (150 mg, 396.81 µmol), and N, N-Diisopropylethylamine (DIPEA) (113 µL, 661.35 µmol) in DMF and stirred for 12 h. The crude product was purified by flash chromatography (Method S7) to yield 250 mg of **16c**. Yield 83%, light brown solid, mp 60 – 61 °C. IR (ATR, ṽ [cm^{-1}]): 3074 (w, Ar–H), 2980 (w, C–H), 2971 (w, C–H), 2891, 1749 (s, ester C═O), 1664 (vs, amide

	C═O), 1213 (s, ether C–O), 1064 (vs, alc. C–O). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.51 (s, 1H), 7.36 – 7.26 (m, 9H), 7.23 – 7.21 (m, 2H), 7.16 – 7.00 (m, 3H), 6.62 (dd, *J* = 7.9, 1.1 Hz, 1H), 5.29 – 5.22 (m, 2H), 5.18 – 5.13 (m, 3H), 4.93 – 4.90 (m, 1H), 4.59 – 4.53 (m, 2H), 4.37 – 4.32 (m, 2H), 4.25 (dd, *J* = 12.3, 4.7 Hz, 1H), 4.17 – 4.13 (m, 1H), 3.77 – 3.68 (m, 4H), 2.05 (s, 6H), 2.01 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.8, 170.5, 169.6, 155.1, 150.4, 149.7, 136.0, 135.1, 134.7, 130.0, 129.2, 128.8, 128.4, 127.8, 127.2, 123.2, 120.7, 110.8, 100.8, 72.7, 72.2, 71.3, 68.6, 62.1, 56.3, 52.3, 49.6, 20.9, 20.9, 20.8. ESI-MS: $[M+H]^+$ = 910.24 *m/z* ($C_{44}H_{45}Cl_2N_3O_{14}$), HPLC purity: 85% (Method 1), Rf = 0.44 (silica gel, 100 % EtOAc + 0.1% TEA).
17a	*benzyl (3,5-dichlorophenyl)(2-((3-methoxy-4-(((2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)benzyl)(propyl)amino)-2-oxoethyl)carbamate (**17a**)* According to general procedure **A**, compound **16a** (182 mg, 211.21 µmol) was treated with approx. 0.5 %w/v solution of $Mg(OMe)_2$ in dry MeOH (20 mL) for 5 h to yield 127.44 mg of **17a**. Yield 87%, off-white semisolid, IR (ATR, ṽ [cm^{-1}]): 3390 (br, O–H), 3078 (w, Ar–H), 2980 (w, C–H), 2970 (w, C–H), 2933, 1714 (s, ester C═O), 1652(vs, amide C═O), 1223 (s, ether C–O), 1065 (vs, alc. C–O). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.31 – 7.25 (m, 5H), 7.22 – 7.15 (m, 4H), 6.94 (d, *J* = 8.1 Hz, 1H), 6.79 (s, 1H), 6.65 (d, *J* = 7.9 Hz, 1H), 5.11 (s, 2H), 4.77 (d, *J* = 6.8 Hz, 1H), 4.47 – 4.29 (m, 4H), 4.10 (q, *J* = 7.1 Hz, 1H), 3.81 – 3.64 (m, 8H), 3.39 – 3.25 (m, 6H), 1.52 – 1.47 (m, 2H), 0.82 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.4, 168.0, 155.1, 150.4, 150.1, 145.9, 145.7, 136.0, 134.9, 134.8, 133.2, 133.2, 128.7, 128.4, 127.9, 117.8, 102.4, 76.4, 76.1, 73.4, 69.7, 68.2, 61.9, 60.6, 56.2, 21.3, 21.0, 14.4, 11.5, 11.4. ESI-MS: $[M+H]^+$ = 693.10 *m/z* ($C_{33}H_{38}Cl_2N_2O_{10}$), HPLC purity: 92% (Method 1), Rf = 0.46 (silica gel, EtOAc/MeOH 9:1 v/v).
17b	*benzyl (3,5-dichlorophenyl)(2-((3,4-dimethoxyphenethyl)(3-methoxy-4-(((2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)benzyl)amino)-2-oxoethyl)carbamate (**17b**)* According to general procedure **A**, compound **16b** (240 mg, 243.94 µmol) was treated with approx. 0.5 %w/v solution of $Mg(OMe)_2$ in dry MeOH (20 mL) for 5 h to yield 181.07 mg of **17b**. Yield 91%, white solid, IR (ATR, ṽ [cm^{-1}]): 3400 (br, O–H), 3074 (w, Ar–H), 2980 (w, C–H), 2971 (w, C–H), 2888, 1714 (s, ester C═O), 1652 (vs, amide C═O), 1229 (s, ether C–O), 1068 (vs, alc. C–O). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.30 – 7.27 (m, 4H), 7.22 – 7.16 (m, 5H), 6.95 (d, *J* = 7.9 Hz, 1H), 6.80 (s, 1H), 6.73 – 6.50 (m, 4H), 5.13 (s, 1H), 5.06 (s, 1H), 4.77 (d, *J* = 6.9 Hz, 1H), 4.48 (s, 1H), 4.29 (s, 1H), 4.18 (s, 1H), 4.10 (q, *J* = 7.1 Hz, 1H), 4.00 (s, 1H), 3.80 – 3.63 (m, 15H), 3.56 – 3.48 (m, 2H), 3.38 – 3.34 (m, 3H), 2.76 – 2.69 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.8, 154.9, 149.2, 149.0, 148.2, 135.9, 134.6, 128.5, 128.2, 120.7, 112.1, 111.5, 111.4, 69.4, 68.0, 61.6, 60.4, 56.1, 56.0,

	55.9, 21.1, 14.2. ESI-MS: $[M+H]^+$ = 815.15 *m/z* ($C_{40}H_{44}Cl_2N_2O_{12}$), HPLC purity: 99% (Method 1), Rf = 0.67 (RP-18, MeCN/H_2O 3:2 v/v).
17c	*benzyl (3,5-dichlorophenyl)(2-((3-methoxy-4-(((2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)benzyl)(pyridin-4-ylmethyl)amino)-2-oxoethyl)carbamate (**17c**)* According to general procedure **A**, compound **16c** (230 mg, 252.54 µmol) was treated with approx. 0.5 %w/v solution of $Mg(OMe)_2$ in dry MeOH (20 mL) for 5 h to yield 120 mg of **17c**. Yield 64%, white solid, mp 107 – 108 °C. IR (ATR, ṽ [cm^{-1}]): 3372 (br, O–H), 3018 (w, Ar–H), 1705 (s, ester C═O), 1654 (vs, amide C═O), 1221 (s, ether C–O), 1066 (vs, alc. C–O). ^1H NMR (400 MHz, $CDCl_3$) δ 8.35 (d, *J* = 5.1 Hz, 1H), 7.28 – 7.26 (m, 4H), 7.23 – 7.15 (m, 4H), 6.96 – 6.86 (m, 3H), 6.76 (s, 1H), 6.54 (d, *J* = 7.9 Hz, 1H), 5.11 (s, 2H), 4.79 (s, 1H), 4.52 – 4.41 (m, 4H), 4.32 – 4.25 (m, 3H), 4.10 (q, *J* = 7.1 Hz, 1H), 3.82 (s, 3H), 3.67 (s, 7H), 3.42 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 168.7, 155.1, 150.3, 149.6, 136.0, 135.0, 128.8, 128.8, 123.3, 102.1, 76.4, 73.4, 69.9, 68.4, 61.9, 60.6, 56.3, 56.2, 52.3, 51.7, 49.1, 21.3, 14.4. ESI-MS: $[M+H]^+$ = 724.45 *m/z* ($C_{36}H_{37}Cl_2N_3O_{10}$), HPLC purity: >99% (Method 1), Rf = 0.11 (silica gel, EtOAc/MeOH 9:1 v/v)

Table S2: Purification methods for compounds purified by flash chromatography

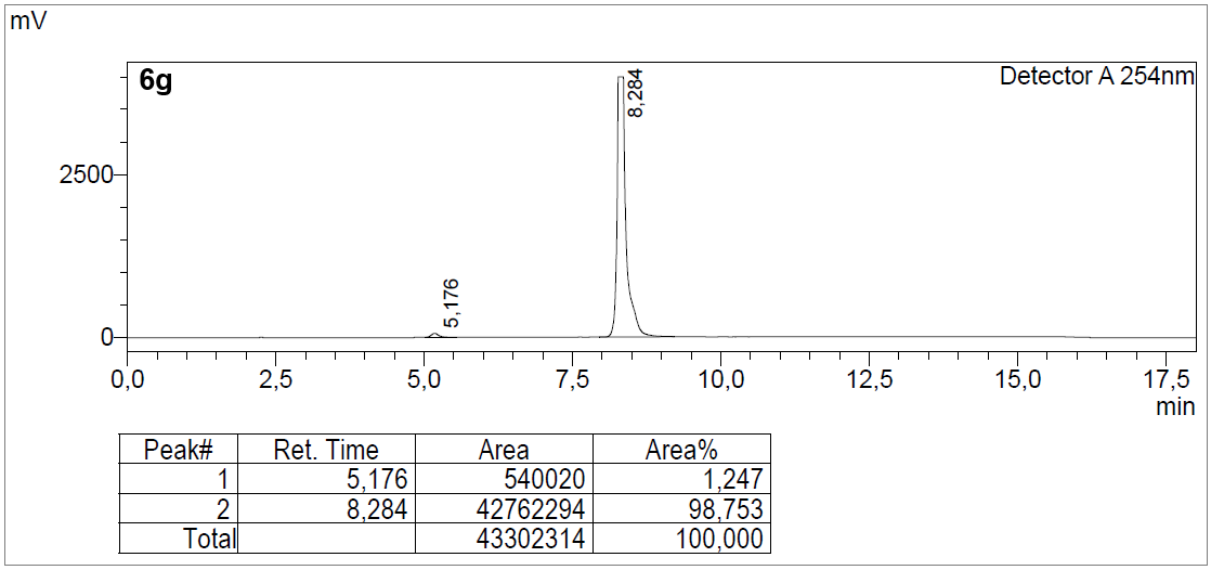
Cpd.	Method	Stationary phase	Elution system
6l	Method S1	CHROMABOND® Flash RS 25 C_{18} ec (Macherey-Nagel GmbH Co. KG, Dueren, Germany)	Solvents: MeOH + 0.1% TEA (A): H_2O + 0.1% TEA (B) Gradient elution: 20 – 50% A (5 min.), 50 – 50% A (25 min.), 50 – 95% A (3 min.), 95 – 95% A (5 min.), 95 – 80% A (1 min.), 80 – 80% A (3 min.)
6m	Method S2	CHROMABOND® Flash RS 25 C_{18} ec (Macherey-Nagel GmbH Co. KG, Dueren, Germany)	Solvents: MeOH + 0.1% TEA (A): H_2O + 0.1% TEA (B)
14	Method S3	CHROMABOND® Flash RS 25 C_{18} ec (Macherey-Nagel GmbH Co. KG, Dueren, Germany)	MeCN/H_2O 1:1 v/v (isocratic)
15	Method S4	CHROMABOND® Flash RS 25 C_{18} ec (Macherey-Nagel GmbH Co. KG, Dueren, Germany)	Solvents: MeCN (A): H_2O (B) Gradient elution: 30 – 30% A (5 min.), 30 – 50% A (5 min.), 50 – 50% A (5 min.), 50 – 55% A (2 min.), 55 – 55% A (15 min.), 55 – 95% A (2 min.), 95 – 95% A (5 min.), 95 - 80% A (1 min.), 80 – 80% A (3 min.)
16a	Method S5	Puriflash® F0025, silica gel 50 µm (Interchim, Los Angeles CA, United States)	Solvents: EtOAc (A): pet. ether (B) Gradient elution: 20 – 20% A (5 min.), 20 – 60% A (2 min.) 60 – 60% A (15 min.), 60 – 95 % A (1 min.), 95 – 95% (5 min.).
16b	Method S6	Puriflash® F0025, silica gel 50 µm (Interchim, Los Angeles CA, United States)	Solvents: EtOAc (A): pet. ether (B) Gradient elution: 50 – 50% A (10 min.), 50 – 60% A (2 min.) 60 – 60% A (20 min.), 60 – 95 % A (2 min.), 95 – 95% (5 min.).
16c	Method S7	Puriflash® F0025, silica gel 50 µm (Interchim, Los Angeles CA, United States)	Solvents: EtOAc + 0.1% TEA (A): pet. ether + 0.1% TEA (B) Gradient elution: 50 – 50% A (20 min.), 50 – 60% A (5 min.) 60 – 60% A (5 min.), 60 – 100 % A (2 min.), 95 – 95% (30 min.).
18a	Method S8	CHROMABOND® Flash RS 25 C_{18} ec (Macherey-Nagel GmbH Co. KG, Dueren, Germany)	Solvents: MeCN (A): H2O (B) Gradient elution: 30 – 30% A (4 min.), 30 – 50% A (30 min.), 50 – 50%A (15 min.) 50 - 80% A (1 min.), 80 – 80% A (3 min.)
18b	Method S9	CHROMABOND® Flash RS 25 C_{18} ec (Macherey-Nagel GmbH Co. KG, Dueren, Germany)	Solvents: MeCN (A): H_2O (B) Gradient elution: 30 – 30% A (4 min.), 30 – 55% A (30 min.), 55 –

			55% A, (15 min.), 55 - 80% A (1 min.), 80 – 80% A (3 min.)
18c	Method S10	CHROMABOND® Flash RS 25 C_{18} ec (Macherey-Nagel GmbH Co. KG, Dueren, Germany)	Solvents: MeCN + 0.1% TEA (A): H_2O + 0.1% TEA (B) Gradient elution: 30 – 30% A (4 min.), 30 – 60% A (25 min.), 60 – 60% A, (10 min.),60 - 80% A (2 min.), 80 – 80% A (3 min.)

Table S3: Solvents and their respective concentrations in working and final test conditions for all tested compounds based on their respective solubilities

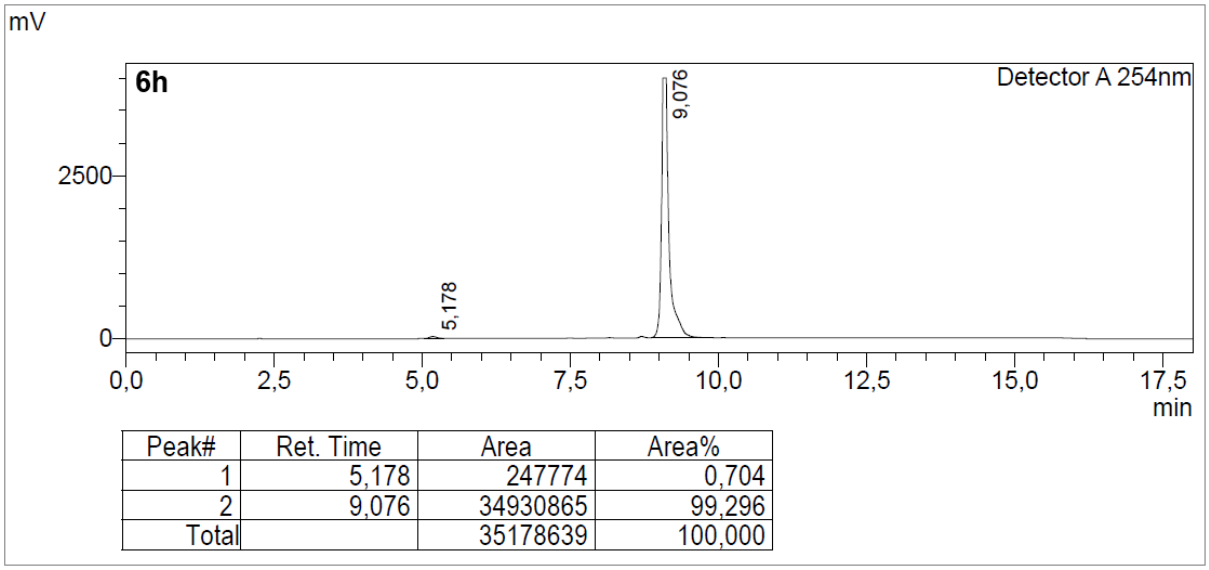
Cpd.	Solvent for preparation of stock solution	Conc. of the stock solution (mg/mL)	Conc. on of the solvent in working solution (before serial dilution) (%v/v)	Conc. range of test compound (µg/mL)	Final/maximum conc. of the solvent under test conditions (%v/v)
4	DMSO	10	5.12	0.5 – 256	2.05
6a-d&g 6i-m	DMSO	20	5.12	1 – 512	2.05
6f&h	DMSO	5	10.24	0.5 – 256	5.12
6e 8b-d 12a&c 18a-c	DMSO	10	5.12	0.5 – 256	2.05
8a 12b&d	acetone	2.048	25.6	0.5 – 256	12.5

HPLC-UV_{254} Chromatograms of key (active) target compounds



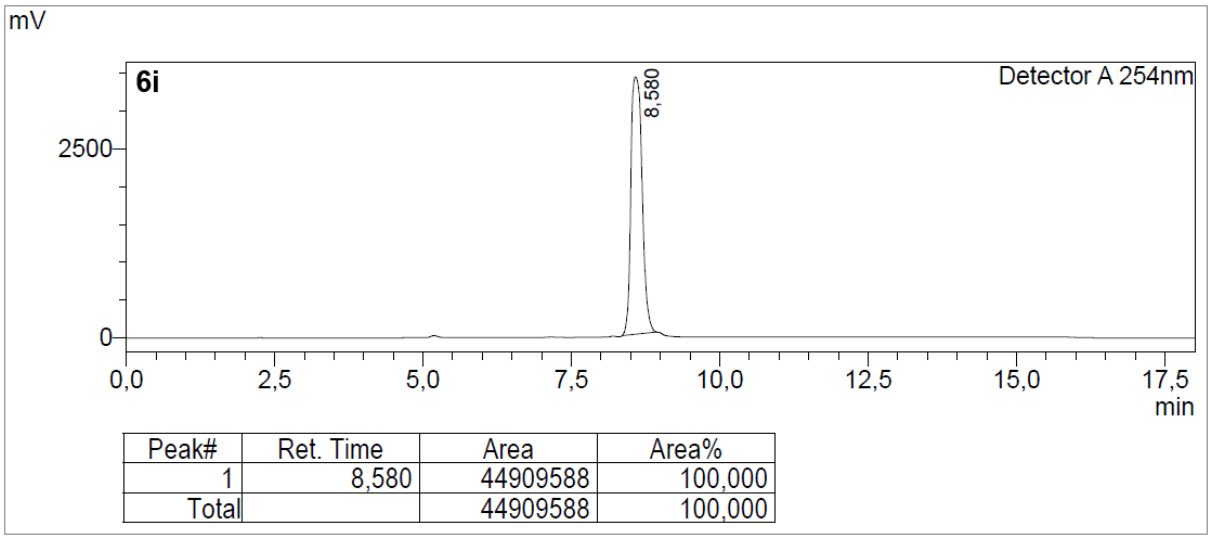
Peak#	Ret. Time	Area	Area%
1	5,176	540020	1,247
2	8,284	42762294	98,753
Total		43302314	100,000

Figure S1: HPLC-UV_{254} chromatogram of compound **6g**



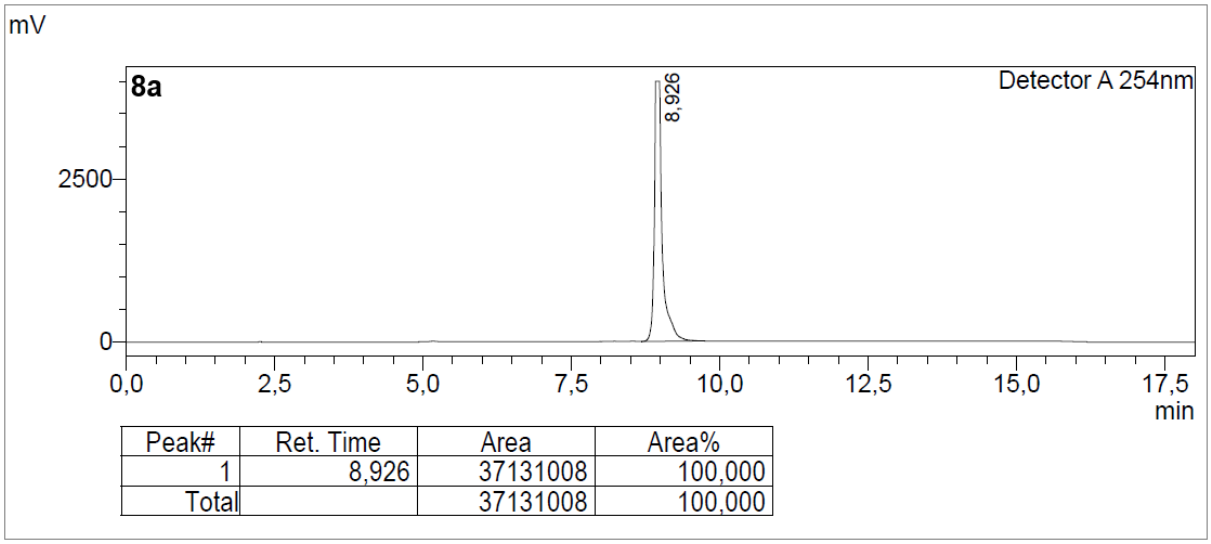
Peak#	Ret. Time	Area	Area%
1	5,178	247774	0,704
2	9,076	34930865	99,296
Total		35178639	100,000

Figure S2: HPLC-UV$_{254}$ chromatogram of compound **6h**



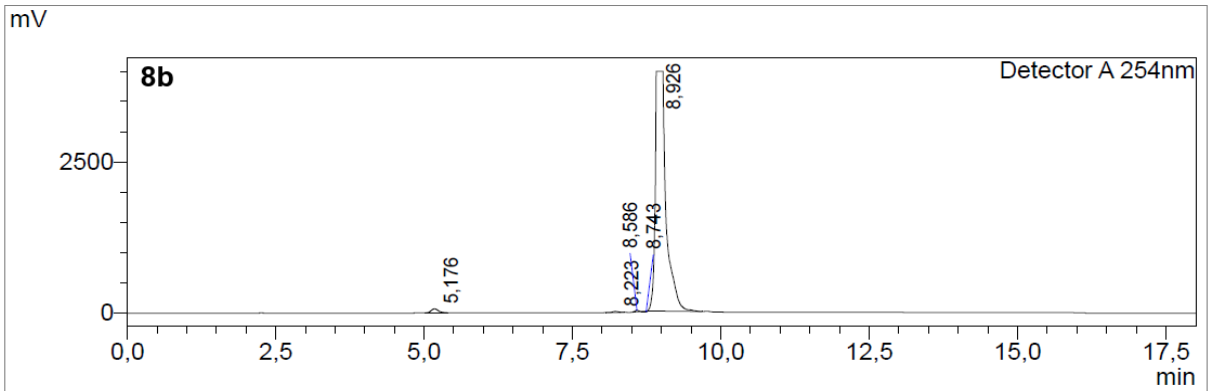
Peak#	Ret. Time	Area	Area%
1	8,580	44909588	100,000
Total		44909588	100,000

Figure S3: HPLC-UV$_{254}$ chromatogram of compound **6i**



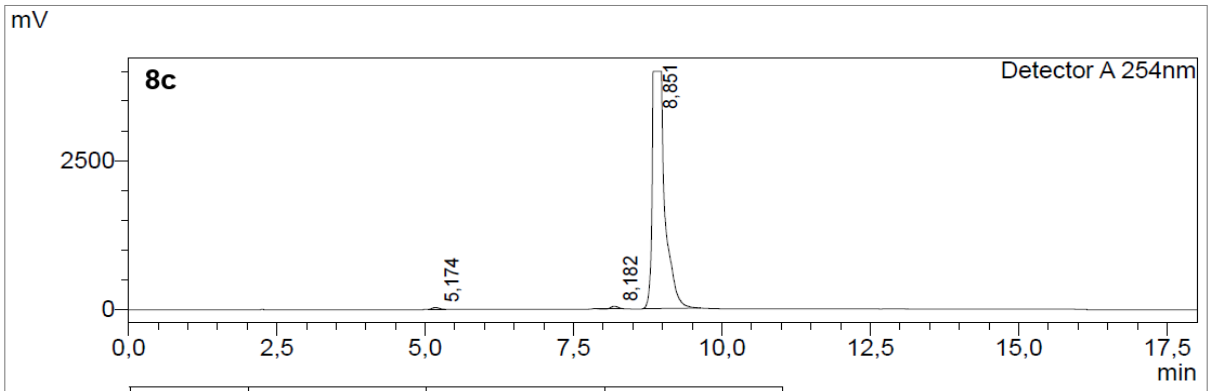
Peak#	Ret. Time	Area	Area%
1	8,926	37131008	100,000
Total		37131008	100,000

Figure S4: HPLC-UV$_{254}$ chromatogram of compound **8a**



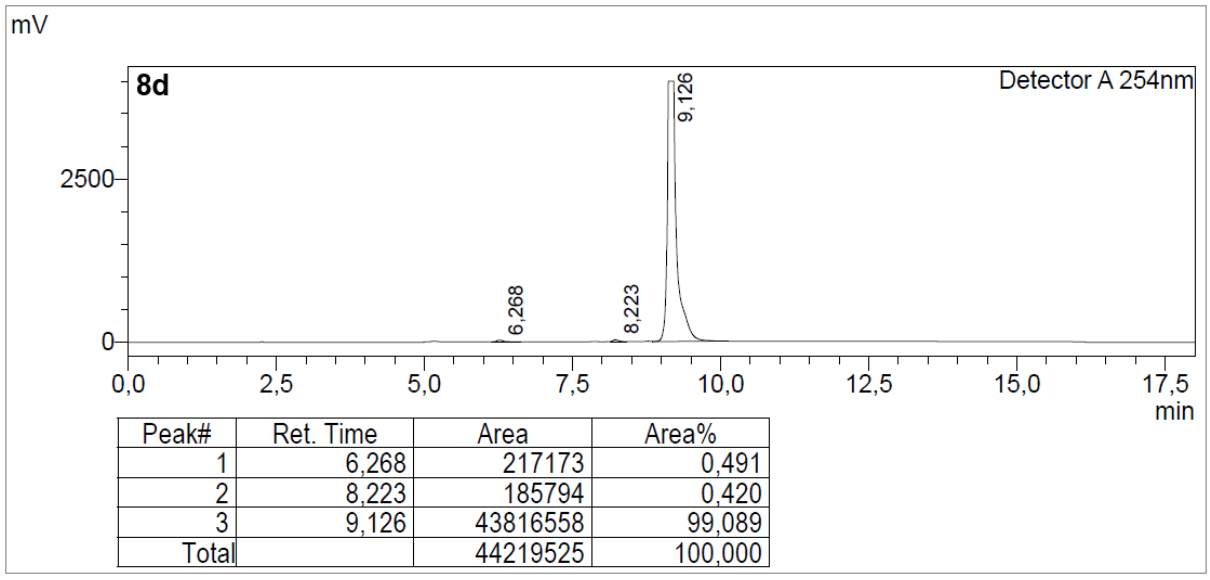
Peak#	Ret. Time	Area	Area%
1	5,176	562441	1,095
2	8,223	127242	0,248
3	8,586	42176	0,082
4	8,743	712	0,001
5	8,926	50611175	98,573
Total		51343747	100,000

Figure S5: HPLC-UV$_{254}$ chromatogram of compound **8b**



Peak#	Ret. Time	Area	Area%
1	5,174	267531	0,447
2	8,182	173151	0,289
3	8,851	59476285	99,265
Total		59916967	100,000

Figure S6: HPLC-UV$_{254}$ chromatogram of compound **8c**



Peak#	Ret. Time	Area	Area%
1	6,268	217173	0,491
2	8,223	185794	0,420
3	9,126	43816558	99,089
Total		44219525	100,000

