Université catholique de Louvain
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Identification of natural molecules as antimicrobials or β-lactam resistance modifying agents against *Staphylococcus aureus*

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-2018-

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Thesis submitted to fulfil the requirements for the degree of PhD in Pharmacy and Biomedical Sciences

Aucun de nous, en agissant seul, ne peut atteindre le succès.

– Nelson Mandela

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Aknowledgements

Au terme de cette thèse de doctorat, je tiens à remercier chaleureusement les personnes qui m'ont aidée, soutenue et qui ont contribué de manière directe ou indirecte à la réalisation de ce travail.

En premier lieu, je souhaite remercier le **Professeur Joëlle Quetin-Leclercq**, pour m'avoir accueillie au sein de son laboratoire et m'avoir montré la voie de la recherche dès mon stage d'étudiant chercheur. Je lui suis extrêmement reconnaissante de m'avoir permis de commencer une nouvelle thématique de recherche et de m'avoir accordé la liberté de voyager et d'orienter mes recherches comme je le sentais. Merci pour votre soutien sans faille, votre disponibilité, vos corrections rapides et efficaces et aussi, merci de m'avoir fait confiance pour encadrer les TPs de pharmacognosie.

Ensuite, je souhaite exprimer toute ma reconnaissance au **Professeur Françoise van Bambeke** pour avoir accepté d'être ma co-promotrice ainsi que pour l'accueil au sein de son laboratoire afin de réaliser la partie «microbiologie» de ce travail. Merci pour votre disponibilité et vos précieux conseils tout au long de ces 6 années.

Je tiens également à exprimer toute ma gratitude à l'ensemble des membres de mon jury pour leur suivi et leurs remarques judicieuses et constructives tout au long de ce doctorat. Merci aux membres UCL de mon jury. Le **Professeur Giulio Muccioli**, Président de jury, pour avoir encadré les différents comités ainsi que la défense privée de cette thèse sans m'avoir tenu rigueur des petits soucis associés à mes oublis administratifs… Le **Professeur Marie-Paule Mingeot**, pour son accompagnement depuis le début de mon doctorat, ses conseils bienveillants et sa « positive attitude » à toute épreuve. Merci également pour votre accueil au sein de votre laboratoire pour le stage d'un mois en 3ème bac de pharmacie. C'était à l'époque mon premier contact avec la recherche et votre accessibilité ainsi que les discussions sur votre parcours m'ont donné l'envie de continuer dans cette direction. Je souhaite également remercier le **Professeur Véronique Préat** pour ses conseils, son expertise « galénique » et sa présence depuis le début de ce travail. Merci également aux deux membres extérieurs de mon jury de thèse, les **Professeurs Pierre Duez et Manuel Simões** d'avoir pris le temps de lire ce manuscrit et de se déplacer pour ma défense privée. Un grand merci pour vos remarques et commentaires qui ont contribué à l'amélioration de la qualité de ce document.

Durant cette thèse, j'ai eu l'occasion de collaborer avec de nombreuses personnes qui m'ont permis de progresser dans mes travaux de recherche.

Je tiens à remercier la commission franco-américaine ***Fulbright***, pour l'octroi de la bourse de recherche m'ayant permis de réaliser un séjour de recherche de 6 mois aux Etats-Unis. Et dans ce cadre, je souhaite remercier chaleureusement de nombreuses personnes du **laboratoire du professeur Victor Nizet**, à San Diego :

*First, many thanks to **Victor**, for having welcomed me in his lab. I am so grateful for his availability, listening and useful advice. Thanks to **Sharon**, for her smile, kindness and efficiency in all administrative tasks. Thanks to **Jason**, **Doris** and **Monika** for having shared their bench ideally situated near the snack drawer (on the other hand, my scales do not thank you ☺). I am really grateful to **Josh O**. for all the help during the mice experiments as well as for having taken care of my security through a shuttle service to perform injections at night. Many thanks to **Ross** for having shared his expertise on neutrophils and **Fatemeh** for having taught me how to isolate MVS. I also want to express all my gratitude to **George** for having shared his passion for research, his advice and his staunch optimism. Tanks also to **Jaqueline**, **Tamara, Alison**, **Katy**, **Janet**, **Angelica, Simon, Stéphanie S., Nina, Chris**, **Samira** and **Josh S**. for all the social moments we spent outside the lab. From a rodeo to a treck through a bachelorette party or a zoo visit, my stay was really busy. I am really glad to have been a part of the Nizet lab and I won't forget all the moments shared with you. Don't forget to come visit me in Belgium!*

J'ai également eu l'occasion de me rendre au Portugal pour deux semaines intenses de microscopie de fluorescence. Dans ce contexte, j'aimerai remercier chaleureusement le **Professeur Mariana de Pinho** et le **Docteur Nathalie Reichmann**.

*I am really grateful to **Mariana** for having given me the opportunity to join her lab at ITQB Nova, in Portugal for a short stay. Many thanks also to **Nathalie**; for her availability, her optimism and her imagination. Thanks to her, I learned many things in a really short period of time.*

Je remercie également tous les membres de **l'équipe FACM** pour leur accueil, leur bonne humeur et leur aide lors des manips.

Merci à l'**équipe BPBL** pour les moments sociaux agréables et les repas partagés dans notre petite cafet**.** Un merci particulier à **Julien** pour ses visites éclairs (presque) quotidiennes en GNOS, égaillant nos journées.

Merci aussi à tous **les assistants** côtoyés de près ou de loin pendant la thèse, pour m'avoir permis de sortir un peu la tête de la recherche.

Je tiens ensuite à remercier tous **les chercheurs du LDRI** avec qui j'ai partagé ces quelques années. La vie de doctorant n'est pas toujours facile mais la partager avec eux l'a souvent adoucie ! Un merci particulier à **Tamara**, **Ahalieyah**, et **Alice** pour votre amitié et votre soutien durant ces six années. Merci aussi à **Fred**, **Vas**, **Eugénie**, **Hariri**, **Micheline**, **Sandrine**, **Hélène** pour les moments de détente partagés**.**

Un immense MERCI à toute l'équipe GNOS pour les discussions quotidiennes, l'ambiance du labo et également l'aide aux manips.

Tout d'abord, merci aux ex-GNOS que j'ai eu la chance de croiser et plus particulièrement : **Manu** pour m'avoir encadrée lors de mon mémoire et m'avoir transmis le goût de la recherche. **Emilie**, pour ta bonne humeur, ton amitié, ton soutien aussi bien professionnel que privé et tous les bons moments partagés. **Cynthia,** pour ton sourire, ta disponibilité et toute ton aide pour les analyses RMN**. Jojo** pour ton exemple de parcours, ta présence au quotidien pendant une bonne partie de cette thèse☺, tes conseils et tous les moments hors labo.
Merci également aux étudiants, **Ruosi** et **Li** qui ont contribué à une partie de ces recherches.
Ensuite, un grand merci à **Claire** pour avoir partagé mon bureau très ordonné pendant ces 8 années. Tout a commencé quand nous avons décidé de faire un stage d'un mois au Benin. On s'est ensuite laissé tenter par la recherche et par l'assistanat pour 6 ans de vie commune. Ton écoute et ton soutien au quotidien m'ont permis d'y arriver. MERCI ! Many thanks to **Binh** for her smile, kindness and her presence in our office.Merci à toute l'équipe technique, **Marie-Christine**, **Jean-Paul**, **Maude**, **Kelly**, **Lucie**, **Amandine** pour votre aide, votre gentillesse, votre présence et votre soutien au quotidien. Merci à **Marie-France** pour ton aide pour les analyses de masse, tous ces moments partagés au labo mais également tous les vendredis de TPs, merci pour tous ces fous-rires et beaux souvenirs. Finalement, merci **Françoise**, pour ton amitié, ta disponibilité, tes encouragements, ton soutien et ton écoute.

Je souhaite également remercier **mes amis hors thèse** qui ont toujours été là, **ma famille et belle famille** pour leur présence et leurs encouragements pendant toutes ces années.

Finalement, merci **Julien** pour ton écoute, ton soutien au quotidien et ta capacité à me divertir dans tous ces moments de stress. Merci aussi de m'avoir permis de réaliser ces séjours de recherche à l'étranger qui étaient importants à mes yeux même si un peu plus difficiles à accepter pour toi. Merci d'être à mes côtés !

A vous tous qui avez contribué à ce travail, MERCI !

TABLE OF CONTENTS

ABBREVIATION LIST 19

AIM OF THE THESIS 23

INTRODUCTION 27

1. *STAPHYLOCOCCUS AUREUS* INFECTIONS AND TREATMENTS 29

1.1 MAIN CHARACTERISTICS 30
1.1.1 Cell wall composition 30
1.1.2 Cell membrane composition 32
1.1.3 Pathogenesis 33
1.2 ANTI-STAPHYLOCOCCAL AGENTS 35
1.2.1 β-lactam antibiotics 37
1.2.1.1 MODE OF ACTION 41
1.2.1.2 RESISTANCE TO β-LACTAM ANTIBIOTICS 42
1.2.1.2.1 β-lactamases-mediated resistance 43
1.2.1.2.2 PBP2a-mediated resistance 47
MRSA Epidemiology 47
mecA gene 48
Penicillin binding protein 2a 49

2. TRADITIONAL MEDICINE AS A SOURCE OF NEW SOLUTIONS TO FIGHT BACTERIA AND ANTIMICROBIAL RESISTANCE 55

3. PENTACYCLIC TRITERPENES 61

3.1 BIOSYNTHESIS 61
3.1.1 The mevalonate pathway 62
3.1.2 The GAP/pyruvate pathway 62
3.1.3 Head-to-tail and tail-to-tail condensations 64
3.2 MAIN BIOLOGICAL ACTIVITIES 67
3.2.1 Anti-tumor, anti-inflammatory and antioxidant activities 67
3.2.2 Antiparasitic activities 69
3.2.2.1 Antiplasmodial activity 70
3.2.2.2 Antitrypanosomial activity 73
3.2.2.3 Antileishmanial activity 75
3.2.3 Antibacterial activities 77

4.NATURAL AND HEMI-SYNTHETIC PENTACYCLIC TRITERPENES AS ANTIMICROBIALS AND RESISTANCE MODIFYING AGENTS AGAINST *STAPHYLOCOCCUS AUREUS*: A REVIEW 80

4.1 INTRODUCTION 81
4.2 PENTACYCLIC TRITERPENES AS ANTI-STAPHYLOCOCCAL COMPOUNDS 83
4.2.1 In vitro antimicrobial susceptibility testing 83
4.2.2 Pentacyclic triterpenes with anti-staphylococcal activities 84
4.2.2.1 Ursane derivatives 85
4.2.2.2 Oleanane derivatives 95
4.2.2.3 Lupane derivatives 105
4.2.2.4 Discussion 111
4.3 PENTACYCLIC TRITERPENES AS RESISTANCE MODIFYING AGENTS 116
4.3.1 In vitro antimicrobial combinations tests 116

4.3.2 Pentacyclic triterpenes with staphylococcal resistance modifying activities...117
4.3.3 Discussion ...124
4.4 MECHANISM OF ACTION...125
4.5 CONCLUSION ...128

RESULTS – PART 1 ... 131

Preliminary evidences of the direct and indirect antimicrobial activity of 12 plants used in traditional medicine in Africa

1. INTRODUCTION... 134

2. MATERIALS AND METHODS ... 135

2.1 PLANT MATERIAL ...135
2.2 PREPARATION OF CRUDE PLANT EXTRACTS AND TANNINS FREE FRACTIONS ...136
2.3 THIN LAYER CHROMATOGRAPHY (TLC) ANALYSIS ...136
2.4 BACTERIA, CELLS AND MEDIA ...138
2.5 ANTIBIOTICS AND ANTIBIOTIC SUSCEPTIBILITY TESTING ...138
2.6 CYTOTOXICITY ASSAY ...139

3. RESULTS AND DISCUSSION... 140

3.1 *ALLOPHYLUS AFRICANUS* P. BEAUV (SAPINDACEAE) ...147
3.2 *ANCHOMANES DIFFORMIS* (BLUME) ENGL. (ARACEAE) ...147
3.3 *ANTHOCLEISTA SCHWEINFURTHII* (GILG.) (GENTIANACEAE) ...149
3.4 *CASSIA SIEBERIANA* DC (CAESALPINIACEAE)...150
3.5 *CISSAMPELOS MUCRONATA* A RICH. (MENISPERMACEAE) ...150
3.6 *COLA GIGANTEA* A. CHEV. VAR. *GIGANTEA* (STERCULIACEAE) ...151
3.7 *HOLARRHENA FLORIBUNDA* (G. DON) DURAND & SCHINZ (APOCYNACEAE) ...152
3.8 *PROTEA ELLIOTII* C. H. WRIGHT (PROETEACEAE) ...153
3.9 *SCHRANKIA LEPTOCARPA* DC. (MIMOSACEAE) ...154
3.10 *SPIROSPERMUM PENDULIFLORUM* THOUARS (MENISPERMACEAE) ...155
3.11 *TAPINANTHUS BANGWENSIS* (ENGL. & KRAUSE) DANSER (LORANTHACEAE) ...156
3.12 *VITELLARIA PARADOXA* C.F. GAERTN. SSP. *PARADOXA* (SAPOTACEAE)...157

4. CONCLUSION ... 158

5. ACKNOWLEDGEMENTS ... 159

RESULTS – PART 2 ... 161

Isolation and antistaphylococcal activities of major compounds from the leaf dichloromethane extract of Vitellaria paradoxa

1. INTRODUCTION... 164

2. MATERIALS AND METHODS ... 165

2.1 PLANT MATERIAL ...165
2.2 PREPARATION OF EXTRACTS ...165
2.3 CRUDE DCM EXTRACT HPLC-UV-MS/MS ANALYSIS...165

2.4 Isolation of compounds 166
2.5 Bacteria, cells and media 167
2.6 Antimicrobial susceptibility testing....168
2.7 Cytotoxicity assays 168

3. RESULTS AND DISCUSSION 169

3.1 Crude DCM extract HPLC-UV-MS/MS analysis....169
3.2 Isolation and structure identification....173
3.3 *In vitro* antistaphylococcal activity....177

4. CONCLUSIONS 179

RESULTS – PART 3 181

Synergy between UA and OA from Vitellaria paradoxa leaf extract and β-lactams against MRSA: in vitro and in vivo activity and underlying mechanisms

1. INTRODUCTION 184

2. MATERIALS AND METHODS 186

2.1 Plant material 186
2.2 Preparation of extracts 186
2.3 Reference compounds 186
2.4 UA and OA identification 186
2.5 UA and OA quantification....187
2.6 BacteriaL strains....188
2.7 MIC determination....188
2.8 FICI determination 189
2.9 Time-kill Assay....189
2.10 Effect of compounds on PBP2 localization....190
2.11 Effect of compounds on PBP2 and PBP2a expression 190
2.12 Evaluation of β-lactamase activity using the nitrocefin test....191
2.13 Murine model of subcutaneous infection 192
2.14 Statistical Analysis....193

3. RESULTS AND DISCUSSION 194

3.1 Analysis and quantification of major components of the *Vitellaria paradoxa* crude extract 194
3.2 Antimicrobial activity of the VP crude extract major components....195
3.3 Effect of OA and UA on PBP2 recruitment to the septum. 202
3.4 Evaluation of b-lactamase Activity. 203
3.5 Murine Model of Subcutaneous Infection. 207

4. ACKNOWLEDGEMENTS 211

RESULTS – PART 4 213

Antiparasitic activities of major compounds isolated from the leaf dichloromethane extract of Vitellaria paradoxa

1. INTRODUCTION 216

2. MATERIALS AND METHODS 217

2.1 PLANT MATERIAL 217
2.2 PREPARATION OF EXTRACTS AND COMPOUNDS ISOLATION 218
2.3 PARASITES, CELLS AND MEDIA 218
2.4 *IN VITRO* ANTIPARASITIC ACTIVITIES 219
2.5 CYTOTOXICITY ASSAYS 220
2.6 ANIMALS 220
2.7 *IN VIVO* ACUTE TOXICITY TEST 220
2.8 *IN VIVO* ANTITRYPANOSOMAL ACTIVITY 221
2.9 STATISTICAL ANALYSES 221

3. RESULTS AND DISCUSSION 221

3.1 *IN VITRO* ANTIPARASITIC ACTIVITIES 221
3.2 *IN VIVO* ACUTE TOXICITY 227
3.3 *IN VIVO* ANTITRYPANOSOMAL ACTIVITY 228

4. CONCLUSIONS 231

GENERAL DISCUSSION & PERSPECTIVES 233

1. TRADITIONAL USE OF SELECTED PLANT SPECIES 236

1.1 UNDETECTED ACTIVITY 236
1.2 EXTRACTS CONTAINING TANNINS 238

2. RATIONALE TO COMBINE PLANT EXTRACTS WITH COMMON ANTIBIOTICS (E.G. AMPICILLIN AND OXACILLIN) 240

3. INTEREST OF UA OR OA AS B-LACTAMS ADJUVANTS 242

3.1 GENERAL ADVANTAGES AND LIMITATIONS 242
3.1.1 Widespread occurrence 242
3.1.2 Toxicity 243
3.1.3 Bioavailability 244
3.1.4 Multiplicity of biological activities 245
3.2 POTENTIAL IN ANTISTAPHYLOCOCCAL THERAPY 247
3.2.1 Antistaphylococcal target 247
3.2.2 In vitro synergy 251
1.1.1 In vivo synergy 254

4. POTENTIAL OF 3-O-*P-Z/E*-COUMAROYLTORMENTIC ACIDS IN ANTI-TRYPANOSOMAL THERAPY 258

5. SHORT TERM PERSPECTIVES 263
6. CONCLUSION 264
REFERENCES..... 267

ABBREVIATION LIST

α-hla	α-toxin or α-hemolysin
ATCC	American Type Culture Collection
BA	Betulinic acid
CA	Corosolic acid
caMHB	cation-adjusted Mueller-Hinton broth
CA-MRSA	Community-acquired Methicillin-resistant *Staphylococcus aureus*
CFU	Colony forming unit
CLSI	Clinical Laboratory Standards Institute
DCM	Dichloromethane
DMAPP	Dimethylallyl diphosphate
DMSO	Dimethylsulfoxide
EUCAST	EUropean Committee on Antimicrobial Susceptibility Testing
FMM	Functional Membrane Microdomain
FICI	Fractional Inhibitory Concentration Index
GAP	Glyceraldehyde-3-phosphate
GAPDH	Glyceraldehyde-3-phosphate-deshydrogenase
GRA	Glycyrrhetinic acid
GR-SU	Disodium 3-succinyloxy β-glycyrrhetinate
HA-MRSA	Healthcare-acquired Methicillin-resistant *Staphylococcus aureus*
IL	Interleukin
INF-γ	Interferon-γ
IPP	Isopentenyl pyrophosphate
Lmm	*Leishmania mexicana mexicana*
MA	Maslinic acid
MIC	Minimum Inhibitory Concentration
MSSA	Methicillin-sensitive *Staphylococcus aureus*
MSCRAMM	Microbial Surface Components Recognizing Adhesive Matrix Molecules
MRSA	Methicillin-resistant *Staphylococcus aureus*
MTCC	Microbial Type Culture Collection
MVA	Mévalonate
NAG	N-acetylglucosamine
NAM	N-acetylmuramic acid

NBRC	NITE (National Institute of Technology and Evaluation) Biological Resource Center
OA	Oleanolic acid
PBP	Penicillin binding protein
PVL	Panton Valentin leucocidin
Pf	*Plasmodium falciparum*
QS	Quorum sensing
SAR	Structure activity-relationship
RMA	Resistance modifying agents
SCC*mec*	Staphylococcal Cassette Chromosome *mec*
Tbb	*Trypanosoma brucei brucei*
TLC	Thin Layer Chromatography
TSST	Toxic-Shock Syndrome Toxin
UA	Ursolic acid
UDP	Uridine Diphosphate
VLC	Vacuum Liquid Chromatography

AIM OF THE THESIS

Staphylococcus aureus is an aggressive pathogen causing significant public health threats including invasive endocarditis, osteomyelitis or skin and soft tissues infections. β-lactam antibiotics are often considered as the first line of treatment. However, inappropriate use of this class of antibiotics has led to an increasing prevalence of bacterial resistance. The main mechanisms of β-lactams resistance in *S. aureus* consist in (a) the production of specific enzymes inactivating the antibiotics (β-lactamases) and (b) the production of PBP2a, an additional penicillin-binding protein with decreased affinity for methicillin and all conventional β-lactam antibiotics, associated to the so-called Methicillin-Resistant *S. aureus* (MRSA) phenotype. A number of drugs still remain active against MRSA, including glycopeptides (e.g., vancomycin and teicoplanin), linezolid, tigecycline, daptomycin, and even some new beta-lactams, such as ceftaroline and ceftobiprole. However, MRSA has shown outstanding versatility at emerging resistance and spreading in different epidemiological settings over time. This antimicrobial resistance crisis is global, reflecting the worldwide over- and mis-use of these drugs and the lack of development of new antibiotic agents. There is thus an urgent need to develop new and original strategies to fight these bacteria.

Plant extracts constitute potential source of new antibiotics. For many centuries, plants are a key source of drugs and an alternative medicine to fight against diseases. They are still widely used in ethnomedicine all over the world. Moreover, plants rarely produce a single defense mechanism against predation. In contrast, they synthetize a variety of molecules such as quinones, tannins, terpenoids, flavonoids, alkaloids or polyphenols that often act synergistically to produce an enhanced effect against pathogens.

Hence, the aim of this thesis is to search for new natural products that can act as antistaphylococcal agents or as adjuvants restoring the activity of β-lactams against *Staphylococcus aureus* resistant strains.

First of all, we performed a screening of medicinal African plants selected on a bibliographical and ethnopharmacological basis. Using *in vitro* tests, we evaluated their direct (alone) or indirect (in combination with β-lactams) antistaphylococcal activities and further selected the most active extract.

Our next goal was to identify the active constituents of this extract using LC-MS but also NMR analyses of isolated compounds. We then quantified and assessed the *in vitro* activity of its two major compounds, in combination with β-lactams against *S. aureus*. Then, we tested the combination of the most active compound and a β-lactam in a murine model of subcutaneous MRSA infection.

Our last goal was to valorize some isolated compounds found as inactive or with low activity against *S. aureus*. Thanks to the expertise of our lab in the search of new antiparasitic compounds (antimalarial, antitrypanonomial and antileishmanial) we knew that similar pharmacophores had previously shown interesting antiparasitic activities. We thus studied the *in vitro* antiparasitic activities of all isolated compounds as well as in an *in vivo* model for the most promising compound.

INTRODUCTION

This introduction is divided into four parts:

1. *STAPHYLOCOCCUS AUREUS* INFECTIONS AND TREATMENTS……21

2. TRADITIONAL MEDICINE AS A SOURCE OF NEW SOLUTIONS TO FIGHT BACTERIA AND ANTIMICROBIAL RESISTANCE 43

3. PENTACYCLIC TRITERPENES 49

4.NATURAL AND HEMI-SYNTHETIC PENTACYCLIC TRITERPENES AS ANTIMICROBIALS AND RESISTANCE MODIFYING AGENTS AGAINST *STAPHYLOCOCCUS AUREUS*: A REVIEW 68

1. *Staphylococcus aureus* infections and treatments

The term *Staphylococcus,* from the Greek expression *staphylé* (= bunch of grapes) was originally introduced by Sir Alexander Ogston to describe grape-like clusters of bacteria responsible for inflammation and suppuration [1]. Staphylococci are gram-positive cocci (0.5 to 1.5 µm in diameter), non-motile, catalase-positive and facultative anaerobe. The genus *Staphylococcus* contains more than 30 species but only a few are pathogenic for humans [2]. *S. epidermidis*, for example, is responsible for device-related infections like prosthetic valve endocarditis while *S. saprophyticus* is considered as the second most frequent cause of acute cystitis or pyelonephritis in young, sexually active females [3]. However, the most pathogenic *Staphylococcus* in humans and animals is undeniably the coagulase-positive and yellow-pigmented *Staphylococcus aureus*.

Figure 1- 1. *Staphylococcus aureus colonies on nutrient agar*



S. aureus is usually found in the nose or on the skin of approximately 30% of the human population without causing any damage to his host. Nonetheless, this microorganism can also induce relatively mild skin infections which can evolve to life-threatening, invasive diseases (**Table 1-1**) [4–6].

***Table 1- 1**. Clinical manifestations associated with S. aureus infections (adapted from* [7]*)*

Source of infection	Disease
Skin and soft tissues	Impetigo, abscesses, cellulitis, folliculitis, surgical and traumatic wound infections
Bone and joints	Arthritis, osteomyelitis
Respiratory tract	Lung abscess, pneumonia, otitis, sinusitis…
Heart	Endocarditis, pericarditis
Central nervous system	Meningitis, brain abscess
Blood stream	Bacteremia, sepsis
Toxin-mediated disease	Food poisoning, scaled skin syndrome, staphylococcal toxic shock

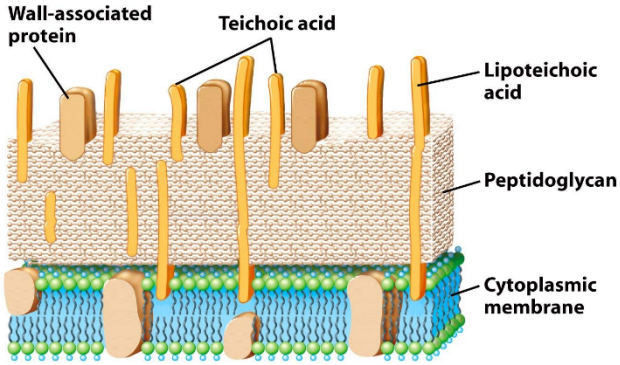
1.1 Main characteristics

S. aureus genome consists of a single circular chromosome of around 2.8 Mb predicted to encode approximately 2600 genes, coupled with an assortment of extrachromosomal accessory genetic elements: conjugative and non-conjugative plasmids, prophages as well as mobile elements (transposons, insertion sequences,…) [8]. About 78% of the genes are conserved between strains and constitute the 'core genome'. The entire 'core genome' is divided into stable core and core variable genomes [9]. While the first is mainly composed of genes required for growth or survival, the second mainly encodes virulence factors, ranging from cell wall components such as the capsular polysaccharide or peptidoglycan, to secreted proteins such as exotoxins [9]. Thanks to genetic mobile elements, combination of mutations, recombinations and gene transfers, this pathogen possesses the ability to acquire potentially useful genes facilitating the evolution of virulent and drug-resistant strains [10].

1.1.1 Cell wall composition

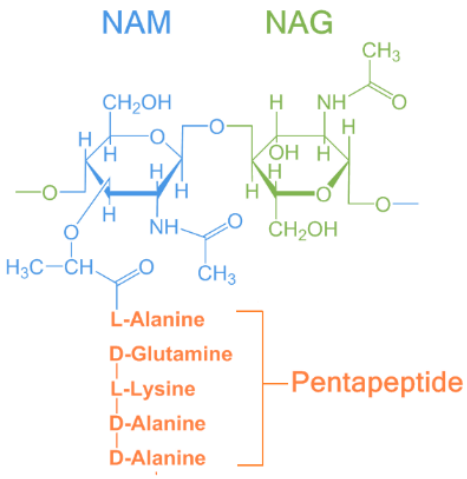
The bacterial cell wall is a strong protective coat conferring the characteristic shape of bacteria. It appears as an amorphous layer, 20-50 nm thick, external to the cytoplasmic membrane (**figure 1-2**) [11].

***Figure 1- 2** : Staphylococcus aureus cell wall [12]*



The major component of the cell wall is the peptidoglycan, which represents up to 50% of the cell wall mass [13]. It is constructed of repetitions of polysaccharide subunits of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM), together with pentapeptide side chains linked to the NAM (**figure 1-3**). Extension and reticulation of the peptidoglycan require the activity of transpeptidases and transglycosidases [14].

***Figure 1- 3** Peptidoglycan monomer*



Another important cell wall constituent, representing up to 40% of the cell wall mass, is a group of phosphate-containing polymers called teichoic acids. These polymers are either covalently bound to peptidoglycan (cell wall teichoic acids) or inserted in the lipids of the bacterial cell membrane (membrane teichoic acids) [14]. Teichoic acid, conferring a negative net charge to the staphylococcal cell surface, is described to be an essential factor for *S. aureus* nasal colonization [15].

The rest of the cell wall consists in surface proteins, exoproteins and peptidoglycan hydrolases (autolysins). Some of these components are involved in attaching the bacteria to surfaces and are virulence determinants (eg. protein A or fibronectin-binding proteins) [16,17]. The cell wall encases the entire bacterium as a single molecule and its integrity is indispensable to the survival of the organism [18].