Figure S7: HPLC-UV$_{254}$ chromatogram of compound **8d**

^{1}H and ^{13}C NMR spectra of all final compounds

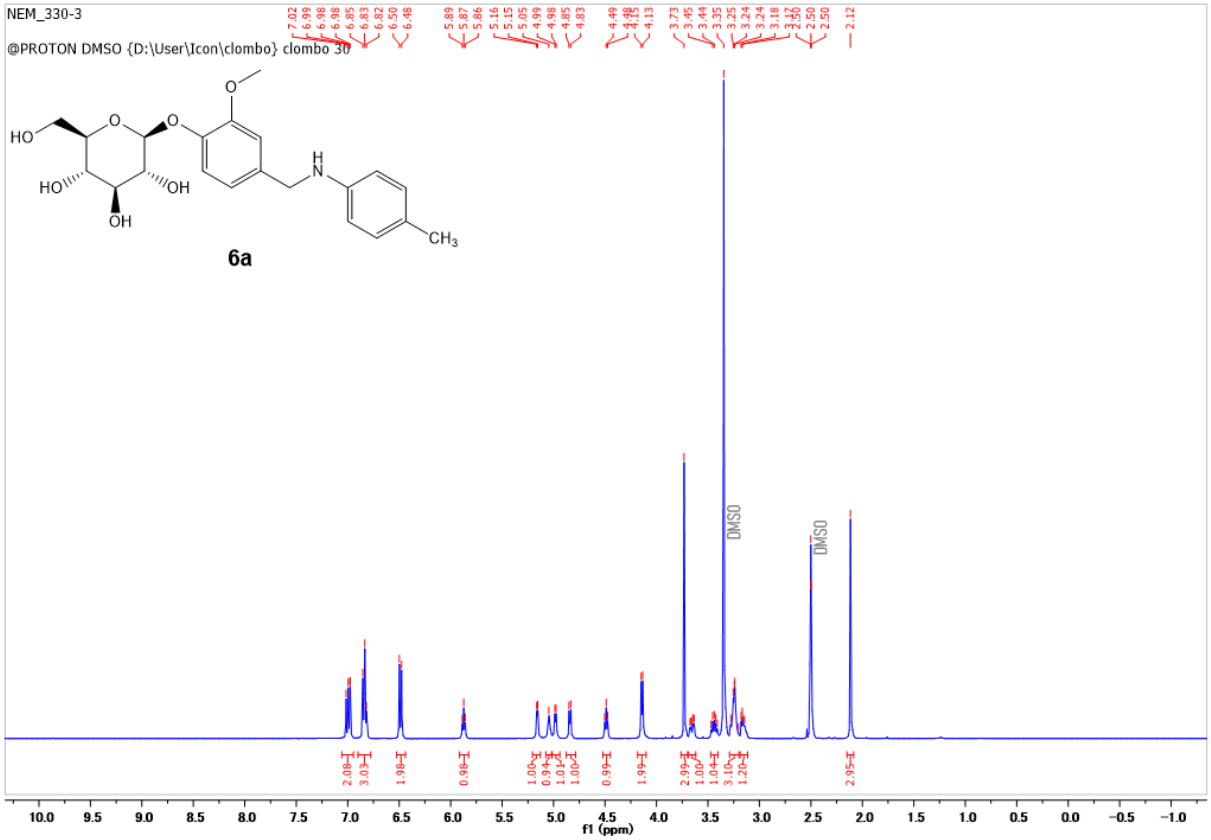


Figure S8a: ^{1}H NMR Spectrum of compound **6a**

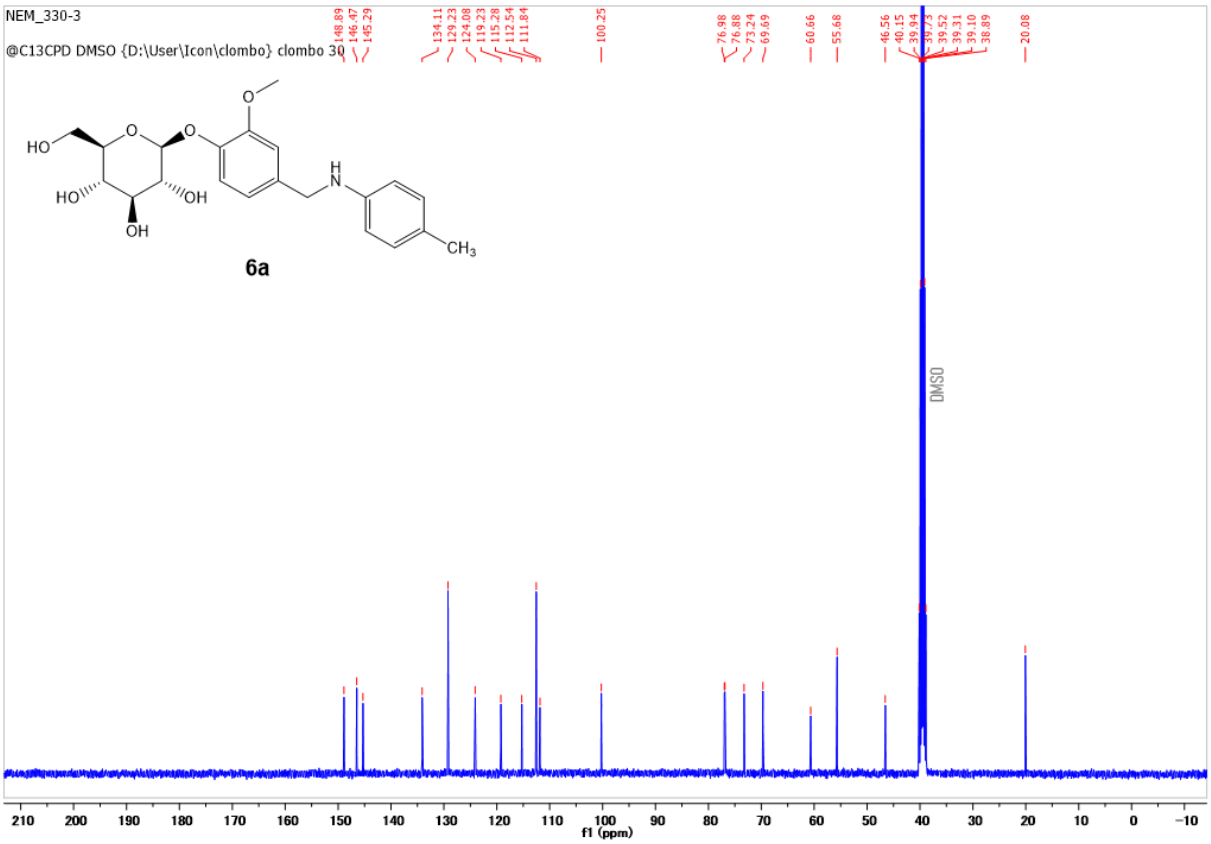


Figure S8b: ^{13}C NMR Spectrum of compound **6a**

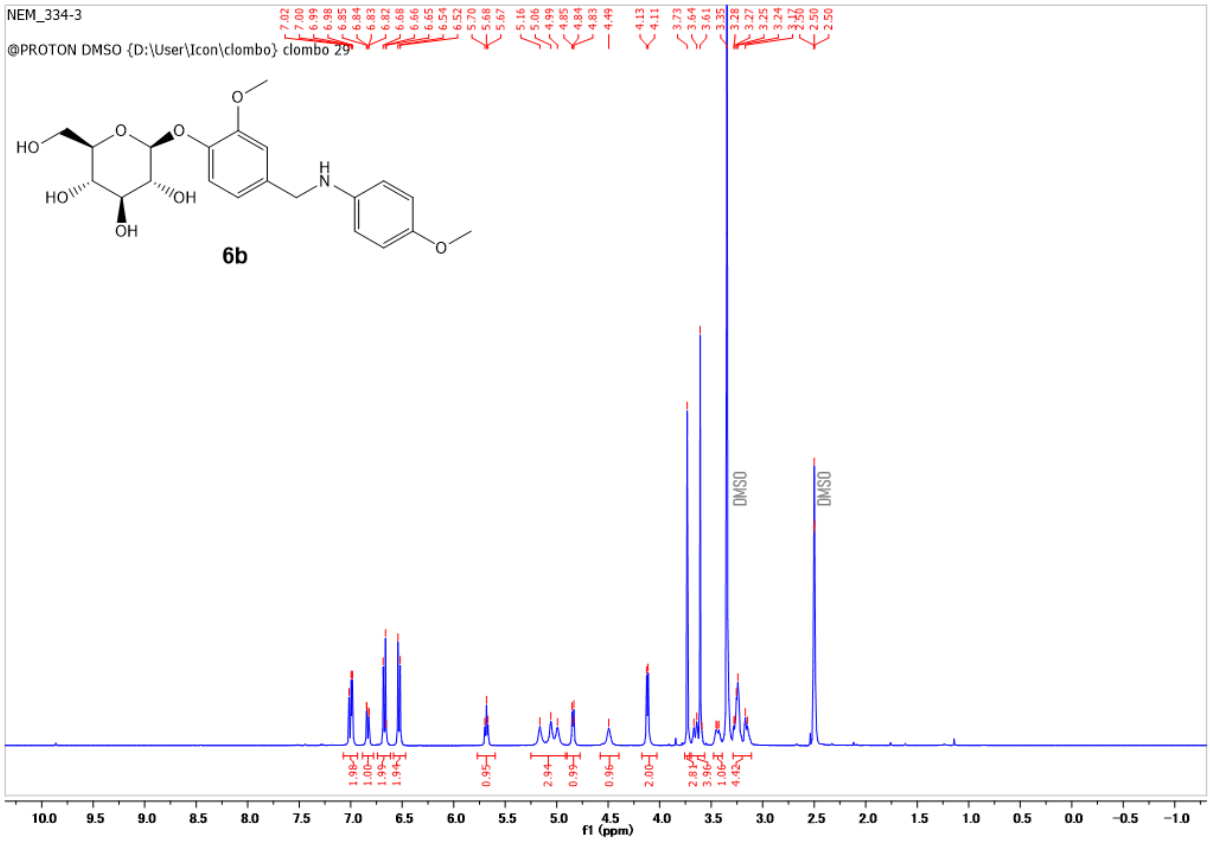


Figure S9a: ^{1}H NMR Spectrum of compound **6b**

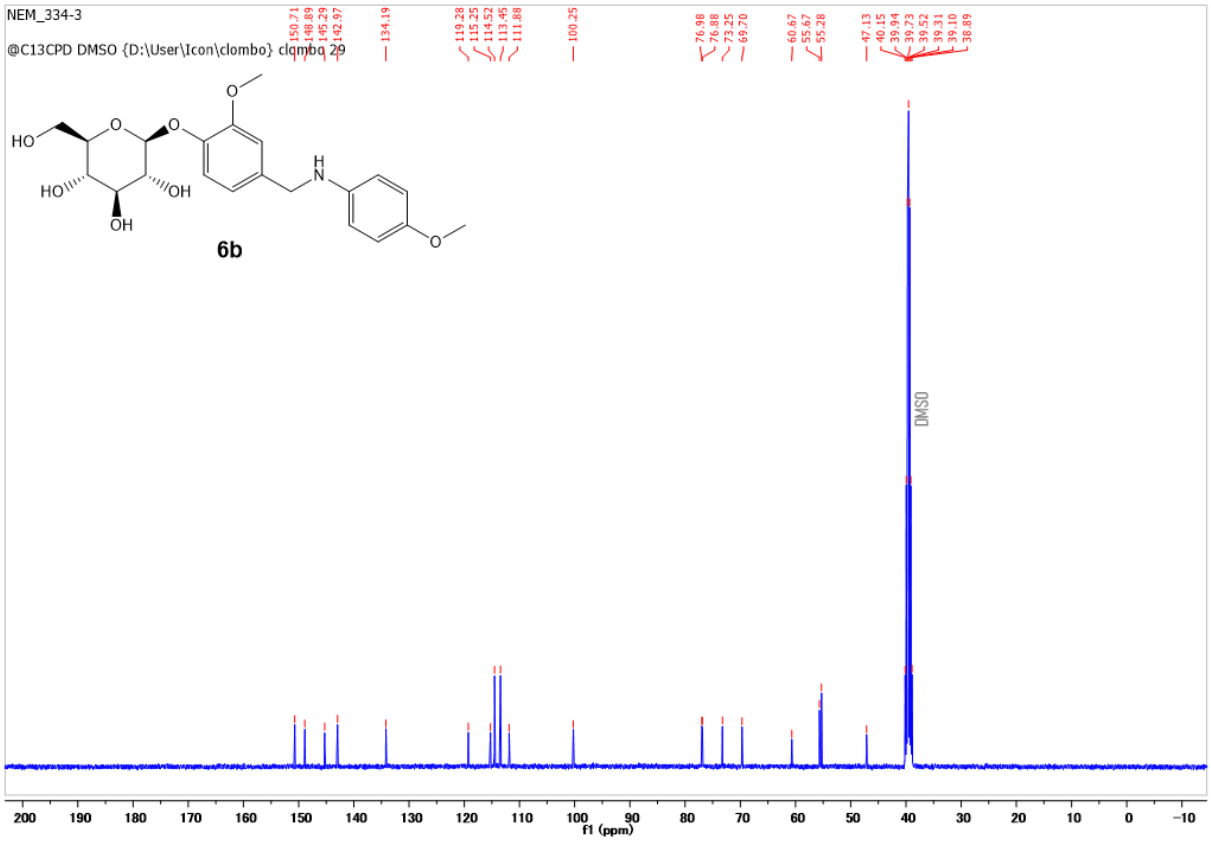


Figure S9b: ^{13}C NMR Spectrum of compound **6b**

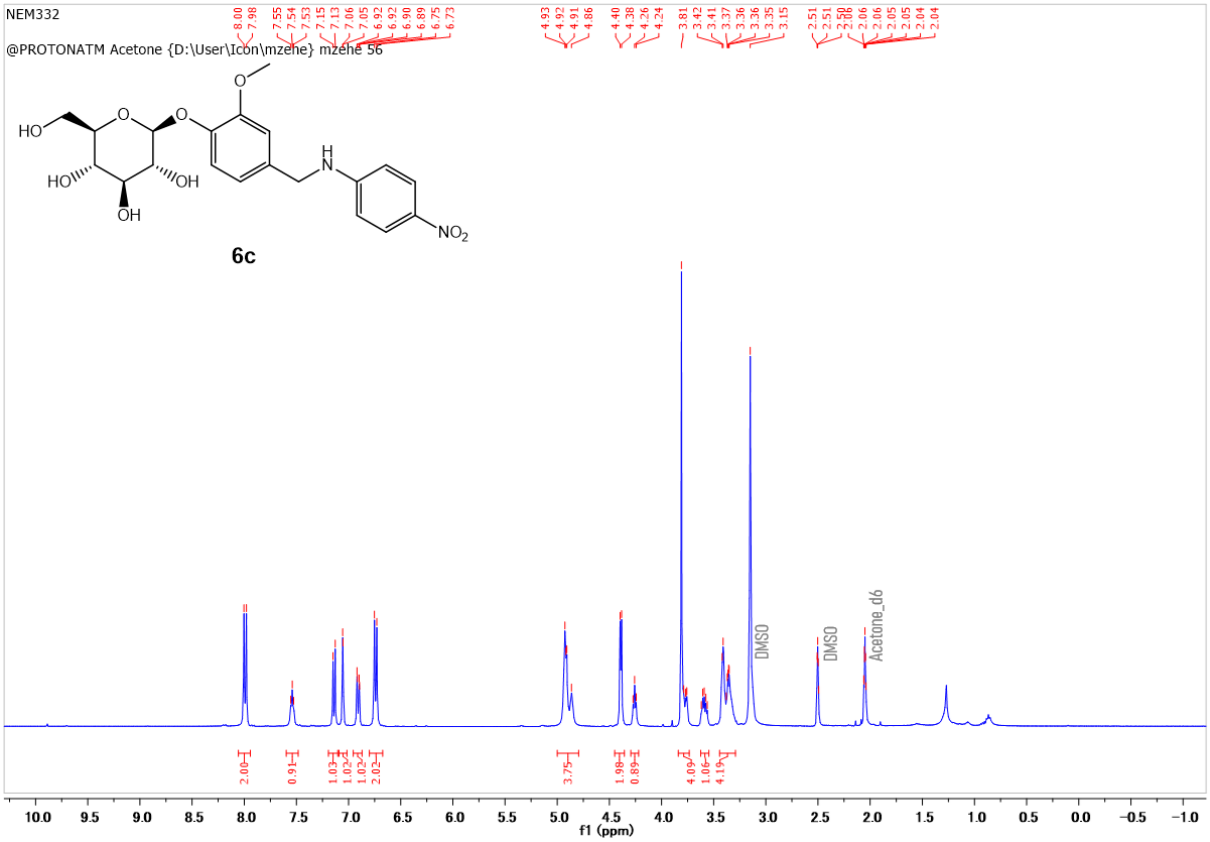


Figure S10a: ^{1}H NMR Spectrum of compound **6c**

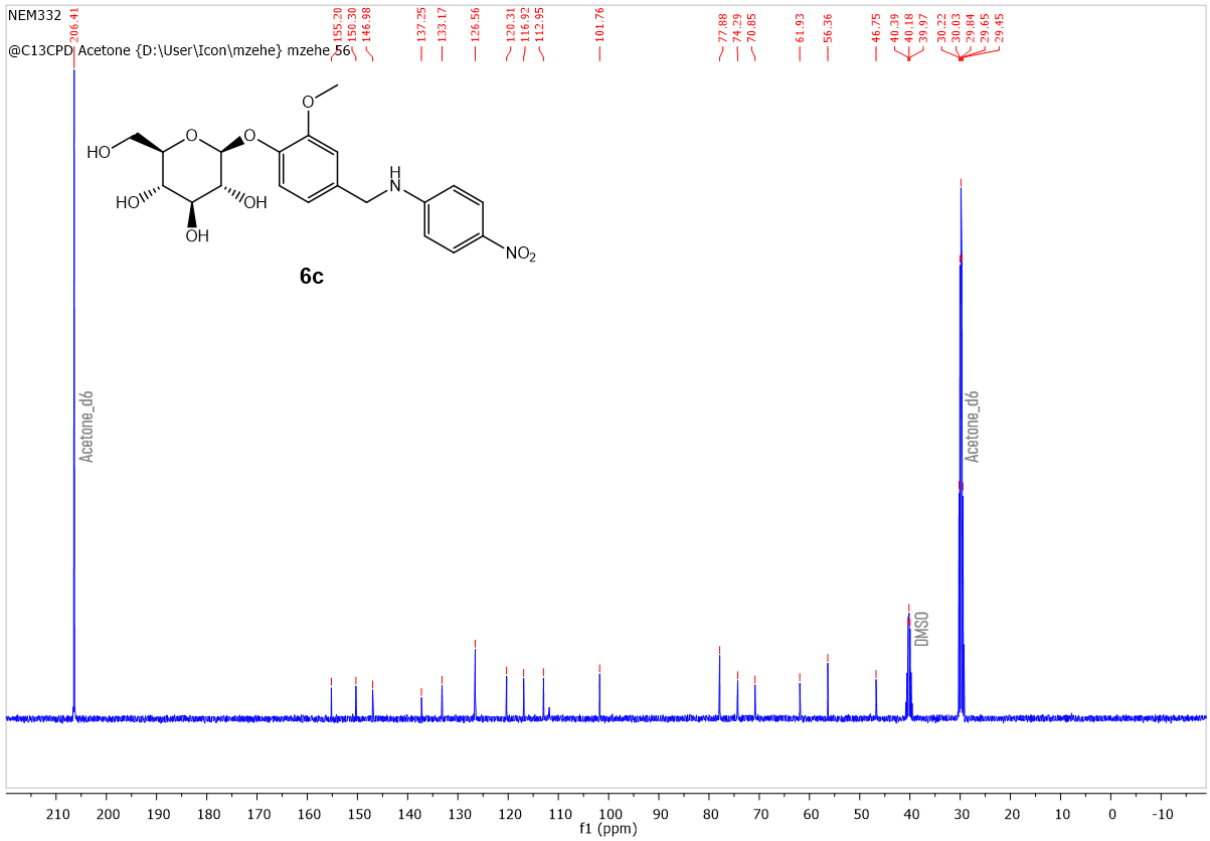


Figure S10b: ^{13}C NMR Spectrum of compound **6c**

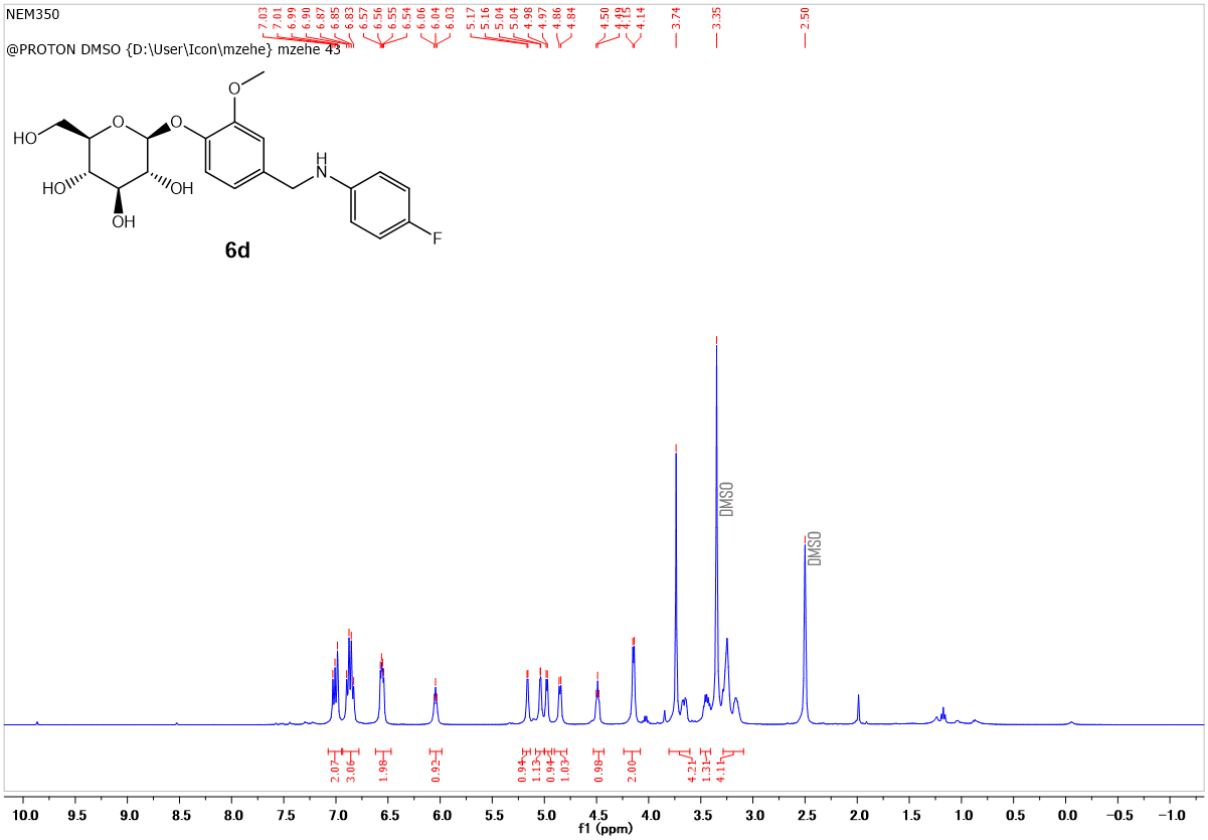


Figure S11a: ^{1}H NMR Spectrum of compound **6d**

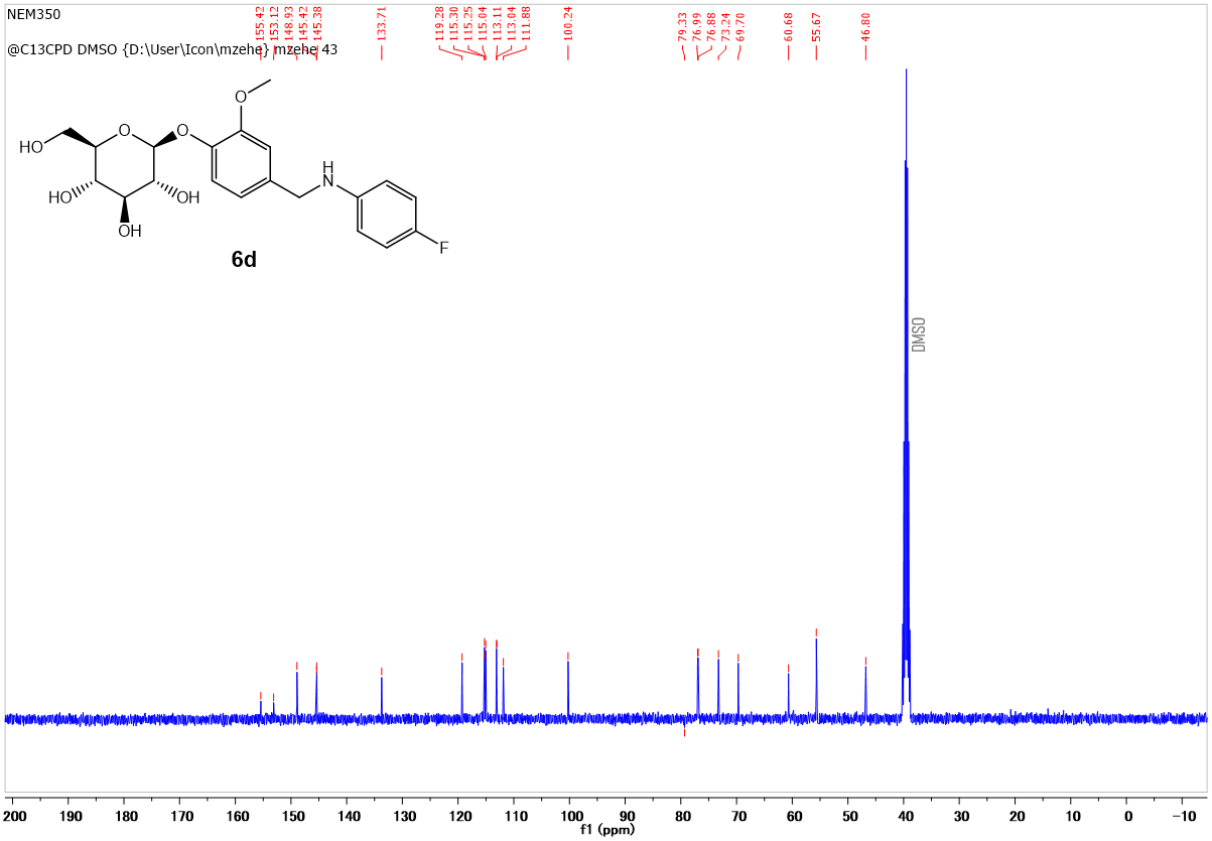


Figure S11b: ^{13}C NMR Spectrum of compound **6d**

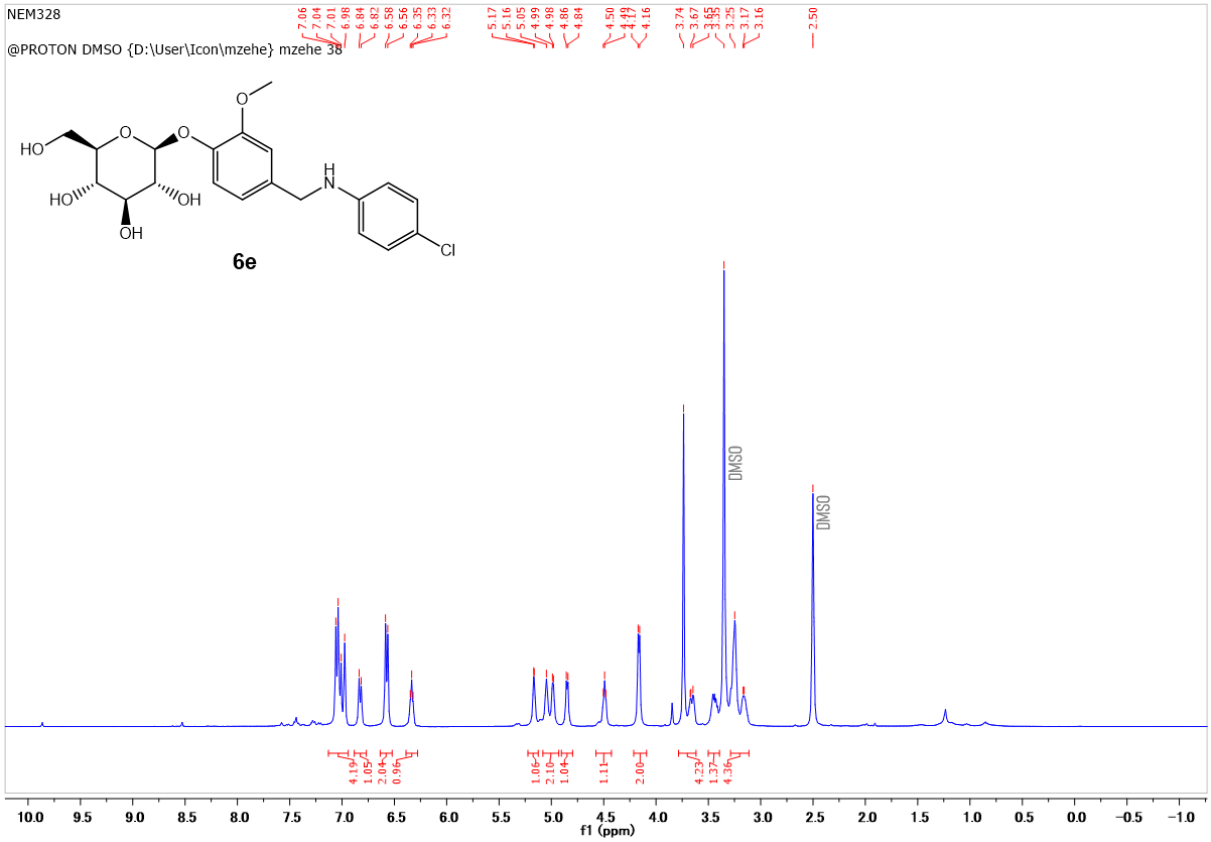


Figure S12a: ^{1}H NMR Spectrum of compound **6e**

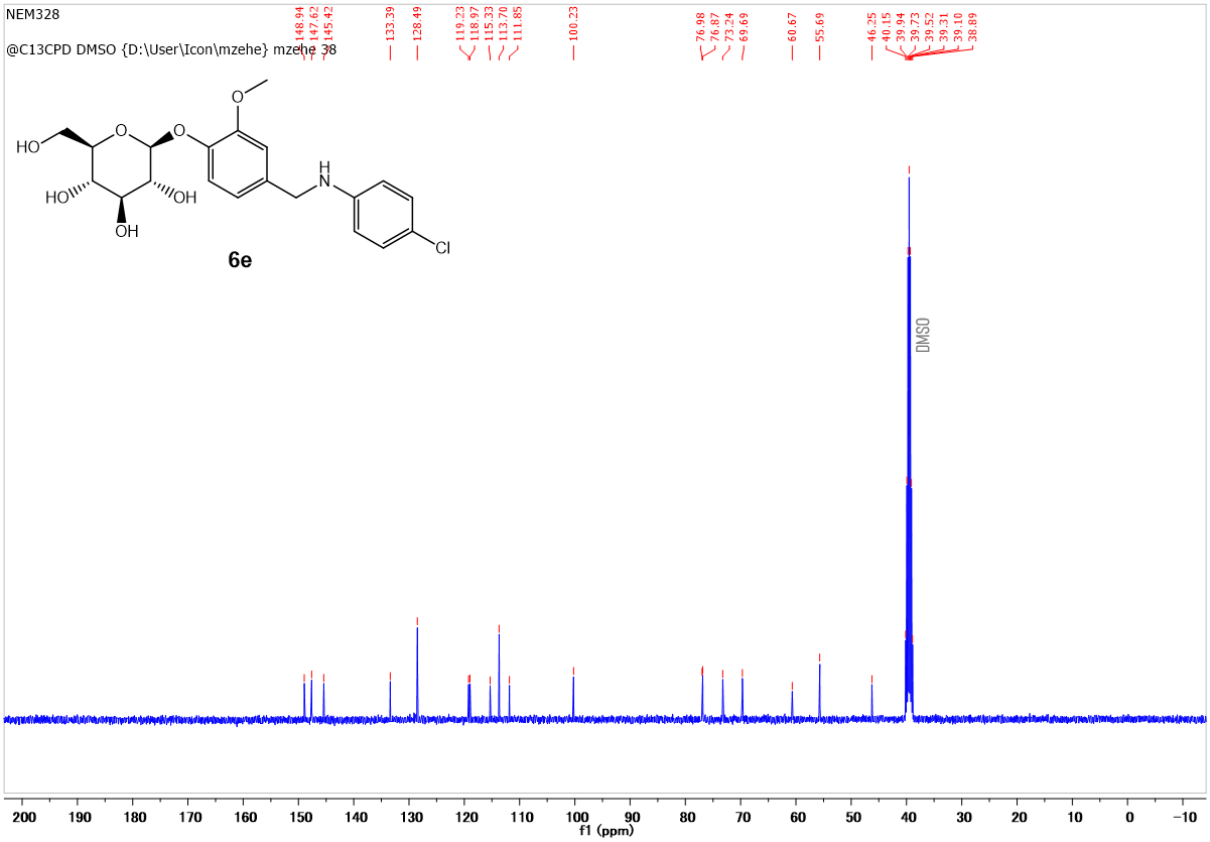


Figure S12b: ^{13}C NMR Spectrum of compound **6e**

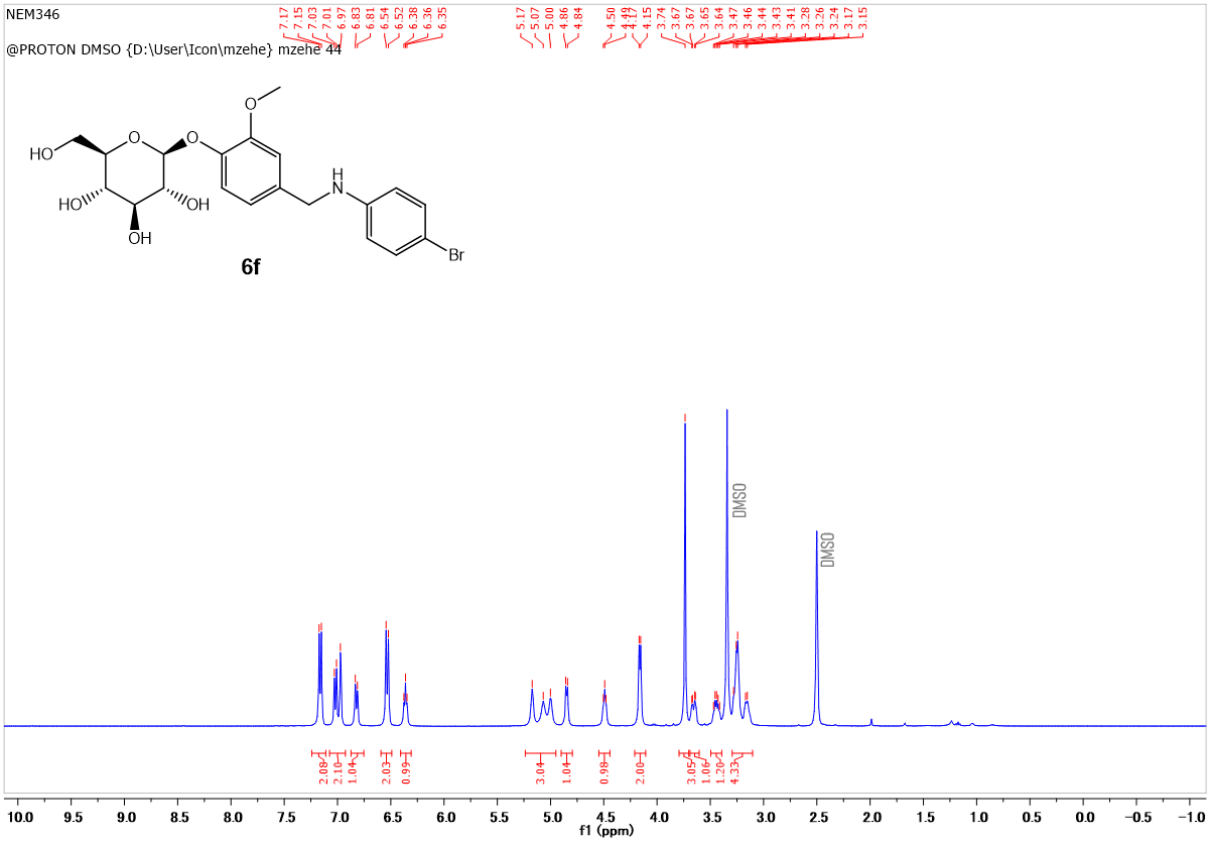


Figure S13a: ^{1}H NMR Spectrum of compound **6f**

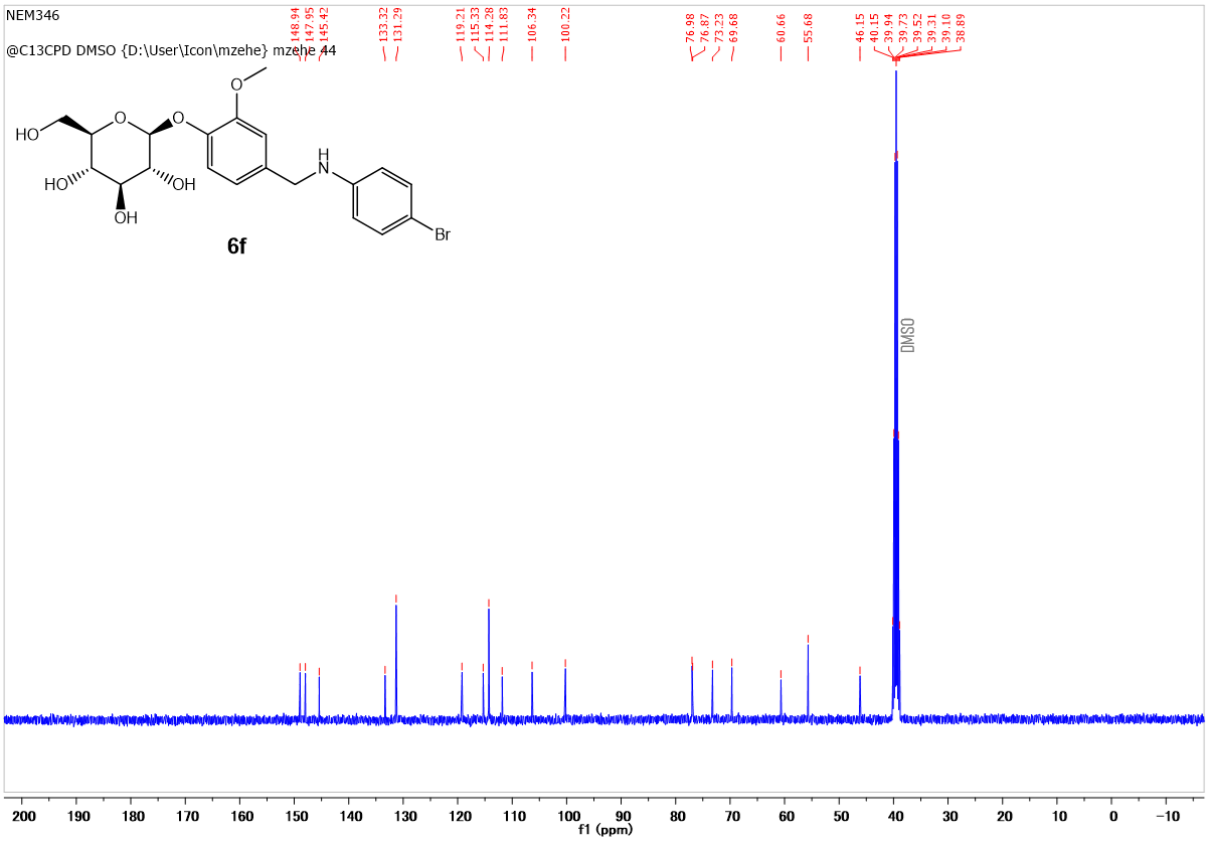


Figure S13b: ^{13}C NMR Spectrum of compound **6f**

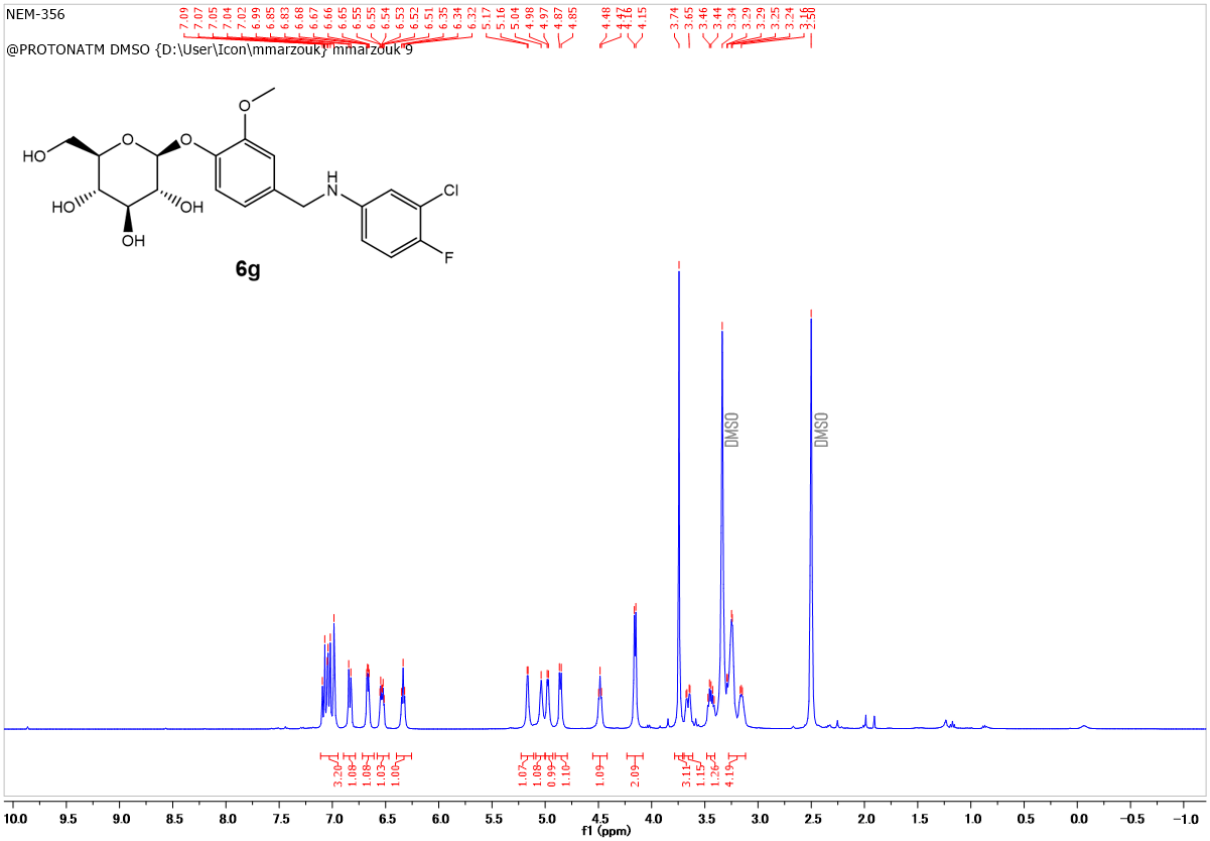


Figure S14a: ^{1}H NMR Spectrum of compound **6g**

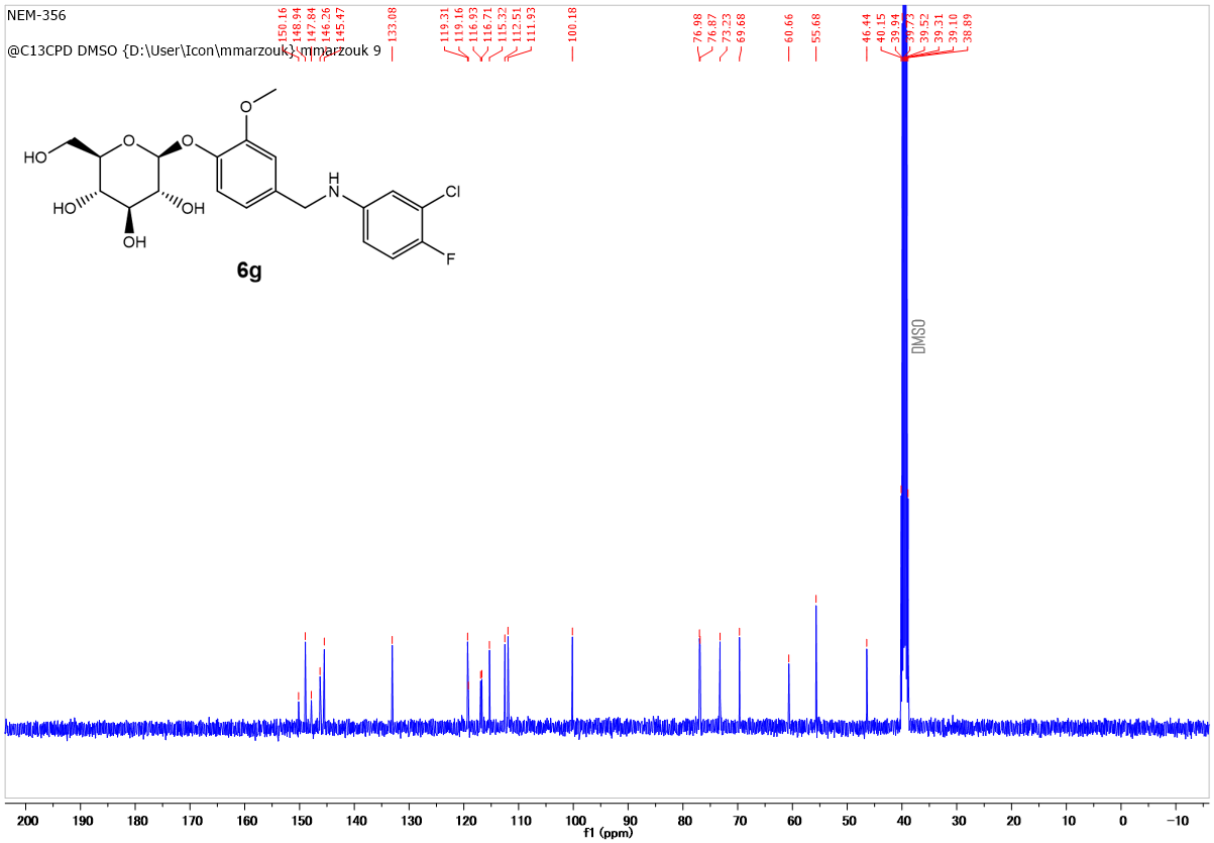


Figure S14b: ^{13}C NMR Spectrum of compound **6g**

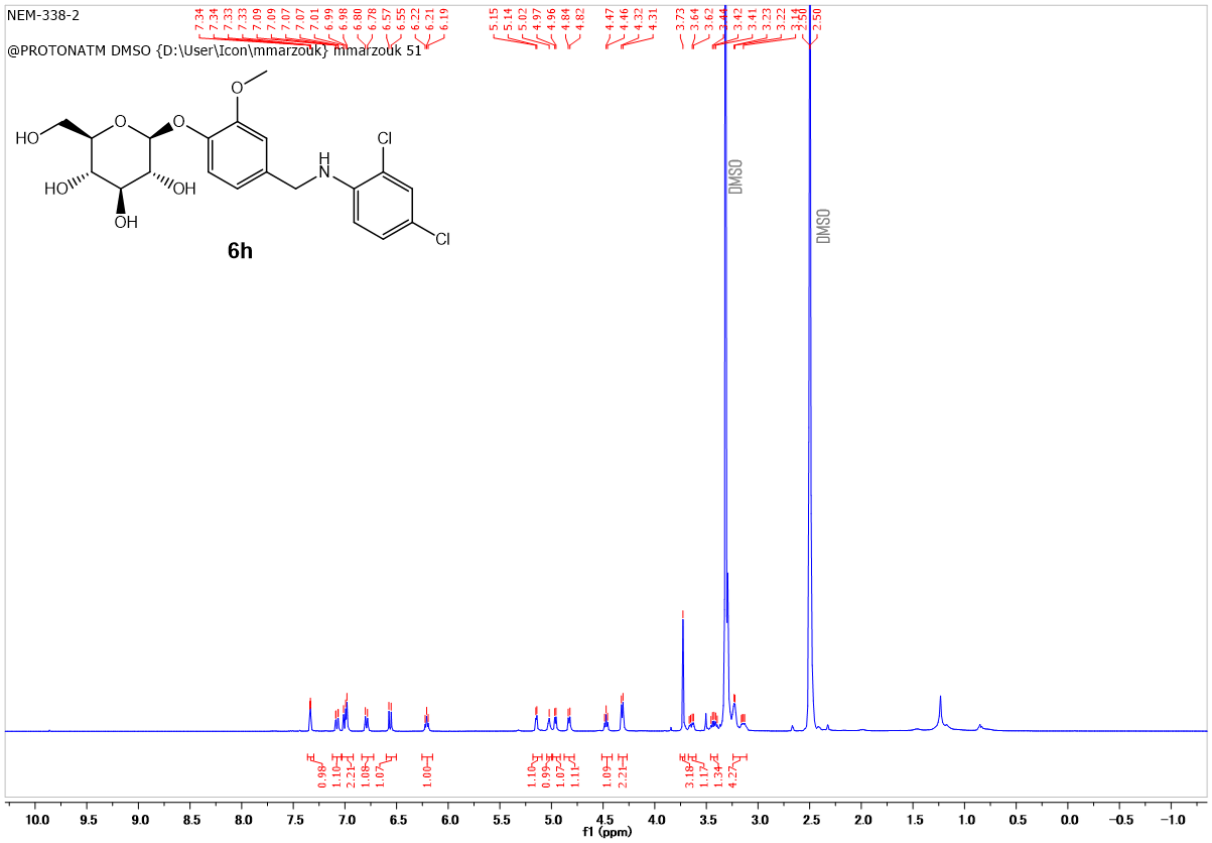


Figure S15a: ^{1}H NMR Spectrum of compound **6h**

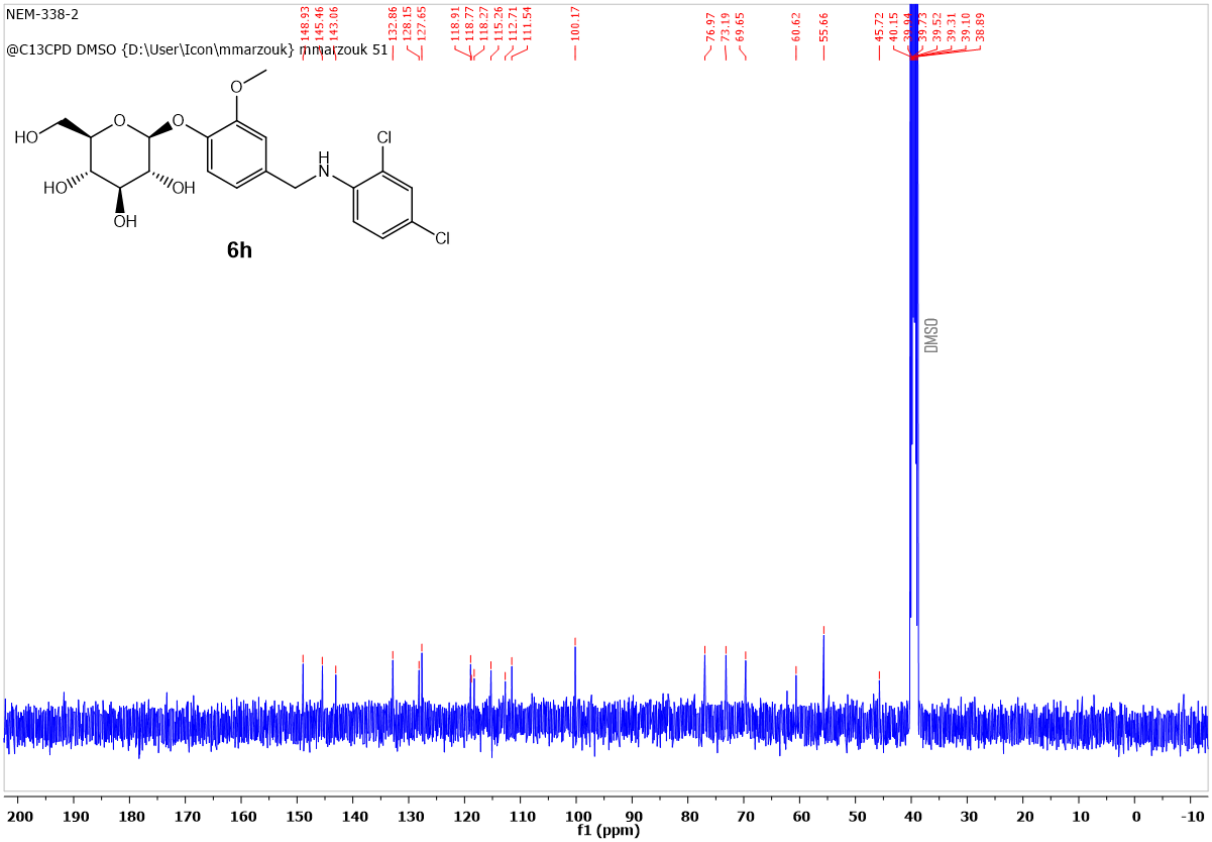


Figure S15b: ^{13}C NMR Spectrum of compound **6h**

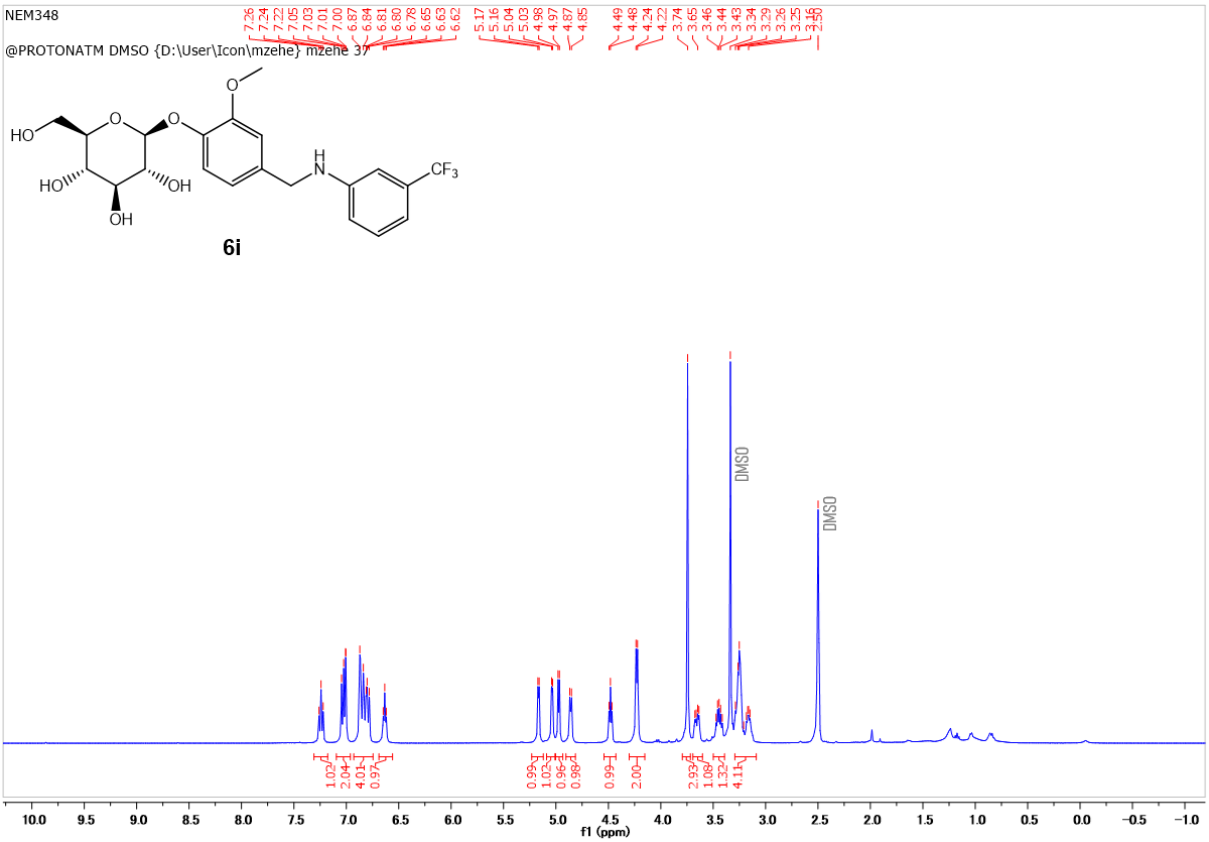


Figure S16a: ^{1}H NMR Spectrum of compound **6i**

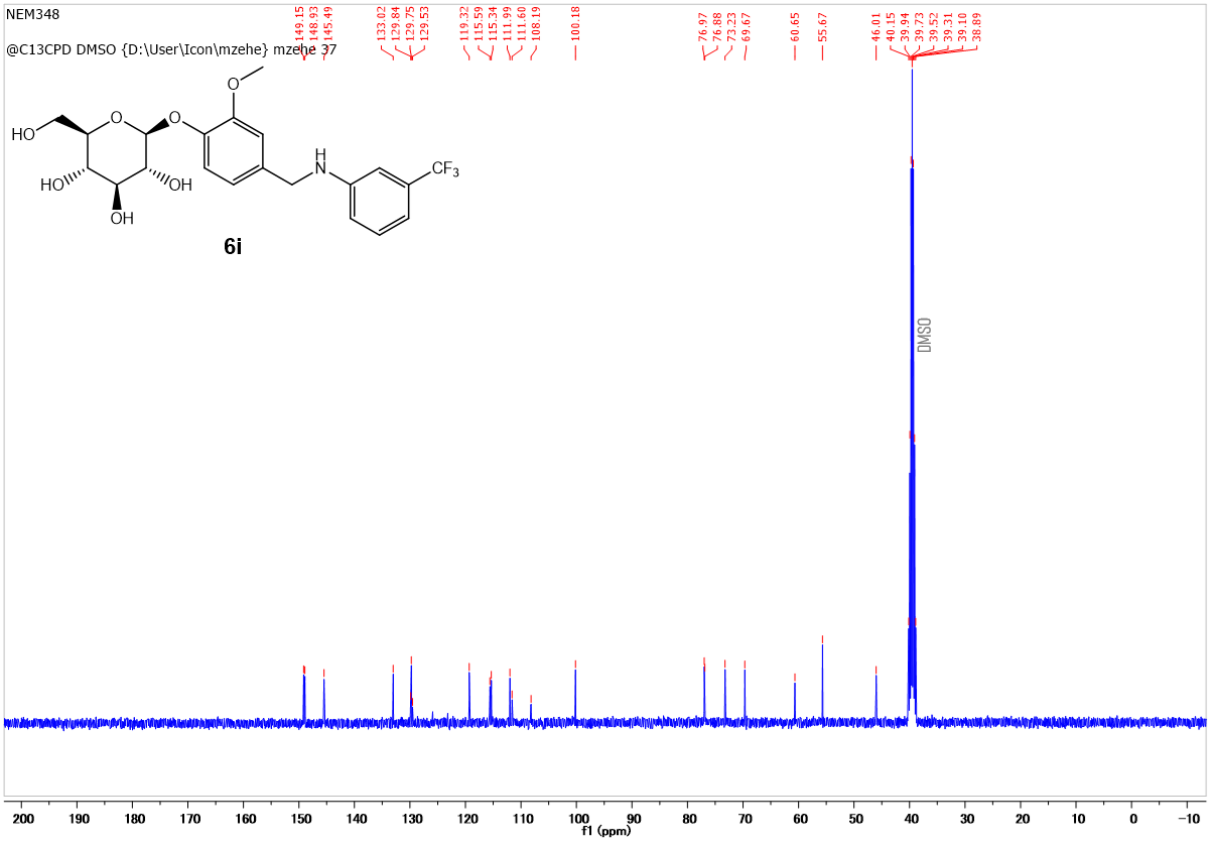


Figure S16b: ^{13}C NMR Spectrum of compound **6i**

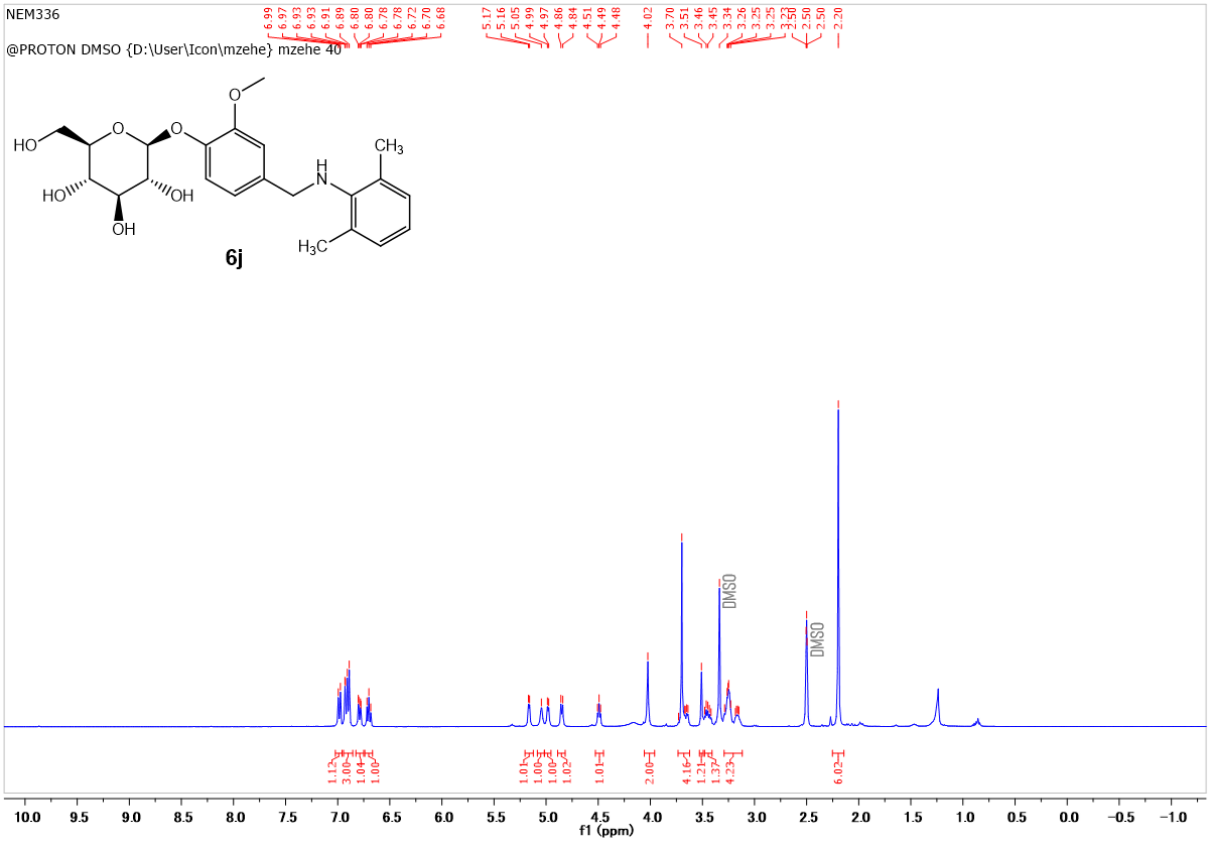


Figure S17a: ^{1}H NMR Spectrum of compound **6j**

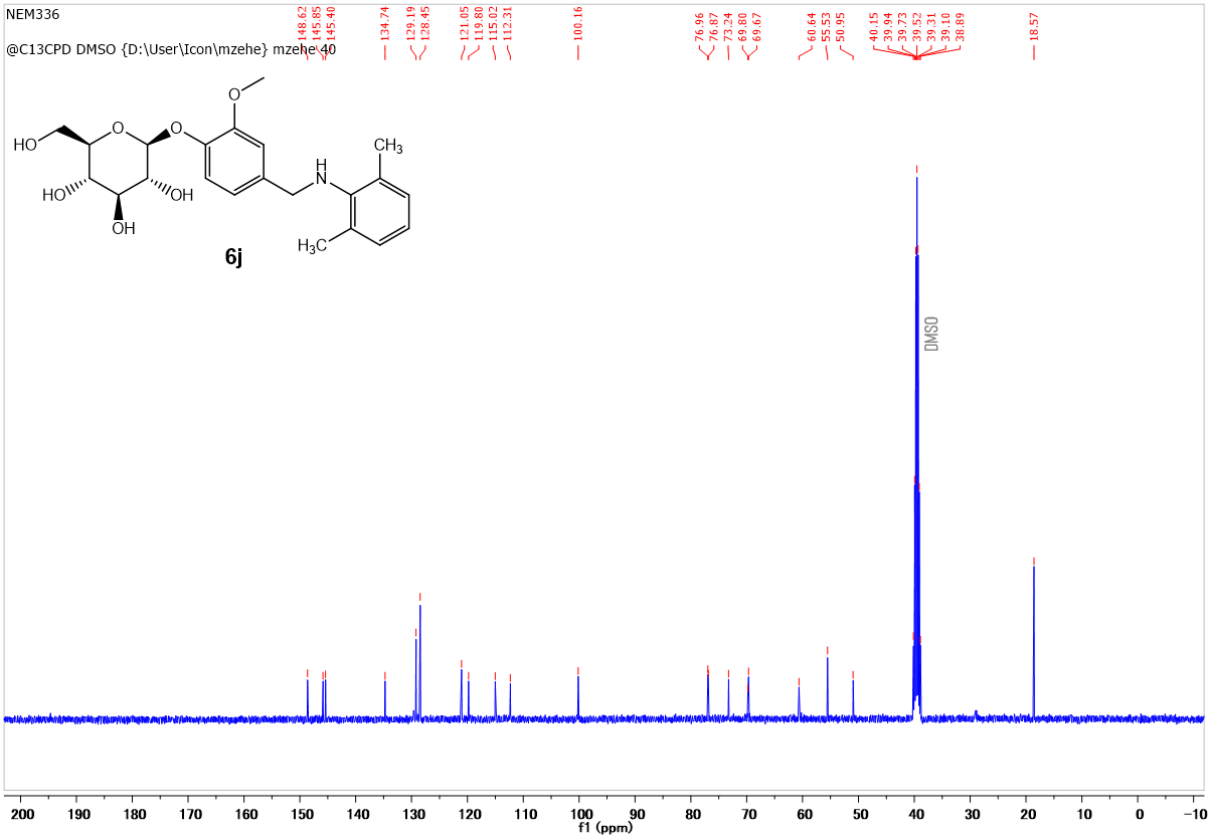


Figure S17b: ^{13}C NMR Spectrum of compound **6j**

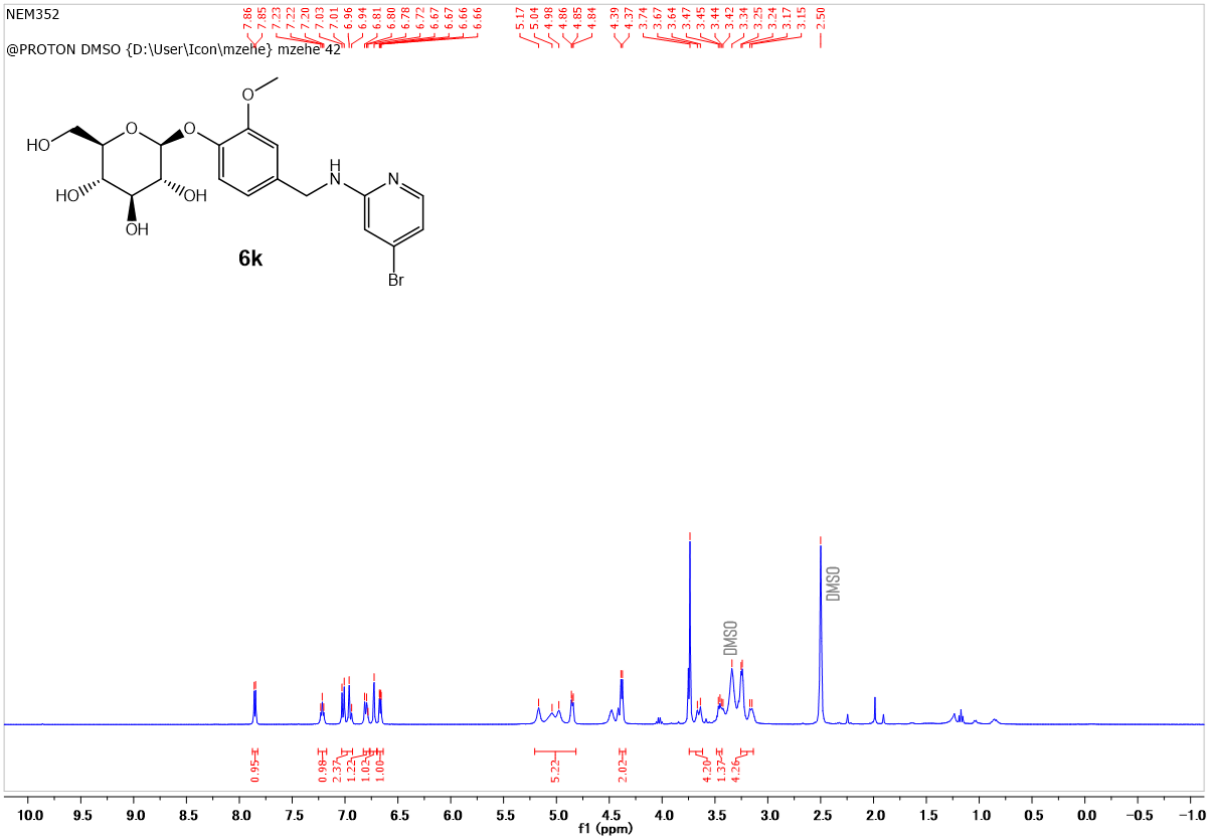


Figure S18a: ^{1}H NMR Spectrum of compound **6k**

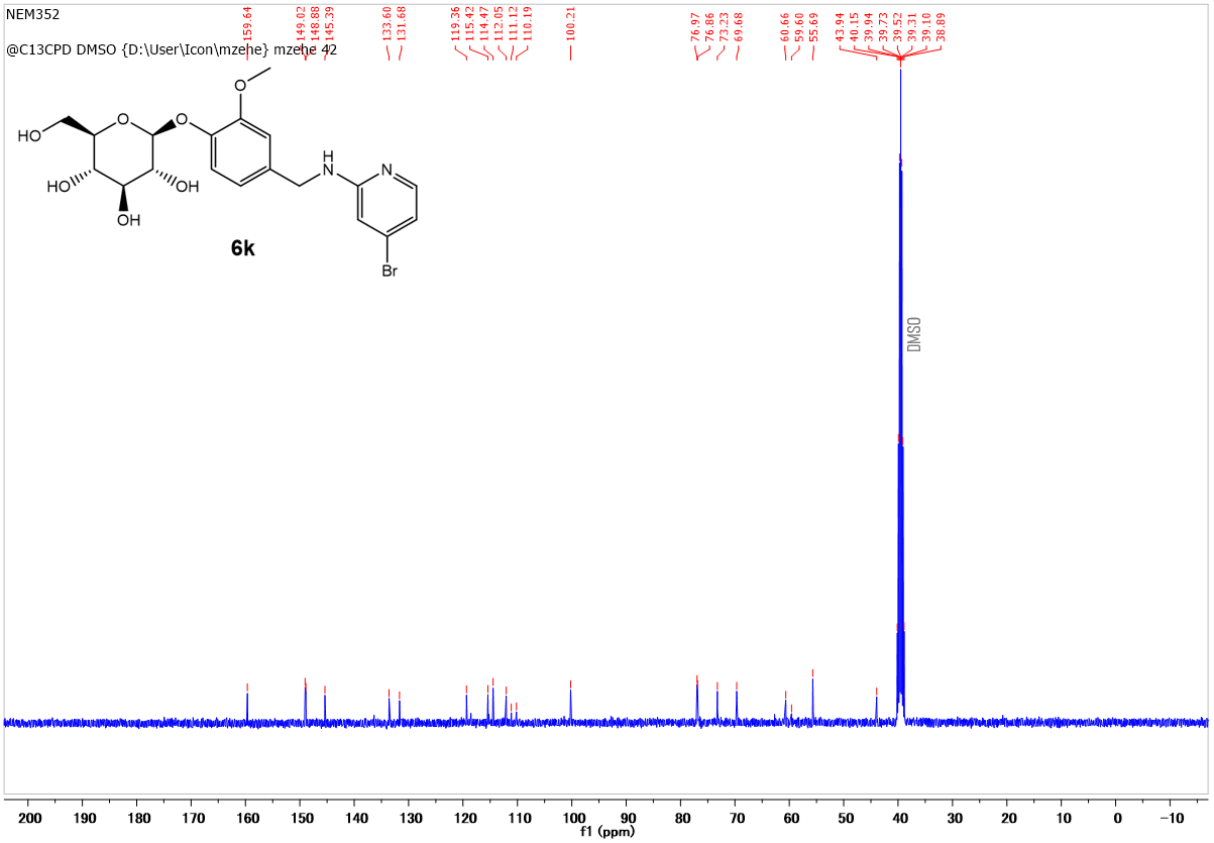


Figure S18b: ^{13}C NMR Spectrum of compound **6k**

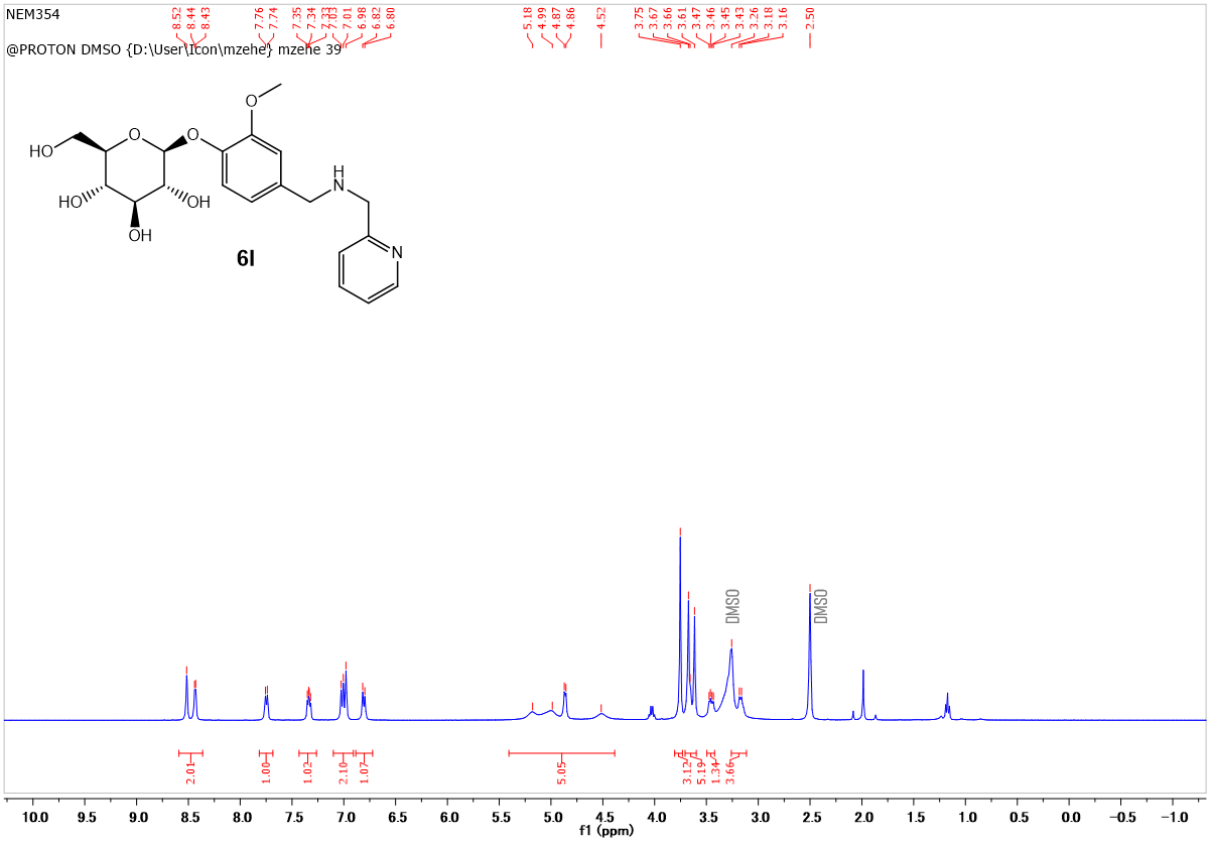


Figure S19a: ^{1}H NMR Spectrum of compound **6l**

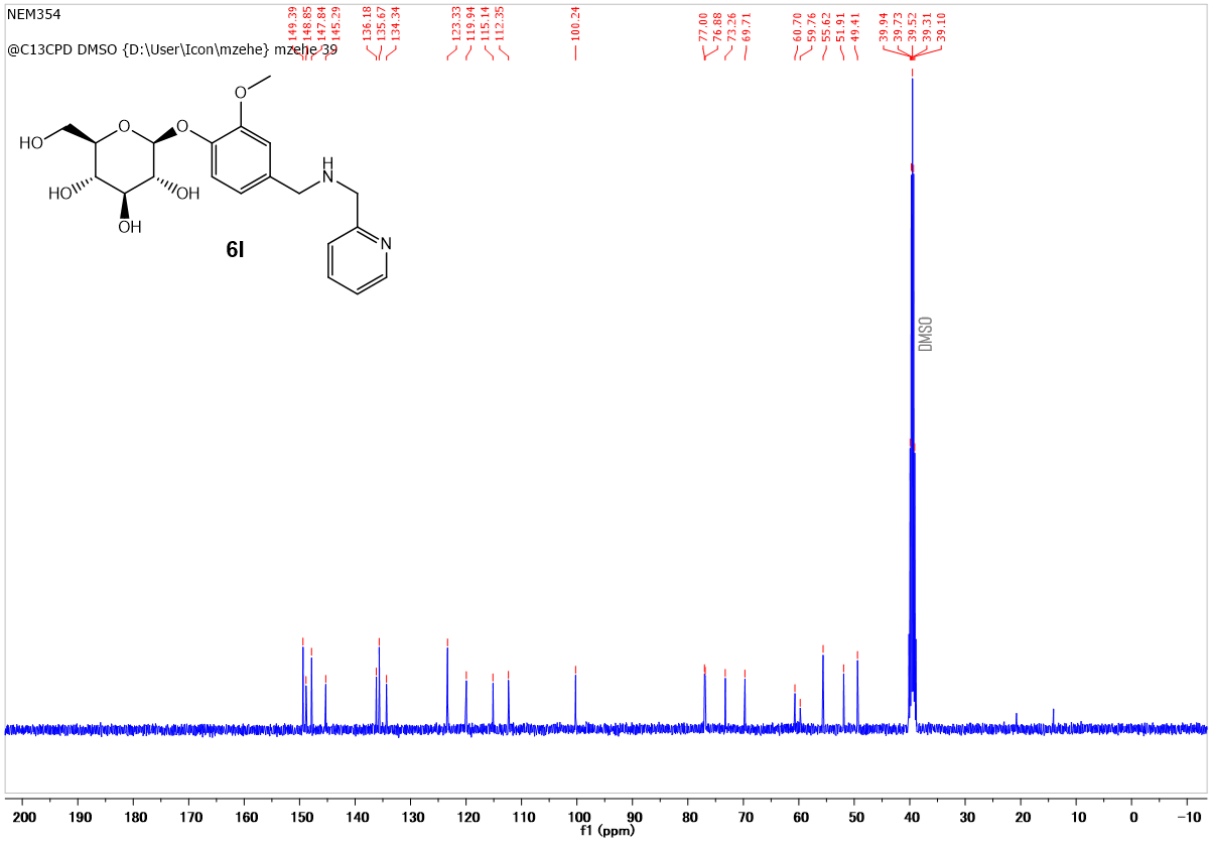


Figure S19b: ^{13}C NMR Spectrum of compound **6l**

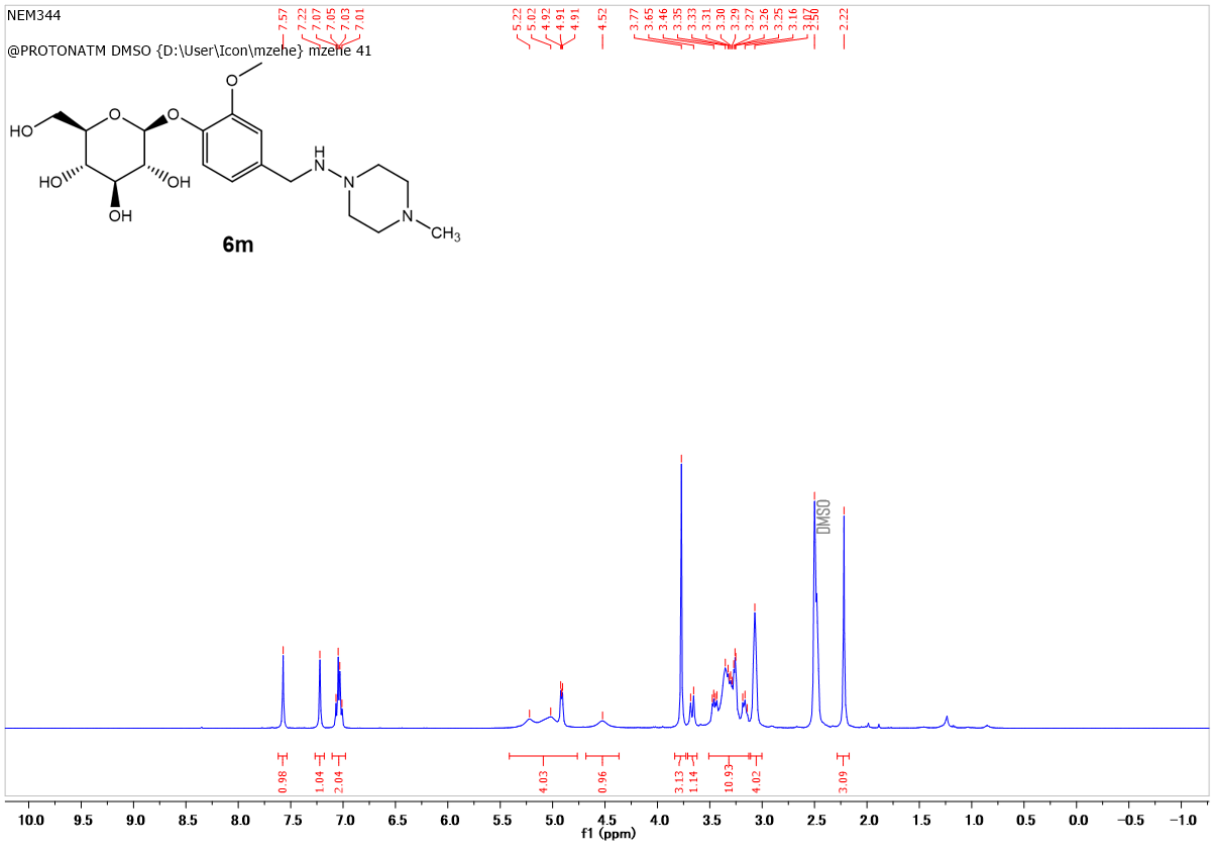


Figure S20a: ^{1}H NMR Spectrum of compound **6m**

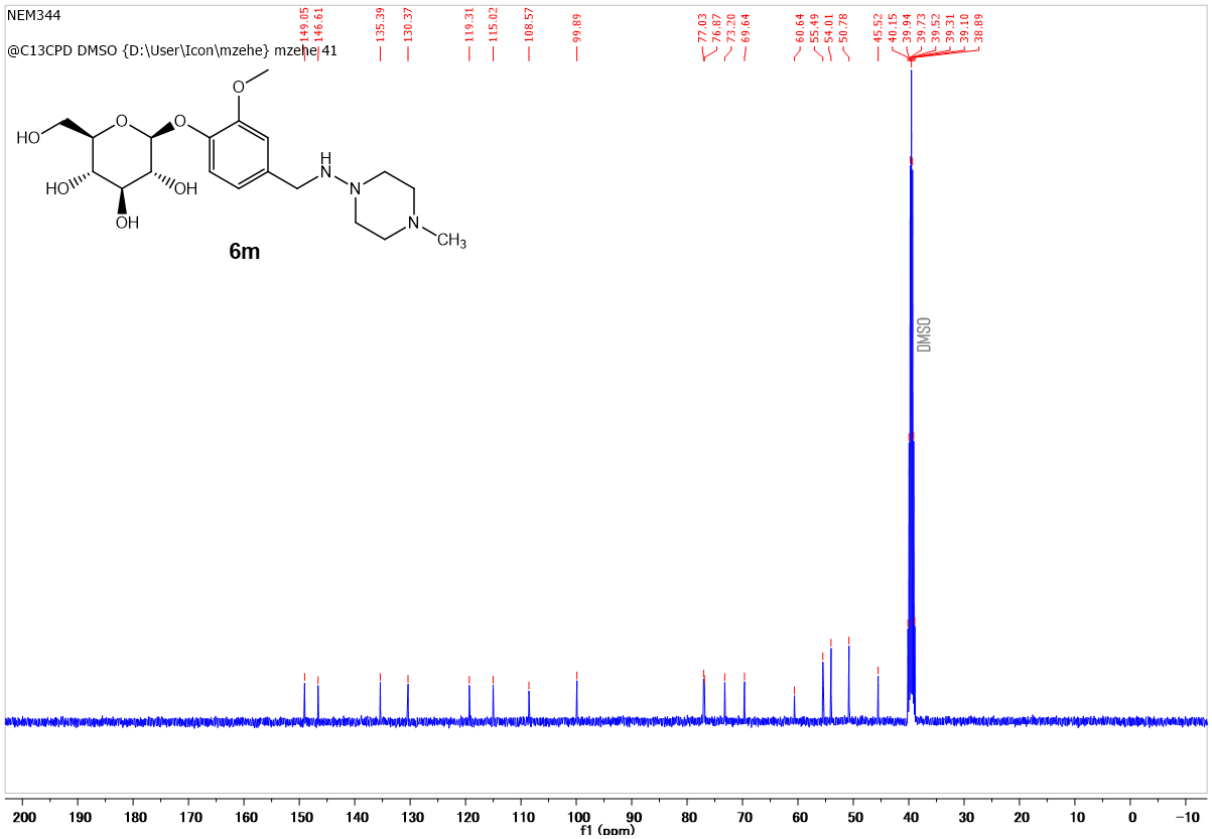


Figure S20b: ^{13}C NMR Spectrum of compound **6m**

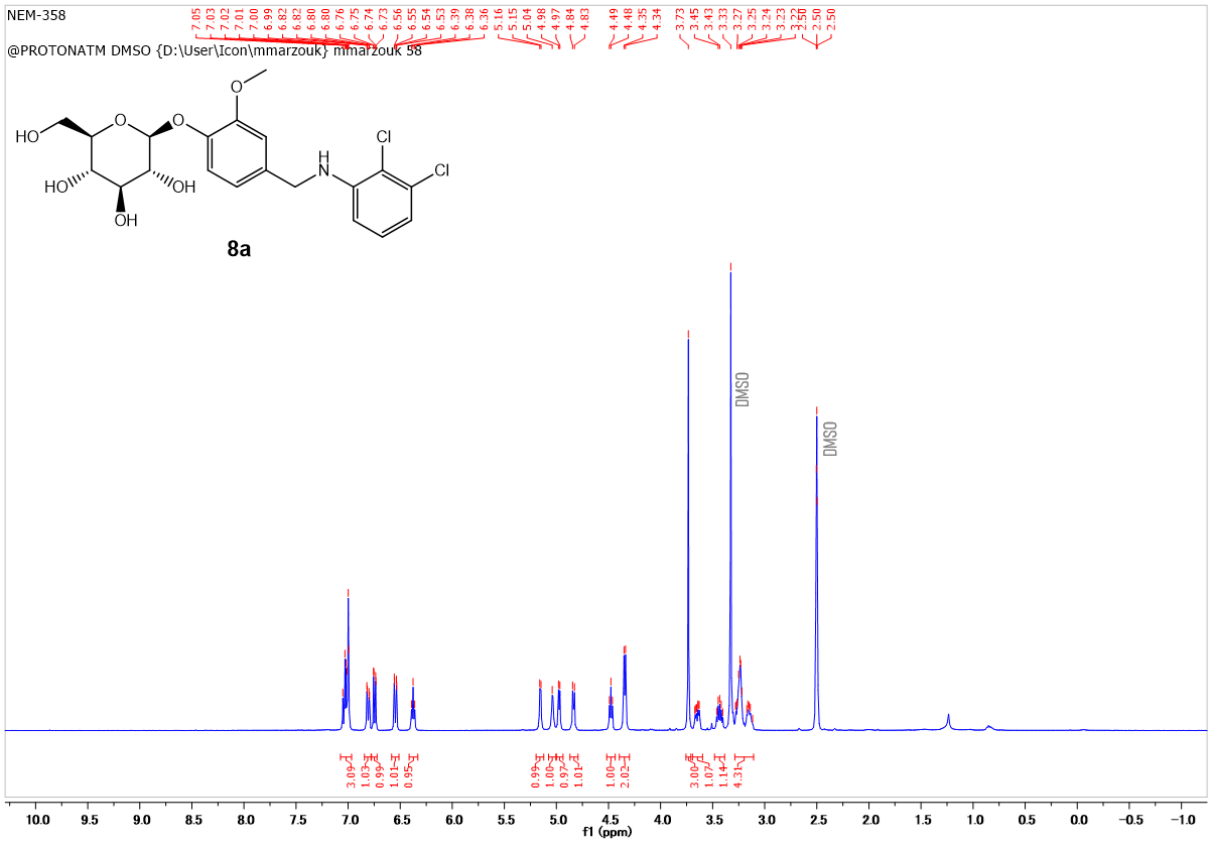


Figure S21a: ^{1}H NMR Spectrum of compound **8a**

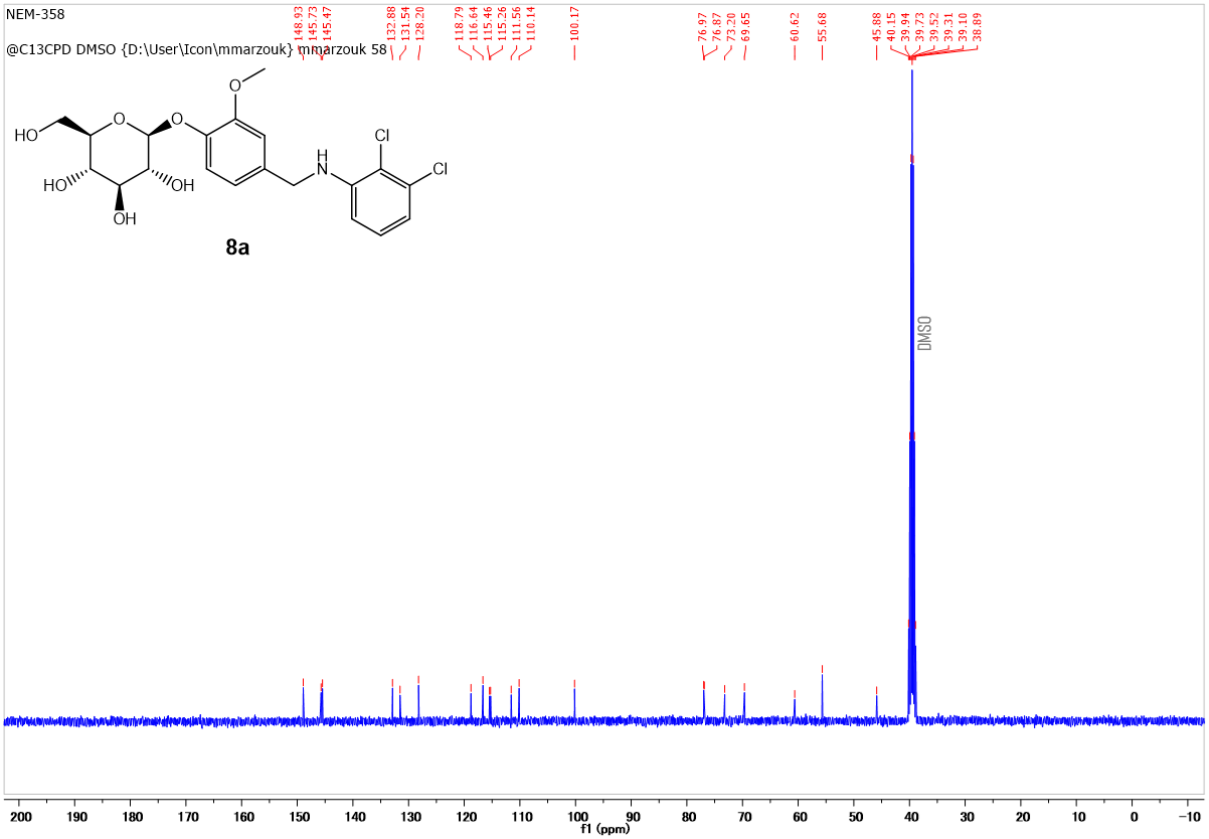


Figure S21b: ^{13}C NMR Spectrum of compound **8a**

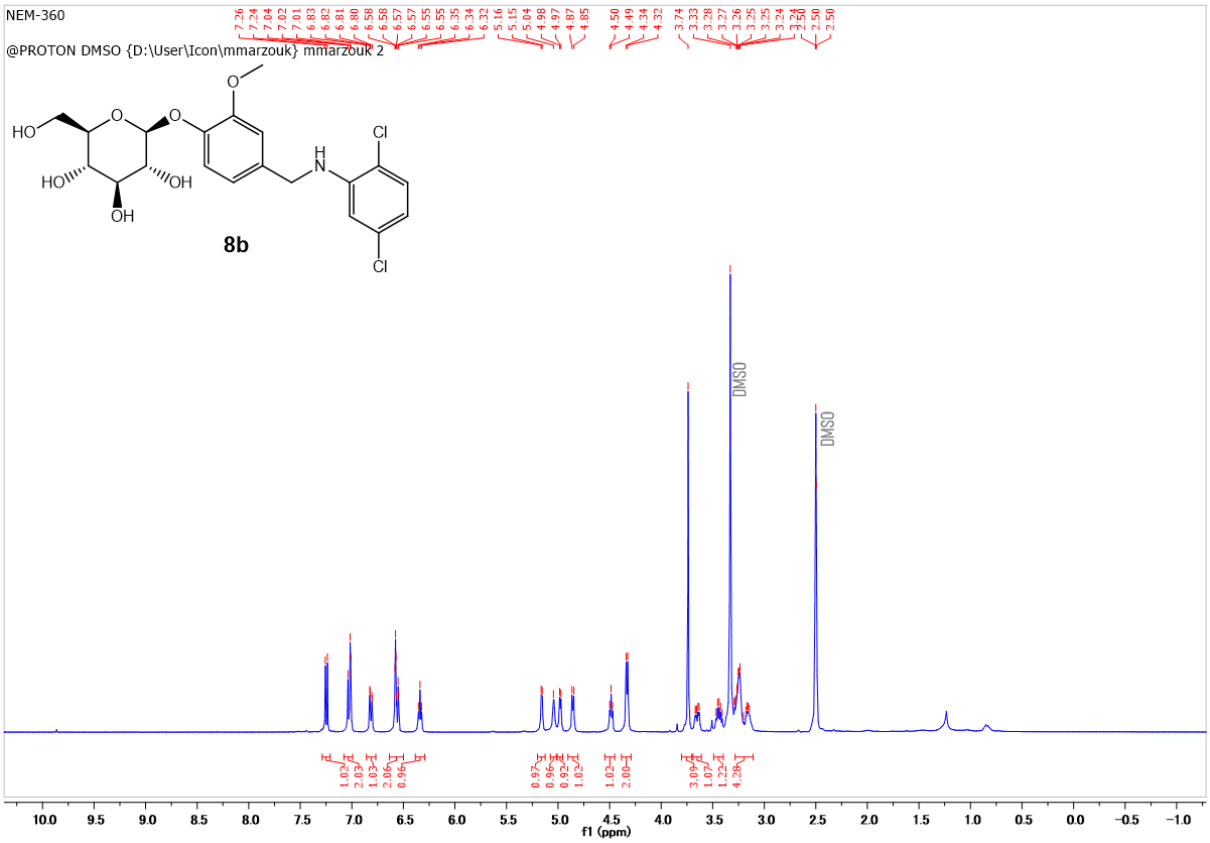


Figure S22a: ^{1}H NMR Spectrum of compound **8b**

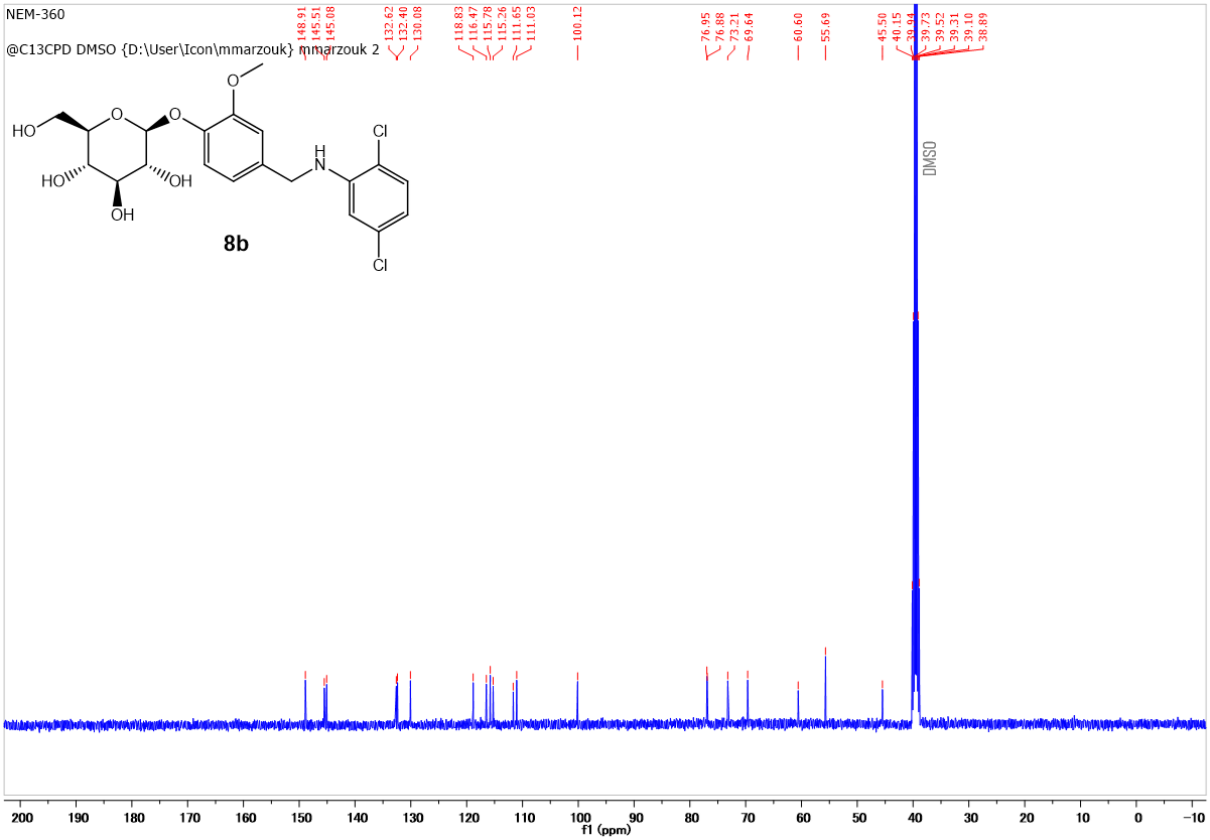


Figure S22b: ^{13}C NMR Spectrum of compound **8b**

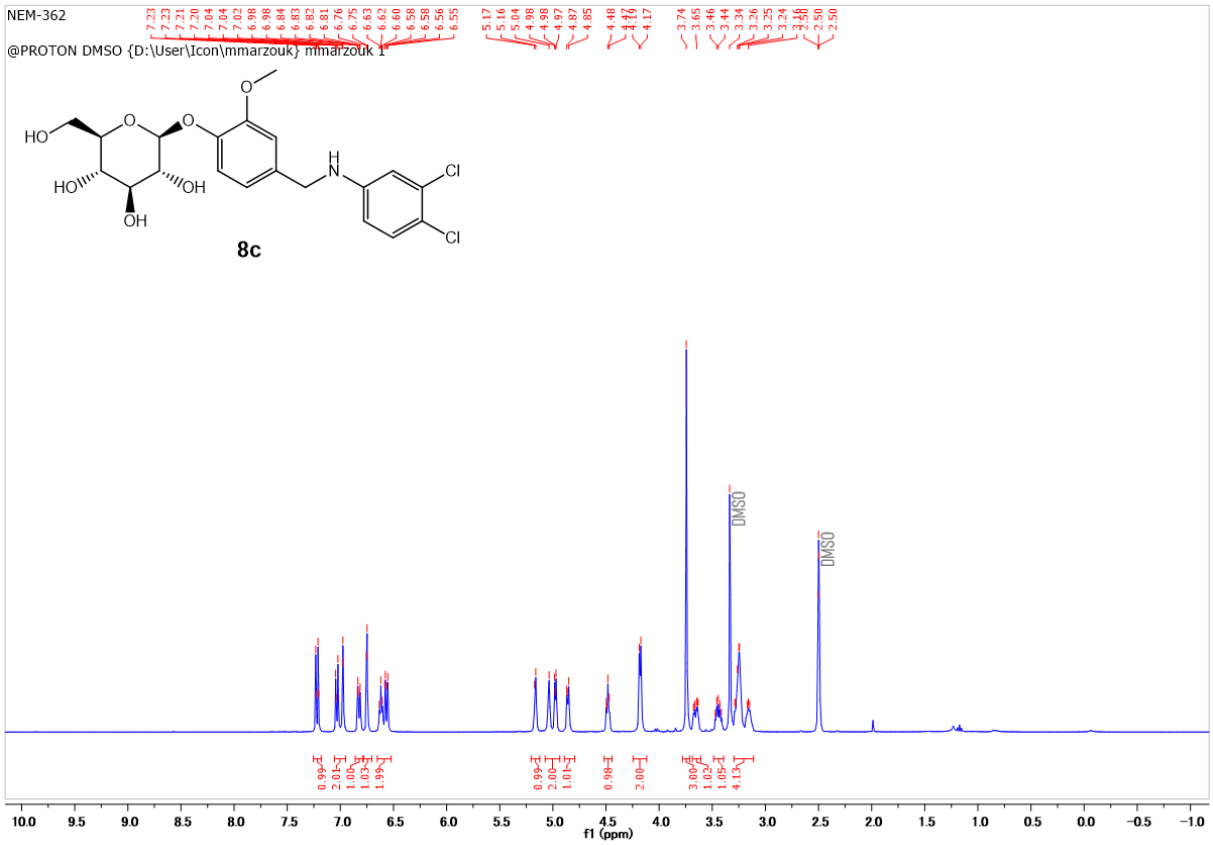


Figure S23a: ^{1}H NMR Spectrum of compound **8c**

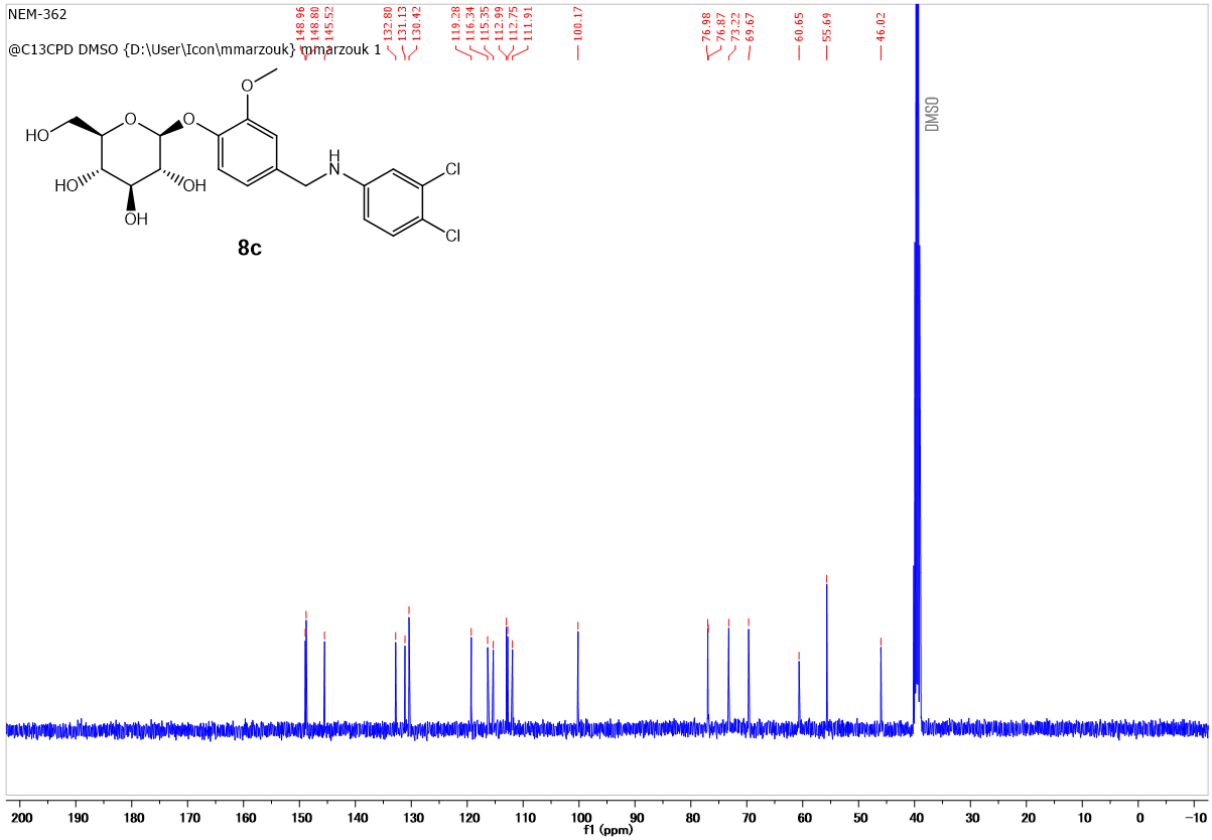


Figure S23b: ^{13}C NMR Spectrum of compound **8c**

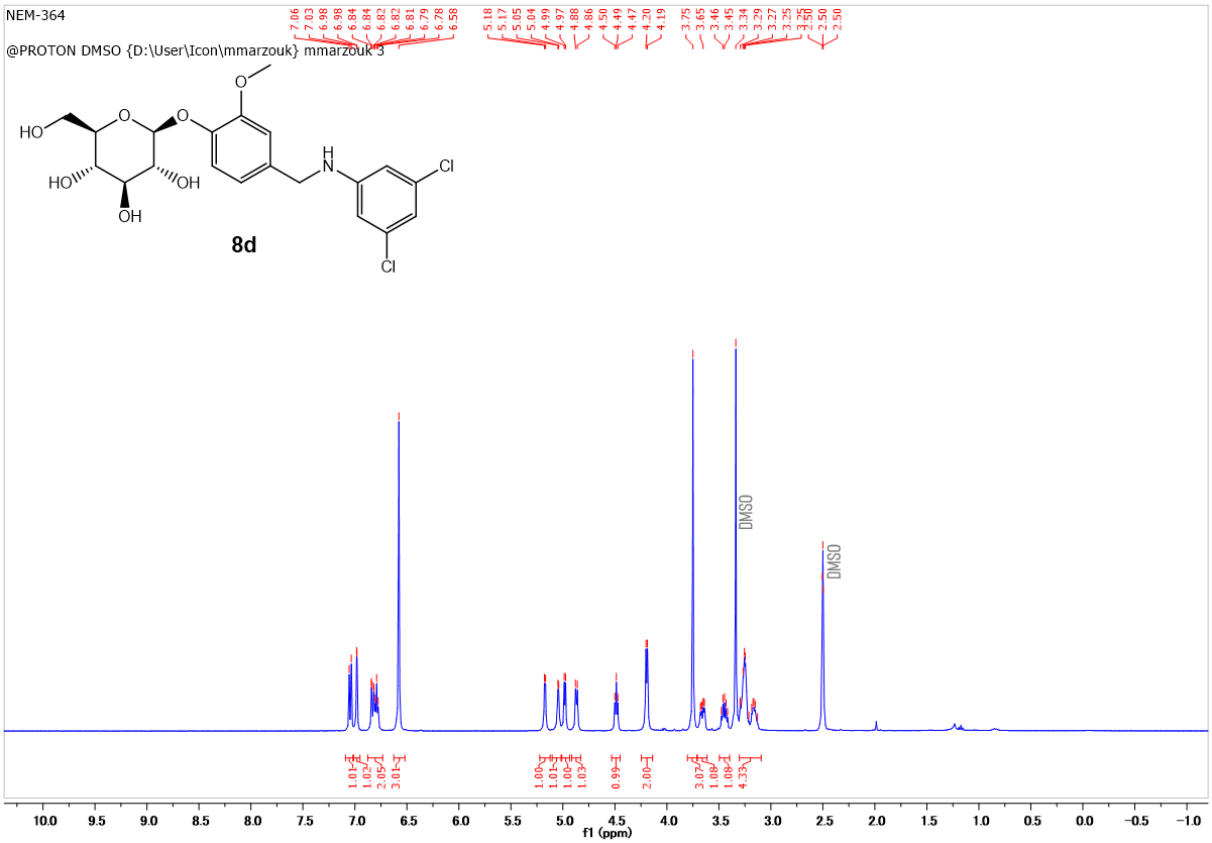


Figure S24a: ^{1}H NMR Spectrum of compound **8d**

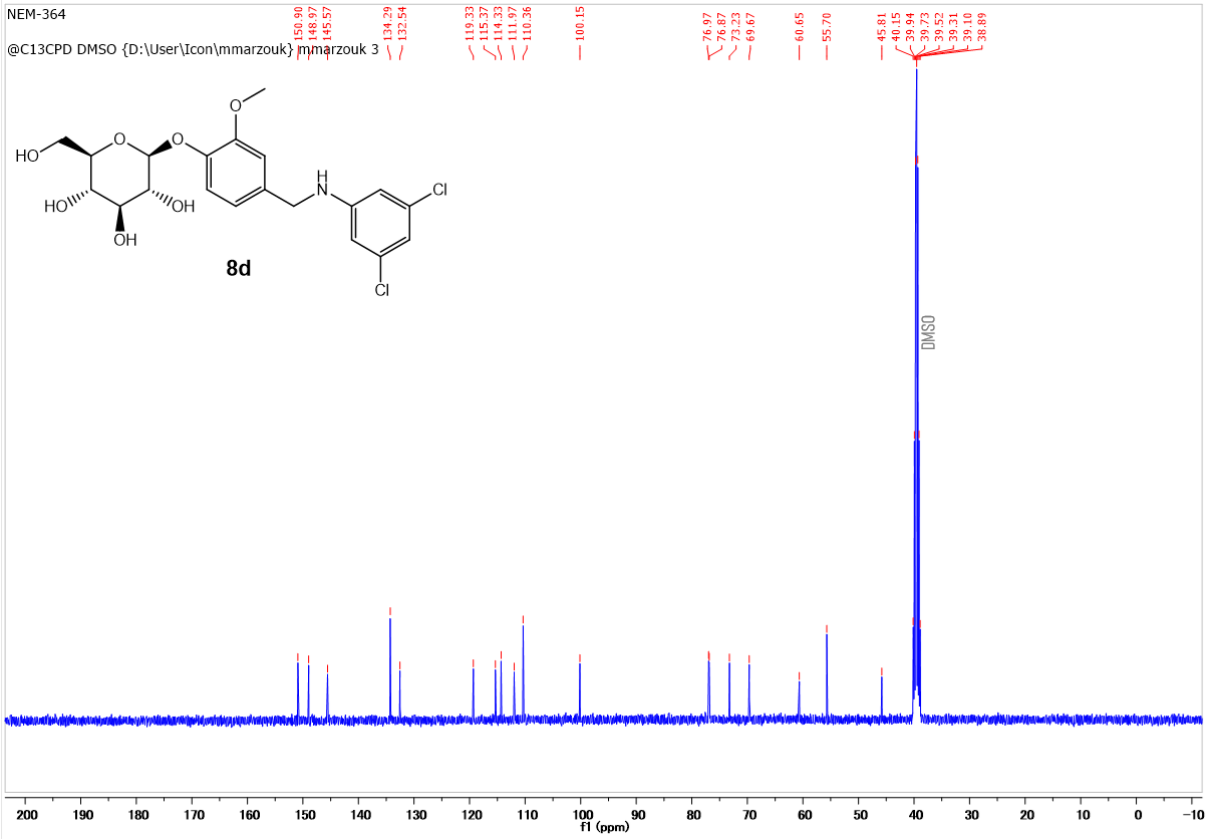


Figure S24b: ^{13}C NMR Spectrum of compound **8d**

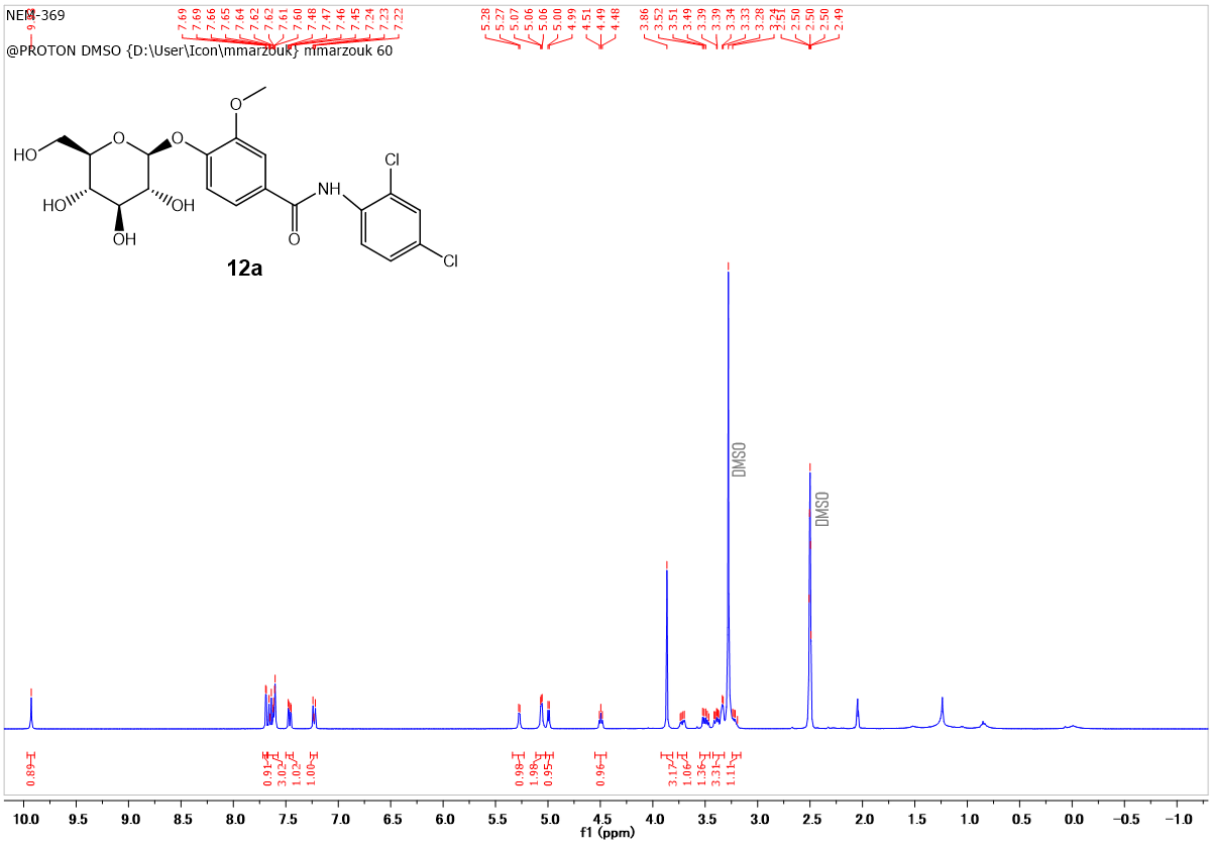


Figure S25a: ^{1}H NMR Spectrum of compound **12a**

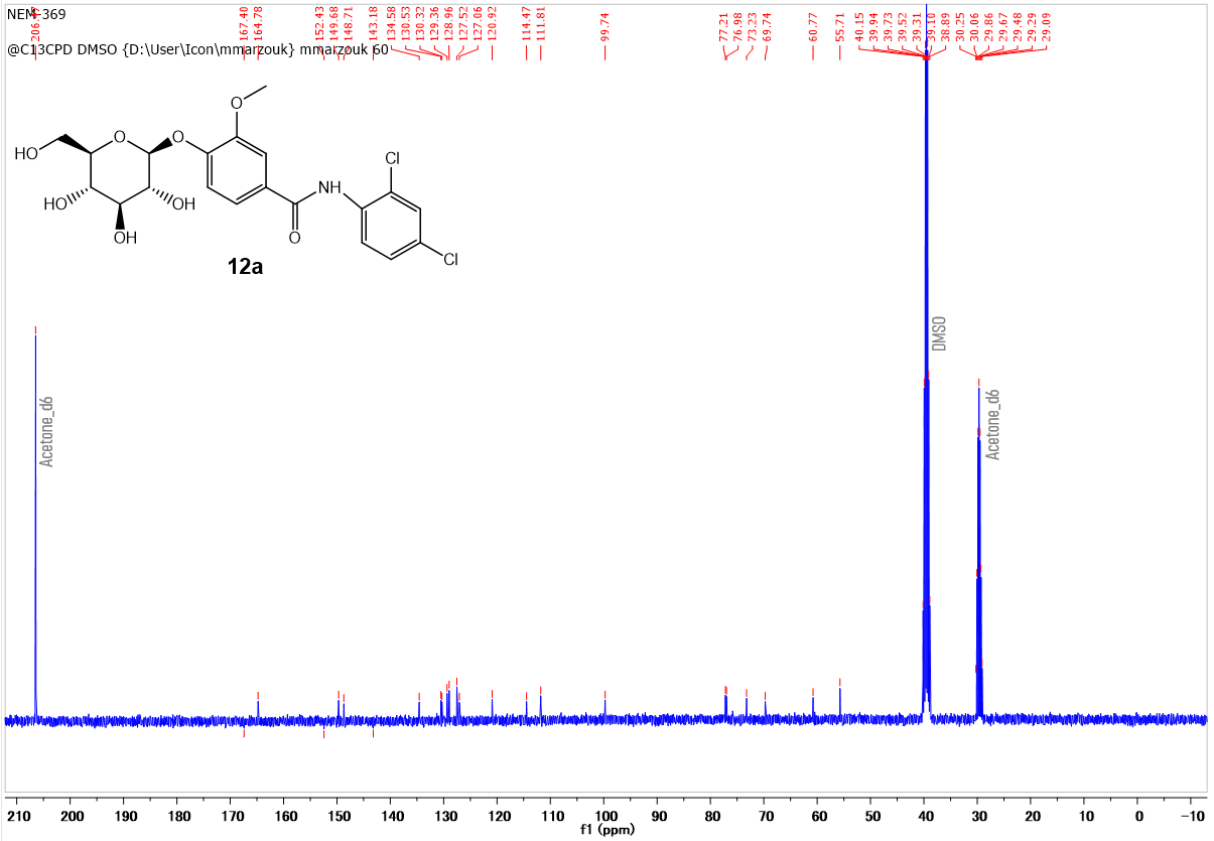


Figure S25b: ^{13}C NMR Spectrum of compound **12a**

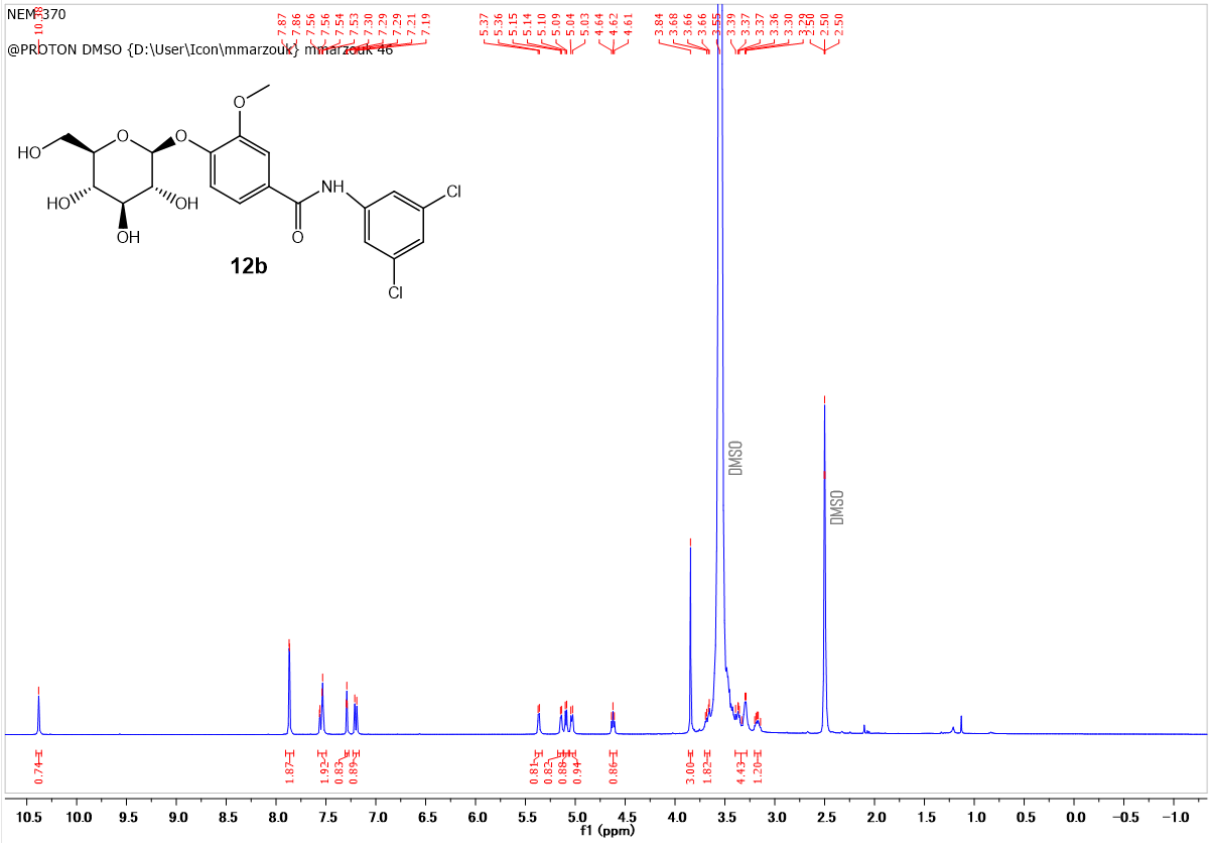


Figure S26a: ^{1}H NMR Spectrum of compound **12b**

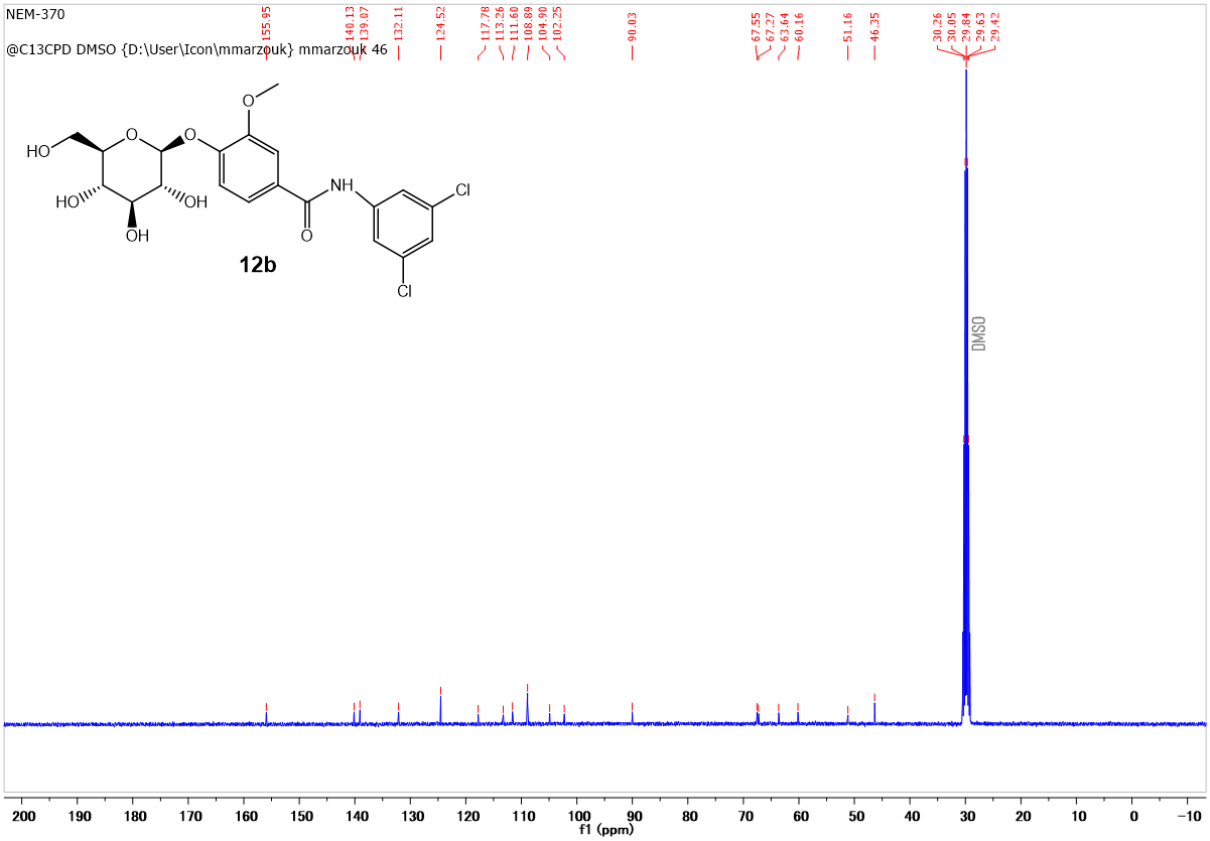


Figure S26b: ^{13}C NMR Spectrum of compound **12b**

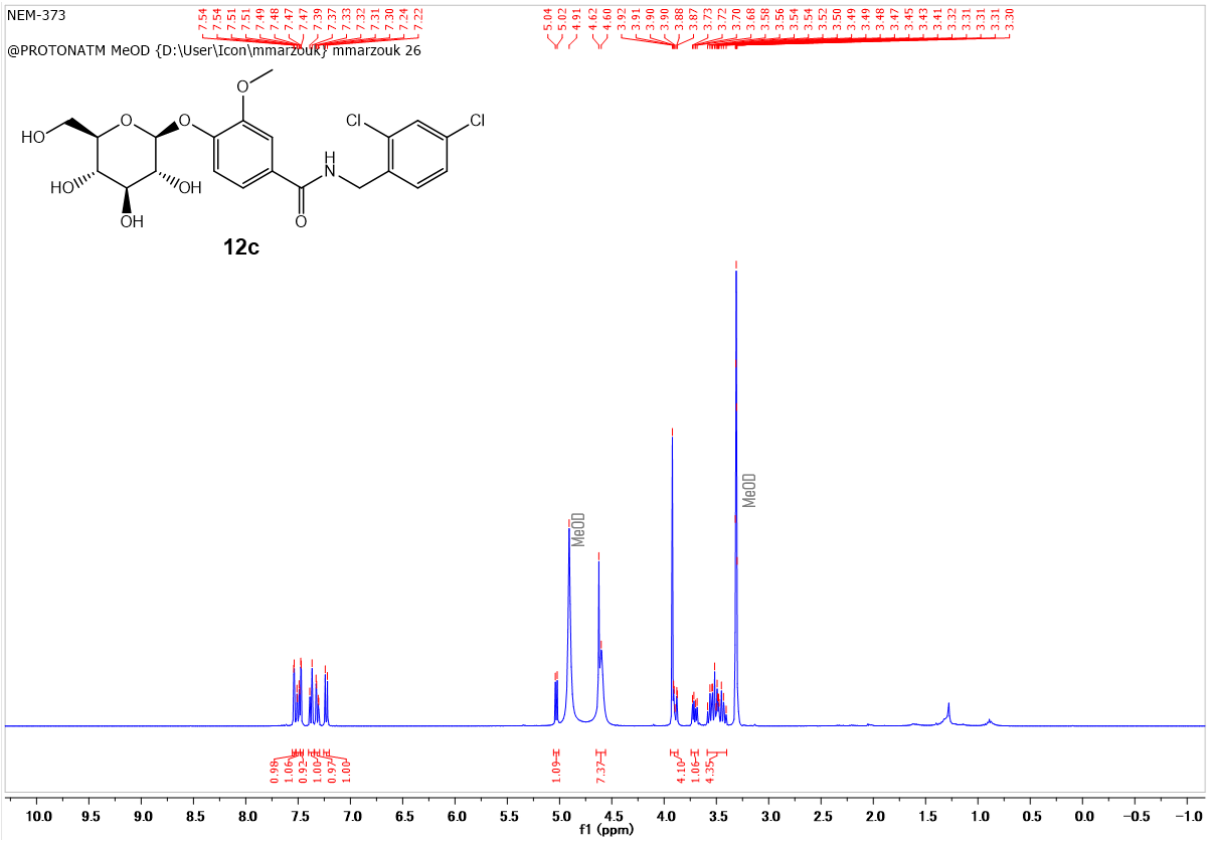


Figure S27a: ^{1}H NMR Spectrum of compound **12c**

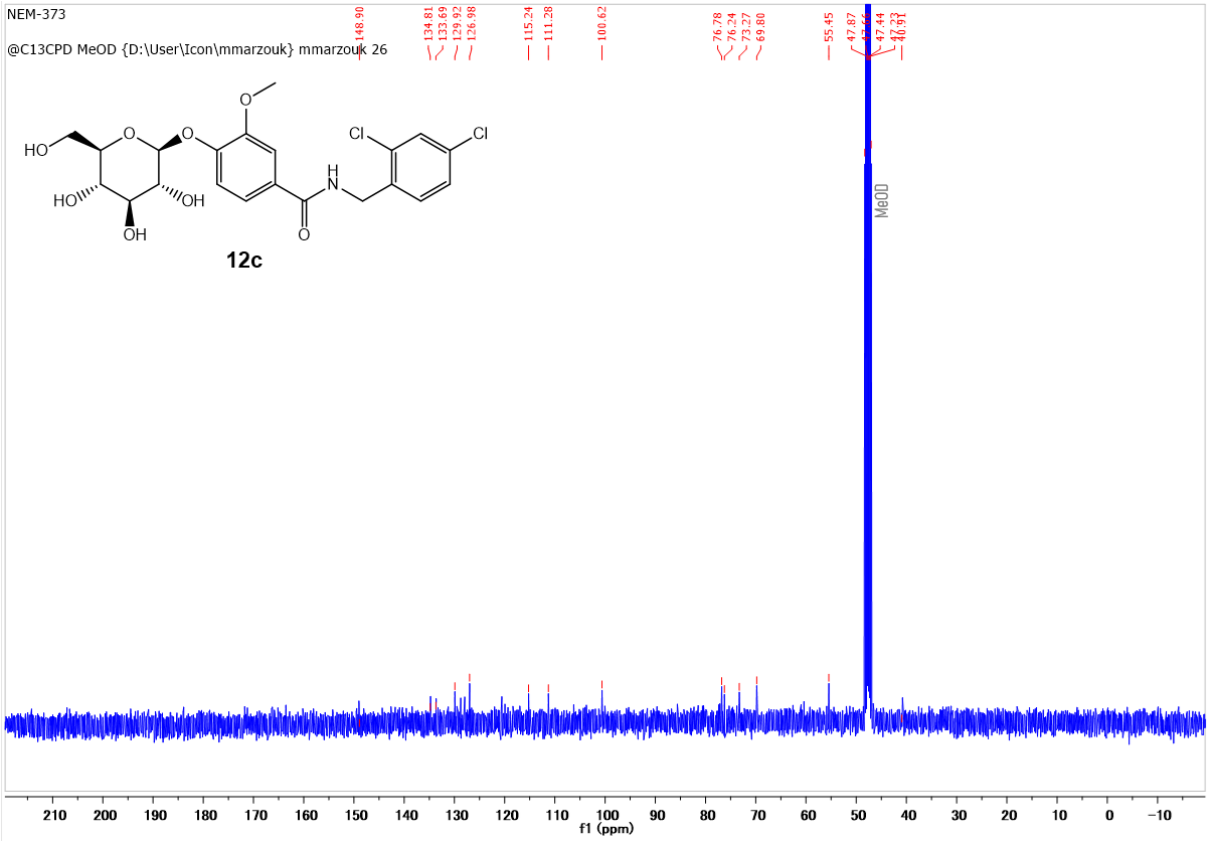


Figure S27b: ^{13}C NMR Spectrum of compound **12c**

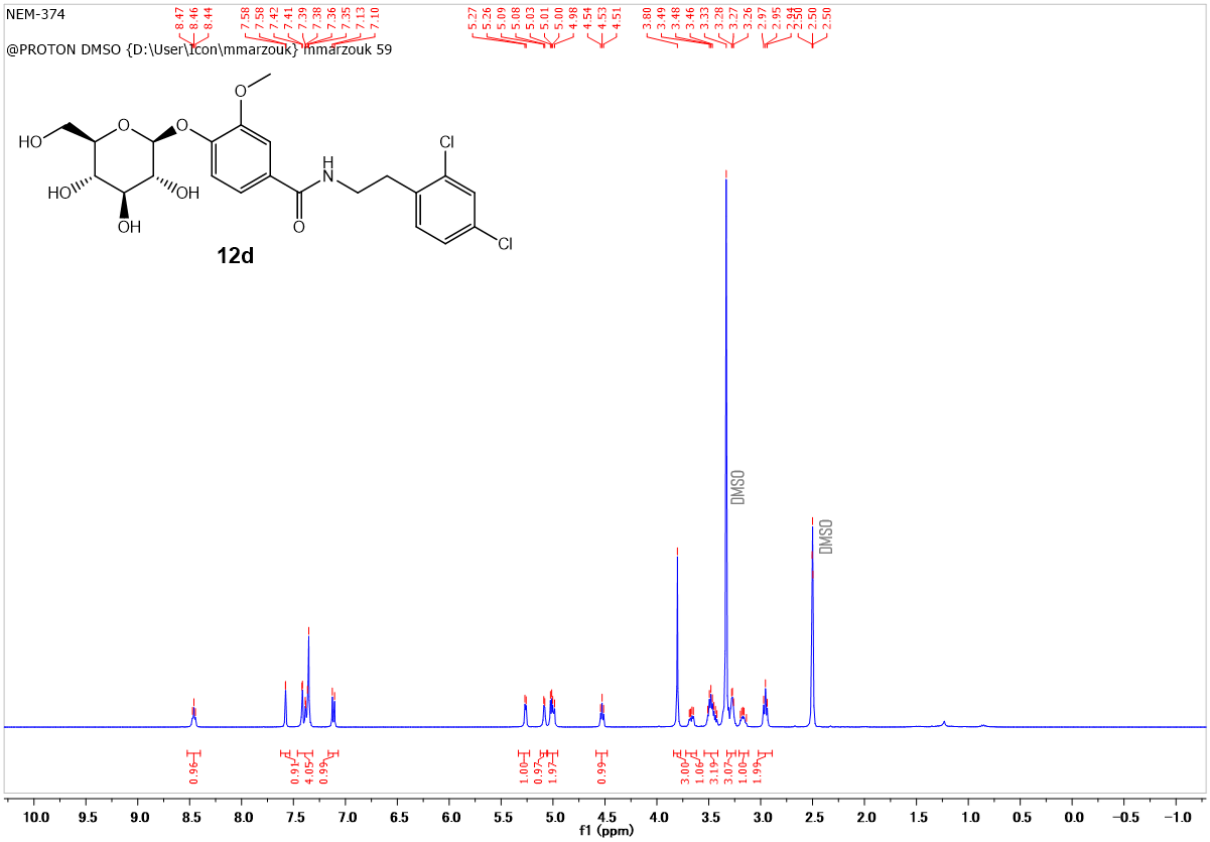


Figure S28a: ^{1}H NMR Spectrum of compound **12d**

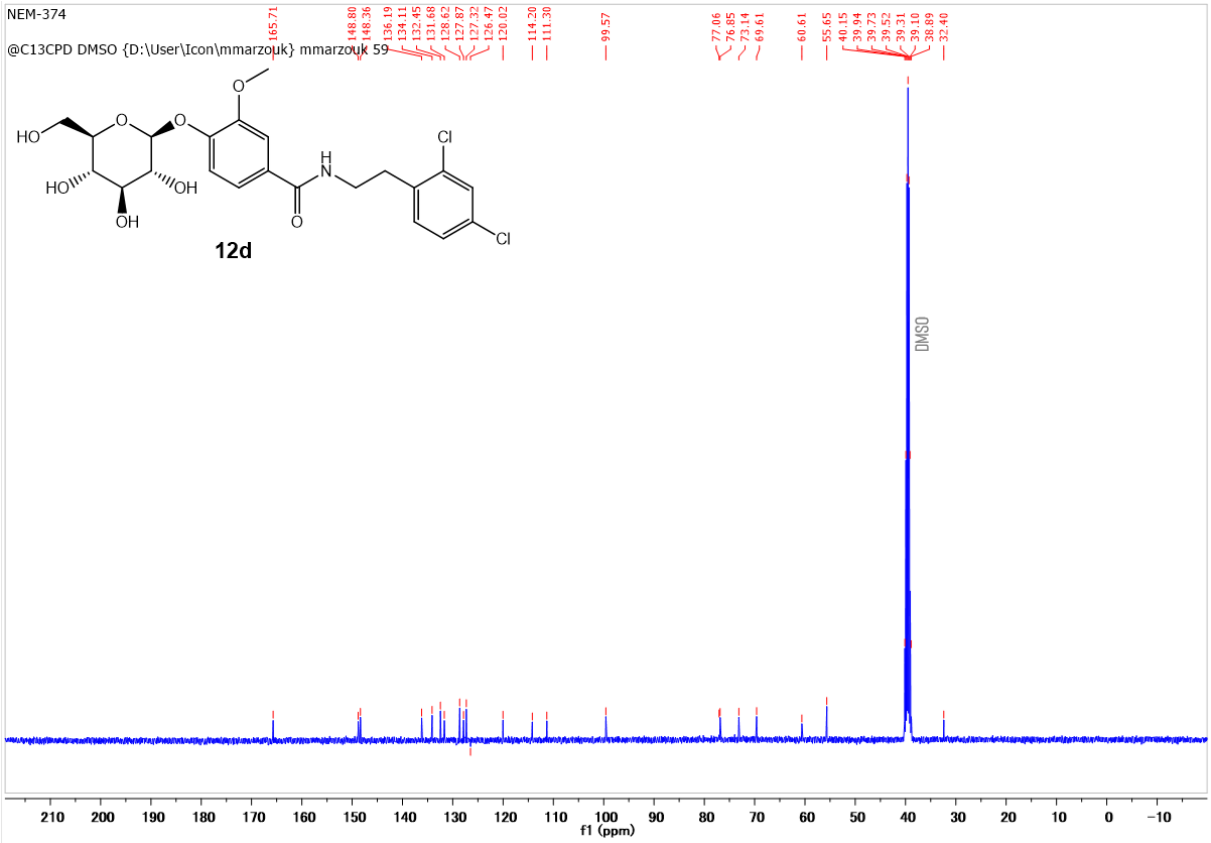


Figure S28b: ^{13}C NMR Spectrum of compound **12d**

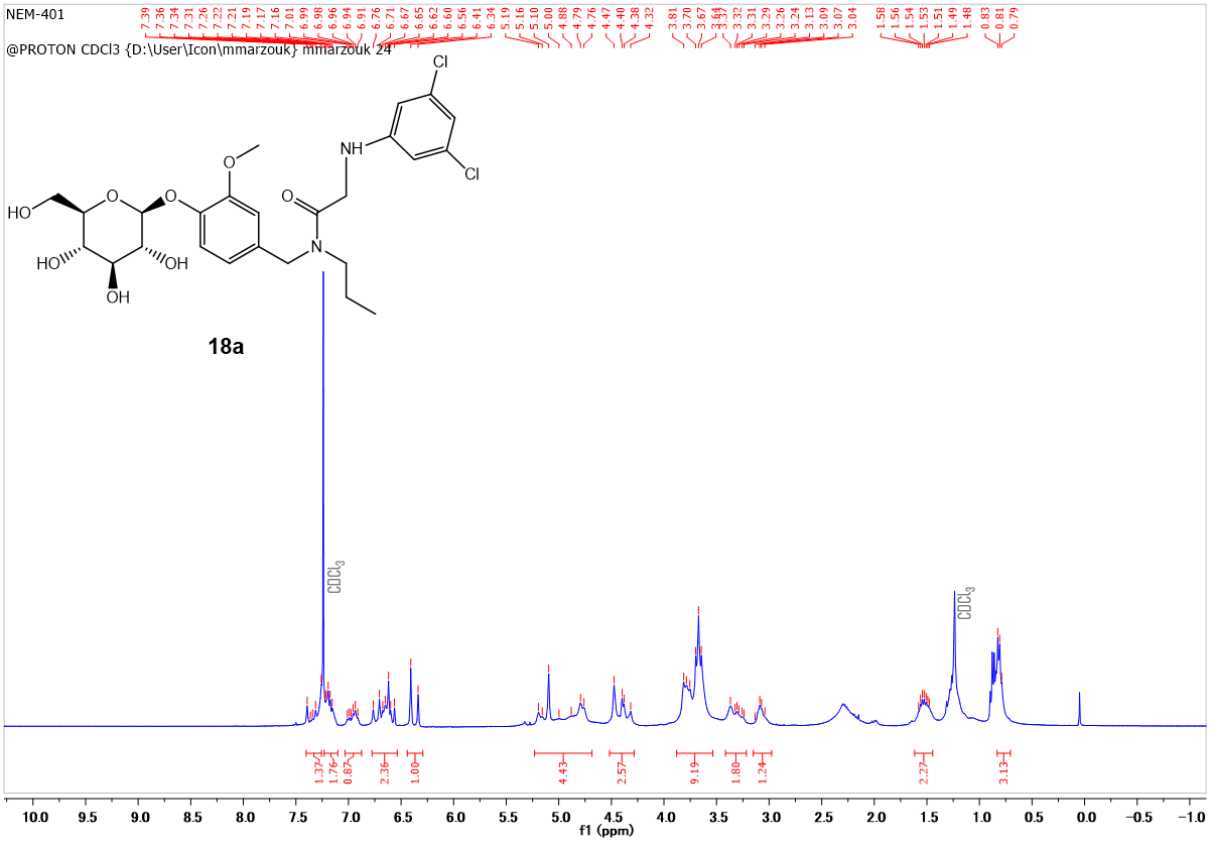


Figure S29a: ^{1}H NMR Spectrum of compound **18a**

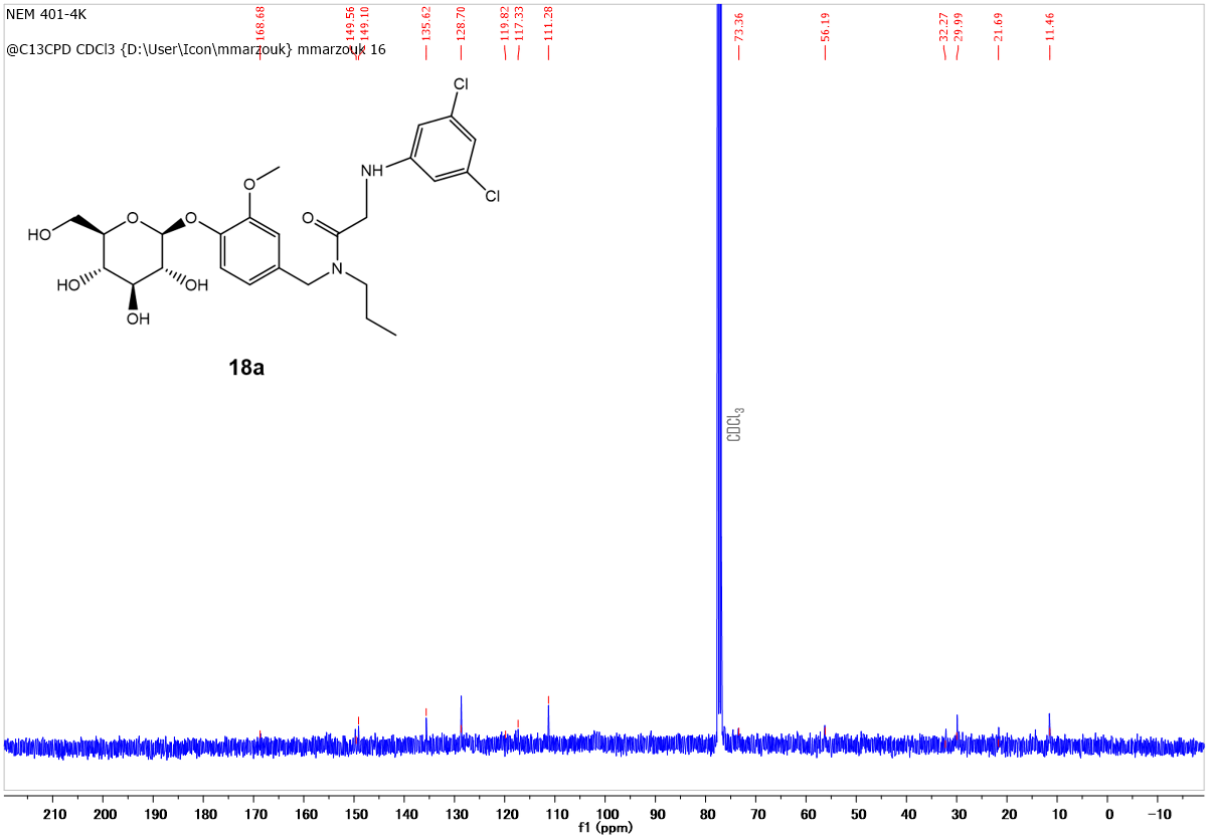


Figure S29b: ^{13}C NMR Spectrum of compound **18a**

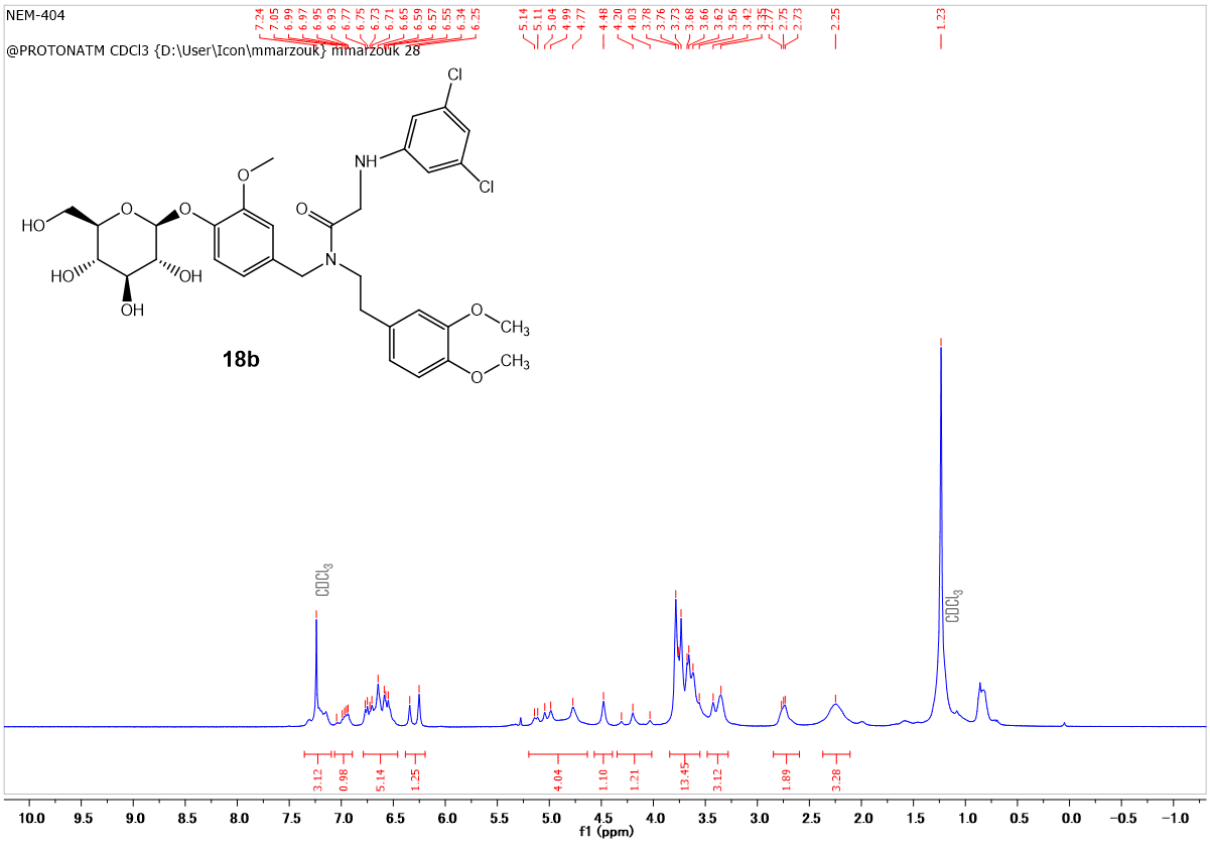


Figure S30a: ^{1}H NMR Spectrum of compound **18b**

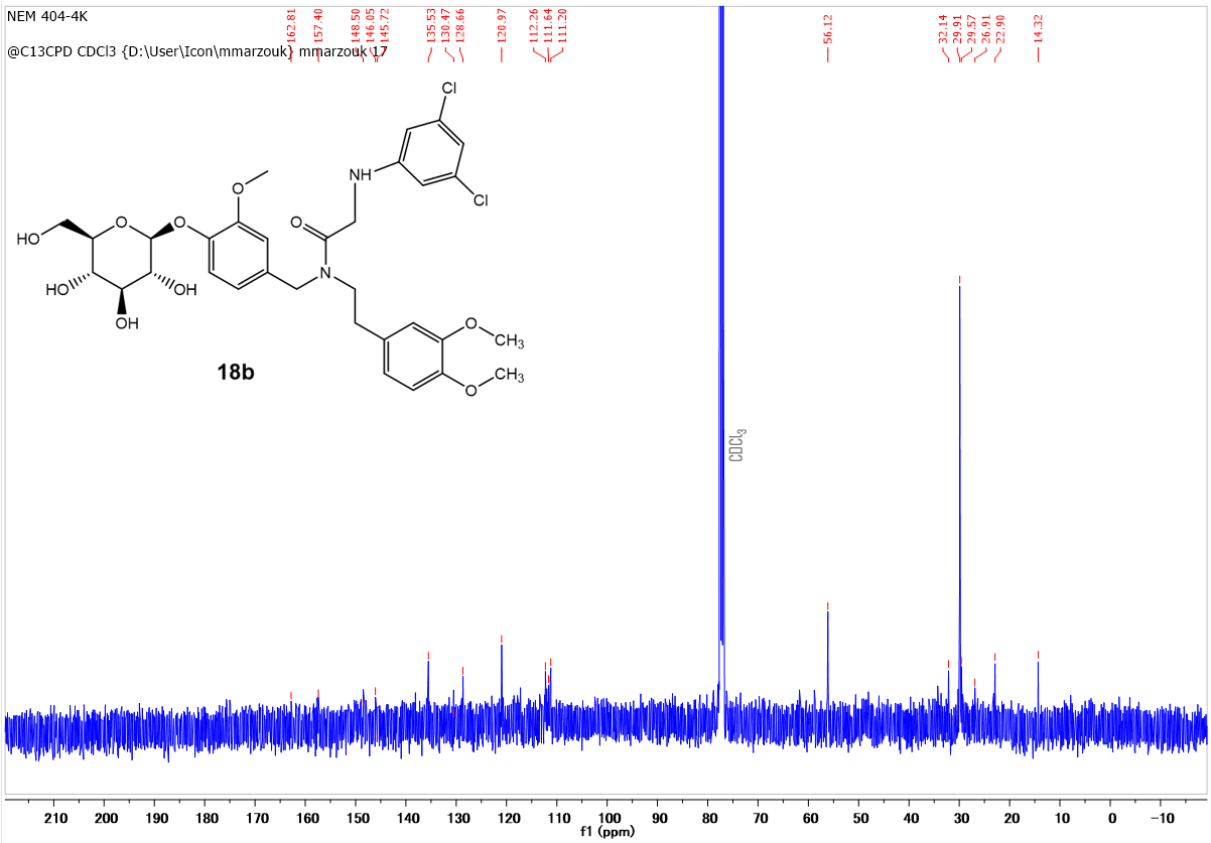


Figure S30b: ^{13}C NMR Spectrum of compound **18b**

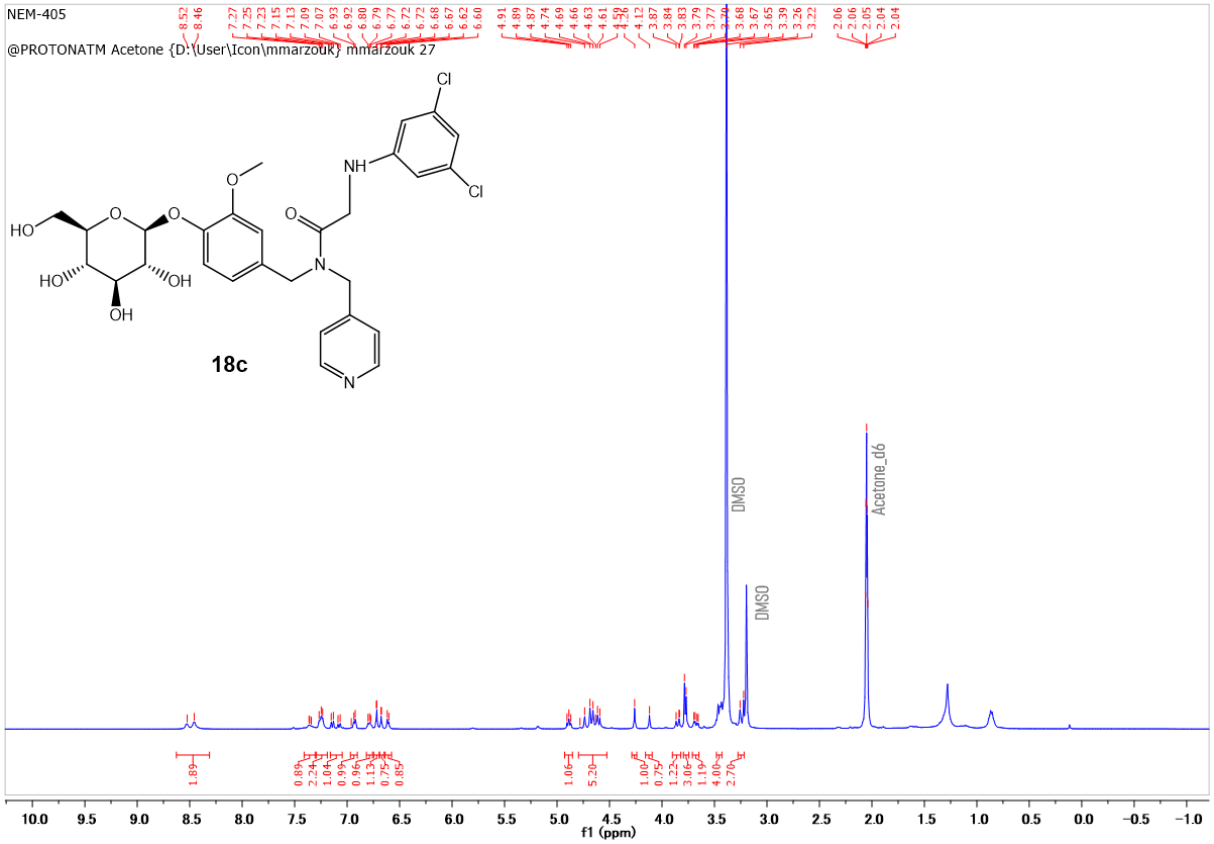


Figure S31a: ^{1}H NMR Spectrum of compound **18c**

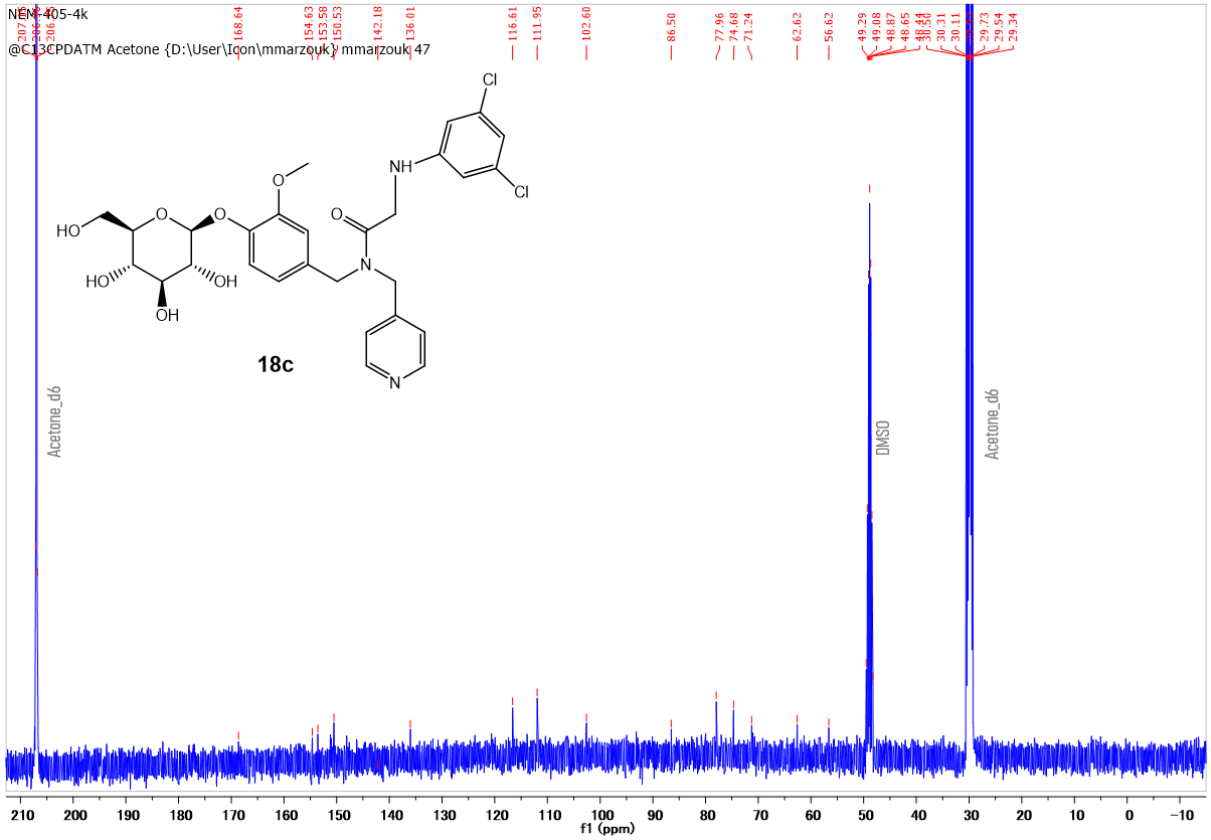


Figure S31b: ^{13}C NMR Spectrum of compound **18c**

Chapter V

Final Discussion

5. FINAL DISCUSSION

Studies in this thesis aimed at searching for new effective agents against Multidrug-resistant (MDR) strains of *Escherichia coli* and *Klebsiella pneumoniae* (Enterobacteriaceae). The approaches employed in the search for such agents led to the development of libraries of plant species and phytochemicals with previously reported good activities against the two bacteria. Moreover, based on the essence of an extraction step in the study of compounds from plant sources, an efficient extraction method employing microwave assistance at low temperatures and high pressure was developed. Studying the plant species identical or related to those in the prepared library showed low chances of reproducing antibacterial activities previously reported in plant extracts, hence calling for collective actions to address the associated challenges. Nevertheless, the isolation and characterization of galloylglucoses with potential activities against numerous MDR strains of the two bacteria were achieved. Ultimately, using synthetic approaches, it was possible to prepare one phytochemical from the assembled library along with its derivatives. The synthesized compounds exhibited moderate broad-spectrum activities against the targeted bacteria.

Creation and evaluation of active plants and phytochemicals

Apart from the lack of clinically approved antibiotics of plant origin, many plant species have been screened and reported for their activities against a broad range of bacteria and other microorganisms [1, 2]. Those studies are usually associated with subsequent fractionation, isolation, and characterization of the actual antibacterial compounds from the complex mixtures within the crude extracts. Altogether, these efforts have led to valuable reports on phytochemicals with broadly ranging antibacterial activities.

Considering these advances, a library of 128 plant species with reported activities of ≤128 µg/mL against *E. coli* and/or *K. pneumoniae* was created following an extensive review of the literature. To enhance objective comparison and higher success rates during the follow-up studies, it was essential to include only studies reporting high activities as determined by broth dilution assays. Based on the highlighted patterns with respect to plant families, tissues, and the nature of the extracting solvents, the library can serve as a guidance and time-saving opportunity in studies by other researchers. Such libraries reduce the challenges in the screening of the bulky literature to obtain specific and detailed information on potential plants and the associated experimental aspects.

Following a similar approach, a library of 122 phytochemicals with previously reported MICs of ≤100 µg/mL against *E. coli* and/or *K. pneumoniae* was created. Further evaluation of this library revealed interesting patterns between the phytochemicals' antibacterial activities, classes, molecular weights, numbers of hydrogen bonds donors/acceptors, total polar surface area, lipophilicity, molecular flexibility, globularity, as well as the number of heavy atoms. In addition to

highlighting potential natural scaffolds, the specialized library grants an opportunity for closer evaluations, and a deeper understanding of properties necessary for targeting the studied bacteria, and possibly other Gram-negative bacteria.

Efficiency and selectivity of low-temperature PMAE and prolonged maceration

Pressurized MAE over a short duration (30 min) and low-temperature conditions (40 – 45 °C) was demonstrated to be capable of attaining yields comparable to those of cold maceration over a 24h duration. The observed efficiency under PMAE is linked to the abrupt rise in temperature, characteristic of microwave heating. This is followed by the rapturing of plant cells due to the sudden evaporation of solvent and residual water molecules [3]. This effect is potentiated by the unidirectional heat and mass transfer attained under microwave heating as opposed to conventional heating [3]. Additionally, a pressurization approach employed in this study was essential in pushing the solvent's molecule through the plant matrices, hence increasing the solubility of the phytochemicals in the extraction solvent [4-7]. Since most microwave-assisted extractions are typically conducted at higher temperatures (60–120 °C) [8-10], findings from this study are important in expanding the suitability and applicability of MAE. This is particularly important as the loss of some valuable compounds due to thermal degradation or cross-reactions is more likely when extractions are performed at high temperatures. Further, since doubling of extraction temperature (from 40 °C to 80 °C) under PMAE did not show a substantial increase in yields, the perceived benefits of high temperatures in most MAE applications should be reconsidered.