1.1.2 Cell membrane composition

The main lipid constituents of bacterial membranes are phospholipids whose composition and relative abundance greatly vary between species and may be different under various environmental conditions and growth phases [19,20]. The two major constituents of Gram positive membranes are phosphatidylglycerol (PG) and cardiolipin (CL), two negatively charged phospholipids imparting anionic properties to the membrane surface [20–22]. *S. aureus* can also produce lysyl-phosphatidylglycerol (L-PG), a cationic lipid, which leads to a partial neutralization of the membrane surface [20,23] Conversely, Gram negative bacteria membranes are mainly rich in phosphatidylethanolamine (PE), a zwitterionic lipid [21].
How phospholipid synthesis is regulated in staphylococci is only superficially understood, but Kuhn and collaborators recently published a review about synthesis, regulation and function of the major phospholipids in *S. aureus* [24]

In addition to phospholipids, *S. aureus* membrane also contains cyclic isoprenoids (hopanoids, analogues of cholesterol) and noncyclic isoprenoids (carotenoids) [25] Of note, *S. aureus* synthesizes staphyloxanthin, a golden-colored membrane-bound carotenoid responsible for its typical yellow coloration [26]

Beside its composition, the cell membrane organization plays a key role for the correct functionality of diverse cellular processes [25]. Indeed, it is well known that membranes of eukaryotic cells organize signal transduction proteins into membrane rafts that are enriched in particular lipids, necessary to maintain cell integrity. While the existence of such membrane domains has been exclusively attributed to eukaryotic cells for a long time, it has been recently discovered that bacteria organize many signal transduction, protein secretion and transport processes in functional membrane microdomains (FMM) which are equivalent to the lipid rafts of eukaryotic cells (see [25] for review).

FMM formation in bacteria involves the biosynthetis and aggragation of isoprenoid membrane lipids and their colocalization with flotillin homolog proteins. Flotillin proteins are membrane bound chaperones that localize in FMM where they may recruit the proteins that need to be localized in domains to be active, and facilitate their interaction and oligomerization. It has recently been shown that *S. aureus* expresses a single flotillin, FloA and that the constitutive lipids of *S. aureus* FMM are staphyloxanthin derivatives [27]

1.1.3 Pathogenesis

In order to colonize host tissues and evade host immune response, *S. aureus* synthesizes an extraordinary repertoire of toxins and virulence factors (**Table 1-2**). At the first stage of infection, *S. aureus* adheres and invades host cells thanks to surface-bound adhesins that recognize host surface structures. These molecules

are collectively termed Microbial Surface Components Recognizing Adhesive Matrix Molecules or MSCRAMM. This colonization is then followed by further growth of the bacteria and secretion of enzymes and toxins such as hemolytic toxins (α-, β-, and γ-toxins), leucotoxins, staphylococcal enterotoxins, epidermolytic toxins, several proteases [28–30]. Although in most cases infections remain localized, once *S. aureus* enters the blood, it is one of the most human lethal pathogens due to its ability to cause difficult-to-treat endovascular infections, pericarditis, pneumonia, meningitis, lung abscesses…[7]

***Table 1- 2** : Some S. aureus virulence factors (adapted from [28,29])*

Virulence factors and toxins	**Function**
Teichoic acid, adhesins, fribronectin-binding proteins	Adhesion to host cells
α-, δ-, γ-hemolysins, Panton-Valentine leukocidin	Lysis of host cells
Protein A, clumping factor A, capsular polysaccharide	Inhibition of phagocytosis
Carotenoid pigment, staphyloxanthin, catalase, hydroperoxide reductase	Survival in phagocytes
Staphylokinase, aureolysin, clumping factor A	Inhibition/modulation of host immune response
Toxic Shock Syndrome Toxin 1 (TSST-1), enterotoxins (A-E), exfoliative toxins	Contribution to symptoms of septic shock

Another key component of the *S. aureus* pathogenesis consists in its ability to form biofilms. Indeed, it is known that *S. aureus* preferentially attaches to various surfaces using a self-produced extracellular polymeric matrix, which forms a biofilm [31]. Representing a reservoir for continuing bacterial dissemination within the body, staphylococcal biofilms are considered as the main reason for persistence and/or recurrence of infections like endocarditis, osteomyelitis… [32]. Due to their inherent resistance to antimicrobial agents and immune defenses, biofilms are difficult to eradicate and play thus a critical role in antibiotic resistance [33,34].

The secretion of virulence factors or the production of biofilms are highly regulated by the quorum sensing (QS), a bacterial cell-cell communication process used by many bacteria to detect their critical cell density by producing and perceiving

diffusible signal molecules in order to coordinate a common behavior [35]. *S. aureus* uses a two component QS system encoded by the *agr* locus [35].

1.2 Anti-staphylococcal agents

The discovery of antibiotics, marked by the introduction of penicillin in the 1940s, reduced the high mortality rate associated with *S. aureus* bacteremia. However, the over- and mis- use of antibiotics combined with the lack of new drug development enhances an antibiotic resistance crisis and the lack of improvement in patient outcomes [5].

Antibiotics used as antistaphylococcal agents are directed against five main targets, namely the synthesis of cell wall, DNA, RNA, proteins, or folic acid. Table (**1-3**) summarizes the targets and mode of action of the most used antibiotic classes against *S. aureus* strains as well as the resistance mechanisms developed by the bacteria against them.

***Table 1- 3** : Targets, mode of action and main resistance mechanisms of common antibiotics*

Target	Class	Mode of action	Main Resistance Mechanisms in *S. aureus*
Cell-wall	**β-lactams**	Inhibiting transpeptidases by irreversible binding to the active site of these enzymes	**Drug inactivation** : β-lactamases **Target modification** : PBP2a with reduced affinity
	Glycopeptides	Inhibiting transpeptidases and transglycosylases by binding with high affinity to the D-Ala-D-Ala precursor	**Target modification** : thickening of the cell wall **Target elimination** : synthesis of a precursor D-Ala-D-Lac instead of D-Ala-D-Ala
	Lipoglycopeptides	Same as glycopeptides + destabilizing cell membrane	Same as glycopeptides but less affected thanks to their dual mode of action
	Lipopeptides	Damaging cell membrane	**Target modification** : phenotypic changes in cell membrane
DNA synthesis	**Quinolones**	Inhibiting DNA gyrase and topoisomerase IV	**Drug efflux:** NorA, NorB, NorC **Target mutations**

RNA synthesis	**Ansamycins**	Inhibiting RNA polymerase	**Target mutations**
Proteins synthesis	**Macrolides**	Binding 50S ribosomal subunit	**Drug efflux** **Target modification:** ribosome methylation **Drug inactivation** : esterases
	Lincosamides		**Target modification**: ribosome methylation
	Streptogramins		**Drug efflux** **Target modification**: ribosome methylation **Drug inactivation** : hydrolases, acetyl-transferases
	Chloramphenicol		**Drug inactivation** : acetyl-transferases
	Oxazolidinones		**Target modification**: ribosome methylation or mutations
	Fusidanes		**Target modification**
	Tetracyclines	Binding 30S ribosomal subunit	**Drug efflux** **Target modification**
	Aminoglycosides		**Drug inactivation:** acetyltransferases, nucleotidyltransferases, phosphotransferases
Folic acid synthesis	**Trimethoprim**	Inhibiting dihydrofolate reductase and tetrahydrofolic acid synthesis	**Target modification**
	Sulfonamides	Inhibiting dihydropteroate synthase and folate biosynthesis	**Target modification**

Although several guidelines have been published in order to improve the management of *S. aureus* infections [5,36–39], choosing an appropriate anti-staphylococcal agent remains difficult given the ever-changing resistance pattern of this organism.

Today, β-lactams, and more precisely penicillinase-stable penicillins (nafcillin, oxacillin) or cephalosporins (cefazolin, cephalexin), are still considered as a first-line of treatment of Methicillin-Susceptible *S. aureus* (MSSA) infections. On the other hand, the first therapeutic option for Methicillin-Resistant *S. aureus* (MRSA) is vancomycin followed by linezolid and daptomycin [40] (**table 1-4**).

***Table 1- 4** : Antibiotics recommended to treat S.aureus infections (adapted from [40])*

Pathogen	Antibiotics
MSSA	**First regimens** ***β-lactams*** Penicillinase-stable penicillins : nafcillin, oxacillin, dicloxacillin Cephalosporins : cefazolin, cephalexin **Alternative regimens** ***Glycopeptides :*** vancomycin ***Lipopeptides :*** daptomycin ***Oxazolidinone :*** linezolid ***Lincosamides :*** clindamycin ***Trimethoprim plus sulfonamide :*** trimethoprim/sulfamethoxazole
MRSA	**First regimens** ***Glycopeptides :*** vancomycin ***Oxazolidinones :*** linezolid ***Lipopeptides :*** daptomycin ***Lipoglycopeptides :*** telavancin ***Broad spectrum cephalosporins :*** ceftaroline **Alternative regimens** ***Glycopeptides :*** teicoplanin Sometimes in conjunction with : **Ansamycin** (rifampicin) **Fusidane** (fusidic acid) ***Tetracyclines :*** minocycline, doxycycline ***Lincosamides :*** clindamycin ***Streptogramins :*** quinupristin/dalfopristin ***Lipoglycopeptides*** **:** dalbavancin, oritavancin

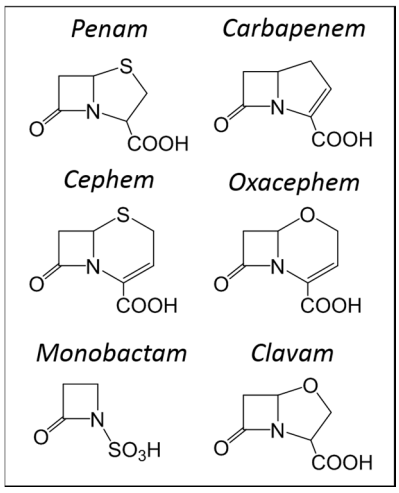
1.2.1 β-lactam antibiotics

In 1928, Alexander Fleming's observed that a *Penicillium* mold was able to inhibit the growth of *Staphylococcus.* Ten years later, after having successfully purified and concentrated penicillin, Sir Howard Walter Florey and Ernst Boris Chain administered this drug for the first time to humans [41]. Since this discovery, β-lactam antibiotics have led the fight against bacterial infections.

All β-lactams share a common pharmacophore, a four-membered cyclic amid (β-lactam ring) which is fused with a five- or six-membered cycle, except for monobactams. Nowadays, they can be divided into 6 subclasses according to the nature of these cycles and/or the presence of a heteroatom. We distinguish penams (penicillins), carbapenems (e.g. imipenem), cephems or oxacephems (cephalosporins), as well as monobactams (e.g. aztreonam), which can be

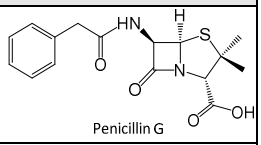
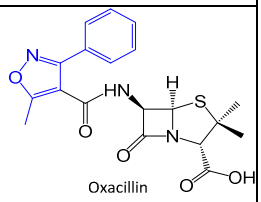
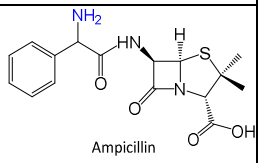
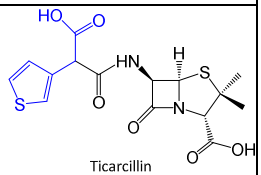
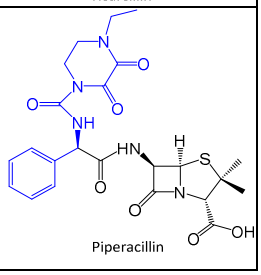
associated with β-lactamases inhibitors or clavams (**figure 1-4**). They all share a common mechanism of action but differ by their spectrum of antibacterial activity [42].

***Figure 1- 4** : Main ring structure of β-lactams subclasses approved for clinical use*



Among the penicillin group, we distinguish 5 subclasses namely natural penicillins, penicillinase-stable penicillins, aminopenicillins, carboxypenicillins and ureidopenicillins (**table 1-5**).

***Table 1- 5** : Penicillin classification (adapted from* [42]*)*

	Molecules	Antibacterial spectrum	β-lactamases resistance	Example of structure
Natural penicillins	Penicillin G Penicillin V	Gram-positives, anaerobes and some Gram-negatives	-	 Penicillin G
Penicillinase-stable penicillins	Cloxacillin Dicloxacillin Flucloxacillin Methicillin Nafcillin Oxacillin	Penicillin-resistant, methicillin-susceptible staphylococci (MSSA) and some streptococci	+	 Oxacillin
Aminopenicillins	Amoxicillin Ampicillin Bacampicillin	Same as penicillin G + β-lactamase-negative Gram-negative bacilli and some *Enterobacteriaceae*	-	 Ampicillin
Carboxypenicillins	Ticarcillin	Some ampicillin-resistant Gram-negatives including *Pseudomonas aeruginosa*	-	 Ticarcillin
Ureidopenicillins	Piperacillin	Similar to carboxypenicillins	-	 Piperacillin

Natural penicillins, such as penicillin G, were used primarily for the treatment of selected Gram-positive and Gram-negative infections [41]. However, the penicillin resistance, attributed to the synthesis of a penicillinase, rapidly appeared (see *β-lactamases-mediated resistance* section)[43].

To fight against this resistance mechanism, structural modifications in the lateral chain of the natural penicillins provided enough steric hindrance to make them very poor substrates for most staphylococcal β-lactamases. Penicillinase-stable penicillins, such as oxacillin or nafcillin, have been extensively used for the treatment of staphylococci infections prior the emergence of widespread resistance among staphylococci due to the acquisition of a low-affinity penicillin target, the penicillin-binding protein 2a (PBP2a) attributed to the so-called MRSA phenotype (see *PBP2a-mediated resistance* section). Of note, penicillinase-stable penicillins continue to be active against other Gram-positive organisms and, as shown in **table 1-4**, remain agents of choice in treating MSSA infections [42]. These two first penicillin subclasses are characterized by a narrow spectrum of activity, being mainly active on Gram-positive bacteria.

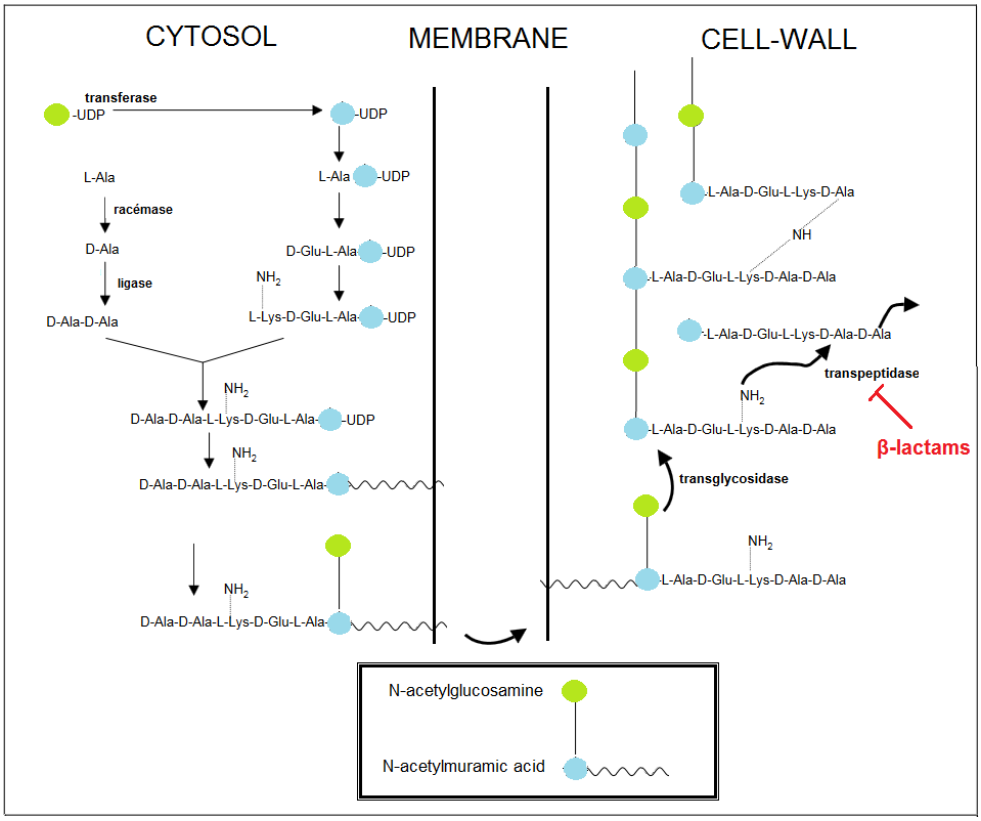
Thanks to the addition of an amino group to the lateral chain, aminopenicillins, such as ampicillin or amoxicillin, possess a broader spectrum of action because they have a better ability to penetrate through the pores in the outer membrane of Gram-negative bacteria [44]. When used in combination with β-lactamases inhibitors, they have a good activity against aerobic Gram-positive (including MSSA), Gram-negative and anaerobic organisms that produce penicillinases [42].

Finally, carboxypenicillins (ticarcillin) and ureidopenicillins (piperacillin), also called antipseudomonal penicillins, retain most of the antibacterial activity of the natural penicillins and the aminopenicillins, but with an added acitivity against Gram-negative bacilli [42].

1.2.1.1 MODE OF ACTION

β-lactams impair the cell wall synthesis by inhibiting transpeptidases (specialized acyl serine transferases), also called penicillin-binding proteins (PBPs) (**figure 1-5**). Thanks to a similar distance between the carboxylate or sulfonate (monobactams) and the cyclic amid, they mimic the D-Ala-D-Ala sequence in the nascent peptidoglycan and act as false substrates for the D-alanyl-D-alanyl transpeptidase. The serine residue of the transpeptidase reacts with the carbonyl of the β-lactam ring to give an inactive acylated enzyme. This inhibition is thus the direct results of the covalent binding between the antibiotic and the membrane-bound enzyme.

Figure 1- 5 *: Mode of action of β-lactams. Peptidoglycan units are constructed inside the cytosol of the bacteria, by binding a short peptide to uridine diphosphate (UDP)-N-acetylmuramic acid. In order to cross the membrane, this precursor in bound to a lipid carrier and then to N-acetylglucosamine. At the cell surface, peptidoglycan units are reticulated thanks to transglycosylases (catalyzing the polymerization between sugars) and transpeptidases (catalyzing the polymerization between peptidic residues). Adapted from* [45].



In contrast to *Escherichia coli* or *Bacillus subtilis* having 12 or 16 PBPs respectively, *S. aureus* only possesses 4 native PBPs (**Table 1-6**), among which, only two (PBP1 and 2) are required for the normal growth and normal shape of *S. aureus* [46,47]. In addition to these proteins, this bacterium also possesses two monofunctional transglycosylases and two "auxiliary" transpeptidases (FmtA and FmtB) [48]. Of note, in this spherical bacteria, cell wall synthesis follows only one mode (no elongation) and takes place at the septum [49].

***Table 1- 6** : Penicillin binding proteins of S. aureus*

Protein	Molecular mass (kd)	Origin	Enzyme type	Role
PBP1	85	Native	Transpeptidase	Cell division (septation) and cell separation [50,51]
PBP2	81	Native	Transpeptidase Transglycosidase	Cell division and β-lactam resistance [49,52]
PBP2a (or 2')	78	Acquired (extraspecies source)	Transpeptidase	β-lactam resistance [53]
PBP3	75	Native	Transpeptidase	Not fully understood. Possible role in the autolysis [54]
PBP4	45	Native	« Secondary » transpeptidase	Secondary cross-linking of the peptidoglycan [55]

By inhibiting cell wall synthesis, β-lactams are usually active against rapidly dividing bacteria only [56]. In addition, they are able to induce cell-lysis through indirect mechanisms (mostly the activation of autolytic enzymes, peptidoglycan hydrolases) which explain their bactericidal effect [57].

1.2.1.2 RESISTANCE TO β-LACTAM ANTIBIOTICS

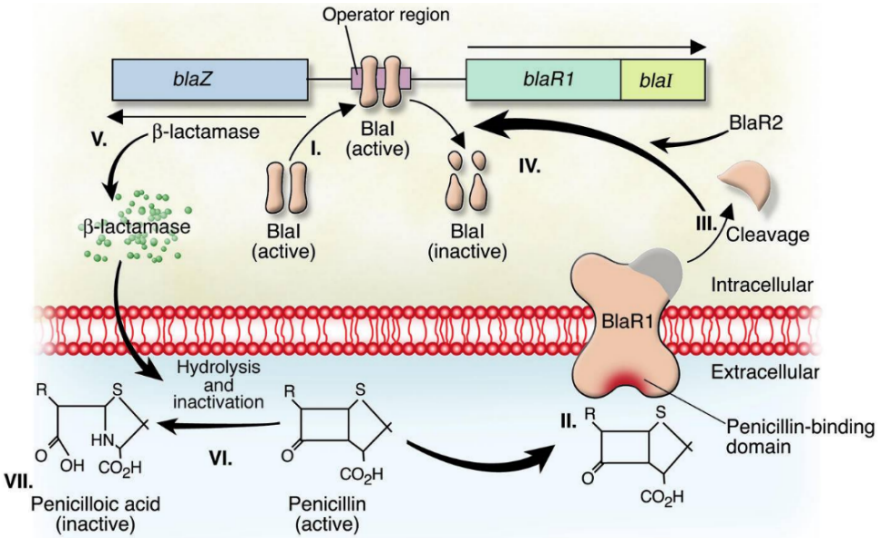
The main mechanisms by which *S. aureus* resists to the action of β-lactam antibiotics are (**a**) the production of β-lactamases degrading the antibiotic and (**b**) the production of an altered target, PBP2a, with a decreased affinity for β-lactams.

1.2.1.2.1 β-lactamases-mediated resistance

Shortly after the introduction of penicillin in the early 1940s, penicillin-resistant *S. aureus* were described, first in hospitals and subsequently in the community. This resistance pattern, attributed to the production of β-lactamases, spread so quickly that by the late 1960s, more than 80% of both community- and hospital- acquired staphylococcal isolates were resistant to penicillin [58]. Actually, the existence of enzymes able to inactivate penicillin was discovered before the introduction of penicillin, in 1940, by Sir Abraham and Chain in a strain of *Escherichia coli* [43]. However, this enzyme was not thought to be clinically relevant since penicillin was targeted to treat staphylococcal infections. To date, more than 90% of staphylococcal isolates resist to β-lactams thanks to the production of penicillinases [58].

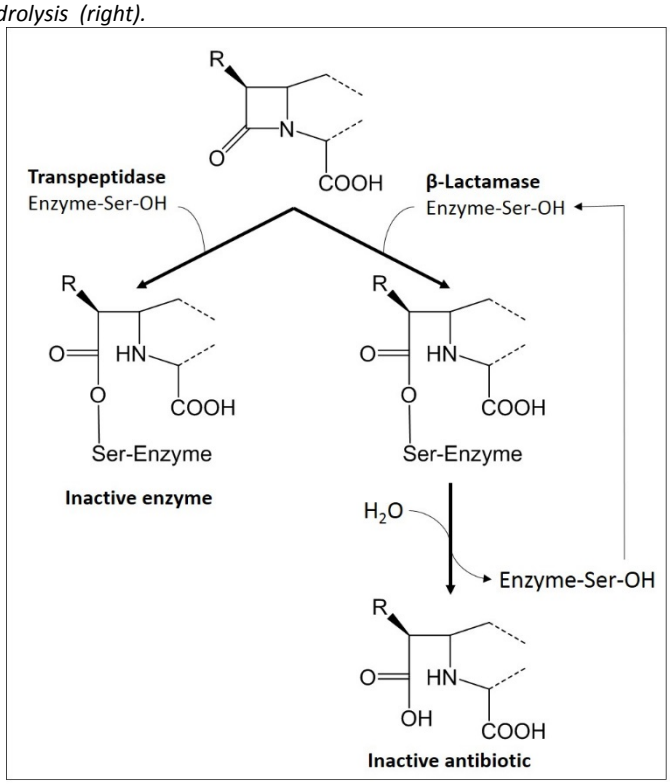
This β-lactam resistance is mediated by the *blaZ* gene that encodes a predominantly extracellular enzyme able to hydrolyze the β-lactam ring of penicillinase-susceptible β-lactams (penicillin, ampicillin,…). The expression of *blaZ* is inducible and regulated by two adjacent sequences, the repressor *blaI* and the antirepressor *blaR1,* coding for BlaI and BlaR1 proteins respectively [59] (**figure 1-6**). In the absence of antibiotic, β-lactamases are only expressed at low levels: the DNA-binding protein BlaI binds to the operator region and represses RNA transcription from both *blaZ* and *blaR1-blaI* (**I**). When exposed to β-lactams, the membrane protein BlaR1 is activated (**II-III**) and binds to the inducer BlaI which is cleaved into inactive fragments (**IV**). Subsequently, this results in the dissociation of BlaI from the operator site and allow the transcription of *blaZ*, encoding the β-lactamases proteins (**V**) [58].

Figure 1- 6 *: Induction of staphylococcal β-lactamase synthesis in the presence of penicillin [58]*



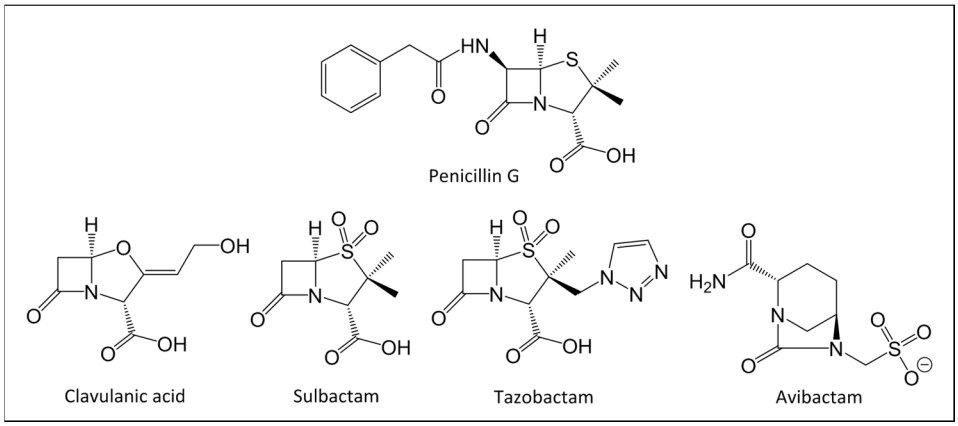
B-lactamases are serine or zinc (Zn^{2+}) proteases having high affinity for β-lactams and cleaving their amide bond. Although most β-lactamases open the β-lactam ring in the same way as transpeptidases, the hydrolysis rate of the acyl-enzyme is far quicker than in PBPs, resulting in the regeneration of the enzyme and the production of an irreversibly inactivated antibiotic (**figure 1-7**) [56]. Moreover, their turn-over is much more rapid (1000 times per second vs one per hour for PBPs) [56].

***Figure 1- 7** : β-Lactam antibiotics as substrates for transpeptidases and β-lactamases* [56]*. A β-lactam covalently binds to transpeptidases and hydrolysis of this acylated enzyme is very slow, making the enzyme inactive (left). The same reaction occurs in the case of serine β-lactamases but hydrolysis of the acylated enzyme is very rapid, making the antibiotic inactive and regenerating the enzyme for a new cycle of hydrolysis (right).*



In order to counteract this resistance mechanism, it is possible to use β-lactamases inhibitors among which clavulanic acid, isolated from *Streptomyces clavuligerus* in the 1970s, was the pioneer (**figure 1-8**) [60]. Two other inhibitors, also sharing a structural similarity with penicillin, sulbactam and tazobactam, were developed later by the pharmaceutical industry as synthetic compounds in 1978 and 1980, respectively [61]. They react with the β-lactamase enzyme to form a covalent acyl-enzyme intermediate. More recently (2015), the use of avibactam, an extended-spectrum β-lactamase inhibitor that does not contain any β-lactam ring but keeps an acidic function and a cyclic amid function, was approved by the *Food and Drug Administration* (FDA) [62].

***Figure 1- 8** : Structure of penicillin G and three β-lactamases inhibitors*



The activity of these inhibitors is depending on the type of β-lactamase produced by the bacteria. Indeed, *S. aureus* is able to synthesize four different classes of β-lactamases, namely A, B, C and D classes, differing for their substrate and inhibitor specificities [61,63]. Of note, producers of types A and C β-lactamases predominate amongst *S. aureus* isolates [64]. In general, clavulanic acid, sulbactam and tazobactam are mainly effective against organisms expressing class A β-lactamases while avibactam is characterized by a broader activity spectrum [61,65].

Of note, while combinations of β-lactam + β-lactamases inhibitors like *ampicillin/clavulanate*, *ampicillin/sulbactam* or *piperacillin/tazobactam* were recommended in previous guidelines in order to treat β-lactamases producers *S. aureus* infections, penicillinases-stable penicillins (*oxacillin* or *nafcillin*) are preferred today (see **table 1-4** for *antibiotics recommended to treat S. aureus infections*) [40,66].