The observed plant tissue-related variations in the obtained yields under PMAE were linked to the overall amounts of residual moisture contents present in the dried plant materials [11, 12]. Due to its higher polarity, water content increases the overall performance of extraction efficiency under PMAE. Nevertheless, other factors such as differences in cell and tissue morphologies might play a role.

Moreover, it was evident that different quantities of the same phytochemicals can be extracted based on the employed extraction method [8, 9, 13]. These observations point out the possibility of extracting different amounts of phytochemicals of interest based on the method of extraction. In addition to quantitative differences, notable variations in the qualitative compositions of the obtained extracts were observed. In general, extraction under maceration was noted to hold a higher likelihood of extracting additional compounds. Ensuring prolonged extraction durations can, therefore, maximize the types of phytochemicals extracted from a particular plant matrix [5, 14, 15]. Based on this study, the use of PMAE at low-temperature conditions is advocated towards the attainment of high yields in a short duration, while avoiding cross-reactions and sparing heat labile phytochemicals [10, 16, 17]

Challenges in reproducing antibacterial activities previously reported from plant extracts

Chances of reproducing antibacterial activities previously reported from plant extracts were found to be very low. Generally, ensuring the use of procedures and conditions similar to those implicated in the respective literature did not guarantee similar results. Nevertheless, this study underscored the usefulness of exploring closely related plant species for bioactivities of interest. Based on the prevailing similarities in the underlying biosynthetic genes and phenotypes, this approach can increase the chances of ascertaining plant species hosting activities of interest [18, 19]. For instance, through exploration of the antibacterial activity of *Paeonia officinalis*, activities better than those previously reported in *Paeonia broteroi* were obtained in this study [20].

Differences in phytochemical compositions due to geographical, sampling, climatic and ecological variations are most implicated to cause the discrepancies in bioactivities among plant sources [21-24]. However, the role played by undeclared or faulty plant-related and experimental details highlighted in this study play a curial role as well. Non-reporting of key details like the plant's maturity state, the season of collection, part/tissue collected, and geographical location, highly impairs objectivity during the follow-up studies, hence lowering the chances of reproducing previous results [22-25]. Additionally, to enable better comparison of studied extracts, the practice of determining and reporting the profiles of the studied crude extracts using commonly available techniques like Thin Layer Chromatography (TLC) is highly encouraged.

Furthermore, the widespread use of unidentified bacterial strains together with the limited availability of experimental details were noted as potential factors limiting the reproducibility of previous findings. Due to genetic and phenotypic differences even among bacteria within the same species, proper reporting of the identities of studied bacteria is crucial. Several studies have indicated lesser susceptibilities of clinical isolates and antibiotic-resistant bacteria to tested plant extracts [26-28]. Additionally, variations in the way of conducting assays for MIC determinations represent another dimension of this challenge. Such variations are mostly contributed to the low adherence to the available standard testing methods [29-31]. Addressing these challenges by ensuring the use of well-identified bacterial strains and following reliable testing guidelines are, therefore, viable ways of mitigating the reproducibility challenge.

Solubilization of plants' crude extracts for investigation of their biological activities is a pivotal hurdle. Surrounding this obstacle are other challenges like the use of solvents toxic to bacteria (e.g., methanol and ethanol) or applying solubilizing agents beyond their recommended concentrations, al leading to false-positive results [18, 29, 32]. Findings from this study have underscored the suitability of acetone as an alternative solubilizing agent in cases where other agents like DMSO cannot provide optimum solubilities. Moreover, despite the quantitative and qualitative differences noted in the