1.2.1.2.2 PBP2a-mediated resistance

The emergence of penicillin resistance in *S. aureus* stimulated the search and the development of new penicillinase-stable penicillins. In 1959, methicillin, the first semi-synthetic penicillin, resistant to the action of β-lactamases thanks to a steric hindrance, was introduced for clinical use. However, shortly after that, reports from United Kingdom describe isolates with an acquired resistance to methicillin, referring to the so-called methicillin-resistant *S. aureus* (MRSA) [67]. This methicillin resistance pattern confers resistance to methicillin but also to most other β-lactams excepting ceftobiprole and ceftaroline, recently brought to the clinical use [68,69]. Today, MRSA are found worldwide and most are drug multi-resistant [70].

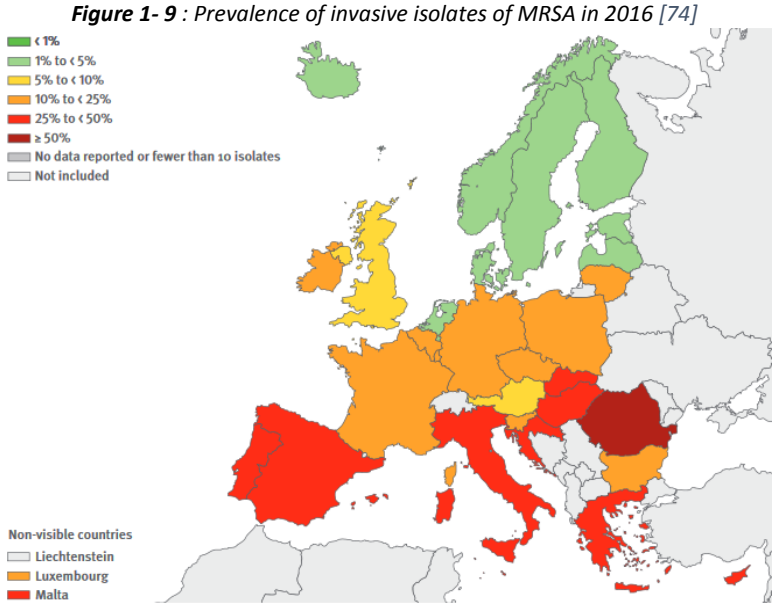
MRSA Epidemiology

MRSA has historically been considered as a problem within hospital settings. However, in the past years, a dramatic increase in the prevalence of MRSA infections in home facilities, as well as in the community and in animals has been noticed. Two terms are generally used to describe MRSA infections: HA-MRSA refers to healthcare-acquired MRSA infections while CA-MRSA, which are less common in Europe than in the USA, defines the infections acquired in the community [71,72]. In order to eradicate this bacterium, clinicians used not only β-lactams but also a huge variety of different antibiotics. As a result of this antibiotics pressure, HA-MRSA developed resistance to β-lactams but also to other classes of antibiotics (quinolones, macrolides, tetracyclines, rifampicin, clindamycin and trimethoprim/sulfamethoxazole) or even to multiple classes of antibiotics. In contrast, the resistance of CA-MRSA strains stays, most often, limited to β-lactams [73].

In Europe, the prevalence of MRSA infections varies significantly among countries (**figure 1-9**). Due to the high prevalence of invasive MRSA isolates, many countries

have introduced national recommendations and guidance documents on the prevention of spread of MRSA in recent years, focusing on both improved infection prevention and control, as well as careful antimicrobial use. Thanks to those efforts, a significantly decreasing trend in the prevalence of invasive MRSA has been noted between 2013 and 2016 [74]. However, an increase in the transfer of healthcare associated clones into the community has been noted [75]. Moreover, as the levels of resistance continue to be high in some countries and combined resistance to other antimicrobial groups is common, MRSA remains an important pathogen in Europe [74].

Figure 1- 9 *: Prevalence of invasive isolates of MRSA in 2016 [74]*



mecA gene

Methicillin resistance in MRSA strains is due to the acquisition of the *mecA* gene via horizontal transfer from an unidentified species. This gene encodes a 78kDa penicillin-binding protein, called PBP2a or PBP2'. In comparison to other PBPs, PBP2a possesses an unusually low affinity for methicillin but also for all the other conventional β-lactam antibiotics.

The *mecA* gene is located on a mobile genetic element, designated as the Staphylococcal Cassette Chromosome *mec* (SCC*mec*). SCC*mec* elements have two essential components: the *ccr* gene complex (*ccr*) and the *mec* gene complex (*mec*). The identification of several *mec* and *ccr* allotypes among SCC*mec* elements has led to the classification of SCC*mec* types I, II, III, IV and V [76]. While on SCC*mec* types I, IV and V, we only find the antibiotic resistance determinants carried by *mecA* (β-lactams), types II and III contain multiple determinants for non-β-lactam antibiotic resistance. These genetic elements explain the difference observed between HA- and CA-MRSA, multidrug resistance being often documented in MRSA isolates circulating in healthcare environments. Indeed, HA-MRSA typically have SCC*mec* subtypes I to III, contrarily to CA-MRSA mainly associated with types IV and V [77].

The expression of *mecA* is regulated by two regulatory elements namely *mecR1* and *mecI* (both located on SCC*mec*) similarly to the regulation of *blaZ* by the *blaR1* and *blaI* upon exposure to β-lactams. Indeed, MecR1 having a sequence homology to the penicillin sensor-transducer BlaR1, acts as a signal transducer in the β-lactam induction of *mecA* thanks to its anti-repressor activity. MecI acts as a repressor of *mecA* transcription and shares sequence homology with BlaI. Thus, in the absence of β-lactam, MecR1 is not induced and *mecA* transcription is tightly repressed by mecI [78]. The sequence homology of *mecI-mecR1* with the *blaI-blaR1* results in the regulation of *mecA* expression by a dual control [79].

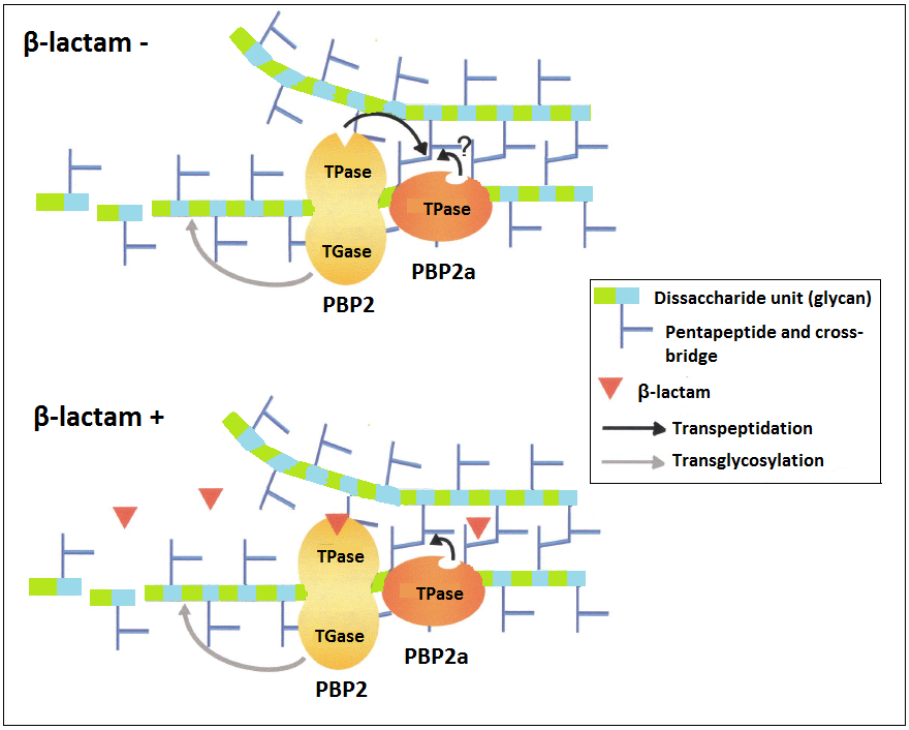
Penicillin binding protein 2a

Many papers describe the specific role that PBP2a confers to MRSA to circumvent the antibacterial effect of β-lactams [58,70,80–82]. PBP2a was shown to have a transpeptidase activity. When the transpeptidase domains of native PBPs (PBP 1, 2, 3 and 4) are acetylated and inactivated by β-lactams, the acquired low-affinity PBP2a protein substitutes to their function and continues the synthesis of the

peptidoglycan. Moreover, it has been shown that in the presence of β-lactams, the assembly of the MRSA peptidoglycan requires the cooperation between the PBP2a transpeptidation and the PBP2 transglycosylation, PBP2 being the only native PBP2 having a dual function (transpeptidase and transglycosidase) (**figure 1-10**) [52].

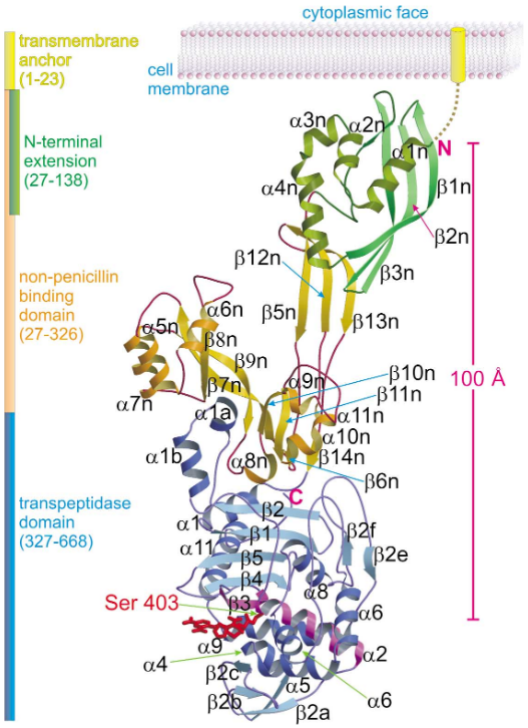
Of note, Pinho and Errington showed in 2005 that PBP2, which normally localizes at the division septum (the main place of cell wall synthesis in *S. aureus*) is dispersed over the entire surface of the cell upon addition of oxacillin. They also showed that the normal localization at the septum seems to require binding to a transpeptidation substrate [49].

***Figure 1- 10**: Cooperative functioning between the transpeptidase domain PBP2a and the transglycosylase domain of PBP2 in MRSA. In the absence of antibiotics, both the transpeptidase and transglycosylase domains of PBP2 participate in the cell-wall biosynthesis while the participation of PBP2a remains elusive until now. Conversely, in the presence of β-lactams, the transpeptidase domain of PBP2 is acetylated and no longer active while its penicillin-insensitive transglycosylase domain remains active and cooperates with the transpeptidase activity of PBP2a for an effective cell-wall synthesis and bacterial growth of the organism in the presence of β-lactams (adapted from* [52]*).*



In 2002, Lim and Strynadka published the first crystal structure of soluble PBP2a [81]. They showed that this protein (overall dimension: ~ 130 x 60 x 58 Å) consists of (i) a N-terminal extension, (ii) a centralized bilobal non penicillin-binding domain, and (iii) a C-terminal transpeptidase domain containing the active-site serine (Ser-403). The protein also contains a transmembrane anchor segment (residues 1-23) (**figure 1-11**).

Figure 1- 11 *: Structure of S.aureus PBP2a [81]*



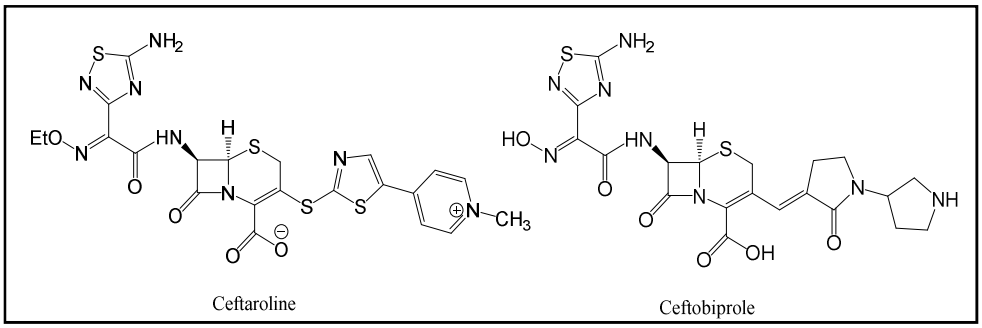
The main difference between native PBP and PBP2a is the active site motif of the nucleophilic serine which is located on an α-helix sequestered within an extended narrow groove in the case of PBP2a; this closed conformation of the active site prevents the access of an intact β-lactam molecule [81]. Of note, exposition of bacteria to an acidic pH causes an opening of the enzymatic cavity [83]. Recently published papers describe PBP2a as an allosteric enzyme [84–87].

Indeed, it has been shown that PBP2a possesses actually two substrate binding sites: one at the active site, where binding of antibiotic or peptidoglycan is known to take place, and another, called allosteric site, within the non-penicillin-binding domain, both localized in major grooves [84].

Binding of muropeptide of peptidoglycan to the allosteric site of the PBP2a potentiates a conformation change displacing the peptide loop enabling access of the substrate for cell-wall synthesis [84].

The two most recently approved cephalosporins, ceftaroline and ceftobiprole have favorable low MIC against MRSA (**figure 1-12**) [88–91]. Resistant to the *S. aureus* β-lactamases activity, they bind with high affinity to *S. aureus* PBPs, including PBP2a [88]. Thanks to their hydrophobic moiety on their lateral chain, these two molecules are able to reach the groove and bind to the allosteric site of the PBP2a, which forces a conformational change resulting in the opening of the active site. This allows the binding of a second antibiotic molecule that inactivates the enzyme [84]. Of note, the increase lipophilicity of these two antibiotics necessitates their administration as prodrugs (fosamyl for ceftaroline and medocaril for ceftobiprole).

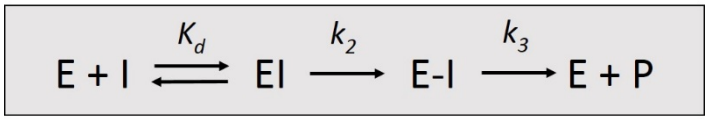
***Figure 1- 12** : Structure of anti-MRSA cephalosporins ceftaroline and ceftobiprole*



While these two new fifth generation cephalosporins have good activity against MRSA strains, resistance has emerged. In general, mutants exhibited diminished capacity to undergo the conformational change that is the requisite to acylation within the active site. Mutants thus resist to the antibiotic action by altering the allosteric communication [87].

The kinetic mechanism of PBP2a is described by a three-step pathway, common with other PBPs and β-lactamases (**figure 1-13**). The interaction of PBP2a with a β-lactam inhibitor begins with the rapid, irreversible formation of a non-covalent Michaelis complex, described by the dissociation constant K_d. This step is followed by the nucleophilic attack by the active-site serine Ser^{403} on the β-lactam ring, to give a relatively stable covalent acyl-enzyme complex, described by the rate constant k_2. Finally, a slow hydrolytic deacylation step, described by the rate constant k_3, release the inactivated antibiotic and regenerate the active enzyme [81,92].

Figure 1- 13 *: Kinetic scheme of the interaction between PBP (E) and a β-lactam agent (P).*



E = enzyme; I = inhibitor; EI = non covalent pre-acylation complex; E-I = covalent acyl-enzyme complex; P = hydrolysis product; K_d = dissociation constant; k_2 = acylation rate constant; k_3 = deacylation rate constant

In comparison to β-lactam sensitive-PBPs, PBP2a is characterized by a higher K_d, which could be explained by the diminished accessibility of the β-lactams to the PBP2a active site [92]. Another important difference is the PBP2a acylation rate constant (k_2) which is two to four orders of magnitude lower than the corresponding rate constant for penicillin-sensitive PBPs. This results in a k_2/K_d ratio, corresponding to the overall binding efficiency of PBPs, which is 10 000 fold smaller for PBP2a compared with susceptible PBP.

Due to the inexorable emergence of resistance to any antibiotic, the need for new antibiotics is still urgent. The new strategies are focusing on the search of new non-β-lactam antibiotics targeting PBP2a [93], new targets [94], combination therapy in which compounds of various types and origins synergize with conventional antibiotics to fight resistance [95–97] or new drug scaffolds. These two last strategies could be achieved using plant origin compounds, which are developed in the next part of this introduction.

2. Traditional medicine as a source of new solutions to fight bacteria and antimicrobial resistance

« An apple a day keeps the doctor away »

Since millennia, herbal medicines are widely used around the world. Many ancient medical texts from different cultures describe the use of plant ingredients for human health. They include for example the *Ebers Papyrus*, an Egyptian medical papyrus describing herbal knowledge from the era 1500 BC. This is also the case of a pharmacopoeia written by Discorides in the first century*, De Materia Medica,* covering about 600 plants and medicines that can be obtained from them [98]. All these texts describe plant-based remedies for a broad number of ailments, many of which could be attributed to infectious diseases.

The use of traditional medicine still continues today and depends on a number of factors such as culture, historical significance but also limitations in availability or accessibility of conventional medicine-based health services [99]. For many millions of people, herbal medicines, traditional treatments, and traditional practitioners remain the main source of health care, and sometimes the only source of care [99].

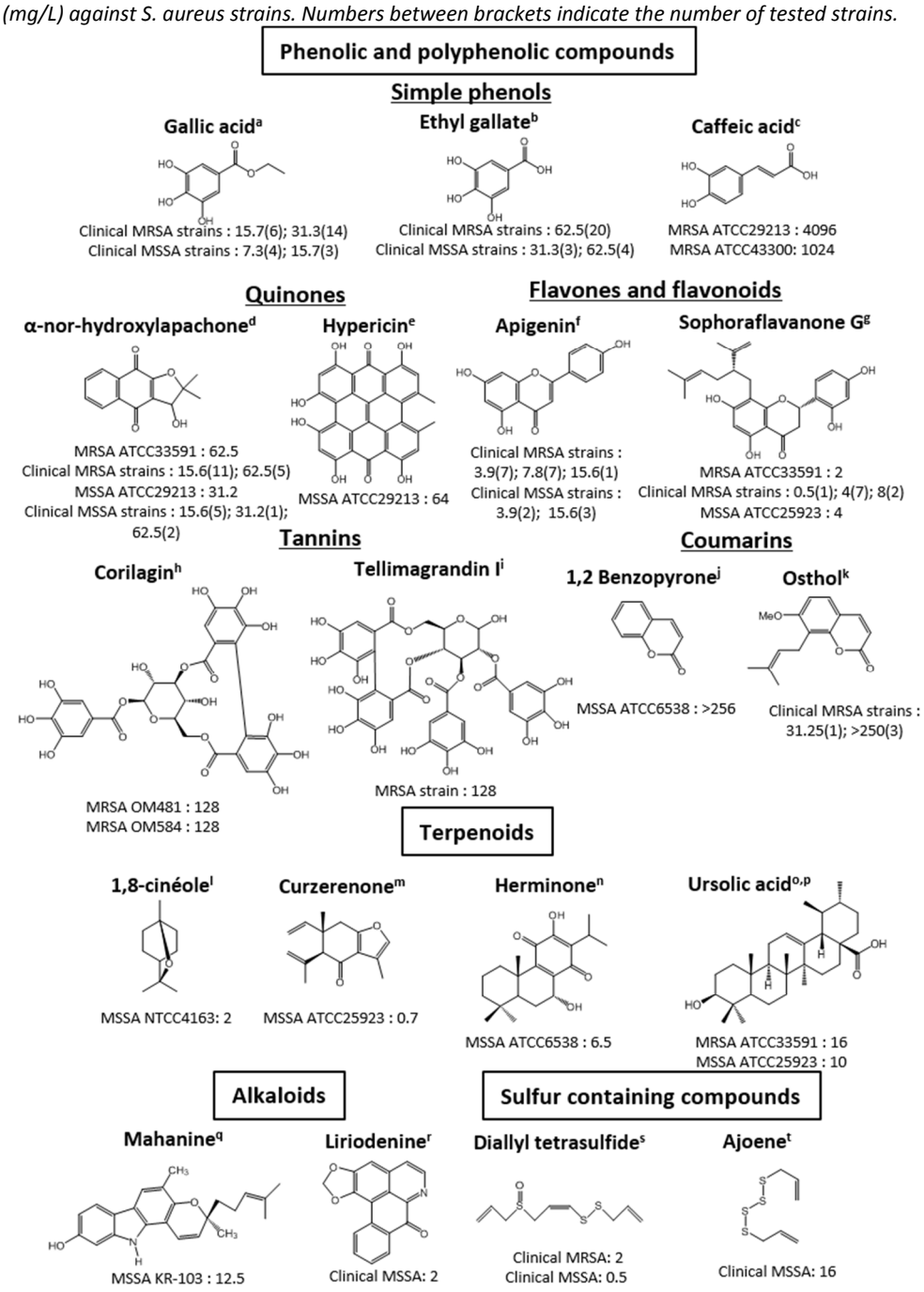
Despite this knowledge of traditional use and the huge biodiversity, the percentage of plants of the current repertoire of FDA-approved antibacterial drugs remain really tiny. However, the research of new phytochemicals is increasing and some reviews about their potential use as antimicrobials or resistance modifying agents were published recently [98,100–104].

Indeed, plants have to defend themselves against outside attacks and therefore produce plenty of structurally diversified compounds, which could potentially also be active against human pathogens. There are four major groups of secondary metabolites synthesized by plants: phenols and derivatives, terpenoids, as well as amino acids derivatives namely alkaloids and sulfur-containing products. Some

compounds belonging to each of these classes having been tested on *S. aureus* strains are reported in **figure 1-14** with their respective minimal inhibitory concentration (MIC). Of note, according to several authors, antimicrobial activity is considered as significant if MIC ≤ 10 mg/L, moderate if 10 < MIC ≤ 100 mg/L, and low or negligible if MIC > 100 mg/L [105–108]. Some of these reported compounds possess thus a good antistaphylococcal activity with MIC below 10 mg/L while, for example, both reported coumarins and tannins possess a negligible activity.

Moreover, plants can produce non-antimicrobial compounds that can for example improve solubility, absorption or stability of active compounds. Indeed, in most cases, plants extracts contain complex mixtures of secondary metabolites and their combined action can yield an enhanced effect [109]. Another strategy to combat antimicrobial resistance, is the use of natural products to enhance or restore the activity of common antibiotics. Such compounds are called *resistance modifying agents* (RMA).

Figure 1- 14 *: Major groups of plants secondary metabolites with selected examples and their MIC (mg/L) against S. aureus strains. Numbers between brackets indicate the number of tested strains.*



[a,b] [110]; [c] [111]; [d] [112]; [e] [113]; [f] [114]; [g] [115]; [h] [116]; [i][117]; [j] [118]; [k] [119]; [l] [120]; [m] [121]; [n] [122]; [o] [123]; [p] [124]; [q] [125]; [r] [126]; [s] [127]; [t] [128]

A common way to evaluate interactions between an antibiotic and a natural compound is the use of the checkerboard method allowing the calculation of the Fractional Inhibitory Concentration Indexes, FICI. It is calculated as the sum of the Fractional Inhibitory Concentrations (FICs) of each combined compound as shown in **figure 1-15** [129]. According to the European Committee for Antimicrobial Susceptibility Testing, the combination is considered as synergistic for FICI ≤ 0.5, additive for 0.5 < FICI ≤ 1, indifferent for 1 < FICI ≤ 4 and antagonistic for FICI > 4 *(see section 4.3.1 of this introduction for more detailed information)*.

***Figure 1- 15** : Calculation of the fractional inhibitory concentration index (FICI) and meaning of its value according to EUCAST [129,130]*

$$FIC_A = \frac{CMI_A \text{ (in combination)}}{CMI_A \text{ (alone)}} \qquad FIC_B = \frac{CMI_B \text{ (in combination)}}{CMI_B \text{ (alone)}}$$

$$FICI = FIC_A + FIC_B$$

FICI	Effect
≤ 0.5	Synergy
> 0,5 et < 1	Addition
> 1 et ≤ 4	Indifference
> 4	Antagonism

In the *S. aureus* context, some natural RMA are described to enhance the activity of β-lactam antibiotics; they showed an additive or synergistic effect in combination with this class of antibiotics. Their main mechanisms of action are in general an inhibition of β-lactamases activity or/and an inhibition of PBP2a activity (**table 1-7**).

Table 1- 7 *: Example of natural compounds enhancing the activity of β-lactams against S. aureus strains with their minimum inhibitory concentrations (MICs in mg/L), their Fractional Inhibitory Concentration Indices (FICI) and their proposed mechanism of action (adapted from [102])*

Compound	Phytochemical class	Plant source	Strain	MIC	Antibiotic potentiated and FICI	Proposed mechanism of action
Baicalein [131]	Flavonoid	*Scuttelaria amoena*	MRSA OM481	100	Oxacillin : < 0.25 Ampicillin : < 0.25	PBP2a inhibition, binding to peptidoglycan
			MRSA OM584	100	Oxacillin : < 0.25 Ampicillin : < 0.25	
Berberine [132]	Alkaloid	*Berberis spp.*	MSSA ATCC 25923	128	Ampicillin : 0.63 Oxacillin: 0.76	Intercalation into DNA, increase membrane permeability
			MRSA OMS7	64	Ampicillin : 0.63 Oxacillin: 0.50	
Corilagin [116]	Hydrolysable tannin	*Arctostaphylos uva-ursi*	MRSA OM481	128	Oxacillin: 0.13	Inhibition of PBP2a activity or synthesis
			MSSA 209P	128	Oxacillin: 0.75	
Catechin gallate [133,134]	Phenol	*Camellia sinensis*	MRSA strain	125	Penicillin: 0.63-2.1 Oxacillin: 0.38-2.1	β-lactamases inhibition, PBP2a synthesis inhibition, binding to peptidoglycan
			EMRSA-15	64	Oxacillin: 0.47	
Epicatechin gallate [134]	Phenol	*Camellia sinensis*	EMRSA-15	64	Oxacillin: 0.42	β-lactamases inhibition, PBP2a synthesis inhibition, binding to peptidoglycan
	Phenol	*Camellia sinensis*	MRSA strains (18)	62.5-125	Penicillin: 0.19-0.63 Oxacillin 0.13-0.57	

Epigallocatechin gallate [135]						β-lactamases inhibition, PBP2a synthesis inhibition, binding to peptidoglycan
			MSSA ATCC 25923	62.5	Penicillin: 0.38-0.56 Oxacillin 0.25-0.38	
Ethyl gallate [136]	Phenol	*Caesalpinia spinosa*	MRSA (18 strains)	62.5-125	Oxacillin : 0.16-1.20	Restriction of substrate diffusion for PBPs by alteration of membrane fluidity
			MSSA (8 strains)	62.5-125	Oxacillin: 0.30-2.05	
Isoamyl gallate	Phenol	*Caesalpinia spinosa*	MRSA (18 strains)	62.5	Oxacillin : 0.33-2.10	Restriction of substrate diffusion for PBPs by alteration of membrane fluidity
			MSSA (8 strains)	62.5	Oxacillin: 0.10-0.45	
Tellimagrandin I [117]	Hydrolysable tannin	*Rosa canina* L.	MRSA OM481	128	Oxacillin: 0.39	Inactivation of PBP, particularly PBP2a
			MSSA 209P	128	Oxacillin: 0.39	

During this thesis, we mainly worked on one class of natural compounds, namely pentacyclic triterpenes that are introduced in the next pages.

3. Pentacyclic triterpenes

Triterpenoids belong to the group of terpenes, one of the largest class of plant secondary metabolites with approximately 30 000 structures identified to date [137]. All terpenes are derived from C_5 isoprene units and, according to the number of these units, are classified as hemiterpenes (C_5), monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), sesterterpenes (C_{25}), triterpenes (C_{30}) or tetraterpenes (C_{40}) [138]. Since 1995, Connelly and Hill have published an annual review on the newly isolated triterpenoids from the plant kingdom [139]. Indeed, the majority of triterpene diversity occurs in plants, although other organisms are also able to produce triterpenes [140]. These compounds are notably present in plant surfaces such as fruit peel, stem bark or leaves, where, together with plant cuticle components, they protect the plant from biotic and abiotic stresses [141,142]. According to the diverse features of their carbon skeletons, triterpenes are divided into acyclic, mono-, bi-, tri-, tetra- or pentacyclic triterpenes. Among pentacyclic triterpenes, those possessing ursane, oleanane, and lupane carbon skeletons have received much attention from a biological point of view [137].

3.1 Biosynthesis

Triterpenes share the same biosynthetic route as sterols, both deriving from isoprene units. The first precursor of their biosynthesis is the isopentenyl pyrophosphate (IPP) synthesized *via* the mevalonate pathway or via an alternative pathway (the glyceraldehyde-3-phosphate (GAP)/pryruvate pathway) [143].