working solutions prepared from both solvents, only slight differences were noted in their antibacterial activities. This might be caused by good solubilities of the actual antibacterial compounds within the extracts to both solvents, particularly in cases where some activities were observed. Based on the observed patterns, the use of acetone can generally be beneficial in solubilizing extracts obtained from less polar extractants and in dissolving antibacterial phytochemicals of low to intermediate polarities [18, 33-35].

Isolation and characterization of antibacterial galloylglucoses

Extraction isolation and characterization

This study has highlighted the suitability of bioautography-guided isolation of bioactive compounds from complex mixtures of phytochemicals. Using bioautography allowed the ascertainment of antibacterial fractions and compounds at lesser efforts and shorter durations. Moreover, the use of distinct separation techniques was essential in isolating the rather structurally similar galloylglucoses at good levels of purity [36]. Following their characterization, it was evident that three of the isolated galloylglucoses were reported from *P. officinalis* for the first time. Since the availability of biological data regarding purified and characterized galloylglucoses is limited, the techniques reported here are of great relevance.

In vivo and in vitro antibacterial activities

Besides, this study has demonstrated bacteriostatic potentials of four galloylglucoses against susceptible and MDR strains of *E. coli* and *K. pneumoniae* in both *in vitro* and *in vivo* models. As no antibacterial activities of three of the isolated compounds were previously reported, these findings have added to the known antibacterial galloylglucoses and their spectrum of activity against MDR bacteria [37-39]. Additionally, findings on the good *in vivo* antibacterial profiles based on *Galleria mellonella* larvae encourage further investigations on the therapeutic relevance of these compounds. This is due to the low availability of data on *in vivo* antibacterial and other biological activities of these compounds in higher animals [37, 40].

Reported modes of action of galloylglucoses

Various modes of action have been related to the antibacterial potentials of the known galloylglucoses. Among them are their potentials to bind to macromolecules and metal cations using hydrogen bonds as well as covalent, and ionic or electrostatic interactions [37, 40-42]. Through these interactions, galloylglucoses can inhibit bacterial enzymes, sequestrate vital substrates, increase cell membrane permeability, and inhibit cell wall synthesis [37, 40, 41, 43-48]. Additionally, a wide range of anti-virulence activities including inhibition of biofilm formation, quorum sensing mechanisms, and bacteria motility were reported among galloylglucoses [41].

The antibacterial activities of galloylglucoses are also dependent on the prevailing physicochemical conditions and the type of bacteria. For example, the addition of iron or bovine serum albumin was shown to reverse their antibacterial activities [39, 47].

Besides, bacteria from the genera Bifidobacterium and Lactobacillus do not depend on heme-containing enzymes for their metabolism and were insensitive to iron sequestration induced by galloylglucoses [39, 49]. Moreover, siderophores produced by some bacteria tend to compete with galloylglucoses for iron in the surrounding environment, making siderophore-producing bacteria more susceptible to galloylglucoses [40].

Variation of activities based on the nature of enzymes expressed by the bacteria
Notably, higher antibacterial activities were observed among the MDR strains expressing resistance enzymes with higher contents of aromatic amino acids and/or net negative charges. This observation was closely related to the nature of galloylglucoses to exhibit stronger interactions with proteins hosting higher contents of aromatic amino acids, as well as electrostatically adsorbing to oppositely charged macromolecules [42, 44, 45, 50-53]. Based on these findings, it is further hypothesized that the existence of favourable interactions between galloylglucoses and resistance enzymes yielded higher concentrations of the compounds around the bacterial cells, leading to better activities against the respective strains [42, 44, 45, 51-53].

Structure of galloylglucoses in relation to their antibacterial properties