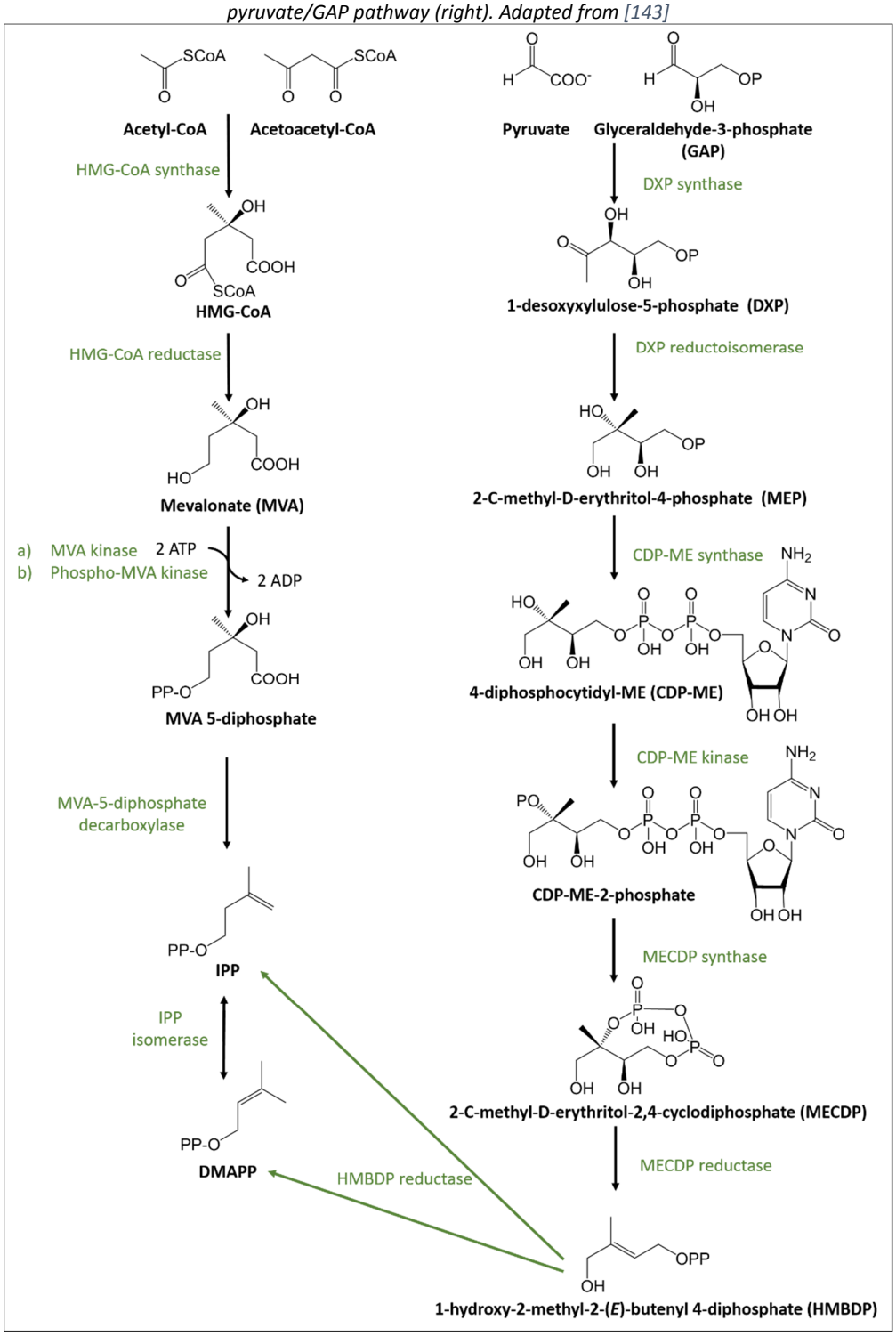
3.1.1 The mevalonate pathway

The first identified pathway to synthetize the triterpenes precursor namely, isopentenyl pyrophosphate (IPP) is the acetate/mevalonate (MVA) pathway given in **figure 1-14 (left)** [143]. This synthesis pathway mainly occurs in the cytoplasm or in mitochondria and is the classical pathway through the terpenes synthesis in all living organisms.

3.1.2 The GAP/pyruvate pathway

Forty years after the discovery of the classical mevalonate pathway, a totally different IPP biosynthesis route has been discovered in eubacteria, green algae and higher plants. This pathway involved glyceraldehyde-3-phosphate (GAP) and pyruvate as precursors of IPP (**figure 1-16 (right)**). Conversely to the mevalonate pathway linked to the cytoplasmic compartment, this alternative route is apparently bound to the plastidic compartment [144]. Of note, while the mevalonate pathway only produces IPP and requires the IPP isomerase function to produce dimethylallyl diphosphate (DMAPP), the GAP/pyruvate pathway generates IPP and DMAPP at a 6:1 ratio [145].

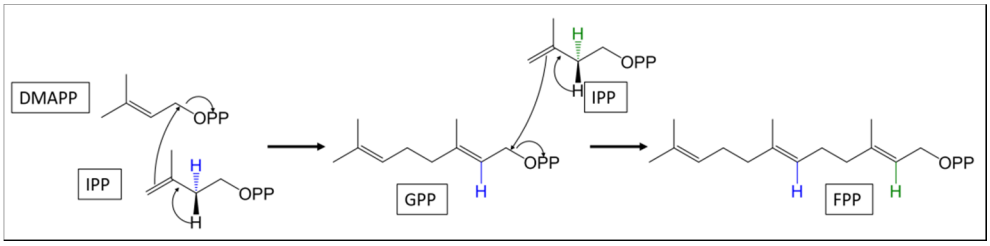
***Figure 1- 16**: Origin of isopentenyl pyrophosphate (IPP): the mevalonate pathway (left) and the pyruvate/GAP pathway (right). Adapted from [143]*



3.1.3 Head-to-tail and tail-to-tail condensations

The addition of DMAPP on the IPP double bound is catalyzed by a prenyltransferase and leads to the formation of geranyl pyrophosphate (GPP). A second addition of an IPP on GPP yields farnesyl pyrophosphate (FPP). The condensation of these three building blocks (DMAPP + IPP + IPP) follows a 'head-to-tail' mode (**figure 1-17**).

***Figure 1- 17** : Farnesyl pyrophosphate synthesis following 'head-to-tail' condensations of DMAPP and IPP (adapted from [143])*



Two FPP molecules will then condense in a 'tail-to-tail' mode to yield a squalene molecule which will be further oxidized into 2,3-époxysqualène. This molecule is the last common precursor of the triterpenoid and steroid pathways [140,146,147]. According to the conformation of this substrate, the chair-boat-chair (CBC) or the chair-chair-chair (CCC) conformation, its cyclization will lead either to sterols or triterpenoids [140]. In the triterpenoids biosynthesis, the chair-chair-chair conformation of 2,3-epoxyquialene will cyclizes in a dammarane cation (**figure 1-18**).

Most frequently, the dammarane cation will be further rearranged to form a supplementary cycle yielding to pentacyclic triterpenoids: oleananes, ursanes, lupanes, friedelanes, taraxastanes… (**figures 1-19**) [143].

Figure 1- 18 *: Biosynthetic route to sterols and triterpenes. Enzymes catalyzing each step are indicated in boxes. CBC: chair-boat-chair; CCC: chair-chair-chair. (adapted from [140])*

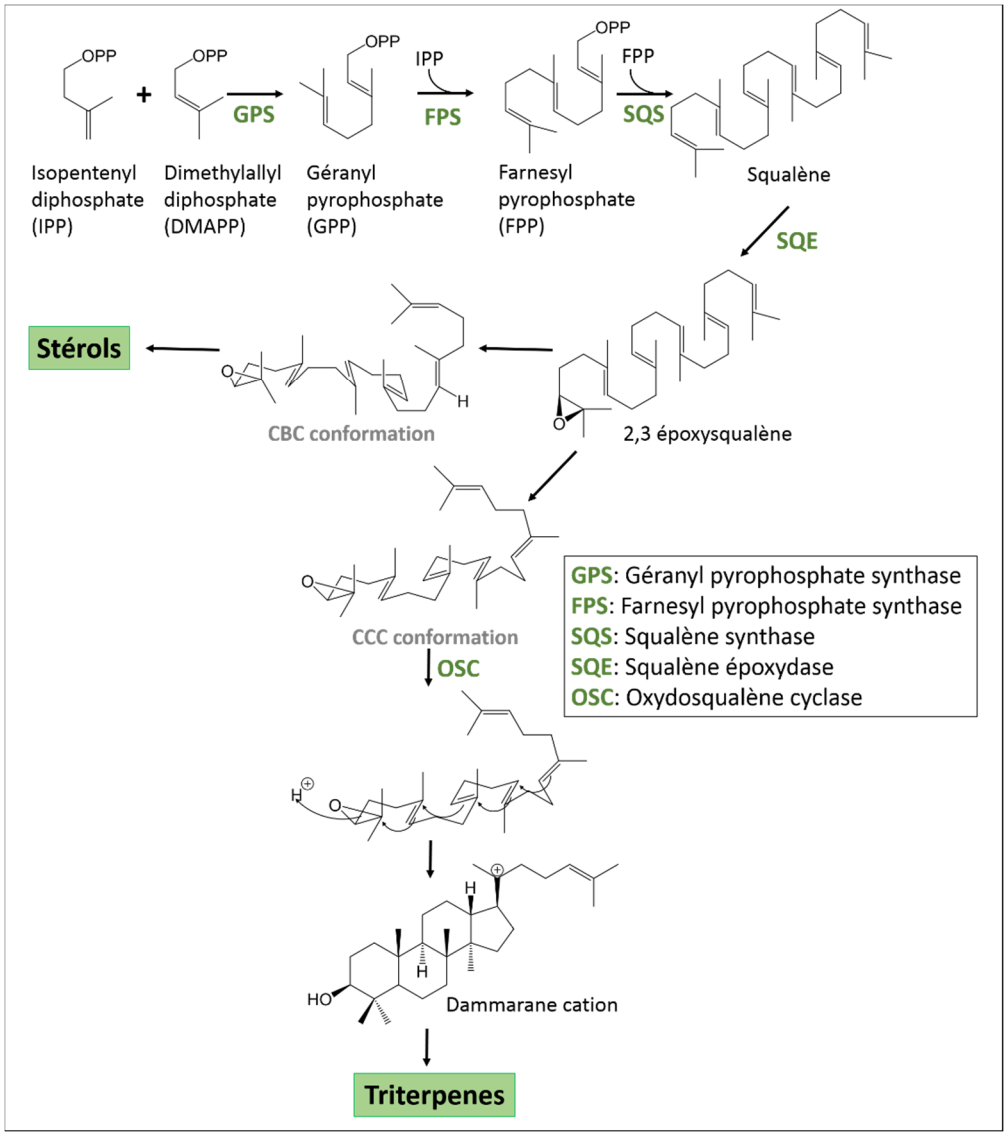
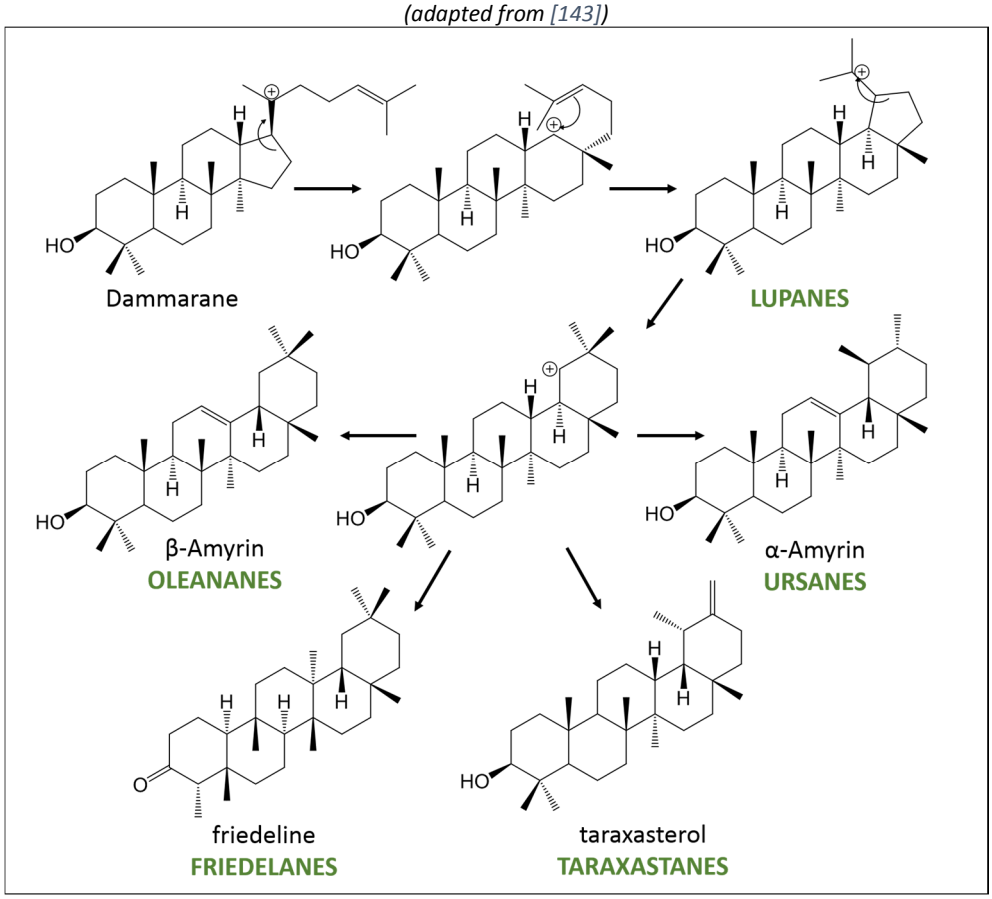


Figure 1- 19 *: Rearrangement of the dammarane squeleton to form pentacyclic triterpenes (adapted from [143])*



Pantacyclic triterpenes can occur as free or as conjugated forms. They may indeed be acylated, or glycosylated forming triterpenoid saponins, thereby leading to enormous structural diversity.

During this work, we focused on free pentacyclic triterpenes and more precisely on the three main classes: lupane, oleanane and ursane, each including important bioactive compounds.

3.2 Main biological activities

From a biological point of view, pentacyclic triterpenes have received much attention evidenced by the increasing number of publication on the subject. Indeed, the number of citations in the database *Pubmed* exceeded 154 067 for the keyword terpenes and 8 054 for triterpenes on the 3rd January 2006 [137]. These numbers were increased up to 294 061 and 26 075, respectively on the 21st of February 2018. This class of compound presents several biological activities including anti-tumor [148], anti-inflammatory [149], antioxidant [150], cardio- [151] and hepato-protective[152], anti-diabetic [153] , anti-viral [154], as well as antiparasitic [155] and antibacterial [156] activities, also summarized in several reviews [137,157–159]. These interesting biological activities resulted in the evaluation of some triterpenes in clinical trials [160–162] as well as an increased number of filled patents [163–165].

3.2.1 Anti-tumor, anti-inflammatory and antioxidant activities

These three biological activities are actually closely linked. Indeed, it has been shown that inflammation plays a clear role in the development and progression of cancer [166]. It is also the case for the production of reactive oxygen species (ROS) for which an increased level was shown to disrupt cell membrane integrity by oxidation of unsaturated membrane lipids [167]. Moreover, free radicals can cause DNA damage which may result in tumor initiation and promotion [168].

Anticancerous and anti-inflammatory activities of pentacyclic triterpenes are the most studied and some molecular mechanisms have been proposed to explain these activities (**Table 1-8**). Pentacyclic triterpenes are reported to influence pro-inflammatory cytokine levels known to be regulated by the transcription factor NF-κB [169–172]. It thus constitutes an interesting target knowing that NF-κB is commonly overexpressed in cancer cells [173]. However, several papers report

controversial data concerning the effect of pentacyclic triterpenes on NF-κB [174,175]. Actually, these observed differences could be explained by concentration-dependent effects as well as an influence of the medium or the cell status [148]. Indeed, the observed effects will be different on stimulated cells in comparison to resting cells [171,176].

Concerning, ROS production, most papers report an antioxidant potential for pentacyclic triterpenes [150,177–180]. They act by activating the enzymatic system, increasing the activity of superoxide dismutase, catalase as well as glutathione S-transferase and glutathione peroxidase [148]. However, in the context of apoptosis, it is reported that oleanolic and betulinic acids enhance ROS generation [181,182]. The hypothesis of Laszczyk and collaborators to explain these discrepancies is that pentacyclic triterpenes could enhance the generation of ROS that triggers the antioxidative cascade [148].

Finally, the last important target of pentacyclic triterpenoids explaining their anti-inflammatory activity is the phospholipase A2 (PLA_2). This enzyme hydrolyzes glycerolipids in order to release arachidonic acid, substrate for cyclooxygenase and 5-lipoxygenase in the eicosanoids (leukotrienes and prostaglandins) synthesis pathway. Pentacyclic triterpenes are reported to inhibit PLA_2 and downstream cyclooxygenase as well as 5-lipoxygenase [183–185,185–190]. The most efficient compound was betulinic acid which is currently under evaluation as a topical agent in a phase II clinical trial for the treatment of dysplastic nevi with the potential to transform into melanoma [191].

***Table 1- 8** : Main reported mechanism of action explaining the anti-cancerous and anti-inflammatory properties of pentacyclic triterpenes.*

Biological activity	Proposed Mechanisms
Anti-cancerous	Activation/Inhibition of NF-κB pathway [175,192,193]
	Induction of reactive oxygen species (ROS) and activation of caspase 3 [181,182]
	Increase the mitochondrial membrane permeability causing the release of cytochrome c and apoptosis inducing factor (AIF) with the resulting activation of caspases 3 and 8. [194–198]
	Inhibition of topoisomerases I and II [137]
Anti-inflammatory	Inhibition of NF-κB pathway [175,199]
	Inhibition of ROS [178–180]
	Inhibition of phospholipase A2 [184,189,190]
	Inhibition of cyclooxygenase 2 [183,187,188]
	Inhibition of 5-lipoxygenase and human leukocyte elastase [184–186,200,201]
	Inhibition of complement mediated-inflammation [202–205]

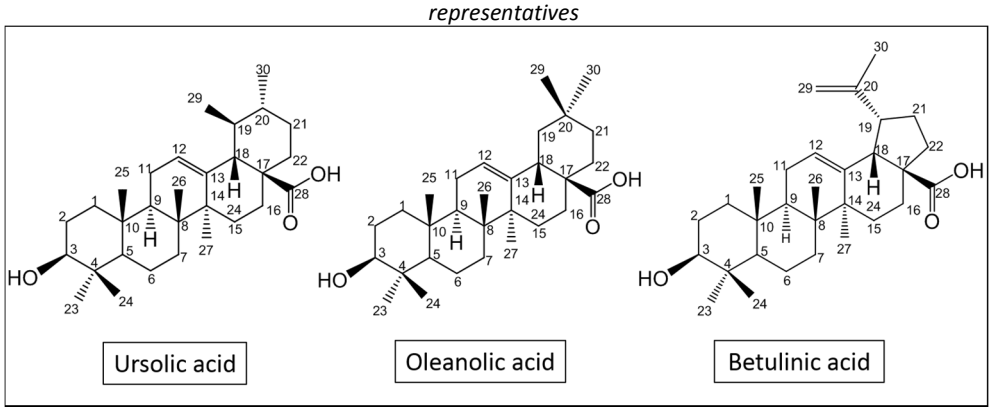
The antioxidant and anti-inflammatory activities of triterpenes may also explain their cardio- and hepato-protective effects. Indeed, lipid peroxidation is commonly related with chronic inflammation or autoimmune diseases [206] as well as hepatic and cardiovascular diseases [207].

3.2.2 Antiparasitic activities

Two years ago, Isah et al. published a review reporting the high activity of 85 pentacyclic triterpenes against different species of *Plasmodium*, *Trypanosoma*, *Leishmania* as well as *Nematoda* highlighting the great interest for this phytochemical group [155].

Given the expertise of our lab in the study of these three first parasites, and the tests realized in the last chapter of our work, we report here, when available, the activity of the most active pentacyclic triterpenic acids as well as the most studied, namely ursolic acid (UA), oleanolic acid (OA) and betulinic acid (BA) which are often considered as reference compounds for the ursane, oleanane and lupane derivatives respectively (**figure 1-20**).

Figure 1- 20 *: Structures of ursolic, oleanolic and betulinic acids; major ursane, oleanane and lupane representatives*



3.2.2.1 Antiplasmodial activity

Protozoan of the genus *Plasmodium* cause malaria. They are transmitted from the blood of an infected person and passed to a healthy human by an infected female mosquito of the genus *Anopheles* (**figure 1-21**). There are several species of *Plasmodium* responsible for human malaria, *P. falciparum* causing the most severe cases.

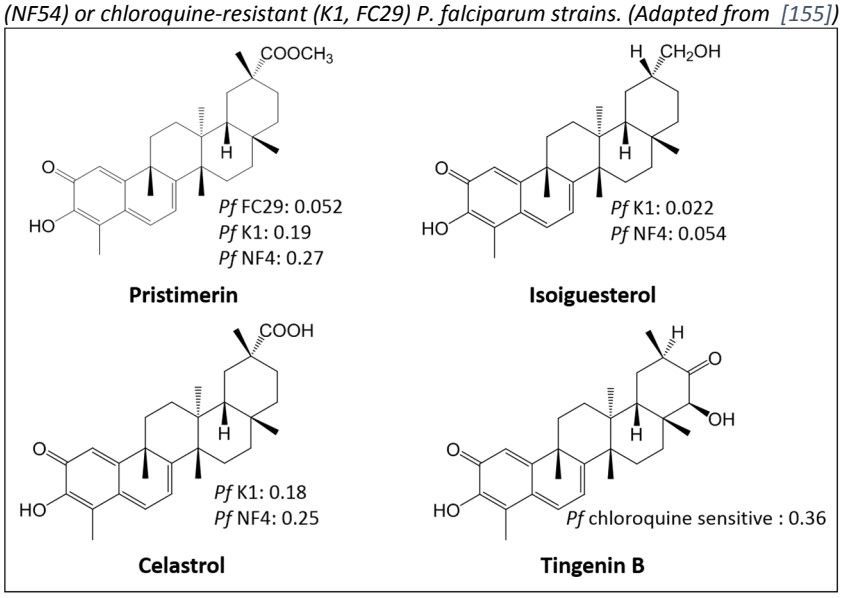
With about 200 million people affected each year and 445 000 reported deaths in 2016, malaria remains, since its discovery in 1880, the most severe parasitic disease worldwide [208]. The disease control is hard due to the development of resistance against quinine derivatives, but also artemisinin and derivatives, the best molecules currently on the market.

Figure 1- 21 *: Anopheles gambiae : malaria vector [209]*



The most *in vitro* active anti-plasmodial pentacyclic triterpenes belong to the small group of quinone methides, among which pristimerin, isoiguesterol, celastrol or tingenin B for example, possess very low IC_{50} values (< 0.5 µg/mL) against *P. falciparum* (**figure 1-22**).

Figure 1- 22 *: Structures and IC_{50} (mg/L) of quinone methide derivatives against chloroquine-sensitive (NF54) or chloroquine-resistant (K1, FC29) P. falciparum strains. (Adapted from [155])*



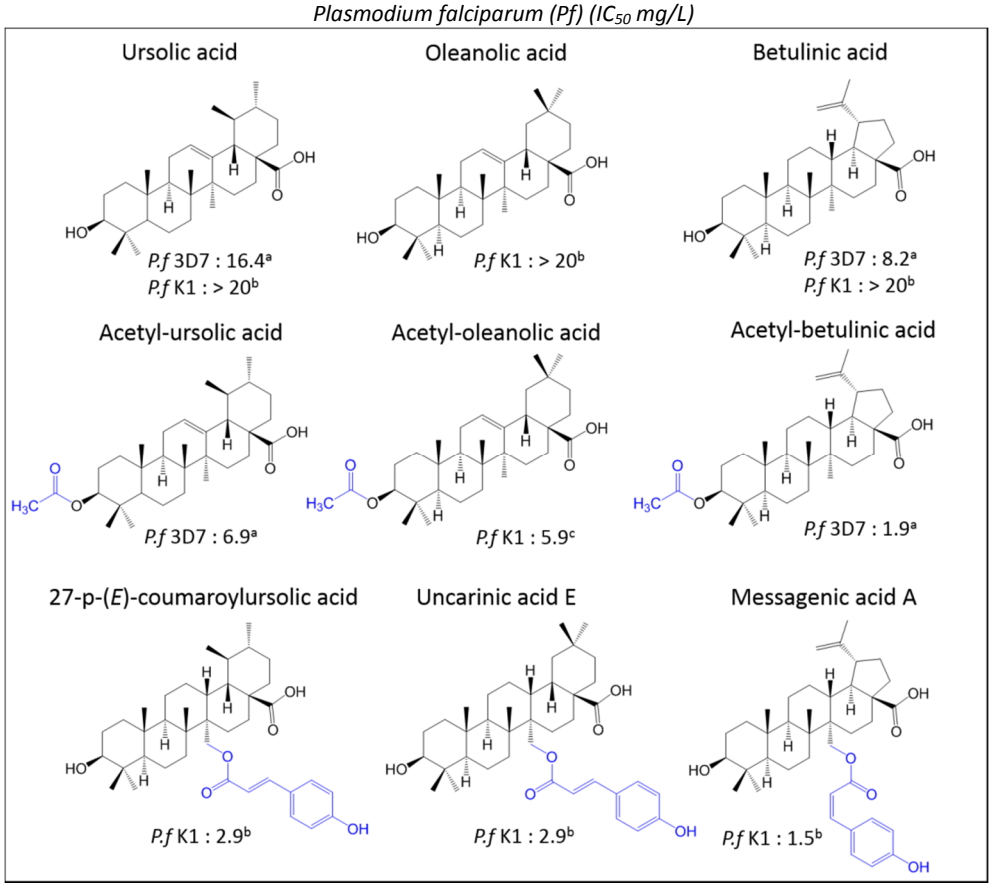
Several authors tested UA, OA or BA against various chloroquine sensitive *P. falciparum* (D6, 3D7 and T9-93) and chloroquine resistant (K1) strains. According to them, it seems that these three pentacyclic triterpenes can be classified following their antiplasmodial potential as BA > UA > OA (**table 1-9**).

Table 1- 9 *: Comparison between the antiplasmodial activiy of UA, OA and BA*

***P. falciparum* Strains**	**IC_{50} (mg/L)**		
	UA	**OA**	**BA**
K1 [210]	36.5 ± 2.2	88.8 ± 6.1	19.6 ± 6.1
K1 [211]	4.9 ± 0.1	9.3 ± 0.2	-
D6 [212,213]	1	2.1	-
3D7 [211]	12.7 ± 4.6	34.5 ± 1.5	-
3D7 [214]	16.4	-	8.2
3D7 [215]	14.8	27.1	-
nd [216]	3.1 ± 1.3	15.2 ± 3.4	-
T9-96 [210]	28.0 ± 3.0	70.6 ± 2.8	25.9 ± 6.2

Of note, some structural modifications of these triterpenic acids are known to enhance the antiplasmodial activity. It is the case for an esterification in C-3 with a short side chain or a C-27 esterification with a coumarate or a ferulate moiety which are illustrated with some examples on **figure 1-23**.

Figure 1- 23 *: Structures of UA, OA and BA derivatives with enhanced antiplasmodial activity against Plasmodium falciparum (Pf) (IC_{50} mg/L)*



A proposed mechanism of the anti-plasmodial activity of pentacyclic triterpenes is their incorporation into the erythrocytes membrane thereby modifying accessibility of the parasites into the cells [217,218]. It was also shown that an oleanolic acid derivative (maslinic acid) inhibits a number of *proteases* essential for the parasite growth and the cell invasion [219].

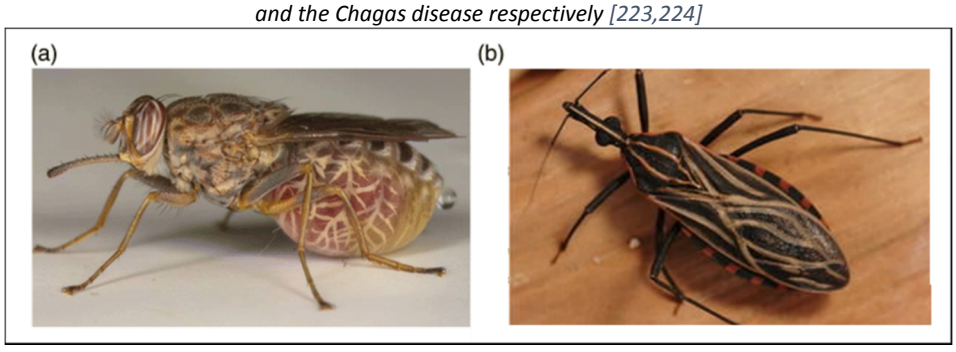
3.2.2.2 Antitrypanosomial activity

According to the parasitic species involved, trypanosomiasis may lead to sleeping sickness, also called African trypanosomiasis (*Trypanosoma brucei*) or to Chagas disease, the American trypanosomiasis (*Trypanosoma cruzi*) which cause important health problems and may be lethal if untreated [220].