Structurally the antibacterial activities of galloylglucoses are attributed to the roles of the number and degree of flexibility of the galloyl groups, phenolic oxygen atoms, and aliphatic hydroxyl groups on the core glucose. As stated above, galloyl groups enable the interactions mainly through hydrogen bonding and hydrophobic interactions. An increase in the number of galloyl units was reported to improve galloylglucoses' antimicrobial activity, enzyme inhibition and interactions with macromolecules [44, 52, 54]. Still, the optimum antibacterial activities are implicated to occur within a limited count of galloyl units. For example, antibacterial activities against *Bacillus subtilis* and *Salmonella typhimurium* were optimum in galloylglucoses with 6 – 7 galloyl units, whereas lower activities were observed among those with lesser or more units [41, 55]. The decline in antibacterial activity with an increase in the number of galloyl units was linked to the resulting increases in their molecular weight, steric hindrance, and hydrophobicity [41, 55].

Galloylglucoses with free galloyl units generally bind to a broader range of proteins (based on proteins' flexibilities) as compared to those in which two or more units are covalently joined (ellagitannins). As a result, ellagitannins were reported to have lower antibacterial activities compared to compounds with free/non-joined galloyl groups [37, 41, 42, 46, 53]. The ortho-ortho-related hydroxyl groups on the galloyl units are necessary for the metal binding (complexation and chelation) activity of galloylglucoses. The phenolic oxygen atoms with pairs of non-bonding electrons constitute strong Lewis bases which can interact with metal cations via coordinate bonds and/or surface adsorption. Moreover, the presence of the third hydroxyl group was stated to increase the stability of the resulting complexes or chelates [42, 44, 45]. Further, some galloylglucoses have residual (non-galloylated) aliphatic hydroxyl

groups on the glucose core which can be esterified by carboxylic acid groups on amino acids like glutamic and aspartic acid [42].

Limitations and possible applications

Apart from the reported findings on a range of biological activities of galloylglucoses, efforts to develop them into therapeutic candidates are low. On the one hand, this is due to their limited bioaccessibility and bioavailability while on the other hand, their lack of target selectivity presents safety concerns. Toxicological data on galloylglucoses is generally scarce, partly due to their very limited oral bioavailability. Galloylglucoses and related compounds were found to exhibit antinutritive effects through their interactions with other nutrients in the gastrointestinal tract [56]. Nevertheless, this challenge could be avoided via the application of proper dosage regimens of the compounds with respect to animal feeds [42]. Furthermore, *in vitro* studies have revealed the inhibition of iron-dependent human enzymes like alpha-ketoglutarate-dependent dioxygenase (ALKBH2) by galloylglucoses. Iron chelation rather than competitive inhibition was observed in the prevailing mode of ALKBH2 inhibition [44].

Even so, usages of tannin-rich extracts in the management of different conditions in humans and animals are not uncommon [41]. For example, tannin-rich extracts and commercial tannins generally prevented post-weaning diarrhoea caused by enterotoxigenic induced by *E. coli* in piglets and *Clostridium perfringens*-induced necrotic enteritis in poultry [37, 57]. Looking at other avenues, gallotannins inhibited the growth of all food-borne Gram-positive bacteria and some Gram-negative bacterial species while sparing lactic acid bacteria. This highlights their potential use as food preservatives which can co-exist with starter, proactive or probiotic cultures of lactic acid bacteria [39, 42]. Additionally, the compounds can be used treatment of septic wounds and other topical infections, as well as in the prevention of dental carries, and as biopesticides [41, 58].

Nature-inspired synthesis of glucovanillin derivatives

Deriving inspiration from natural compounds is a promising approach towards synthetically combining diverse natural and synthetic scaffolds in the preparation of novel compounds. Since target-based discovery via synthetic chemical libraries has shown very low success in delivering new antibiotics, tapping on the proven success of natural products is vital [59]. Through the combination of nature-derived scaffolds and synthetic ones, the existing limitations in the individual approaches can be overcome. Along these aspects, this study highlighted the dynamics around the selection and synthetic modifications of a phytochemical to yield various amines, amides, and capsaicin-like derivatives of glucovanillin.

Among the synthesized compounds, antibacterial activities between 128 – 512 µg/mL against *K. pneumoniae*, MRSA and *E. faecium* (VRE) were noted only among the amine derivatives of glucovanillin. Efforts to replace the amine moiety with amides or