In the Human African trypanosomiasis the protozoan parasites belonging to the genus *Trypanosoma*, are transmitted by tsetse flies (*Glossina* genus) bites which have acquired their infection from infected humans (**Figure 1-24 (a)**). It exists two forms of sleeping sickness, depending on the parasite involved: *T. b. gambiense* causes a chronic infection while *T. b. rhodiense* causes an acute infection. A third subspecies, *T. b. brucei* is pathogenic for wild and domestic animals but not for humans [221]. The sleeping sickness occurs in two stages. The early, haemo-lymphatic stage and the later, meningo-encephalitic stage [221].

In the Chagas disease, *T. cruzi* is transmitted to humans by a triatomine bug, also known as "kissing bug" (**Figure 1-24 (b)**). This disease also occurs in two successive phases: an acute phase most often asymptomatic and a chronic phase with cardiac, digestive and/or neurological symptoms which can be life threatening if left undiagnosed and untreated [222].

Figure 1- 24 *: tsetse fly (a) and triatomine bug (b) vectors for the transmission of the sleeping sickness and the Chagas disease respectively [223,224]*



As against *Plasmodium*, the most active antitrypanosomal pentacyclic triterpene belongs to quinone methide derivatives: tingenin B (**figure 1-20**) is reported to have an IC_{50} < 0.25 mg/L against *T. brucei brucei* and *T. cruzi* [155,225].

Table 1-10 reports the IC_{50} of UA, OA and BA against *T. brucei brucei* or *T. cruzi*. Concerning their antitrypanosomal activity, except from the results reported by Cunha et al. [226], it seems that UA is more active than OA followed by BA, unlike the antiplasmodial activity.

***Table 1- 10** : Comparison between the antitrypanosomal activities of UA, OA and BA*

	IC50 (mg/L)		
***Trypanosoma* Species**	**UA**	**OA**	**BA**
T. brucei rhodesiense [211]	1.5 ± 0.7	5.1 ± 0.2	-
T. brucei brucei [227]	1.0 ± 0.2	2.9 ± 0.4	14.9 ± 2.9
T. brucei brucei [228]	2.5 ± 0.5	7.3 ± 3	11.2 ± 6.9
T. cruzi [226]	7.8	5.8	-
T. cruzi [229]	11.5	20.6	

According to Isah et al., the carboxylic group at C17 as well as the OH group at C3 are important for the antitrypanosomal activity of triterpenic acids [155]. Indeed, uvaol (aldehyde group replacing C17 COOH of UA), as well as α-amyrin and β-amyrin (CH_3 replacing the C17 COOH of UA and OA respectively) were less active than the parent compound [227]. Moreover, the addition of an acetyl-group in UA, OA or BA is accompanied by a loss of the antitrypanosomal activity in comparison to the parent compounds [230,231]. However, these structure-activity relationships stay limited to a few studies and need to be confirmed.

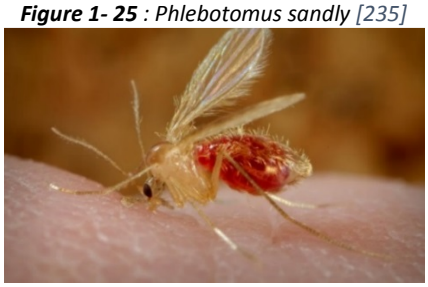
Concerning their mechanism of action, it is reported that UA, OA and BA are able to inhibit the *T. brucei* GAPDH, a specific glycolytic enzyme essential for the survival of the bloodstream stage of trypanosomes [228,232]. However, these effects can only explain one part of their antitrypanosomal activity.

3.2.2.3 Antileishmanial activity

Leishmania parasites are the causal agents of leishmaniasis, a group of protozoan diseases characterised by four main clinical manifestations: localised cutaneous leishmaniasis, diffuse cutaneous leishmaniasis, mucosal leishmaniasis and visceral leishmaniasis [233]. These diseases are associated with 0.7 to 1 million of new cases and cause 20 000 to 30 000 deaths annually [234].

Leishmania parasites are transmitted to mammals by phlebotomine sandflies (**figure 1-25**). More than 20 *Leishmania* species (*L. donovani*, *L. mexicana*, *L. major*,…) have about 70 natural reservoir hosts and vectors including animals, such as dogs or rodents, and human bodies, and are transmitted by more than 90 sandfly species [234]. The parasite exists as two different forms: flagellated promastigotes in insects and amastigotes, intracellular parasites in humans [233].

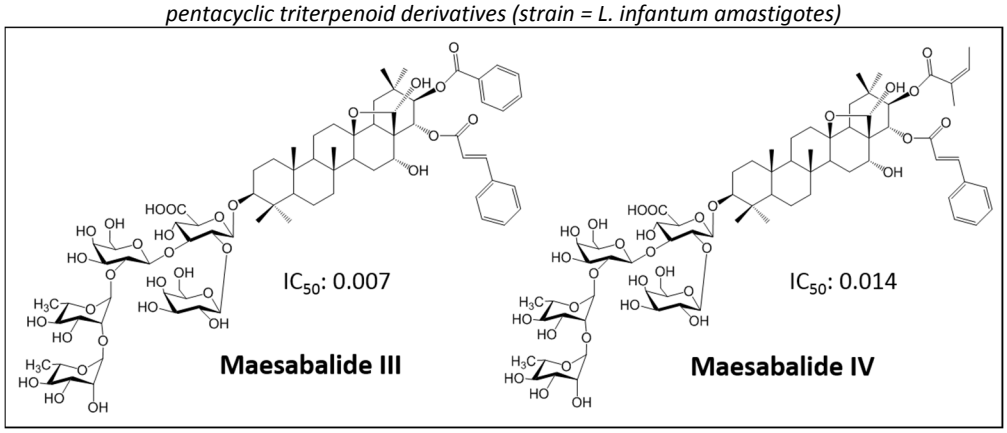
***Figure 1- 25** : Phlebotomus sandly [235]*



Existing drugs (pentavalent antimonials, amphotericin B,…) have serious drawbacks in terms of safety, efficacy, cost, difficulty in administration as well as the emergence of resistance [236]. As for malaria and trypanosomiasis, the screening of natural products has been shown to be a valuable approach to discover new antileishmanial leads [236].

The best antileishmanial activity among pentacyclic triterpenes was obtained for a range of saponin glycosides belonging to the oleanane group isolated from *Maesa balansae.* We can cite for example maesabalide III and IV which showed very low IC_{50} against *Leishmania infantum* amastigotes (**figure 1-26**).

***Figure 1- 26** : Structures and IC_{50} (mg/L) obtained for the strongest detected antileishmanial pentacyclic triterpenoid derivatives (strain = L. infantum amastigotes)*



The antileishmanial activities of UA and OA were compared in three studies while only one study compares the activities of the three acids (**table 1-11**) [237–239]. It seems that, as for the antitrypanosomal activity, UA is more active than OA, itself more active than BA.

***Table 1- 11** : Comparison between the antileishmanial activities of ursolic, oleanolic and betulinic acids*

Leishmania Species	IC50 (mg/L)		
	UA	OA	BA
L. donovani (promastigotes) [240]	0.040	0.040	-
L. donovani (amastigotes) [240]	0.005	0.030	-
L. donovani (amastigotes) [237]	1.1 ± 0.0	2.4 ± 0.0	> 4.5
L. amazonensis (amastigotes) [238]	11 ± 0	27 ± 1	-
L. amazonensis (promastigotes) [238]	5 ± 0	99 ± 1	-
L. amazonensis (promastigotes) [239]	9.1 ± 0.9	> 11.4	-
L. infantum (promastigotes) [239]	> 11.4	> 11.4	-
L. major (promastigotes) [240]	0.003	0.060	-
L. major (amastigotes) [240]	0.006	0.050	-

Concerning structure-activity relationship studies, the 3-O-acetylation of UA seems to reduce its activity against *L. amazoniensis* promastigotes (from 9.41 to > 11.4 mg/L) while the same structural modification increased the activity of OA on the same strain (from > 11.4 to 2.28 mg/L) [239]. Of note these modifications did not induce any change in the antileishmanial activity against another *Leishmania* strain (*L. infantum*) [239]. The 3-O acetylation of BA increased its activity against *L.*

amazonensis (from > 91 to 22.3 mg/L) but did not change its activity against *L. braziliensis* (> 91 mg/L) [231]. Structure-activity relationship studies are thus challenging given the variety of parasites species.

The mechanism of the antileishmanial activity of triterpenoids is not fully understood. It is reported that some pentacyclic triterpenes are excellent inhibitors of *Leishmania* DNA topoisomerase I and II, further inducing apoptosis of the parasite [241,242]. It is also proposed that due to their structure similarity to sterols, they can interfere with sterol metabolism, knowing that *T. cruzi* and *Leishmania* parasites have a strict requirement for specific endogenous sterols (ergosterol and analogues) for survival and growth [243].

3.2.3 Antibacterial activities

As explained before for *S. aureus,* antibiotic resistance is rising to dangerously high levels all over the world. New resistance mechanisms are emerging and spreading globally, threatening our ability to treat common infectious diseases such as tuberculosis, pneumonia, blood poisoning, gonorrhea, and foodborne diseases [6]. The World Health Organization established a global pathogen list of antibiotic-resistant bacteria to help in prioritizing the research and development of new and effective antibiotic treatments (**figure 1-27**). In this context, plant-derived pentacyclic triterpenoids could be an interesting source for the investigation of novel antibacterial compounds.

***Figure 1- 27** : WHO priority pathogen list for R&D of new antibiotics [244]*

PRIORITY 1 : CRITICAL #

Acinetobacter baumannii, carbapenem-resistant
Pseudomonas aeruginosa, carbapenem-resistant
Enterobacteriaceae, carbapenem-resistant, 3rd generation cephalosporin-resistant

PRIORITY 2 : HIGH

Enterococcus faecium, vancomycin-resistant
Staphylococcus aureus, methicillin-resistant, vancomycin intermediate and resistant
Helicobacter pylori, clarithromycin-resistant
Campylobacter, fluoroquinolone-resistant
Salmonella spp., fluoroquinolone- resistant
Neisseria gonorrhoeae, 3rd generation cephalosporin-resistant, fluoroquinolone-resistant

PRIORITY 3 : MEDIUM

Streptococcus pneumoniae, penicillin-non-susceptible
Haemophilus influenzae, ampicillin-resistant
Shigella spp., fluoroquinolone- resistant

Mycobacteria (including *Mycobacterium tuberculosis*), the cause of human tuberculosis, was not included as it is already a globally established priority for which innovative treatments are urgently needed.

UA, OA and BA were tested for their antibacterial activity on a wide range of bacterial strains, summarized in recent reviews [156,245,246]. They were shown to be active on several bacterial species with a higher potency on Gram-positive than on Gram-negative bacteria as well as a better activity for UA in comparison to OA; BA being in general inactive [245] (**table 1-12**).

Very few studies checked the impact of structural modifications on the antibacterial activity which is dependent on the bacterial strain. However, it was shown that the presence of an oxygenated group at C-3 as well as the carboxylic acid function at C-17 are important for the antibacterial activity [246].

Table 1- 12 *: Comparison between the antibacterial activities of ursolic, oleanolic and betulinic acids*

Bacteria Species and Strains		MIC (mg/L)		
		UA	**OA**	**BA**
Mycobacteria				
Mycobacterium tuberculosis	H37-RA [247]	12.5	50	50
GRAM -				
E. coli	TCC25922 [248]	>256	>256	>256
	ATCC35150 [249]	>128	>128	>128
	K12 [250]	>128	>128	>128
K. pneumoniae	MTCC109 [251]	>256	>256	>256
P. aeruginosa	ATCC27853 [248]	256	256	256
	ATCC27853 [249]	>128	>128	>128
	PAO1 [250]	>128	>128	>128
	MTCC429 [251]	>128	>128	>128
S. enterica	ATCC13311 [249]	>128	>128	>128
GRAM +				
B. cereus	ATCC9139 [249]	8	16	128
E. faecalis	ATCC29212 [248]	4	8	>256
	ATCC29212 [249]	1	4	128
	NCTC12201 [250]	4	8	>128
E. faecium	BM4147 [250]	4	8	>128
L.monocytogenes	ATCC7644 [249]	2	8	>128
S. aureus	MSSA ATCC25923 [248]	8	64	>256
	MSSA ATCC29213 [249]	16	>128	>128
	MRSA ATCC43300 [249]	64	>128	>128
S. mutans	MTCC 890 [251]	2	4	>128
S. pneumoniae	R6 [250]	8	16	>128
	MTCC655 [251]	8	16	>128
Vancomycin-resistant enterococci	VRE912 [251]	4	8	>128

As during this work, we mainly focused on *S. aureus* strains, we decided to further study the effect of structural modifications on the antistaphylococcal activity of pentacyclic triterpenes.

4. Natural and hemi-synthetic pentacyclic triterpenes as antimicrobials and resistance modifying agents against *Staphylococcus aureus*: A review

Adapted from Lucy Catteau, Li Zhu, Françoise Van Bambeke and Joëlle Quetin-Leclercq: **Natural and hemi-synthetic pentacyclic triterpenes as antimicrobials and resistance modifying agents against *Staphylococcus aureus*: A review.** *Phytochemistry reviews*, 2018, 1-35. doi:10.1007/s11101-018-9564-2.

Abstract

Staphylococcus aureus infections are considered as seriously problematic for human health and necessitate the development of new medicines and innovative antimicrobial strategies. Plant secondary metabolites have already demonstrated their potential as antibacterials when used alone but also in combination with other antimicrobial agents to potentiate their activity. Triterpenoids are widely distributed in the plant kingdom and known to have many beneficial effects, including anti-inflammatory, immunomodulatory, anti-proliferative, antimycotic, or antimicrobial activities. The aim of this paper is to review the activity of pentacyclic triterpenoids belonging to the ursane, oleanane, or lupane groups against *Staphylococcus aureus*. We summarize their activity as anti-staphylococcal compounds but also as resistance modifying agents when combined with common antibiotics.

4.1 Introduction

Due to the emergence and propagation of antimicrobial resistance and to the difficulties to develop new antimicrobials, innovative strategies to control and combat bacterial infections are highly needed. In this review we focus on the ability of pentacyclic triterpenes to fight *Staphylococcus aureus* infections.

S. aureus is a Gram-positive round shaped bacterium, belonging to the *Staphylococcaceae* family. While *S. aureus* usually colonizes asymptomatically the nose or the skin of approximately 30% of the human population, it can also cause severe diseases in humans [4–6]. *S. aureus* is one of the most common causes of bacteremia and infective endocarditis, of various skin and soft tissue infections, and of infections developing on medical devices [5]. β-Lactam antibiotics are often considered as the first line of treatment. However, inappropriate use of this class of antibiotics has led to an increasing prevalence of bacterial resistance. The main mechanisms of β-lactams resistance in *S. aureus* consist in (a) the production of specific enzymes inactivating the antibiotics (β-lactamases) and (b) the production of PBP2a, an additional penicillin-binding protein with decreased affinity for methicillin and all conventional β-lactam antibiotics. The production of this modified protein (PBP2a) is associated to the so-called MRSA (Methicillin-Resistant *Staphylococcus aureus)* phenotype [252]. Recent epidemiological studies indicate that the prevalence of MRSA is high over the world, representing a significant public health challenge [253,254]. Therapeutic options available for treating MRSA infections have become seriously limited. Indeed, MRSA have also acquired resistance mechanisms to most of the other available antibiotic classes [255,256]. Although ceftaroline, telavancin, tedizolid, dalbavancin, oritavancin and delafloxacin have been recently registered as anti-MRSA drugs, new and original strategies are still needed [257].

Triterpenoids belong to the group of terpenes. They represent a wide class of natural compounds with approximately 30,000 identified structures, mainly found in the vegetal kingdom but also synthetized by other organisms [140,258]. All terpenes are derived from C_5 isoprene units, and, based on the number of units, are classified as hemiterpenes (C_5) monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), sesterterpenes (C_{25}), triterpenes (C_{30}) and tetraterpenes (C_{40}) [138]. In nature, triterpenoids are often found as tetra- or penta-cyclic structures, but acyclic, mono-, bi-, tri- and hexa-cyclic triterpenes also exist [258]. Pentacyclic triterpenes can be divided into three representative classes, namely ursanes, oleananes, and lupanes, which all include interesting bioactive compounds with analgesic, immunomodulatory, anti-inflammatory, anticancer, antioxidant, antifungal as well as antibacterial activities [148,151,177,259].

Moreover, in the past few years, the use of phytochemicals as resistance-modifying agents has become an increasingly active research topic. This strategy of modifying resistance pattern is already used in the clinics, as illustrated by clavulanic acid, a well-known β-lactamases inhibitor, which, despite its weak antimicrobial activity, is remarkably efficient to control a wide range of bacterial infections when combined with amoxicillin [260]. As their targets are often different from those of conventional antibiotics, phytochemicals could be used in combination with them for the treatment of resistant infections. For example, corilagin, tellimagrandin I, rugosin B or baicalein are polyphenols described as synergistic with β-lactams against MRSA due to their capacity to inhibit PBP2a [102,116,117,261]. Given the widespread occurrence and use of pentacyclic triterpene derivatives, this review will focus on the potential of these compounds as anti-staphylococcal agents or as adjuvants capable of restoring the activity of common antibiotics against *Staphylococcus aureus* resistant strains.

4.2 Pentacyclic triterpenes as anti-staphylococcal compounds

4.2.1 In vitro antimicrobial susceptibility testing

Antimicrobial susceptibility tests are used as early screening in the search of new antimicrobial agents. Standard methods include disk diffusion and agar or broth dilution methods [262].

Diffusion methods, mostly the agar disk diffusion method, are commonly used for antimicrobial screening of plant extracts, essential oils and other drugs [263–265]. However, these methods give more qualitative than quantitative results by detecting if the bacteria are susceptible, intermediate or resistant to the tested compound [266]. Moreover, they possess different limitations: poor diffusible compounds will be inactive in these assays and no minimum inhibitory concentration (MIC) can be determined as it is impossible to quantify the amount of the antimicrobial agent having diffused into the agar. Approximate values can though be calculated for some organisms and antibiotics by comparing the inhibition zone with stored algorithms [267].

MICs can be determined using agar as well as micro- or macro-broth dilution techniques. Microdilution method presents different advantages as it avoids the manipulation of several tubes, needs less compound and is cost-saving. To reduce the variations in MIC values due to the change of inoculum size, medium, incubation time,…, broth dilution has been standardized by CLSI and EUCAST [130,262]. The EUCAST broth dilution method is similar to that of CLSI with some modifications concerning specific test parameters such as inoculum size, inoculum preparation and the MIC reading method which is visual in CLSI assay and spectrophotometric in EUCAST guidelines [267].

Of note, to study in more detail the effect of an agent on bacteria, time-kill tests or flow cytofluorometric methods can be used. These methods provide information on

the nature of the inhibitory effect: bactericidal or bacteriostatic (time-dependent or concentration-dependent) and the damage inflicted to the cells [267].

Due to the large panel of methods used and of tested microorganisms, reported activity of natural compounds are highly variable among studies [268]. In this review, we therefore only selected papers reporting MIC of pure compounds as assessed using dilution methods, which is the most commonly used technique.

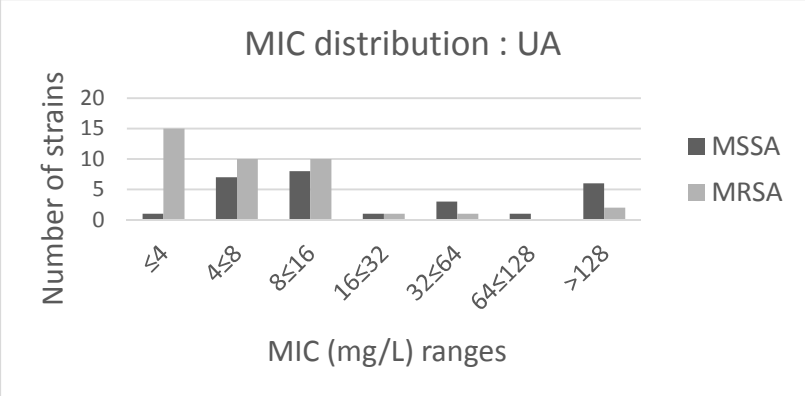
4.2.2 Pentacyclic triterpenes with anti-staphylococcal activities

Bibliographic searches were performed with the key words *triterpenoid*, *triterpene*, *ursolic*, *oleanolic* or *betulinic* together with *Staphylococcus aureus* and *MIC* on three libraries including *Pubmed*, *Scifinder* and *Scopus*. According to several authors, activity is considered as significant if MIC ≤ 10 mg/L, moderate if 10 < MIC ≤ 100 mg/L, and low or negligible if MIC > 100 mg/L [105–108]. Yet as the dilution tests used to determine MIC often use geometric dilutions starting from a concentration of 128 mg/L we considered here that compounds were active if MIC ≤ 16 mg/L, moderately active if 16 < MIC ≤ 128 mg/L, and had low or negligible activity if MIC > 128 mg/L. The 78 pentacyclic triterpenes showing significant antistaphylococcal activity against at least one strain were subdivided in three categories according to their ursane, oleanane or lupane skeletons, differing in the position of the two methyl groups on the E-ring (ursane and oleanane), or a 5-membered E ring (lupane). The most tested compounds in these series were ursolic acid (UA), oleanolic acid (OA) and betulinic acid (BA), which were chosen as reference compounds for each series respectively. First we report and discuss the activity of all compounds showing at least one significant antistaphylococcal activity when tested on reference or clinical methicillin-sensitive (MSSA) and methicillin-resistant (MRSA) strains. When available, we also discuss possible structure-activity relationships (SAR) inside each of the three groups of compounds.

4.2.2.1 Ursane derivatives

The compounds with an ursane skeleton showing a significant antistaphylococcal activity are reported in **table 1-13.** In this group of molecules, UA is the most studied, with MICs ranging from 4 to 1024 mg/L on MSSA or MRSA strains (**figure 1-28**).

***Figure 1- 28** : Representative MIC distribution of UA against MSSA and MRSA strains.*

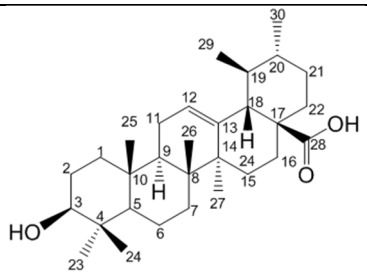


Surprisingly huge variations are observed when testing UA on the same reference strain. The most plausible explanation for these discrepancies is a difference in the origin and purity of the tested product. For example Rizvi et al. (2009), who found high MIC values, did not indicate the purity of UA they extracted from the leaves of *Ficus lyrata* (Moraceae). Likewise, Na et al. (2016) did not mention the origin or the purity of the commercial product they used. Globally, however, MICs of UA are ≤ 16 mg/L for more than half of MSSA (14/25) and the majority of MRSA (30/33) tested. Of note, Kim and coworkers reported MICs of 4 mg/L against 15/19 MRSA.

The other natural or hemi-synthetic compounds with the same skeleton as UA having an MIC ≤ 16 mg/L on at least one *S. aureus* strain are described here under, according to their structural difference with UA.

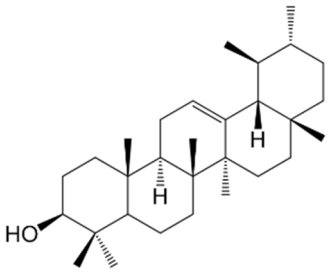
Table 1- 13 *: Minimal inhibitory concentrations (MICs) of ursane derivatives on S. aureus strains. Number between brackets correspond to the number of tested strains.*

Ursolic acid



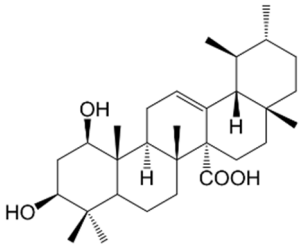
MSSA strains	MIC (mg/L)	MRSA strains	MIC (mg/L)
nd	500[1]	**nd**	1000[6]
ATCC 12624	≥ 1024[2]	**ATCC 33591**	16[15, 33]
ATCC 12692	≥ 1024[2]	**ATCC 43300**	10[22]; 15.62[8]; 16[9]; 64[12]
ATCC 25923	10[3]; 5.7[4]; 62.5[5] ; 500[6] ; 8[19,23]	**MTCC-84**	16[21]
ATCC 29213	12.5[7]; 7.81[8]; 4[9]; 125[10] ; 62.5[11]; 16[12]; 12.5[13] ; 8[23]	**Clinical**	15.7 ± 12.63(8)[9] ; 4(15)[19] ; 8(10)[15,19,33,25]; 16(2)[15,33]; >256[23]
ATCC 6538	32[2] ; 16[15]	**Multi-drug-resistant**	25[24]
ATCC 6571	11.4[14]	**Amoxiclav-resistant**	12.5[13]
CECT976	15.6[16]		
CMCC26001	50[18]		
MTCC 6908	160[20]		
MTCC-737	8[21]		
Clinical	630(1)[17]; 7.8 ± 0.8(8)[9]		
Amoxiclav-sensitive	12.5[13]		

α-amyrin



MSSA strains	MIC (mg/L)	MRSA strains	MIC (mg/L)
ATCC 29213	64[26,27]	**ATCC 43300**	64[26] ; 32-64[27]
16 clinical	4(1)[27] ; 8(1) [27] ; 16(4)[27] ; 32(8)[27] ; 64(2)[27]	**16 clinical**	2(1)[27] ; 4(1)[27] ; 8(1)[27] ; 16(3)[27] ; 32 (6)[27] ; 64(4)[27]

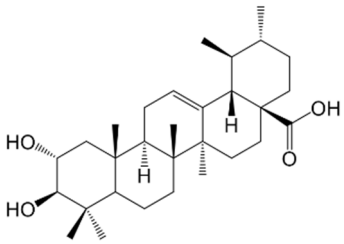
1β,3β-dihydroxyurs-12-en-27-oic acid



MSSA strains	MIC (mg/L)	MRSA strains	MIC (mg/L)
Clinical (strain 375)	4[28]	**Clinical (strain 310)**	4[28]

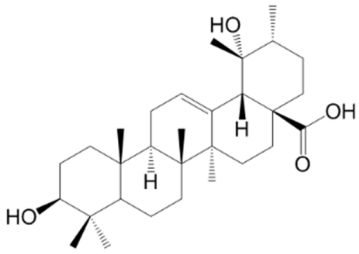
Corosolic acid

(2α-hydroxy ursolic acid)



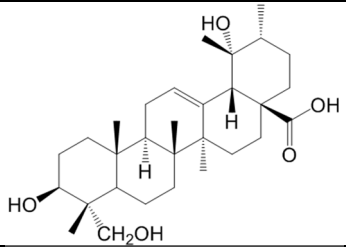
MSSA strains	MIC (mg/L)	MRSA strains	MIC (mg/L)
ATCC 6538	16[15]	**ATCC 43300**	7[22]
ATCC 29213	32[11]	**ATCC 33591**	8[15]
CMCC26001	100[18]	**Clinical (C100459)**	16[15]
		Clinical (C98506)	16[15]

Pomolic acid



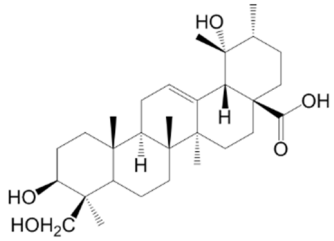
MSSA strains	MIC (mg/L)	MRSA strains	MIC (mg/L)
NBRC 100910	10.52[29]	**-**	-

Rotundic acid



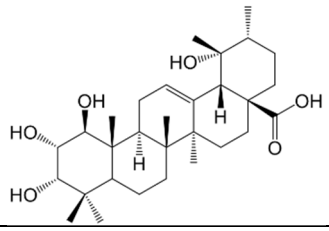
MSSA strains	MIC (mg/L)	MRSA strains	MIC (mg/L)
NBRC 100910	10[30]	**-**	-

Rotungenic acid



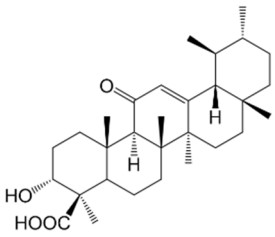
MSSA strains	MIC (mg/L)	MRSA strains	MIC (mg/L)
NBRC 100910	2.5[30]	**-**	-