capsaicin-line moieties resulted in the loss of activities. These findings resonate with previous observations of the antibacterial activities of vanillin-derived amines [60-62]. Furthermore, the predominance of antibacterial activities among compounds containing halogen atoms (chlorine and fluorine) is related to the role of amphiphilic character induced by these moieties in the respective compounds. Other reports have demonstrated the roles of high amphiphilicity, low globularity and less flexibility, and hosting protonatable amino moieties in promoting the penetration and accumulation of antibacterial compounds within the Gram-negative bacteria [63].

Furthermore, the activities exhibited by compounds **6h** and **8d** against drug-resistant strains of both Gram-Positive and Gram-negative nature highlights their broad-spectrum potential against those problematic strains. Since the two compounds exhibited moderate activities towards strains resistant to many known antibiotics, further efforts to improve their activities and other relevant profiles are encouraged. Taken together, findings from this study advocate further efforts to combine natural and synthetic scaffolds towards the development of novel antibiotics. Such endeavours are valuable in the virtue of their potential to address the limitations of individual approaches while collectively benefiting from their strengths.

Conclusion

Findings from the studies in this thesis have shown the feasibility and usefulness of diverse approaches in the search for new antibacterial compounds against *E. coli* and *K. pneumoniae* from both natural and synthetic sources. From the review of literature, new libraries for plant species as well as phytochemicals with good activities against the two bacteria were assembled. The plant family, parts, nature of the extraction solvents, as well as phytochemicals' classes, lipophilicity, flexibility, and globularity, among others, were noted to influence the reported antibacterial activities in diverse ways. These libraries grant quick access to useful data in equipping researchers in different fields involved in the search for new antibiotics.

High extraction efficiency could be achieved using Pressurized Microwave Assisted Extraction (PMAE) method employing low-temperature conditions over a short duration. The observed benefits under PMAE are linked to higher solubilities under high-pressure conditions, rapturing of plant cells, and unidirectional mass and heat transfers during microwave heating. Using PMAE at low temperatures is, therefore, a promising approach to achieving high yields within a short time while potentially sparing heat-sensitive compounds and avoiding cross-reactions among compounds typical of high extraction temperatures.

Apart from the usefulness of previous reports on the antibacterial potentials of plant extracts, reproducing such findings is an outstanding challenge. The challenge is rooted in the great diversity of methodological approaches, as well as plant- and bacteria-related factors. Collective measures are necessary towards the successful

addressing of such challenges. In that respect, possible solutions were suggested and appraised.

Furthermore, adopting a mixture of extractive and chromatographic methods along with contact bioautography is essential towards the successful isolation and purification of hard-to-purify phytochemicals mixtures in crude extracts. In that, contact bioautography can potentially reduce the time needed to repeatedly test multiple (sub)fractions using more laborious assays. Moreover, this study showed the high to moderate *in vitro* and *in vivo* activities of galloylglucoses against susceptible and MDR strains of *E. coli* and *K. pneumoniae*. Interestingly, the nature of resistance enzymes expressed by the MDR bacteria influenced their ultimate susceptibilities to galloylglucoses. Despite being limited by pharmacokinetic challenges, galloylglucoses can find uses in agriculture and food industries, in the treatment of infected wounds and other topical infections, as well as in the prevention of dental carries, and as biopesticides.

Besides, nature-inspired synthesis of antibacterial agents was indicated as a promising approach to capitalize on a broad array of novel scaffolds presented by nature. This way, it was possible to synthesize and evaluate the antibacterial activities of several derivatives of glucovanillin. The expansion of the chemical space around the selected natural scaffold by synthetic means is therefore an essential link to the extensive exploration of antibacterial activities around natural products. Taking note of the current lack of any new antibiotic from the target-based screening of synthetic chemical libraries, re-directing the focus to natural compounds' libraries is a path worthy of following.