1β-hydroxyeuscaphic acid



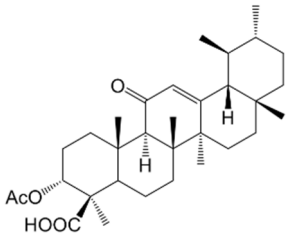
MSSA strains	MIC (mg/L)	MRSA strains	MIC (mg/L)
ATCC 25922	7.81[34]	**-**	-

11-keto-β-boswellic acid



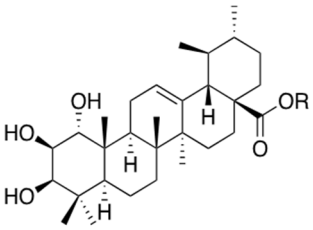
MSSA strains	MIC (mg/L)	MRSA strains	MIC (mg/L)
ATCC 29213	16[31]	**ATCC 33591**	16-32[31]
		50 clinical isolates	16-32[31]

Acetyl-11-keto-β-boswellic acid



MSSA strains	MIC (mg/L)	MRSA strains	MIC (mg/L)
ATCC 29213	2[31]	**ATCC 33591**	2-4[31]
		50 clinical isolates	2-4[31]

Polyhydroxylated esters



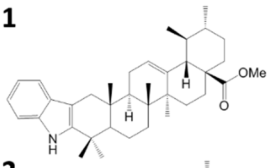
A R = $-CH_3$
B R = $-CH_2CH_3$
C R = $-(CH_2)_2CH_3$
D R = $-CH(CH_3)_2$
E R = $-(CH_2)_3CH_3$

MSSA strains	MIC (mg/L)	MRSA strains	MIC (mg/L)
ATCC 25923	A, C, E : 8[35] B : 2[35] D : 1[35]		

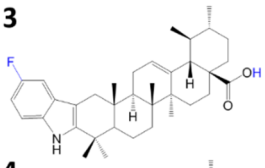
Carbazole derivatives

1-4

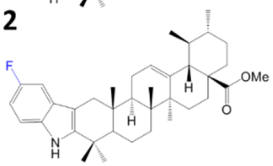
1



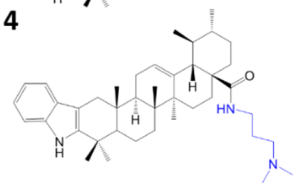
3



2



4

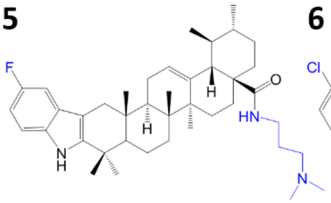


MSSA strains	MIC (mg/L)	MRSA strains	MIC (mg/L)
CGMCC 1.1361	15.6[32]	-	-

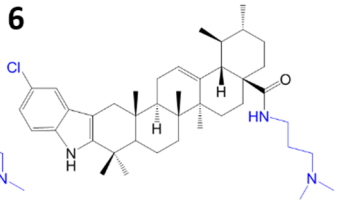
Carbazole derivatives

5-6

5



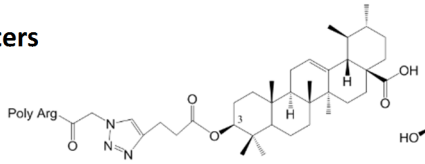
6



MSSA strains	MIC (mg/L)	MRSA strains	MIC (mg/L)
CGMCC 1.1361	7.8[32]	-	-

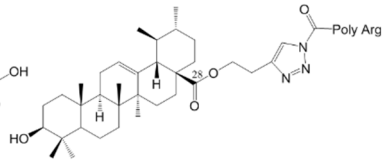
Polyarginine esters

1 : Derivatives UAc-R



UAc-R2, -R3, -R5, -R8

2 : Derivatives UAo-R



UAo-R1, -R5, -R8

R = number of arginine residues in the polyarginine

MSSA strains	MIC (mg/L)	MRSA strains	MIC (mg/L)
ATCC 25923	7.8[5]	-	-

Polyarginine esters		Derivatives UAo-R UAo-R2, -R3 R = number of arginine residues in the polyarginine	
MSSA strains	**MIC (mg/L)**	**MRSA strains**	**MIC (mg/L)**
ATCC 25923	3.9[5]	-	-

[1][271], [2][177], [3][123], [4][272], [5][270], [6][269], [7][273], [8][124], [9][274], [10][108], [11][275], [12][249], [13][276], [14][277], [15][278], [16][279], [17][280], [18][281], [19][282], [20][283], [21][251], [22][284], [23][248], [24][285], [25][250], [26][286], [27][287], [28][288], [29][289], [30][290], [31][291], [32][292], [33][293], [34][294], [35][278]

Loss of the carboxylic acid function: α-amyrin

α-Amyrin has MICs ranging between 4 and 64 mg/L and between 2 and 64 mg/L against MSSA and MRSA strains, respectively [286,287]. Even if no study directly compares α-amyrin to UA, the different data obtained using the same strain (i.e. MSSA ATCC29213) report MIC of 64 mg/L for α-amyrin *vs.* ≤ 16 mg/L (6/8 values) for UA [108,124,248,249,273–276,287]. Furthermore, α-amyrin has a MIC ≥ 16 mg/L on most of the tested strains (30/35). The presence of the carboxylic function of UA seems thus important for its direct activity.

Mono-hydroxylation: 1β,3β-dihydroxyurs-12-en-27-oic, corosolic and pomolic acid

In comparison to UA, 1β,3β-dihydroxyurs-12-en-27-oic acid possesses a carboxylic function in position C-27 instead of C-28 and an additional hydroxyl group in position C-1. UA was not tested in parallel to this compound but its reported MICs are lower than most of the MICs reported for UA, except those from the study of Kim et al. [282].

The antisaphylococcal activity of corosolic acid (2α-hydroxy ursolic acid) was studied in four publications [275,278,281,284] in comparison with UA. Ngezahayo and coworkers observed an improved activity compared to UA for only 1 strain (MRSA ATCC33591), while an equal activity was obtained on MSSA ATCC6538 and

the clinical MRSA isolate C100459 and a lower activity on the MRSA clinical isolate C98506 [278]. Nevertheless, the three other authors showed lower MICs for corosolic acid in comparison to UA, confirming the general positive effect of 2α-hydroxylation of UA [275,281,284].

Pomolic acid (19-hydroxy ursolic acid) was only tested on the MSSA NBRC 100910 [289] with a lower or equivalent MIC than the range previously reported for UA on most MSSA strains in other studies.

Mono-hydroxylation of UA, at least at positions 1, 2 or 19 seems thus to slightly increase the antistaphylococcal activity of UA.

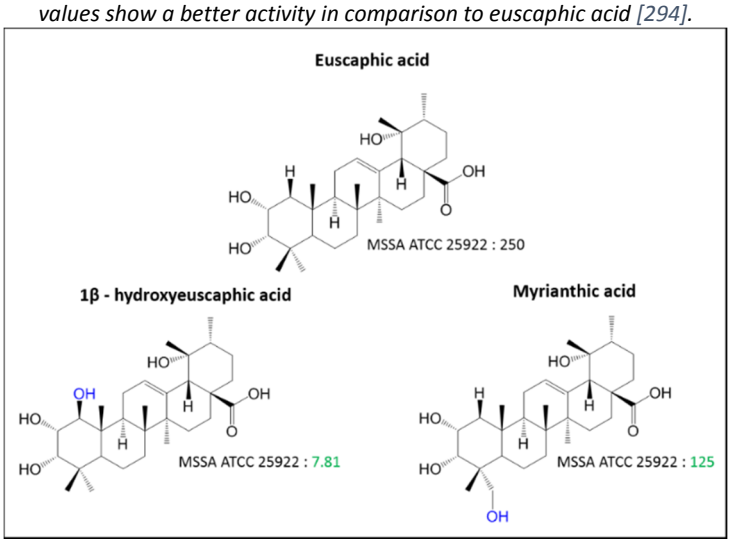
Di-hydroxylation : rotundic and rotungenic acids

Rotundic and rotungenic acids are derivatives of UA presenting a first hydroxyl group in α C-19 and a second one on C-23 (rotundic acid) or on C-24 (rotungenic acid) which were only tested on one MSSA strain. This study shows the importance of the absolute configuration of C-4, the 4S configuration (in rotungenic) conferring higher activity than the 4R [290].

Tri-hydroxylation : 1β-hydroxyeuscaphic acid

This ursane derivative corresponding to UA with three additional hydroxyl groups (βC-1, αC-2 and αC-19) was isolated together with an isomer (myrianthic acid; αC-2 and αC-19, C-23) as well as its dehydroxylated derivative (euscaphic acid; αC-2 and αC-19) from the seeds of *Butyrospermum paradoxum* (Don) Hepper (Sapotaceae) (**figure 1-29**). 1β-hydroxyeuscaphic acid is the only derivative showing a significant antistaphylococcal activity. The authors suggest that the hydroxylation on the first ring could be responsible for an increase in the antistaphylococcal activity as myrianthic acid also showed a slight improvement of the activity compared to euscaphic acid, but this has to be confirmed on other strains [294].

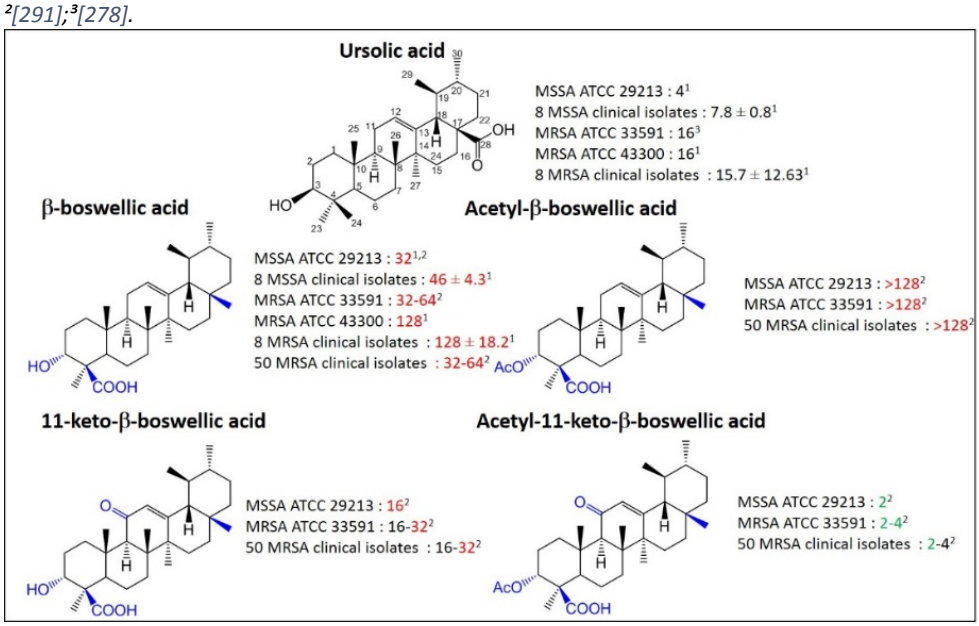
Figure 1- 29 *: Minimal inhibitory concentrations (MIC) in mg/L of euscaphic acid derivatives. Green values show a better activity in comparison to euscaphic acid [294].*



β-Boswellic acid derivatives: 11-keto-β-boswellic and acetyl-11-keto-β-boswellic acids

β-Boswellic acid is a UA derivative having the carboxylic function in C-24 instead of C-28 (for UA) and a configuration change of the C-3 hydroxyl group (α instead of β for UA). Its activity [274] and that of derivatives [291] are shown in **figure 1-30**. β-boswellic acid was tested against 69 strains including 66 clinical and 3 reference *S. aureus* strains and showed lower activity than UA (MICs > 32 mg/L). Acetylation in position 3 completely suppresses its antimicrobial activity (MIC > 128 mg/L). However, when a ketone group is added in position C-11 of both compounds, the MIC values were similar (11-keto-β-boswellic acid) or lower (acetyl-11-keto-β-boswellic acid) as compared to those of UA. Acetyl-11-keto-boswellic acid is the most active in the series (MIC of 2 mg/L) [291]. The addition of a keto-group on the position C-11 seems thus to improve antistaphylococcal activity of boswellic acid derivatives, but no 11-keto derivatives of UA were tested.

Figure 1- 30 *: Minimal inhibitory concentrations (MIC) in mg/L of ursolic acid and β-boswellic acid derivatives. Green/Red values show a better/worse activity in comparison to ursolic acid.[1][274]; [2][291];[3][278].*



Polyhydroxylated esters

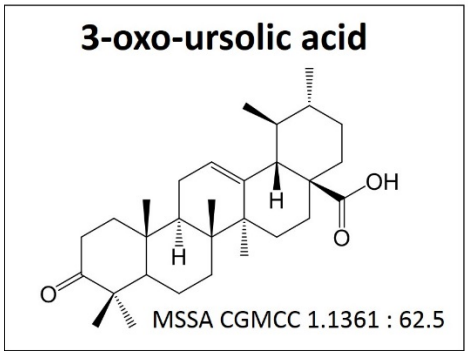
Huang and coworkers synthesized UA derivatives with a polyhydroxylated A-ring as well as an esterification with a short alkyl chain in C-17. Among them, 5 display a significant activity (MIC ≤ 8 mg/L) against MSSA ATCC25923 [295]. During a preliminary evaluation of the antimicrobial activity limited to one MSSA strain, they demonstrate that introducing two hydroxyl groups at positions C-1 and C-2 together with a small alkyl ester group at C-17 of UA enhanced growth-inhibiting activity against *S. aureus* [295]

Semisynthetic derivatives: carbazoles and polyarginin derivatives

Gu and his collaborators synthesized a series of new carbazole derivatives of UA in an attempt to develop potent antimicrobial agents. They tested their compounds on three fungi, two Gram-negative bacteria, and two Gram-positive bacteria including one MSSA strain. The compounds showing significant antistaphylococcal activity are reported in **table 1-13**.

Two limitations of this work are that the activity of the synthesized compounds was not compared to that of UA but to that of an intermediate compound (3-oxo-UA, **figure 1-31**) and that they suggest structure-activity relationships on the basis of the results obtained only against one MSSA strain. Comparing to 3-oxo-UA, SAR indicate that the incorporation of a carbazole moiety was beneficial to the antimicrobial activity as well as a N-(dimethylamino)propyl amide side chain at C-28 position and an addition of a strong electro-attractive substituent on the aromatic ring of the carbazole [292].

Figure 1- 31 *: Structure and MIC (mg/L) of the synthesis intermediate used by Gu et al. (2015) to form carbazoles derivatives [292].*



In another study, Na et al. synthesized 16 UA derivatives conjugated with cationic polyarginine peptides, considered as cell-penetrating peptides which had previously shown to inhibit the growth of *S. aureus* with a moderate potency [270]. These compounds differed by the number of arginine residues fixed (1 to 8) and the position of the conjugation (C-3 or C-28). The majority of synthesized compounds showed a significant antimicrobial activity on the reference strain MSSA ATCC25923 (MICs from 3.9 to 7.8 mg/L), which was better than that of UA (MIC = 62.5 mg/L) [270].

4.2.2.2 Oleanane derivatives

OA and its derivatives having a significant antistaphylococcal activity (MIC ≤ 16 mg/L) are reported in **table 1-14**. OA was the most studied compound in this group, with 19 authors reporting MICs comprised between 1.9 and 3750 mg/L against *S. aureus* strains (**Figure 1-32**). For the reference strain MSSA ATCC25923, MICs vary from 32 to 3750 mg/L [123,282,296–298], the lowest value being obtained for a commercially available compound from *Sigma.* The four other studies used OA isolated from Pomace olive (*Olea europaea L.*) (MIC = 95 mg/L), aerial parts of *Lotus corniculatus* (MIC = 800 mg/L) and of *Salvia chamelaeagnea* (MIC = 3750 mg/L), or from the roots of *Vernonia auriculifera* (MIC = 500 mg/L). None of these papers mentioned the purity of the compound, which, as described for UA, could explain the discrepancies reported in activities. However, excepted the low MIC (1.9 mg/L [299]) reported for one strain, all MIC values obtained for OA are > 8 mg/L suggesting that it is less potent than its isomer UA.

Figure 1- 32 *: Representative MIC distribution of OA against MSSA and MRSA strains.*

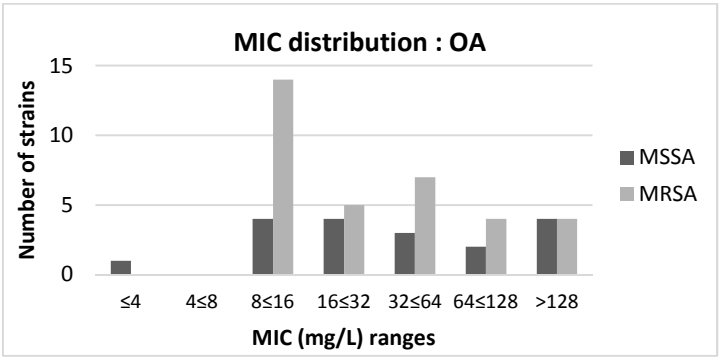
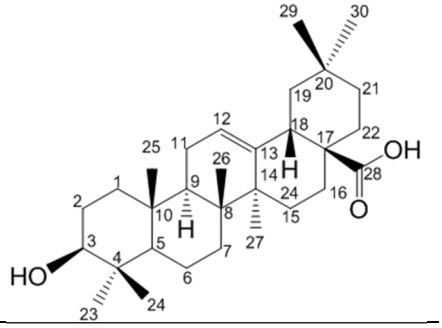


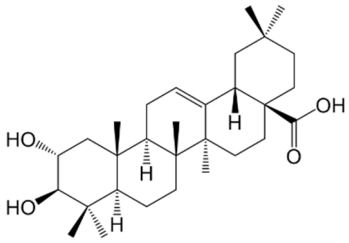
***Table 1- 14** : Minimal inhibitory concentrations (MICs) of oleanane derivatives on S. aureus strains. Number between brackets correspond to the number of tested strains.*

Oleanolic acid



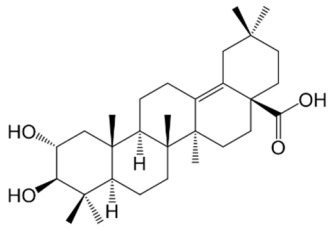
MSSA strains	MIC (mg/L)	MRSA strains	MIC (mg/L)
nd	1,9[1]	**ATCC 43300**	100[3]; 64[6]; >128[8]
ATCC 25923	95 ± 2[2]; 800[3]; 3750[4]; 500[5]; 32[11] ; 64[27]	**ATCC 33591**	32[26]
		MTCC-84	32[12]
ATCC 29213	16 ± 0[6]; 125[7]; >128[8] ; 32[27]	**Clinical**	80 ± 16 (8)[6];16(12)[11]; 32(3)[11] ; 64(3)[11]; 128(1)[11]; 16(2)[19]; 64(3)[26]; 128(1)[26] ; >256[27]
ATCC 6571	22,8[9]	**Multi-drug-resistant**	>9,76[16]; 312,5[17];400[18]
CECT 976	62,5[10]		
MTCC 737	16[12]		
MTCC 96	35[13]		
NBRC 100910	10[14,15]		
Clinical	19,83 ± 3,4 (8)[6]		

Maslinic acid



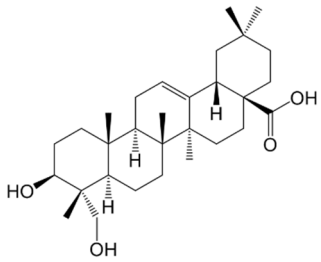
MSSA strains	MIC (mg/L)	MRSA strains	MIC (mg/L)
nd	7,8[1]	**-**	-
ATCC 25923	15[2]		

2α,3β-dihydroxyol-ean-13(18)-en-28-oic acid



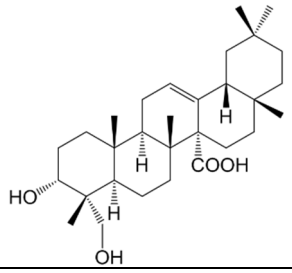
MSSA strains	MIC (mg/L)	MRSA strains	MIC (mg/L)
nd	15,6[1]	-	-

Hederagenin



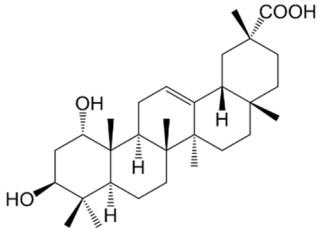
MSSA strains	MIC (mg/L)	MRSA strains	MIC (mg/L)
nd	4[20]	-	-
ATCC 29213	31[7]		

Aceriphyllic acid A



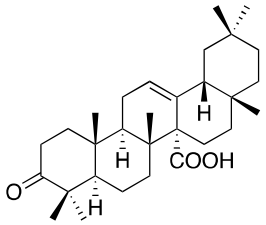
MSSA strains	MIC (mg/L)	MRSA strains	MIC (mg/L)
RN4220	4[21]	**CCARM 3506**	4[21]
503	2[21]	**CCARM 3167**	4[21]
KCTC 1916	4[21]		

1α,3β-hydroxyimberbic acid



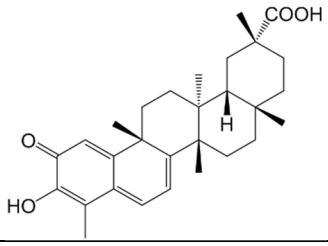
MSSA strains	MIC (mg/L)	MRSA strains	MIC (mg/L)
nd	3,13[23]	**-**	-

3-oxo-olean-12-en-27-oic acid



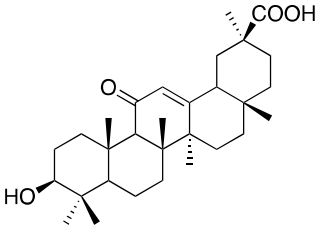
MSSA strains	MIC (mg/L)	MRSA strains	MIC (mg/L)
RN4220	4[21]	**CCARM 3506**	4-8[21]
503	2[21]	**CCARM 3167**	4[21]
KCTC 1916	2[21]		

Celastrol



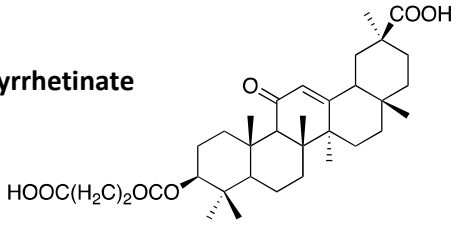
MSSA strains	MIC (mg/L)	MRSA strains	MIC (mg/L)
ATCC 29213	0,9[22]	**Clinical**	0,9[22]

Glycyrrhetinic acid (GRA)

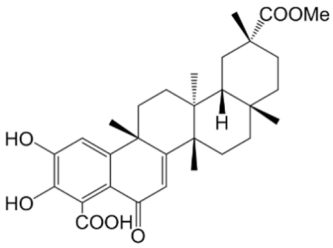
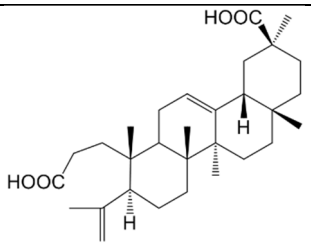


MSSA strains	MIC (mg/L)	MRSA strains	MIC (mg/L)
Clinical	16(1) ; 64(3) ; 128(5) ; 256(7) ; 512(15)	**Clinical**	32(4) ; 64(4) ; 128(3) ; 256(2) ; 512(6)

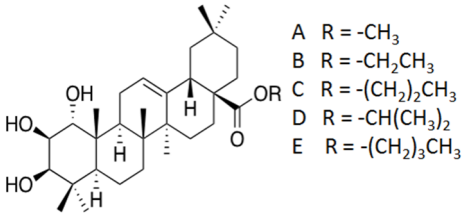
Disodium 3-succinyloxy β-glycyrrhetinate (GR-SU)



MSSA strains	MIC (mg/L)	MRSA strains	MIC (mg/L)
Clinical	16(1) ; 32(4) ; 64(9) ; 128(5) ; 256(12)	**Clinical**	16(2) ; 32(3) ; 64(3) ; 128(9) ; 256(2)

Zeylasterone			
MSSA strains	**MIC (mg/L)**	**MRSA strains**	**MIC (mg/L)**
ATCC6538	5[25]	-	-
Clinical (LMM1)	5-10[25]		
Koetjapic acid			
MSSA strains	**MIC (mg/L)**	**MRSA strains**	**MIC (mg/L)**
ATCC 6535	>6,25[24]	**ATCC 33591**	12,5[24]

OA or maslinic acid semi-synthetic derivatives (see figure 6)

MSSA strains	**MIC (mg/L)**	**MRSA strains**	**MIC (mg/L)**
ATCC 25923	5-15[2]	**-**	**-**
Polyhydroxylated esters			
MSSA strains	**MIC (mg/L)**	**MRSA strains**	**MIC (mg/L)**
ATCC 25923	D, E : 8[28] C : 2[28] B : 1[28] A : inactive[28]		

[1][299], [2][296], [3] [297], [4][123], [5][298], [6][274], [7][108], [8][249], [9][277], [10][279], [11][300], [12][251], [13][301], [14][289], [15][290], [16][302], [17][303], [18][285], [19][250], [20][304], [21][305], [22][306], [23][307], [24][308], [25][309], [26][293], [27][248], [28][295]

Mono-hydroxylation: maslinic acid; 2α,3β-dihydroxyol-ean-13(18)-en-28-oic acid; hederagenin; aceriphyllic acid A and 1α,3β-hydroxyimberlic acid

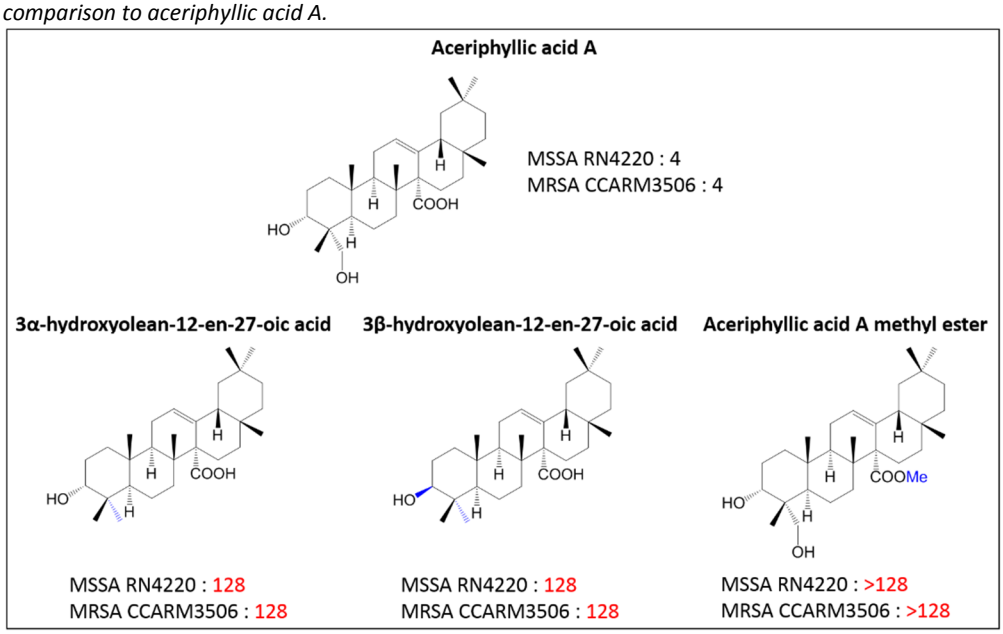
Maslinic acid (MA: 2α-hydroxy oleanolic acid) is, according to the authors, more potent [296] or less potent [299] than OA. More data are thus necessary to determine the effect of this additional hydroxyl group.

2α, 3β-dihydroxyol-ean-13(18)-en-28-oic only differs from MA by the position of the double bond ($\Delta^{13,18}$ against $\Delta^{12,13}$ for MA) . Analyzing its activity on one MSSA strain, Wang et al (2015) suggested that the modification of the position of the double bond has a negative effect on the antistaphylococcal activity as this compound is less effective than MA [299].

Hydroxylation at C-23 of OA (hederagenin) seems to improve the activity as a MIC of 4 mg/L - lower than the mean MIC of OA - was obtained on a MSSA strain [304]. By testing both hederagenin (MIC = 31 mg/L) and OA (MIC = 125 mg/L) on the same strain (MSSA ATCC29213), Ndjateu and coworkers confirmed this hypothesis [108].

Aceriphyllic acid A differs from hederagenin by the configuration of the C-3 hydroxyl group (α instead of β for hederagenin) and by the position of the carboxyl-group (C-27 instead of C-28 for hederagenin). It was isolated together with 2 other OA hydroxyl-derivatives from the roots of *Aceriphyllum rossii* (Saxifragaceae) (**figure 1-33**). The authors also synthetized its methyl-ester. They mentioned the importance of the hydroxyl group in position C-23 as its non-hydroxylated derivatives have a negligible antistaphylococcal activity. Moreover, the presence of the carboxylic group is also important as its methyl-ester is not active on the tested *S. aureus* strains [305].