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Chapter VI

Summary

6. SUMMARY

This thesis aimed at searching for new effective agents against Multidrug-Resistant Enterobacteriaceae. This is necessitated by the urgent need for new and innovative antibacterial agents addressing the critical priority pathogens prescribed by the World Health Organization (WHO). Among the available means for antibiotics discovery and development, nature has long remained a proven, innovative, and highly reliable gateway to successful antibacterial agents. Nevertheless, numerous challenges surrounding this valuable source of antibiotics among other drugs are limiting the complete realization of its potential. These include the availability of good quality data on the highly potential natural sources, limitations in methods to prepare and screen crude extracts, bottlenecks in reproducing biological potentials observed in natural sources, as well as hurdles in isolation, purification, and characterization of natural compounds with diverse structural complexities.

Through an extensive review of the literature, it was possible to prepare libraries of plant species and phytochemicals with reported high potentials against *Escherichia coli* and *Klebsiella pneumnoniae*. The libraries were profiled to highlight the existing patterns and relationships between the reported antibacterial activities and studied plants' families and parts, the type of the extracting solvent, as well as phytochemicals' classes, drug-likeness and selected parameters for enhanced accumulation within the Gram-negative bacteria. In addition, motivations, objectives, the role of traditional practices and other crucial experimental aspects in the screening of plant extracts for antibacterial activities were identified and discussed.
Based on the implemented strict inclusion criteria, the created libraries grant speedy access to well-evaluated plant species and phytochemicals with potential antibacterial activities. This way, further studies in yet unexplored directions can be pursued from the indicated or related species and compounds. Moreover, the availability of compound libraries focusing on related bacterial species serves a great role in the ongoing efforts to develop the rules of antibiotics penetrability and accumulation, particularly among Gram-negative bacteria. Here, in addition to hunting for potential scaffolds from such libraries, detailed evaluations of large pool compounds with related antibacterial potential can grant a better understanding of structural features crucial for their penetration and accumulation. Based on the scarcity of compounds with broad structural diversity and activity against Gram-negative bacteria, the creation and updating of such libraries remain a laborious but important undertaking.

A Pressurized Microwave Assisted Extraction (PMAE) method over a short duration and low-temperature conditions was developed and compared to the conventional cold maceration over a prolonged duration. This method aimed at addressing the key challenges associated with conventional extraction methods which require long extraction durations, and use more energy and solvents, in addition to larger quantities of plant materials. Furthermore, the method was intended to replace the

common use of high temperatures in most of the current MAE applications. Interestingly, the yields of 16 of 18 plant samples under PMAE over 30 minutes were found to be within 91–139% of those obtained from the 24h extraction by maceration. Additionally, different levels of selectivity were observed upon an analytical comparison of the extracts obtained from the two methods. Although each method indicated selective extraction of higher quantities or additional types of certain phytochemicals, a slightly larger number of additional compounds were observed under maceration. The use of this method allows efficient extraction of a large number of samples while sparing heat-sensitive compounds and minimizing chances for cross-reactions between phytochemicals.

Moreover, findings from another investigation highlighted the low likelihood of reproducing antibacterial activities previously reported among various plant species, identified the key drivers of poor reproducibility, and proposed possible measures to mitigate the challenge. The majority of extracts showed no activities up to the highest tested concentration of 1024 µg/mL. In the case of identical plant species, some activities were observed only in 15% of the extracts, in which the Minimum Inhibitory Concentrations (MICs) were 4 – 16-fold higher than those in previous reports. Evaluation of related plant species indicated better outcomes, whereby about 18% of the extracts showed activities in a range of 128–512 µg/mL, some of the activities being superior to those previously reported in related species.
Furthermore, solubilizing plant crude extracts during the preparation of test solutions for Antibacterial Susceptibility Testing (AST) assays was outlined as a key challenge. In trying to address this challenge, some studies have used bacteria-toxic solvents or generally unacceptable concentrations of common solubilizing agents. Both approaches are liable to give false positive results. In line with this challenge, this study has underscored the suitability of acetone in the solubilization of crude plant extracts. Using acetone, better solubility profiles of crude plant extracts were observed compared to dimethyl sulfoxide (DMSO) at up to 10 %v/v. Based on lacking toxicity against many bacteria species at up to 25 %v/v, its use in the solubilization of poorly water-soluble extracts, particularly those from less polar solvents is advocated.

In a subsequent study, four galloylglucoses were isolated from the leaves of *Paeonia officinalis* L., whereby the isolation of three of them from this source was reported for the first time. The isolation and characterization of these compounds were driven by the crucial need to continually fill the pre-clinical antibiotics pipeline using all available means. Application of the bioautography-guided isolation and a matrix of extractive, chromatographic, spectroscopic, and spectrometric techniques enabled the isolation of the compounds at high purity levels and the ascertainment of their chemical structures.
Further, the compounds exhibited the Minimum Inhibitory Concentrations (MIC) in a range of 2–256 µg/mL against Multidrug-Resistant (MDR) strains of *E. coli* and *K. pneumonia* exhibiting diverse MDR phenotypes. In that, the antibacterial activities of three of the isolated compounds were reported for the first time. The observed *in vitro* activities of the compounds resonated with their *in vivo* potentials as determined

using the *Galleria mellonella* larvae model. Additionally, the susceptibility of the MDR bacteria to the galloylglucoses was noted to vary depending on the nature of the resistance enzymes expressed by the MDR bacteria. In that, the bacteria expressing enzymes with higher content of aromatic amino acids and zero or positive net charges were generally more susceptible. Following these findings, a plausible hypothesis for the observed patterns was put forward.
The generally challenging pharmacokinetic properties of galloylglucoses limit their further development into therapeutic agents. However, the compounds can replace or reduce the use of antibiotics in livestock keeping as well as in the treatment of septic wounds and topical or oral cavity infections, among other potential uses.

Using nature-inspired approaches, a series of glucovanillin derivatives were prepared following feasible synthetic pathways which in most cases ensured good yields and high purity levels. Some of the prepared compounds showed MIC values in a range of 128 – 512 µg/mL against susceptible and MDR strains of *Klebsiella pneumoniae*, Methicillin-Resistant *Staphylococcus aureus* (MRSA) and Vancomycin-Resistant *Enterococcus faecium* (VRE). These findings emphasize the previously reported essence of small molecular size, the presence of protonatable amino groups and halogen atoms, as well as an amphiphilic character, as crucial features for potential antibacterial agents.
Due to the experienced limited success in the search for new antibacterial agents using purely synthetic means, pursuing semi-synthetic approaches as employed in this study are highly encouraged. This way, it is possible to explore broader chemical spaces around natural scaffolds while addressing their inherent limitations such as solubility, toxicity, and poor pharmacokinetic profiles.

7. ZUSAMMENFASSUNG

Ziel dieser Arbeit war die Suche nach neuen wirksamen Antiinfektiva gegen multiresistente Enterobacteriaceae. Grund dafür ist der dringende Bedarf an neuen und innovativen antibakteriellen Wirkstoffen gegen die von der Weltgesundheitsorganisation (WHO) als vorrangig eingestuften Krankheitserreger. Unter den verfügbaren Methoden zur Entdeckung und Entwicklung von Antibiotika ist die Natur seit langem ein bewährtes, innovatives und äußerst zuverlässiges Mittel, um erfolgreich zu antibakteriellen Wirkstoffen zu gelangen. Dennoch stehen dieser wertvollen Quelle von Antibiotika und anderen Arzneimitteln zahlreiche Herausforderungen gegenüber, die die vollständige Ausschöpfung ihres Potenzials einschränken. Dazu gehören die Verfügbarkeit qualitativ hochwertiger Daten über die hochpotenten natürlichen Quellen, Einschränkungen bei den Methoden zur Herstellung und zum Screening von Rohextrakten, Engpässe bei der Reproduktion des in natürlichen Quellen beobachteten biologischen Potenzials sowie Hürden bei der Isolierung, Reinigung und Charakterisierung von Naturstoffen mit unterschiedlicher struktureller Komplexität.

Mittels einer umfassenden Durchsicht der Literatur war es möglich, Bibliotheken mit Pflanzenarten und Phytochemikalien zu erstellen, die ein hohes Potenzial gegen *Escherichia coli* und *Klebsiella pneumnonia* aufweisen. Die Bibliotheken wurden profiliert, um die bestehenden Muster und Beziehungen zwischen den berichteten antibakteriellen Aktivitäten und den untersuchten Pflanzenfamilien und -teilen, der Art des Extraktionslösungsmittels sowie den Klassen der Phytochemikalien, der Wirkstoffähnlichkeit und ausgewählten Parametern für eine verstärkte Akkumulation in den gramnegativen Bakterien aufzuzeigen. Darüber hinaus wurden Motivationen, Ziele, die Rolle traditioneller Methoden und andere wichtige experimentelle Aspekte beim Screening von Pflanzenextrakten auf antibakterielle Aktivitäten identifiziert und diskutiert.
Auf der Grundlage der strengen Aufnahmekriterien bieten die erstellten Bibliotheken einen schnellen Zugang zu gut bewerteten Pflanzenarten und Phytochemikalien mit potenziellen antibakteriellen Aktivitäten. Auf diese Weise können weitere Studien in noch unerforschten Richtungen mit den angegebenen oder ähnlichen Arten und Verbindungen durchgeführt werden. Darüber hinaus spielt die Verfügbarkeit von Substanzbibliotheken, die sich auf verwandte Bakterienarten konzentrieren, eine große Rolle bei den laufenden Bemühungen, die Regeln für die Penetration und Akkumulation von Antibiotika zu entwickeln, insbesondere bei gramnegativen Bakterien. Neben der Suche nach potenziellen Molekülgerüsten aus solchen Bibliotheken können detaillierte Bewertungen großer Pools von Verbindungen mit antibakteriellem Potenzial ein besseres Verständnis der strukturellen Merkmale ermöglichen, die für ihre Penetration und Akkumulation entscheidend sind. Da es kaum Verbindungen mit breiter struktureller Vielfalt und Aktivität gegen gramnegative Bakterien gibt, ist die Erstellung und Aktualisierung solcher Bibliotheken nach wie vor ein mühsames, aber wichtiges Unterfangen.

Es wurde eine schnelle mikrowellenunterstützte Extraktionsmethode unter Druck (PMAE) und bei niedrigen Temperaturen entwickelt und mit der herkömmlichen Kaltmazeration mit längerer andauernd verglichen. Mit der PMAE-Methode sollten die wichtigsten Probleme herkömmlicher Extraktionsmethoden gelöst werden, die eine lange Extraktionsdauer erfordern, mehr Energie und Lösungsmittel verbrauchen und zudem größere Mengen an Pflanzenmaterial benötigen. Darüber hinaus sollte die Methode die übliche Verwendung hoher Temperaturen in den meisten der derzeitigen MAE-Anwendungen ersetzen. Interessanterweise lag die Ausbeute von 16 der 18 Pflanzenproben bei der 30-minütigen PMAE zwischen 91 und 139 % der jenigen, die bei der 24-stündigen Extraktion durch Mazeration erzielt wurde. Darüber hinaus wurden bei einem analytischen Vergleich der mit den beiden Methoden gewonnenen Extrakte unterschiedliche Selektivitätsgrade festgestellt. Obwohl jede Methode eine selektive Extraktion größere Mengen oder zusätzlicher Arten bestimmter Phytochemikalien anzeigte, wurde bei der Mazeration eine etwas größere Anzahl an Verbindungen beobachtet. Die Anwendung dieser PMAE-Methode ermöglicht eine effiziente Extraktion einer großen Anzahl von Proben, wobei hitzeempfindliche Verbindungen geschont werden und die Wahrscheinlichkeit von Kreuzreaktionen zwischen Phytochemikalien minimiert wird.

Die weitere Untersuchung von Pflanzenextraktionen haben die geringe Reproduzierbarkeit von antibakteriellen Aktivitäten, die zuvor für verschiedene Pflanzenarten berichtet wurden, aufgedeckt, die Hauptursachen für die schlechte Reproduzierbarkeit identifiziert und mögliche Maßnahmen zur Minimierung dieser Herausforderung vorgeschlagen. Die Mehrheit der Extrakte zeigte bis zur höchsten getesteten Konzentration von 1024 µg/ml keine Aktivitäten. Bei identischen Pflanzenarten wurden nur bei 15 % der Extrakte gewisse Aktivitäten beobachtet, wobei die minimalen Hemmkonzentrationen (MHK) um das Vier- bis 16-fache höher waren als in früheren Berichten. Die Auswertung verwandter Pflanzenarten zeigte geringfügig bessere Ergebnisse, wobei etwa lagen 18 % der Extrakte Aktivitäten in einem Bereich von 128-512 µg/ml aufwiesen; dabei einige der Aktivitäten über denen, die zuvor bei verwandten Arten berichtet wurden.
Darüber hinaus wurde die Löslichkeit von Pflanzenrohextrakten bei der Herstellung von Testlösungen für die Bestimmung der Antimikrobischen Suszeptibilität (AST) als eine der größten Herausforderungen bezeichnet. Bei dem Versuch, diese Herausforderung zu bewältigen, wurden in einigen Studien bakterientoxische Lösungsmittel oder allgemein inakzeptable Konzentrationen gängiger Lösungsvermittler verwendet. Beide Ansätze können zu falsch-positiven Ergebnissen führen. Deshalb hat diese Studie die Eignung von Aceton für die Solubilisierung von Pflanzenrohextrakten unterstrichen. Bei Verwendung von Aceton wurden eine bessere Löslichkeit der Pflanzenrohextrakten im Vergleich zu Dimethylsulfoxid (DMSO) bei bis zu 10 % v/v beobachtet. Aufgrund der fehlenden Toxizität gegen viele Bakterienarten bei bis zu 25 % v/v wird die Verwendung von Aceton für die Solubilisierung schwer wasserlöslicher Extrakte, insbesondere solcher aus weniger polaren Lösungsmitteln, befürwortet.

In der nachfolgenden Untersuchung wurden vier Galloylglucosen aus den Blättern von *Paeonia officinalis* L. isoliert, wobei von drei Substanzen aus dieser Quelle zum ersten Mal berichtet wurde. Die Isolierung und Charakterisierung dieser Verbindungen wurden durch die dringende Notwendigkeit vorangetrieben, die präklinische Antibiotika-Pipeline mit allen verfügbaren Methoden zu füllen. Die Anwendung der bioautographisch gesteuerten Isolierung und einer Matrix aus extraktiven, chromatographischen, spektroskopischen und spektrometrischen Techniken ermöglichte die Isolierung der Verbindungen mit hohem Reinheitsgrad und die Bestimmung ihrer chemischen Strukturen.
Darüber hinaus wiesen die Verbindungen minimale Hemmkonzentrationen (MHK) in einem Bereich von 2-256 µg/ml gegen multiresistente (MDR) Stämme von *E. coli* und *K. pneumonia* auf, die verschiedene MDR-Phänotypen aufweisen. Über die antibakteriellen Aktivitäten von drei der isolierten Verbindungen wurde zum ersten Mal berichtet. Die beobachteten *In-vitro*-Aktivitäten der Verbindungen stimmten mit ihren *In-vivo*-Potenzialen überein, die anhand des *Galleria mellonella*-Larvenmodells ermittelt wurden. Darüber hinaus wurde festgestellt, dass die Empfindlichkeit der MDR-Bakterien gegenüber den Galloylglucosen von der Art der von den MDR-Bakterien exprimierten Resistenzenzyme abhängt. So waren die Bakterien, die Enzyme mit einem höheren Gehalt an aromatischen Aminosäuren und null oder positiven Nettoladungen exprimieren, im Allgemeinen anfälliger. Nach diesen Erkenntnissen wurde eine plausible Hypothese für die beobachteten Muster aufgestellt.
Die allgemein schwierigen pharmakokinetischen Eigenschaften von Galloylglucosen schränken ihre weitere Entwicklung als therapeutischen Wirkstoffen ein. Die Verbindungen können jedoch den Einsatz von Antibiotika in der Tierhaltung sowie bei der Behandlung von septischen Wunden und Infektionen der Haut oder der Mundhöhle ersetzen oder reduzieren, neben anderen potenziellen Anwendungen.

Mit von der Natur inspirierten Ansätzen wurde eine Reihe von Glucovanillin-Derivaten synthetisch hergestellt. Einige der neuen Verbindungen wiesen MHK-Werte im Bereich von 128 - 512 µg/ml gegen empfindliche und MDR-Stämme von *Klebsiella pneumoniae*, Methicillin-resistentem *Staphylococcus aureus* (MRSA) und Vancomycin-resistentem *Enterococcus faecium* (VRE) auf. Diese Ergebnisse unterstreichen die bereits früher berichtete Bedeutung einer kleinen Molekülgröße, des Vorhandenseins protonierbarer Aminogruppen und Halogenatome sowie eines amphiphilen Charakters als entscheidende Merkmale für potenzielle antibakterielle Wirkstoffe.
Da die Suche nach neuen antibakteriellen Wirkstoffen mit rein synthetischen Mitteln bisher nur begrenzt erfolgreich war, sind halbsynthetische Ansätze, wie sie in dieser Studie verwendet wurden, sehr zu empfehlen. Auf diese Weise ist es möglich, größere chemische Räume um natürliche Molekülgerüste herum zu erforschen und gleichzeitig deren inhärente Einschränkungen wie Löslichkeit, Toxizität und schlechte pharmakokinetische Profile zu überwinden.

Appendix

8. APPENDIX

8.1. LIST OF PUBLICATIONS

Review Paper

- Masota N.E., Vogg G., Ohlsen K., Meinel L. and Holzgrabe U. Searching for new agents against Enterobacteriaceae from nature: Approaches, potential plant species, isolated compounds, and their respective properties. (Submitted to Phytochemistry Reviews).

Research Papers

- Masota N.E., Vogg G., Heller E. and Holzgrabe U. Comparison of extraction efficiency and selectivity between low-temperature pressurized microwave-assisted extraction and prolonged maceration. Archiv der Pharmazie, 2020; **353**, p.2000147.

- Masota N.E., Vogg G., Ohlsen K. and Holzgrabe U. Reproducibility challenges in the search for antibacterial compounds from nature. PLos ONE, 2021; **16**, p.e0255437.

- Masota N.E., Ohlsen K., Schollmayer C., Meinel L. and Holzgrabe U. Isolation and characterization of galloylglucoses effective against Multidrug-resistant Strains of *Escherichia coli* and *Klebsiella pneumoniae*. Molecules, 2022; **27**, 5045.

- Masota N.E., Ohlsen K., Meinel L., and Holzgrabe U. Nature-inspired synthesis of antibacterial glucovanillin derivatives. (Submitted to Archiv der Pharmazie).

Other publications

- Saedtler M., Förtig N., Ohlsen K., Faber F., Masota N., Kowalick K., Holzgrabe U., and Meinel L. (2020) Antibacterial Anacardic Acid Derivatives. ACS Infectious Diseases 2020; **6**, 1674–1685.

8.2. CONFERENCE CONTRIBUTIONS

- Masota N.E., Ohlsen K., Schollmayer C., Meinel L. and Holzgrabe U. Hexagallolyglucoses effective against Multidrug-Resistant strains of *Escherichia coli* and *Klebsiella pneumoniae*. Poster presentation, Frontiers in Medicinal Chemistry, March 2022, Freiburg, Germany (Online).

- Masota N.E., Vogg, G., Ohlsen K., and Holzgrabe U., Challenges in reproducing antibacterial activities against *E. coli* and *K. pneumoniae* previously reported from natural sources. Oral presentation, Eureka! Symposium, October 2021, Würzburg, Germany (Online).

8.3. DOCUMENTATION OF AUTHORSHIP

8.3.1. Statement of individual author contributions and of legal second publication rights to manuscripts included in the dissertation

Manuscript 1 (Review)

Masota N.E., Zehe M, Vogg G., Ohlsen K., Meinel L. and Holzgrabe U. Searching for new agents against Enterobacteriaceae from nature: Approaches, potential plant species, isolated compounds, and their respective properties. (*Manuscript submitted to Phytochemistry Reviews*).

Participated in	**Author Initials,** Responsibility decreasing from left to right				
Study Design Methods Development	NEM, UH NEM	KO, LM GV			
Data Collection	NEM	GV			
Data Analysis and Interpretation	NEM, GV	UH	KO, LM, MZ		
Manuscript Writing Writing of Introduction Writing of Materials & Methods Writing of Discussion Writing of First Draft	 NEM NEM NEM NEM	 UH	 KO, LM		

Explanations: Conceptualization: NEM and UH; Methodology: NEM, GV and UH; Software: NEM, MZ; Validation: GV, EH and UH; Formal analysis: NEM; Resources: GV, KO, LM and UH; Data curation: NEM and UH; Visualization: NEM and UH; Writing—original draft preparation: NEM and UH; Writing—review and editing: KO, LM, and UH; Supervision of NEM: KO, LM and UH.

Manuscript 2

Masota N.E., Vogg G., Heller E. and Holzgrabe U. Comparison of extraction efficiency and selectivity between low-temperature pressurized microwave-assisted extraction and prolonged maceration. Archiv der Pharmazie, 2020; **353**, 2000147.

Participated in	**Author Initials,** Responsibility decreasing from left to right				
Study Design Methods Development	NEM, UH NEM,	 GV, EH			
Data Collection	NEM	GV			
Data Analysis and Interpretation	NEM	GV, EH, UH			
Manuscript Writing Writing of Introduction Writing of Materials & Methods Writing of Discussion Writing of First Draft	 NEM NEM NEM NEM	 UH			

Explanations: Conceptualization: NEM and UH; Methodology: NEM, GV and EH; Software: NEM; Validation: GV, EH and UH; Formal analysis: NEM; Resources: GV and UH; Data curation: NEM and GV; Visualization: NEM; Writing—original draft preparation: NEM; Writing—review and editing: EH, UH; Supervision of NEM: UH.

Manuscript 3

Masota N.E., Vogg G., Ohlsen K. and Holzgrabe U. Reproducibility challenges in the search for antibacterial compounds from nature. PLos One, 2021; **16**, e0255437.

Participated in	**Author Initials,** Responsibility decreasing from left to right				
Study Design Methods Development	NEM, UH NEM,	KO GV, KO, UH			
Data Collection	NEM	GV, KO			
Data Analysis and Interpretation	NEM	GV, UH			
Manuscript Writing Writing of Introduction Writing of Materials & Methods Writing of Discussion Writing of First Draft	 NEM NEM NEM NEM	 UH, KO			

Explanations: Conceptualization: NEM, KO, and UH; Methodology: NEM, GV and KO; Software: NEM; Validation: KO and UH; Formal analysis: NEM; Resources: GV, KO, and UH; Data curation: NEM and GV; Visualization: NEM; Writing—original draft preparation: NEM; Writing—review and editing: KO and UH; Supervision of NEM: KO and UH.

Manuscript 4

Masota N.E., Ohlsen K., Schollmayer C., Meinel L. and Holzgrabe U. Isolation and characterization of galloylglucoses effective against Multidrug-resistant Strains of *Escherichia coli* and *Klebsiella pneumoniae*. Molecules, 2022; **27**, 5045.

Participated in	**Author Initials,** Responsibility decreasing from left to right				
Study Design Methods Development	NEM, UH NEM	KO, LM CS			
Data Collection	NEM				
Data Analysis and Interpretation	NEM	CS, KO, UH			
Manuscript Writing Writing of Introduction Writing of Materials & Methods Writing of Discussion Writing of First Draft	 NEM NEM NEM NEM	 UH, KO, LM			

Explanations: Conceptualization: NEM, KO, LM, and UH; Methodology: NEM, KO, CS and UH; Software: NEM and CS; Validation: NEM, CS and KO; Formal analysis: NEM; Resources: KO, LM, and UH; Data curation: NEM and CS; Visualization: NEM; Writing—original draft preparation: NEM; Writing—review and editing: KO, LM, and UH; Supervision of NEM: KO, LM, and UH.

Manuscript 5

Masota N.E., Ohlsen K., Meinel L., and Holzgrabe U. Nature-inspired synthesis of antibacterial glucovanillin derivatives. (*Manuscript submitted to Fitoterapia*)

Participated in	**Author Initials,** Responsibility decreasing from left to right				
Study Design Methods Development	NEM, UH NEM	KO, LM UH, KO, LM			
Data Collection	NEM				
Data Analysis and Interpretation	NEM	UH, KO			
Manuscript Writing Writing of Introduction Writing of Materials & Methods Writing of Discussion Writing of First Draft	 NEM NEM NEM NEM	 UH			

Explanations: Conceptualization: NEM, KO, LM, and UH; Methodology: NEM, KO, LM, and UH; Software: NEM; Validation: CS and KO; Formal analysis: NEM; Resources: KO, LM, and UH; Data curation: NEM and UH; Visualization: NEM; Writing—original draft preparation: NEM; Writing—review and editing: KO, LM, and UH; Supervision of NEM: KO, LM, and UH.

The doctoral researcher confirms that she has obtained permission from both the publishers (copyright) and the co-authors for legal second publication.

The doctoral researcher and the primary supervisor confirm the correctness of the above mentioned assessment.

Nelson Enos Masota		Würzburg	
Doctoral Researcher's Name	Date	Place	Signature

Prof. Dr. Ulrike Holzgrabe			
Primary Supervisor's Name	Date	Place	Signature

8.3.2. Statement of individual author contributions to figures/tables of manuscripts included in the dissertation

Introduction					
Figure	**Author Initials,** Responsibility decreasing from left to right				
1	Adopted with permission				
2	Reprinted with permission				
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4	NEM	UH			
Table	**Author Initials,** Responsibility decreasing from left to right				
1	NEM	UH			

Manuscript 1 (Review)

Masota N.E., Zehe M, Vogg G., Ohlsen K., Meinel L. and Holzgrabe U. Searching for new agents against Enterobacteriaceae from nature: Approaches, potential plant species, isolated compounds, and their respective properties. (*Manuscript submitted to Phytochemistry Reviews*).

Figure	**Author Initials,** Responsibility decreasing from left to right				
Graphical abstract	NEM	UH			
1	NEM	UH			
2	NEM	UH			
3	NEM	UH			
4	NEM	UH			
5	NEM	UH			
6	NEM	UH			
7	NEM	UH			
8	NEM	UH			
Table	**Author Initials,** Responsibility decreasing from left to right				
1	NEM	GV, UH, KO			
2	NEM	UH, KO			
3	NEM	UH			

Manuscript 2

Masota N.E., Vogg G., Heller E. and Holzgrabe U. Comparison of extraction efficiency and selectivity between low-temperature pressurized microwave-assisted extraction and prolonged maceration. Archiv der Pharmazie, 2020; **353**, 2000147.

Figure	**Author Initials,** Responsibility decreasing from left to right				
Graphical abstract	NEM	UH			
1	NEM	GV, EH, UH			
2	NEM	UH			
3	NEM	UH			
4	NEM	UH			
5	NEM	UH			
6	NEM	UH			
7	NEM	UH			
8	NEM	UH			
All figures in the Supporting information	NEM	UH			
Table	**Author Initials,** Responsibility decreasing from left to right				
1	NEM	GV, UH			

Manuscript 3

Masota N.E., Vogg G., Ohlsen K. and Holzgrabe U. Reproducibility challenges in the search for antibacterial compounds from nature. PLoS ONE, 2021; **16**, e0255437.

Figure	**Author Initials,** Responsibility decreasing from left to right				
1	NEM	UH			
2	NEM	UH			
3	NEM	UH			
4	NEM	UH, KO			
Table	**Author Initials,** Responsibility decreasing from left to right				
1	NEM	GV, UH			
2	NEM	KO, UH			
3	NEM	KO, UH			
4	NEM	UH			

Manuscript 4

Masota N.E., Ohlsen K., Schollmayer C., Meinel L. and Holzgrabe U. Isolation and characterization of galloylglucoses effective against Multidrug-resistant Strains of *Escherichia coli* and *Klebsiella pneumoniae*. Molecules, 2022; **27**, 5045.

Figure	**Author Initials,** Responsibility decreasing from left to right				
Scheme 1	NEM	KO, UH			
1	NEM	UH			
2	NEM	CS, UH			
3	NEM	KO, UH			
4	NEM	KO, UH			
5	NEM	KO, UH			
6	NEM	KO, UH			
All figures in the Supporting information	NEM	UH			
Table	**Author Initials,** Responsibility decreasing from left to right				
1	NEM	CS, UH			
2	NEM	CS, UH			
3	NEM	KO, UH			
4	NEM	KO, UH			
All tables in the supporting information	NEM	UH			

Manuscript 5

Masota N.E., Ohlsen K., Meinel L., and Holzgrabe U. Nature-inspired synthesis of antibacterial glucovanillin derivatives. (*Manuscript submitted to Fitoterapia*)

Figure	**Author Initials,** Responsibility decreasing from left to right				
1	NEM	UH			
Scheme 1	NEM	UH			
Scheme 2	NEM	UH			
Scheme 3	NEM	UH			
Scheme 4	NEM	UH			
Scheme 5	NEM	UH			
All figures in the supporting information	NEM	UH			
Table	**Author Initials,** Responsibility decreasing from left to right				
1	NEM	UH, KO			
All tables in the supporting information	NEM	UH			

I also confirm my primary supervisor’s acceptance.

Nelson Enos Masota		Würzburg	
Doctoral Researcher’s Name	Date	Place	Signature

8.4. CURRICULUM VITAE

8.5. AFFIDAVIT

Affidavit

I hereby confirm that my thesis entitled “The Search for Novel Effective Agents Against Multidrug-Resistant Enterobacteriaceae” is the result of my own work. I did not receive any help or support from commercial consultants. All sources and/or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in an identical nor in a similar form

_____ _____

Place, Date Signature

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation „Die Suche nach neuen wirksamen Wirkstoffen gegen multiresistente Enterobacteriaceae” eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegeben Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

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Ort, Datum Signature