Figure 1- 33 *: Minimal inhibitory concentrations (MIC) in mg/L of aceriphyllic acid A and derivatives isolated from Aceriphyllum rossii by Zheng et al. (2008) [305]. Red values show worse activity in comparison to aceriphyllic acid A.*



1α,3β-hydroxyimberbic acid is the last mono-hydroxylated derivative of OA having a significant antistaphylococcal activity [307]. This compound is characterized by a carboxylic function on C-29 and an added α-hydroxyl-group in C-1. It was isolated together with 4 of its glucosides from the leaves of *Combretum imberbe* (Combretaceae). These glucoside derivatives all show interesting antistaphyloccocal activities (MIC ≤ 25mg/L), two of them having a MIC of 6.25 mg/L on the same MSSA strain [307]. However, the activity of these compounds was not compared to that of OA.

Ketonic derivatives : celastrol, 3-oxo-olean-12-en-27-oic acid, glycyrrhetinic acid and disodium 3-succinyloxy β-glycyrrhetinate

3-oxo-olean-12-en-27-oic acid isolated from the roots of *Aceriphyllum rossii* Engler. (Saxifragaceae) shows significant activity against 5 *S. aureus* (both MSSA and MRSA) strains [305].

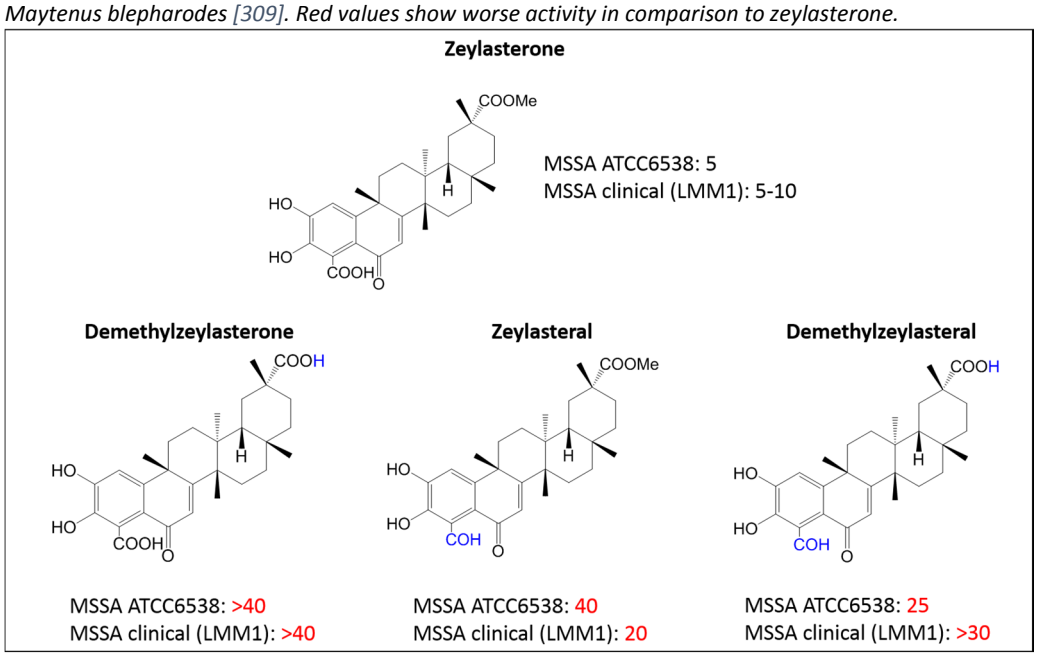
During a screening to identify inhibitors of *S. aureus* biofilm formation, Woo et al. selected celastrol among 1280 tested natural compounds [306]. This pentacyclic triterpene possesses a hydroxyquinone in the A cycle and a carboxylic function on C-29. It showed a lower MIC than that reported by other teams for OA tested against the same strain [108,249,274,306].

Glycyrrhetinic acid (GRA) and disodium 3-succinyloxy β-glycyrrhetinate (GR-SU) were tested on 50 clinical *S. aureus* strains, including 19 MRSA with MICs ranging between 16 and 512 mg/L (GRA) and between 16 and 256 mg/L (GR-SU) [310]. While the authors attribute a strong antistaphylococcal activity to these compounds, only a low proportion of strains actually showed a MIC of 16 mg/L (1/50 for GRA and 3/50 for GR-SU).

Dicarboxylated derivatives: zeylasterone

6-oxophenolic triterpenoids (zeylasteral, demethylzeylasteral, zeylasterone and demethylzeylasterone) from *Maytenus blepharodes* Lundell (Celastraceae) were tested for their antistaphylococcal activities by Kim et al., 2015 [283]. Out of these four compounds (**figure 1-34**), only zeylasterone can be considered as active on staphylococci. The authors attribute the lack of activity of zeylasteral and demethylzeylasteral to the absence hydrogen-bond-donor (HBD) group at C-4. Moreover, they suggest that the lipophilicity, which can be increased by esterification, associated with solubility and permeability through cell membrane, is essential for its activity, explaining the enhanced activity of zeylasterone vs. demethylzeylasterone, but this was not observed for other methyl esters of triterpenic acids (see above: aceriphyllic acid methyl ester).

***Figure 1- 34** : Minimal inhibitory concentrations (MIC) in mg/L 6-oxophenolic triterpenoids from Maytenus blepharodes [309]. Red values show worse activity in comparison to zeylasterone.*



Other natural derivatives: koetjapic acid

Koetjapic acid was isolated from the aerial parts of *Maytenus undata* (Celastraceae) together with 6 other OA derivatives and showed a significant antimicrobial activity against MRSA ATCC33591 (MIC = 12.5 mg/L) and MIC > 6.25 mg/L on MSSA ATCC6535 [308]. 20-Epi-koetjapic acid (C-20 carboxyl group in α), has MICs > 6.25 mg/L and > 3.25 mg/L against the same MRSA and MSSA strains [308].

Semisynthetic derivatives: carbazoles and polyarginin derivatives

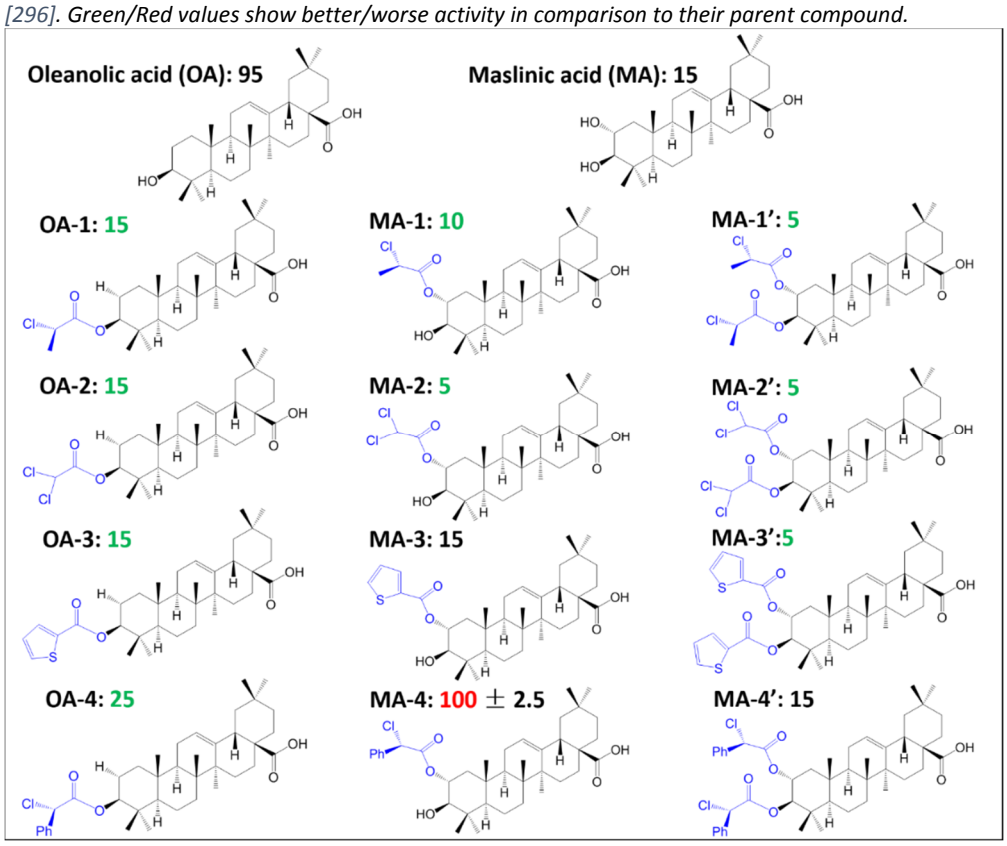
After having successfully isolated OA and MA in large amounts from Pomace olive (Olea europaea L.), Chouaïb and his collaborators synthesized a new series of triterpenic acid esters by incorporating acyl substituents in position C-3 of OA or in positions C-3 and/or C-2 of MA [296]. They observed in general an improved activity when the acyl substituent contained a sulfur or chlorine atom (**figure 1-35)**, particularly for OA derivatives, which is itself less potent than MA.

The only less effective derivative is MA-4. The addition of a second ester containing the same atoms seems to improve the antistaphylococcal activity of these derivatives [296].

Polyhydroxylated esters

Huang and coworkers synthesized OA derivatives with a polyhydroxylated A-ring as well as an esterification with a short alkyl chain in C-17. Among them, 4 display a significant activity (MIC ≤ 8mg/L) against MSSA ATCC25923 [295]. The introduction of two hydroxyl groups at positions C-1 and C-2 together with a small alkyl ester group at C-17 of OA thus enhances activity against *S. aureus* [295].

***Figure 1- 35** : Minimal inhibitory concentrations (MIC) on MSSA ATCC25923 in mg/L of triterpenic esters [296]. Green/Red values show better/worse activity in comparison to their parent compound.*



4.2.2.3 Lupane derivatives

Studies reporting the antistaphyloccal activity of lupane derivatives are reported in **table 1-15**. Betulinic acid (BA) MICs range between 16 and > 5000 mg/L on MSSA strains and between 4 and > 312.5 mg/L on MRSA strains. However, the majority of strains (23/30 MSSA and 16/25 MRSA) showed MICs > 16 mg/L (**figure 1-36**), BA thus being less potent than OA and UA. These values indicate that BA does not seem to have any interesting antistaphylococcal activity.

Figure 1- 36 *: Representative MIC distribution of BA against MSSA and MRSA strains.*

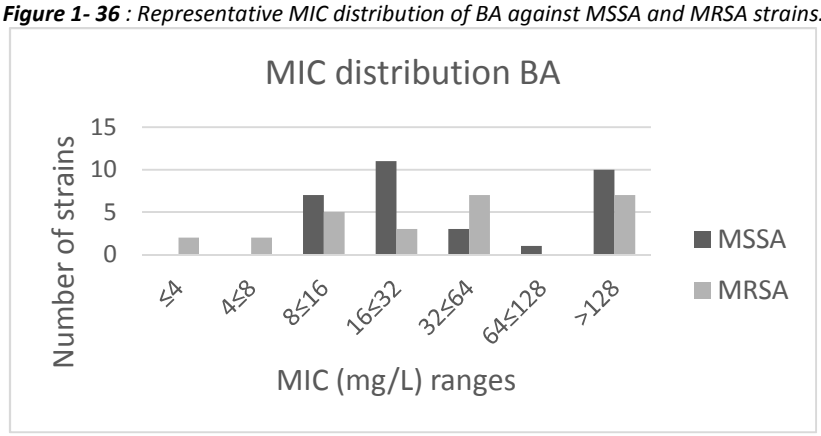
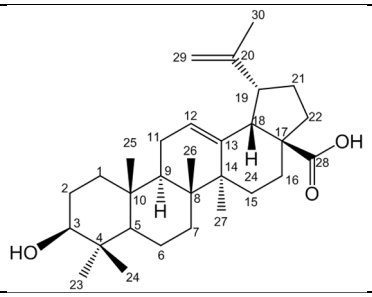


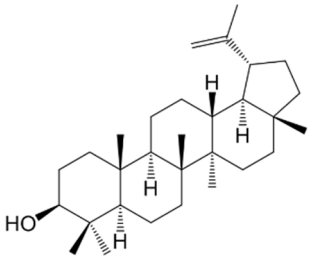
Table 1- 15 *: Minimal inhibitory concentrations (MICs) of lupane derivatives on S. aureus strains. Number between brackets correspond to the number of tested strains.*

Betulinic acid



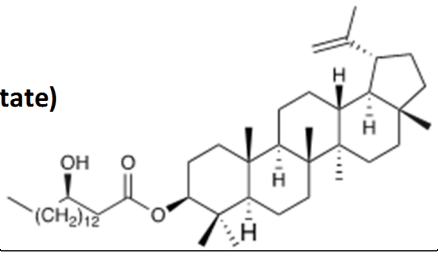
MSSA strains	MIC (mg/L)	MRSA strains	MIC (mg/L)
8325-4	>256[1]	**ATCC 33591**	> 128[5]
ATCC 25923	250[2]; 31.2[3] ; >256[23]	**ATCC 43300**	64[4. 21]; >128[7]
ATCC 29213	64[4. 21]; 128[5]; 250[6]; >128[7] ; >256[22]	**Clinical**	62.5 ± 7.8(9)[3]; 4(2)[4] ; 8(2)[4] ; 16(5)[4] ; 32(3)[4] ; 64(4)[4] ; >128(3) [11. 13] ; >256[22]
ATCC 6538	>250[8]	**Multi-drug-resistant (LMP0206U)**	>312.5[14]
CIP53154	>256[1]		
ITAL SFCO 001	>5000[10]		
MTCC-737	>128[11]		
NBRC 100910	20[12]		
Clinical	60(1)[9]; 16(7)[4] ; 32(9) [4]		

Lupeol



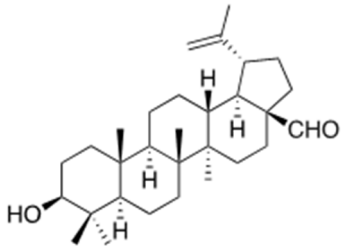
MSSA Strains	MIC (mg/l)	MRSA Strains	MIC (mg/l)
RCMB 010028	0.49[15]	**ATCC 43300**	>128[7]
ATCC 29213	250[6] ; >128[7]	**Clinical RCMB 010028**	3.9[15]
ATCC 25923	>200[16]		
Clinical	200(1)[9]		

lup-20(29)-en-3-(3'-β-hydroxypalmitate)



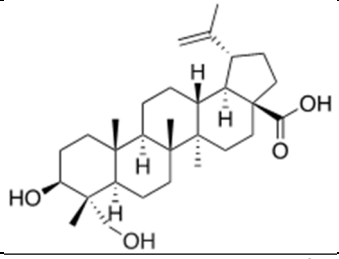
MSSA Strains	MIC (mg/l)	MRSA Strains	MIC (mg/l)
RCMB 010028	15.63[15]	**Clinical RCMB 010028**	62.5[15]

Betulinaldehyde



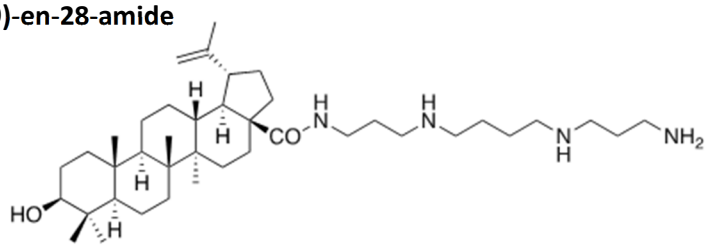
MSSA Strains	MIC (mg/l)	MRSA Strains	MIC (mg/l)
ATCC 29213	512[22] ; 256-512[4]	**ATCC 43300**	256-512[4] ; 512[22]
Clinical	128(6)[4] ; 256(4)[4] ; 512(6)[4]	**Clinical**	8(2)[4] ; 16(2)[4] ; 32(1)[4] ; 64(1)[4] ; 128(4)[4] ; 256(5)[4] ; 512(1)[4]

23 hydroxybetulinic acid



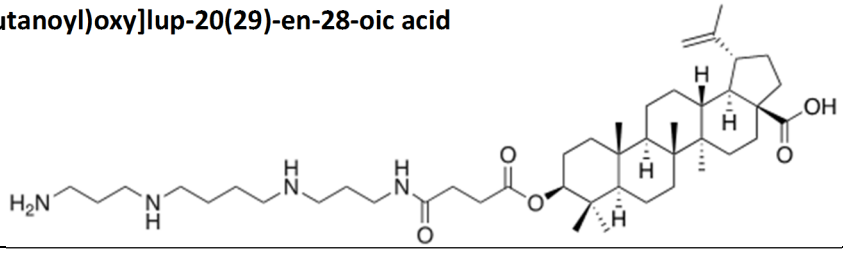
MSSA Strains	MIC (mg/l)	MRSA Strains	MIC (mg/l)
RN4220	16[19]	**USA300**	16[19]

(3β)-N-[3-({4-[(3-aminopropyl)amino]butyl}amino)propyl]-3-hydroxylup-20(29)-en-28-amide

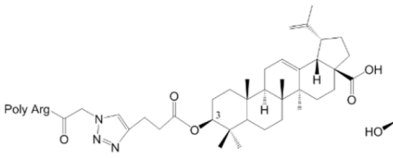
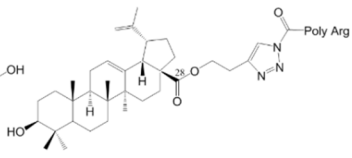


MSSA Strains	MIC (mg/l)	MRSA Strains	MIC (mg/l)
CCM 3953	12.5[18]	-	-

(3β)-3-[(4-{[3-({4-[(3-aminopropyl)amino]butyl}amino)propyl]amino}-4-oxobutanoyl)oxy]lup-20(29)-en-28-oic acid



MSSA Strains	MIC (mg/l)	MRSA Strains	MIC (mg/l)
CCM 3953	3.125[18]	-	-

Polyarginine esters	**1 : Derivatives BAc-R**  **BAc-R2, -R8**	**2 : Derivatives BAo-R**  **BAo-R2, -R5, -R8**	
	R = number of arginine residues in the polyarginine		
MSSA Strains	**MIC (mg/l)**	**MRSA Strains**	**MIC (mg/l)**
ATCC 25923	7.8[2]	-	-

Polyarginine esters	**1 : Derivatives BAc-R** **BAc-R3, -R5**	**2 : Derivatives BAo-R** **BAo-R3**	
	R = number of arginine residues in the polyarginine		
MSSA Strains	**MIC (mg/l)**	**MRSA Strains**	**MIC (mg/l)**
ATCC 25923	3.9[2]	-	-

[1][311], [2][270], [3][312], [4][287], [5][313], [6][275], [7][249], [8][314], [9][280], [10][315], [11][251], [12][290], [13][250], [14][302], [15][316], [16][317], [17][318], [18][319], [19][320], [20][321], [21][286], [22][248]

Loss or substitution of the carboxylic acid function: lupeol, lup-20(29)-en-3-(3'-β-hydroxypalmitate) & betulinaldehyde

While one study reports a significant antistaphylococcal activity for lupeol [316], four other teams showed MICs > 128 mg/L against different *S. aureus* strains [249,275,280,317]. Lupeol and BA were tested simultaneously and found to have both MIC > 128 mg/L on one MSSA strain [249,275] as well as on the MRSA strain ATCC33591 [299]. These results confirm that the loss of the carboxylic acid doesn't improve the antibacterial activity.

Lup-20(29)-en-3-(3'-β-hydroxypalmitate) is a natural compound isolated, together with lupeol, from the seeds of *Acokanthera oppositifolia* Lam. (Apocynaceae). It corresponds to lupeol esterified by a hydroxyl-palmitic acid in C-3 position [316]. Its MIC was higher than the one of lupeol simultaneously tested on the same strains, suggesting a negative effect of C-3 esterification [286].

Chung et al isolated BA and betulinaldehyde together from the bark of *Callicarpa tomentosa* (L.) (Lamiaceae) and compared their antistaphylococcal activities on a

wide range of MSSA and MRSA strains (34 strains in total) [286,287]. The MICs of the aldehyde derivative were higher than those of BA suggesting that the presence of an aldehydic function in C-28 is negative for the antistaphylococcal activity of lupanes [287].

Mono-hydroxylation: 23-hydroxybetulinic acid

23-hydroxybetulinic acid has a significant activity against one MSSA and one MRSA strain [320]. This result show the interest of the hydroxylation to enhance the antimicrobial activity of BA but this hypothesis is limited to this single study in which a direct comparison with BA is lacking.

Semisynthetic derivatives: polyamine and polyarginine derivatives

Among the polyamine derivatives of BA synthesized by Bildziukevich et al., only two showed interesting antistaphylococcal activities, namely (3β)-3-[(4-{[3-({4-[(3-aminopropyl)amino]butyl}amino)propyl]amino}-4-oxobutanoyl)oxy]lup-20(29)-en-28-oic acid and the (3β)-N-[3-({4-[(3-aminopropyl)amino]butyl}amino)propyl]-3-hydroxylup-20(29)-en-28-amide. [319].

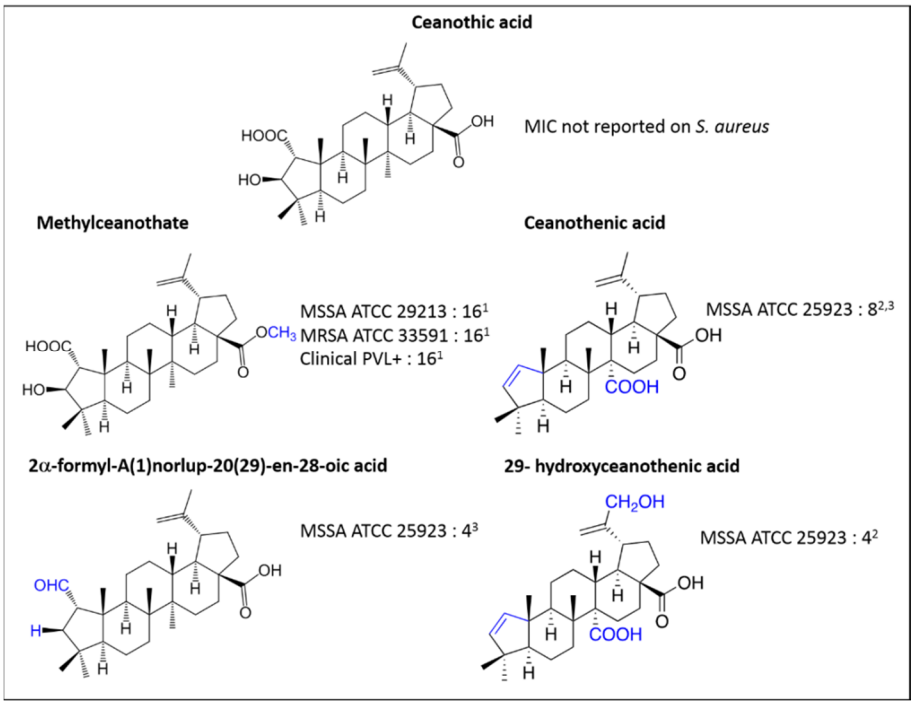
As for UA, Na and collaborators also synthesized polyarginine derivatives of BA by adding a number arginine residues varying between 1 and 8 [270]. The majority of these derivatives have a better activity than BA itself with MICs ranging from 3.9 and 7.8 mg/L on MSSA ATCC25923 in comparison to 250 mg/L for BA [270].

Ceanothic acid derivatives

Ceanothic acid is characterized by the ceanothane skeleton with three 6-membered cycles and two 5-membered cycles. Contrarily to UA, OA or BA, ceanothic acid possesses a second carboxylic acid function on the first cycle. Ceanothane derivatives are sometimes considered as a separate class but as only few compounds were tested, we considered them as lupane derivatives. No MIC against *S. aureus* is

reported for this compound (low activity determined by disk diffusion method [321]). However, four of its derivatives showed a significant activity against *S. aureus* strains (**figure 1-37**).

***Figure 1- 37** : Minimal inhibitory concentrations (MIC) in mg/L of ceanothic acid derivatives.* [1][313], [2][321], [3][318]



Methylceanothate (C-28 methyl ester of ceanothic acid) was extracted from the stem bark of a Brazilian plant, *Zizyphus joazeiro* Mart. (Rhamnaceae) together with epigouanic acid A, another ceanothane derivative [313]. Methylceanothane shows significant antistaphylococcal activity on reference MSSA and MRSA strains while epigouanic acid A did not show any antimicrobial activity [313].

2α-formyl-A(1)norlup-20(29)-en-28-oic acid was isolated from the powdered bark of *Jaffrea xerocarpa* (Baill.) H. C. Hopkins & Pillon (Rhamnaceae) and showed an interesting antistaphylococcal activity on MSSA ATCC25923 [318].

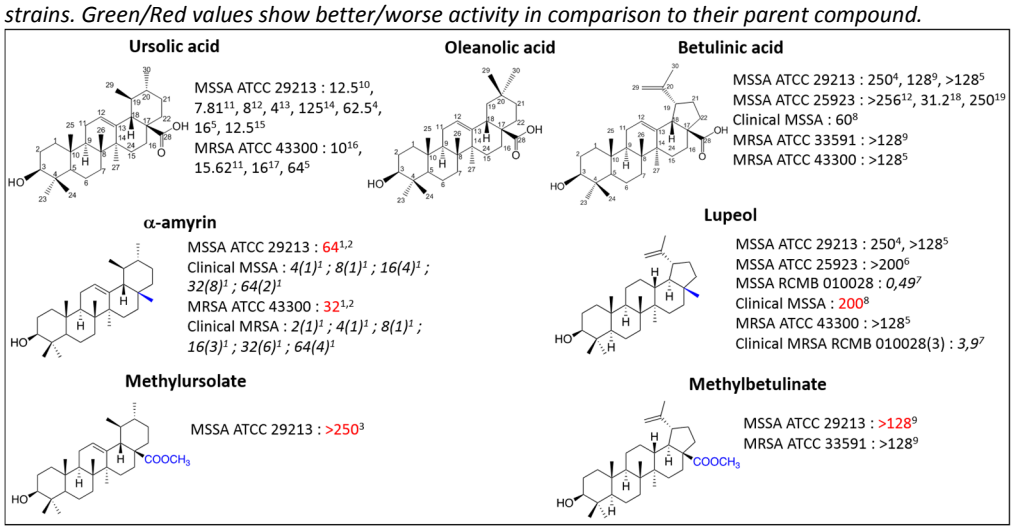
Ceanothenic acid and 29-hydroxyceanothenic acid from the leaves of *Alphitonia xerocarpus* Baill (Rhamnaceae) showed MICs of 8 and 4 mg/L against MSSA ATCC25923 respectively [318,321].

4.2.2.4 Discussion

Comparing the reported antistaphylococcal activities for the three reference acids (UA, OA and BA), UA seems to be globally the most active compound, with MIC ≤ 16 mg/L against more than 50 % of the tested MSSA and MRSA strains. OA most often shows moderate activity on MSSA strains but significant or moderate activity against MRSA strains. BA shows most often moderate or low activity against MSSA strains while its activity is highly variable on MRSA. Comparison of data obtained for these three most studied compounds of the three series remains difficult, due to differences in protocols, strains investigated or purity of the compounds. However, the comparison of MIC ranges obtained for the three compounds tested simultaneously on the same strain confirms that UA is the most active molecule and BA the less active one, OA MICs being in general 1-3 dilutions higher than those of UA.

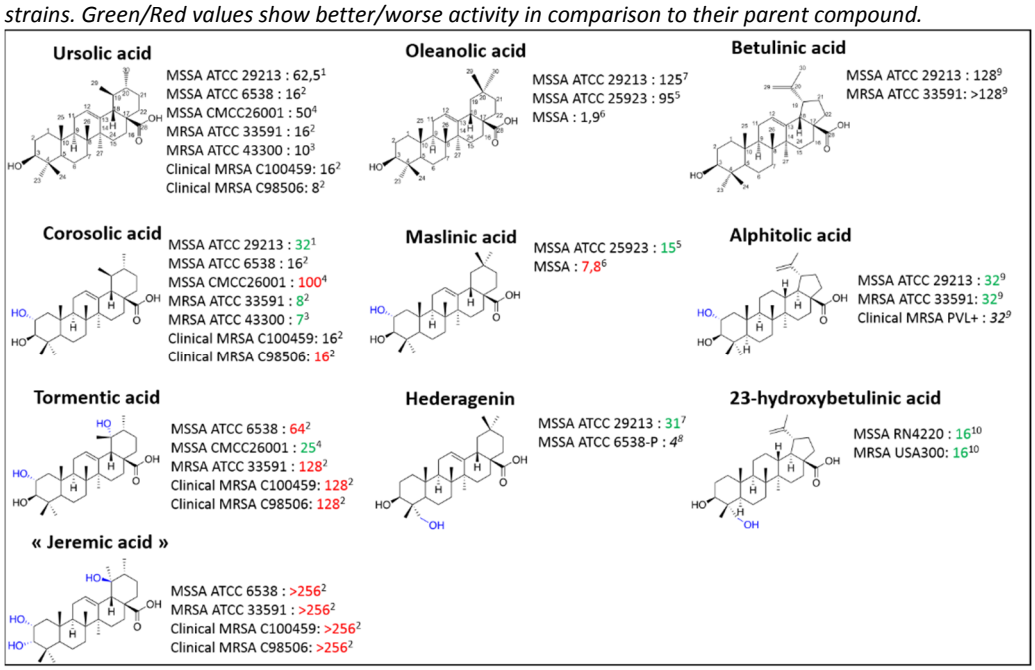
Same difficulties are encountered to determine SAR between series (**figures 1-38** & **1-39**). It seems that the loss of the C-17 carboxylic function, its methylation or change in position are deleterious for the antistaphylococcal activity while hydroxylation of the A-ring on C-2, C-23 or even C-1 seems to have a positive effect. Moreover, further hydroxylations may have different effects.

Figure 1- 38 *: Structures and MIC (mg/L) of UA, OA and BA decarboxylated derivatives against S. aureus strains. Green/Red values show better/worse activity in comparison to their parent compound.*



[1][287], [2][286], [3][322], [4][275], [5][249], [6][317], [7][316], [8][280], [9][313], [10][250], [11][274], [12][124], [13][248], [14][273], [15][276], [16][108], [17][284], [18][312], [19][270]

Figure 1- 39 *: Structures and MIC (mg/L) of UA, OA and BA hydroxylated derivatives against S. aureus strains. Green/Red values show better/worse activity in comparison to their parent compound.*



[1][275], [2][278], [3][284], [4][281], [5][296], [6][299], [7][108], [8][304], [9][313], [10][320]

We report here the MIC of 78 pentacyclic triterpenoids having a significant activity against *S. aureus* strains and suggest some SAR. Of note, out of the 78 reported compounds, 56 were tested on only one *S. aureus* strain. These results should thus be taken as preliminary data as testing the antibacterial activity on a wide panel of bacterial strains is necessary to conclude that a compound has an interesting activity. Among active compounds, 5 UA derivatives (UA, α-amyrin, 1β,3β-dihydroxyurs-12-en-27-oic acid, corosolic acid and acetyl-11-keto-β-boswellic acid) as well as 3 OA derivatives (aceriphyllic acid A, 3-oxo-olean-12-en-27-oic acid and celastrol) showed MIC ≤ 8 mg/L against MRSA strains which are of higher interest for clinical applications than MSSA.

In **table 1-16** we compare the MIC of these most active pentacyclic triterpenic compounds (MIC ≤ 8 mg/L) with that of common antibiotics tested on the same strain. While in most cases the antibiotic MIC is lower than the one of the triterpene, some compounds showed promising MICs (≤ 2 mg/L). Moreover, plants rarely or never produce a single strong antibacterial compound to defend themselves against outside attacks but hundreds of structurally different chemicals with a wide range of activity [323]. Thus, it is highly probable that activity of extracts results from the combined action of several substances, explaining why most pure compounds show in general low activity. Moreover, these extracts can also contain non-antimicrobial derivatives, like saponins for example [324], that can improve solubility, absorption or stability. The second part of this review will therefore focus on the combination of pentacyclic triterpenes with common antibiotics.

***Table 1- 16** : Comparison between MICs of pentacyclic triterpenoids having a significant antistaphylococcal activity (MIC≤ 8 mg/L) and conventional antibiotics.*

***Staphylococcus aureus* strain**	**MIC (mg/L)**	
	Antibiotic	**Triterpene**
		Corosolic acid
ATCC 43300	4[18] ; 4[19] (vancomycin) 0.63[2] (ciprofloxacin)	7[2]
ATCC 33591	2[20] (vancomycin) >64[3] (oxacillin)	8[3]
		Rotungenic acid
MSSA NBRC 100910	5[4] (ampicillin)	2.5[4]
		1β-hydroxyeuscaphic acid
ATCC 25922	31.25[5] (gentamicin)	7.81[5]
		Acetyl-11-keto-β-boswellic acid
ATCC 29213	16[21] (ampicillin) 0.25[22] ; <1[23] (oxacillin) 0.25[6] (ciprofloxacin)	2[6]
ATCC 33591	2[20] (vancomycin) 8[6] (ciprofloxacin)	2-4[6]
		UA (A. C. E) or OA (D. E) polyhydroxylated derivatives
ATCC 25923	4[7] ; 7.8[24] (ampicillin) <1[23] ; 7.8[24] (oxacillin) inactive[7] (streptomycin)	8[7]
		UA (B) or OA (C) polyhydroxylated derivatives
ATCC 25923	4[7] ; 7.8[24] (ampicillin) <1[23] ; 7.8[24] (oxacillin) inactive[7] (streptomycin)	2[7]
		OA or maslinic acid semi-synthetic derivatives : 18. 23
ATCC 25923	4[7] ; 7.8[24] (ampicillin) <1[23] ; 7.8[24] (oxacillin)	1[7]
		Carbazole derivatives 5-6
MSSA CGMCC 1.1361	0.9[8] (amikacin)	7.8[8]
		Sapogenin-polyarginine conjugates : Derived UAc-R (-R2. -R3. -R5. -R8) Derived UAo-R (-R1. -R5. -R8)
ATCC 25923	4[7] ; 7.8[24] (ampicillin) <1[23] ; 7.8[24] (oxacillin)	7.8[9]
		Sapogenin-polyarginine conjugates : Derived UAo-R (-R2. -R3)
ATCC 25923	4[7] ; 7.8[24] (ampicillin) <1[23] ; 7.8[24] (oxacillin)	3.9[9]
		Maslinic acid
nd MSSA strain	3.9[10] (kanamycin sulfate)	7.8[10]
		Hederagenin
nd MSSA strain	1[11] (gentamicin)	4[11]

		Aceriphyllic acid A
MSSA KCTC 1916	0.25[12] (vancomycin) 0.25[12] (oxacillin) 0.25[12] (norfloxacin)	4[12]
MRSA CCARM 3167	1[12] (vancomycin) 500[12] (oxacillin) 8[12] (norfloxacin)	4[12]
		1α.3β-hydroxyimberbic acid
nd MSSA	0.78[13] (streptomycin)	3.13[13]
		3-oxo-olean-12-en-27-oic acid
MSSA KCTC 1916	0.25[12] (vancomycin) 0.25[12] (oxacillin) 0.25[12] (norfloxacin)	2[12]
MRSA CCARM 3167	1[12] (vancomycin) 500[12] (oxacillin) 8[12] (norfloxacin)	4[12]
		Celastrol
ATCC 29213	16[21] (ampicillin) 0.25[22] ; <1[23] (oxacillin) 0.81[14] (telithromycin)	0.9[14]
Clinical MRSA	0.81[14] (telithromycin)	0.9[14]
		Zeylasterone
ATCC 6538	0.5[15] (penicillin) 1.56[15] (vancomycin)	5[15]
Clinical (LMM1)	/	5-10[15]
		OA or maslinic acid semi-synthetic derivatives : MA-2. MA-1'. MA-2'. MA-3'
ATCC 25923	4[7] ; 7.8[24] (ampicillin) <1[23] ; 7.8[24] (oxacillin) 15[16] (imipenem)	5[16]
		(3β)-3-[(4-{[3-({4-[(3-aminopropyl)amino]butyl}amino)propyl]amino}-4-oxobutanoyl)oxy]lup-20(29)-en-28-oic acid
MSSA CCM 3953	/	3.125[17]
		Sapogenin-polyarginine conjugates : Derivatives BAc-R (-R2. -R8) Derivatives BAo-R (-R2. -R5. -R8)
ATCC 25923	4[7] ; 7.8[24] (ampicillin) <1[23] ; 7.8[24] (oxacillin)	7.8[9]
		Sapogenin-polyarginine conjugates : Derivatives BAc-R (-R3. -R5) Derivatives BAo-R (-R3)
ATCC 25923	4[7] ; 7.8[24] (ampicillin) <1[23] ; 7.8[24] (oxacillin)	3.9[9]

[1][288], [2][284], [3][278], [4][290], [5][294] , [6][291], [7][295], [8][292], [9][270], [10][299], [11][304], [12][305], [13][307], [14][306], [15][309], [16][296], [17][319], [18][287], [19][274], [20][325], [21][249], [22][313], [23][248], [24][326]

4.3 Pentacyclic triterpenes as resistance modifying agents

4.3.1 In vitro antimicrobial combinations tests

Combinations of two antimicrobial agents can lead to four different effects: no interaction (the combination has the same effect as the most active compound), additivity (the combination effect equals the sum of the effects of the individual products), synergism (the combination effect exceeds the sum of the effects of the individual products), or antagonism (the combination effect is lower than that of individual compounds) [327]. A number of methods used to detect *in vitro* interactions between antimicrobial agents have been described but the time-kill assay and the checkerboard method are the most widely used techniques [328–330].

The time-kill method determines the bactericidal activity of each individual compound in the combinations, as well as the effect of combinations at different concentration ratios, as a function of time. Synergy is defined as an additional reduction of ≥ 2-$\log_{10}$ in colony counts after 24 h with the combination compared to the effect of the most active single agent alone, indifference as less than 2-$\log_{10}$ difference in colony counts after 24 h between the combination and the most active single agent alone, and antagonism as ≥2-$\log_{10}$ increase in colony counts after 24 h with the combination compared with the most active single agent alone. Additive effects correspond to a decrease in colony number equal to the sum of the reductions observed for each individual agent [331]. The time-kill method is a labor intensive and time-consuming method [332].

The checkerboard method is much easier. It consists in a two-dimensional test plate were drug X is diluted along one dimension and drug Y along the other dimension in 96 wells microtiter plates. Each well thus represents a different combination of two antimicrobial agents. Interactions between antibiotics can be evaluated using the FIC Indices, calculated as the sum of the Fractional Inhibitory Concentrations (FICs) as follows: FICI = FIC X + FIC Y, where FIC X is MIC of the compound X in the

combination/MIC of the compound X alone, and FIC Y is MIC of the compound Y in the combination/MIC of the compound Y alone [129]. The combination is considered as synergistic for FICI ≤ 0.5, additive for 0.5 < FICI ≤ 1, indifferent for 1 < FICI ≤ 4 and antagonistic for FICI > 4 according to the European Committee for Antimicrobial Susceptibility Testing [333].

As the checkerboard is a less fastidious technique, combinations of pentacyclic triterpenes with common antibiotics were mostly evaluated using this technique. To allow the comparison between data, FICI values are reported in this review.

4.3.2 Pentacyclic triterpenes with staphylococcal resistance modifying activities

Out of our bibliographic research with the words *Staphylococcus aureus, synergy* combined with *triterpenoid, triterpen, ursolic, oleanolic* or *betulinic* in three different libraries including *Pubmed, Scifinder* and *Scopus*, we only found 11 references reporting the potential synergy between conventional antibiotics and pentacyclic triterpenoids. All those papers were published between 2011 and 2017 illustrating the recently recognized interest of combining phytochemicals with common antibiotics. Combined antibiotics tested belong to 6 different classes. Their target as well as their mechanism of action and the resistance mechanism developed against them are summarized in **table 1-17**. Most tested antibiotics in combination were chosen because their use is currently limited by increased bacterial resistance. The antibiotics targeting the cell wall (β-lactams and glycopeptides) were the most used. Up to now, only 8 different pentacyclic triterpenoids were tested for their ability to increase the efficiency of common antibiotics; their fractional inhibitory concentration indexes (FICI) are reported in **table 1-18**.

***Table 1- 17** : Targets and mode of action of antibacterial agents tested in combination with pentacyclic triterpenes and main resistance mechanisms in S. aureus.*

Class	Subclass	Antibiotic	Target	Mode of action	Main Resistance Mechanisms in *S. aureus*
β-lactams	Penicillins	Penicillin	Cell wall	Inhibition of transpeptidases by irreversible binding to the active site of these enzymes	● Inactivation of antibiotics : β-lactamases ● Production of less affinity targets : PBP2a
		Amoxicillin			
		Ampicillin			
	Penicillinase-stable penicillins	Methicillin			
		Oxacillin			
	1st generation cephalosporins	Cefradine			
	2nd generation cephalosporins	Cefuroxime			
Glycopeptides		Vancomycin	Cell wall	Inhibition of peptidoglycan synthesis by binding to the D-Ala-D-Ala precursor	● Multiplication of the target ● Alternative cell wall precursors with low affinity for glycopeptides
Aminoglycosides		Tobramycin	Ribosomal subunit 30S	Protein synthesis inhibition	Inactivation of antibiotics : aminoglycoside modifying enzymes (acetyltransferases. nucleotidyltransferases. phosphotransferases)
		Gentamicin			
		Amikacin			
Tetracyclines		Tetracycline	Ribosomal subunit 30S	Protein synthesis inhibition	● Efflux of the antibiotics ● Modification of the target
		Doxycycline			
Macrolides		Erythromycin	Ribosomal subunit 50S	Protein synthesis inhibition	● Efflux of the antibiotics ● Modification of the target
Fluoroquinolones		Ciprofloxacin	topoisomerases	DNA replication inhibition	● Efflux of the antibiotics ● Modification of the target
		Norfloxacin			

Table 1- 18 *: FICI of pentacyclic triterpenes combined with common antibiotics against S. aureus strains*

Triterpenoids	Antibiotics	Strains		FICI
		Type	Name	
Ursolic acid	Ampicillin	MSSA	ATCC 29213	0.36[a]
		MSSA	ATCC 29213	0.38[b]
		MRSA	ATCC 43300	0.38[b]
		MRSA	CECT976	1[c]
		MRSA	ATCC33591	0.38-1[i]
		MRSA	Clinical VUB2	0.50-1[i]
		MRSA	Clinical VUB10	0.37-1[i]
		MRSA	Clinical VUB20	0.37-1[i]
		MRSA	Clinical VUB30	0.56-1[i]
	Oxacillin	MSSA	ATCC 29213	0.90[a]
		MRSA	ATCC33591	0.31-1[i]
		MRSA	Clinical VUB2	0.25-1[i]
		MRSA	Clinical VUB10	0.31-1[i]
		MRSA	Clinical VUB20	0.25-1[i]
		MRSA	Clinical VUB30	0.37-1[i]
	Penicillin	-	Clinical *S. aureus* 220	5[d]
	Cefradine	MSSA	Clinical	0.19[e]
		MRSA	Clinical	0.67[e]
		MSSA	ATCC 29213	0.5[e]
		MRSA	ATCC 43300	0.37[e]
	Vancomycin	MSSA	Clinical	0.17[e]
		MRSA	Clinical	0.46[e]
		MSSA	ATCC 29213	0.33[e]
		MRSA	ATCC 43300	0.38[e]
	Tetracycline	MSSA	ATCC 29213	0.19[b]
		MRSA	ATCC 43300	0.09[b]
		MRSA	CECT976	0.31[c]
	Ciprofloxacin	MRSA	CECT976	0.63[c]
	norfloxacin	MSSA	ATCC 29213	0.75[f]
	Erythromycin	MRSA	CECT976	1[c]
Oleanolic acid	Ampicillin	MSSA	ATCC 29213	0.48[a]
		MRSA	CECT976	1[c]
		MRSA	ATCC33591	0.25-1[i]
		MRSA	Clinical VUB2	0.31-1[i]
		MRSA	Clinical VUB10	0.31-1[i]
		MRSA	Clinical VUB20	0.50-1[i]
		MRSA	Clinical VUB30	0.50-1[i]
	Oxacillin	MSSA	ATCC 29213	0.90[a]
		MRSA	ATCC33591	0.16-1[i]
		MRSA	Clinical VUB2	0.18-1[i]
		MRSA	Clinical VUB10	0.18-1[i]

		MRSA	Clinical VUB20	0.37-1 [i]
		MRSA	Clinical VUB30	0.25-1 [i]
	Cefradine	MSSA	ATCC 29213	0.50[e]
		MSSA	Clinical	0.47[e]
		MRSA	ATCC 43300	0.24[e]
		MRSA	Clinical	0.37[e]
	Vancomycin	MSSA	ATCC 29213	0.375[e]
		MSSA	Clinical	0.52[e]
		MRSA	ATCC 43300	0.375[e]
		MRSA	Clinical	0.44[e]
	Tetracycline	MRSA	CECT976	0.5[c]
	Ciprofloxacin	MRSA	CECT976	0.75[c]
	Erythromycin	MRSA	CECT976	0.38[c]
Betulinic acid	Methicillin	MSSA	ATCC 29213	0.5[g]
		MRSA	ATCC 43300	0.25[g]
	Vancomycin	MSSA	ATCC 29213	0.51[g]
		MRSA	ATCC 43300	0.5[g]
α-Amyrin	Methicillin	MSSA	ATCC 29213	0.27[g]
		MRSA	ATCC 43300	0.08[g]
	Vancomycin	MSSA	ATCC 29213	0.38[g]
		MRSA	ATCC 43300	0.27[g]
β-Boswellic acid	Cefradine	MSSA	Clinical	Ineffective[e]
		MSSA	ATCC 29213	
		MRSA	Clinical	
		MRSA	ATCC 43300	
	Vancomycin	MSSA	Clinical	0.99[e]
		MSSA	ATCC 29213	0.75[e]
		MRSA	Clinical	0.84[e]
		MRSA	ATCC 43300	1[e]
18 β-Glycyrrhetinic acid	Amoxicillin	MRSA	LUH14616	0.6[h]
	Methicillin			0.85[h]
	Cefuroxime			1.1[h]
	Oxacillin	*S.aureus*	Clinical	≤0.5 (2)[j] >0.5-4 (6)[j]
	Doxycycline	MRSA	LUH14616	1.1[h]
	Tetracycline	*S.aureus*	Clinical	>0.5-4 (8)[j]
	Ciprofloxacin	MRSA	LUH14616	0.73[h]
	Ofloxacin	*S.aureus*	Clinical	>0.5-4 (8)[j]
	Erythromycin	MRSA	LUH14616	1.1[h]
		S.aureus	Clinical	>0.5-4 (8)[j]
	Tobramycin	MRSA	LUH14616	0.13[h]
	Gentamicin	MRSA	LUH14616	<0.27[h]
		S.aureus	Clinical	≤0.5(8)[j]
	Amikacin	MRSA	LUH14616	0.23[h]
	Polymyxin B			0.14[h]

Disodium 3-succinyloxy β-glycyrrhetinate	Oxacillin	*S.aureus*	Clinical	>0.5-4 (8)[j]
	Tetracycline	*S.aureus*	Clinical	≤0.5 (1)[j] >0.5-4 (8)[j]
	Ofloxacin	*S.aureus*	Clinical	>0.5-4 (8)[j]
	Erythromycin	*S.aureus*	Clinical	>0.5-4 (8)[j]
	Gentamicin	*S.aureus*	Clinical	≤0.5(8)[j]
Betulinaldehyde	Methicillin	MSSA	ATCC 29213	0.13[g]
		MRSA	ATCC 43300	ns[g]
	Vancomycin	MSSA	ATCC 29213	0.5[g]
		MRSA	ATCC 43300	0.5[g]

[a][334], [b][249], [c][279], [d][285], [e][274], [f][124], [g][286], [h][335], [i][293], [j][310]

UA is the most studied with 7 references reporting its activity in combination with common antibiotics against *S. aureus* strains. Three studies report a synergy between UA and ampicillin on MSSA and MRSA strains while Abreu et al. did not find any effect for this combination on a MRSA strain [249,279,293,334]. The combination of UA and oxacillin was synergistic on various MRSA strains [293] but only additive on one MSSA strain [334]. Surprisingly, one paper reports an antagonism between UA and penicillin against a clinical strain of *S. aureus* (FICI = 5)[285]. UA was also combined with cefradine, a first generation cephalosporin belonging to the β-lactam group and inactive on MRSA (MIC = 45mg/L). UA induced a remarkable decrease in the MIC of this antibiotic on both clinical and reference MSSA or MRSA strains [274].

Synergies were also detected for the combination of UA with vancomycin or tetracycline on MSSA and MRSA strains [249,274,279] while only an additive effect was detected for the combination with erythromycin (macrolide) against one MSSA strain [279]. Finally, two papers report an additive effect of UA and fluoroquinolones (ciprofloxacin and norfloxacin) against two different strains of MSSA [124,279]. Filocamo et al. showed in their paper that the combination of UA with norfloxacin increases its postantibiotic effect (delay before regrowth of bacteria following a short antibiotic exposure) [124]. This may be the sign of an inhibition of efflux by UA as it has been shown that inhibitors of NorA, a membrane-

associated multidrug efflux protein that decreases susceptibility to fluoroquinolones in *S. aureus,* significantly increases post-antibiotic effects of this class of antibiotics [336].

Three papers analyzed in parallel combinations of conventional antibiotics with UA and with its isomer OA. Concerning β-lactams, OA was found to synergize with ampicillin on one MSSA reference strain as well as on various MRSA strains [293,334] while another paper reports that the same combination had no effect on one MRSA strain [279]. An additive effect was reported in combination with oxacillin against MSSA ATCC29213 and a synergistic effect against 5 MRSA strains [293,334]. Synergies between OA and cefradine were also detected on MSSA and MRSA strains [274]. As UA, OA was found to synergize or at least have an additive effect in combination with vancomycin on MSSA and MRSA strains [274]. In 2016, Abreu et al. showed a synergy for the combinations OA/erythromycin as well as OA/tetracycline (FICI ≤ 0.5) on a MRSA strain (CECT976) while the effect was only additive when OA was combined with ciprofloxacin [279].

Only one paper studied the combination of BA and common antibiotics. It shows a synergistic effect between BA and methicillin against one MSSA and one MRSA strains (FICI ≤ 0.5). Its combination with vancomycin is considered as synergistic (FICI = 0.5) on the MRSA strain ATCC43300 but at the limit of the additive effect on the MSSA strain ATCC29213 (FICI 0.51) [286].

α-Amyrin, the non-acidic form of UA, was tested in combination with methicillin and vancomycin and found to act in synergy with both antibiotics on MSSA and MRSA strains [286].

The combination of β-boswellic acid, a derivative of UA, with cefradine was ineffective against all tested strains while the one with vancomycin was additive, except on the reference strain of MRSA [274]. This compound seems thus less interesting than others previously reported here.

De Breij and his collaborators studied the ability of ten amyrin-based pentacyclic triterpenoids (UA, uvaol, asiatic acid, erythrodiol, OA, stearyl glycyrrhetinate, carbenoxolone, glycyrrhizin, and 18α- or 18β-glycyrrhetinic acid (18β-GA)) to potentiate the activity of the aminoglycoside tobramycin against *S. aureus* and reported that 10µM of 18β-GA lowered the MIC of tobramycin from 2 to <0.3mg/L. As reported in **table 1-18**, 18β-GA was also tested in combination with other antibiotics on the strain MRSA LUH14616. They detected synergies for the combination with the three tested aminoglycosides (tobramycin, gentamicin, amikacin) [335]. Another team also reports this interesting synergy between gentamicin and 18β-GA as well as one of its derivative (disodium 3-succinyloxy β-glycyrrhetinate) on 8 clinical *S. aureus* strains [310]. 18β-GA was also found to have an additive effect with two β-lactams (amoxicillin and methicillin) but not with cefuroxime. No activity was detected for the combination of 18β-GA with neither doxycycline nor erythromycin. However, it enhances the activity of ciprofloxacin in an additive manner [310,335]. Unexpectedly, it was also shown that 18β-GA improved the activity of polymyxin B (16-64 fold), a lipopeptide antibiotic usually exclusively used against Gram-negative species [335].

Finally, betulinaldehyde has synergistic effects with methicillin on a MSSA strain while the same combination is reported as non-effective on a MRSA strain. Betulinaldehyde is also shown as a synergistic potentiator of vancomycin against one MSSA and one MRSA strains [286].

4.3.3 Discussion

As already mentioned for the direct antimicrobial activity, the comparison of the indirect activity of pentacyclic triterpenes is also made difficult by the variability in the method, the bacterial strains and the antibiotics used to perform the tests. We though try to suggest here some SAR comparing the activity of pentacyclic tritepenes tested on ATCC reference strains (MSSA ATCC29213 and MRSA ATCC43300) used in five different studies.

The presence of the acid function at the position 17 does not seem to be discriminant for the indirect activity as α-amyrin, the non-acid form of UA, shows the same synergistic effects as the parent compound on both reference strains with methicillin and vancomycin. However, the displacement of this carboxylic group on C-24 (β-boswellic-acid) seems to have a negative effect (no synergy with cefradine nor vancomycin). Reduction to an aldehyde, as in betulinaldehyde, has different effects according to the antibiotic/strain tested: similar effect, such as BA in combination with vancomycin, synergy with methicillin on the MSSA strain but no effect on the MRSA strain.

As research on the combination of this class of natural compounds with common antibiotics is a quite new topic, the data are limited and SAR are difficult to establish. As observed for the direct activity, UA seems to be the more effective > OA > BA while the presence of an acidic function in position 17 seems here not to be necessary. However, more data are needed to confirm these hypotheses.

4.4 Mechanism of action

Plenty of studies were initiated to identify the antibacterial targets and molecular mechanism(s) of action of triterpenoids [300,334,337–339]. In **table 1-19**, we summarized the postulated mechanisms of action of some pentacyclic triterpenoids focusing on *S. aureus* strains, often showing their effect on the bacterial membrane as well as the modulation of gene expression as main targets for antistaphylococcal activity.

Concerning the indirect antimicrobial activity of pentacyclic triterpenes, we can assume that, as their structures are different from the combined antibiotic agent, their targets are probably different [334]. Bacterial resistance against antibiotics can be achieved in several ways: (i) modification of the cellular target of the antibiotic, (ii) hindrance of antibiotic penetration into the cell or enhancement of its efflux from the cell, and (iii) destruction or modification of the antibiotic [334]. Moreover, it has been shown that due to the presence of exopolysaccharide matrix, a slow growth rate, the presence of persistant cells and small-colony variants (SCVs), biofilm-grown cells express an increased resistance to antimicrobials in comparison to planktonic cells [340]. Each of these mechanisms may be influenced by plant-derived compounds.

***Table 1- 19** : Mode of action postulated for some pentacyclic triterpenoids against S. aureus strains.*

Compound	Concentration	Mode of action
Zeylasterone [309]	3 x MIC	- Cell membrane disruption (inhibition of radiolabeled precursor (N-acetylglucosamine) uptake. rapid potassium leakage. inhibition of NADH oxidation and formation of mesosome-like structures around the septa). - Early inhibitory effect on incorporation of radiolabeled precursors: thymidine (DNA), uridine (RNA) and N-acetylglucosamine (peptidoglycan) and later on leucine (protein).
UA [341]	4 x MIC	- Damaging of cell membrane integrity - Inhibition of protein synthesis - Inhibition of metabolic pathway (glycolysis repression and oxidative stress promotion)
Acetyl-11-keto-β-boswellic acid [291]	32 x MIC	- Disruption of bacterial membrane (increased propidium iodine uptake and leakage of 260 and 280 nm absorbing material)
BA **Betulinaldehyde** **α-Amyrin** [342,343]	0.5 x MIC	- Regulation of multiple desirable targets in cell division, ABC transporters, fatty acid biosynthesis, peptidoglycan biosynthesis. aminoacyl-tRNA synthetase, ribosome and β-lactam resistance pathways (proteomic analyzes)
Polyhydroxylated pentacyclic triterpenes derivatives [295]	0.5 x MIC	- Regulation of gene expression associated with peptidoglycan and respiratory metabolisms as well as virulence factors in bacteria
Glycyrrhetinic acid **Disodium 3-succinyloxy beta-glycyrrhetinate** [310]	0.5 x MIC	- Downregulation of genes involved in sugar. amino acid and nucleic acid metabolism - Downregulation of genes encoding for transporters - Downregulation of virulence factors

We showed in **table 1-18**, that except β-boswellic acid and 18β-glycyrrethinic acid, all tested pentacyclic triterpenes synergize with β-lactams and vancomycin, both targeting the cell wall of the bacteria. While the synergistic mechanism of this kind of compounds with vancomycin has never been studied, we can find in the literature some data about the mechanism of synergy between terpenes and β-lactams. It is shown that these compounds are able to target the two main mechanisms of resistance developed by *S. aureus* against β-lactams, namely β-lactamases and production of PBP2a a modified PBP with low affinity for conventional β-lactams.

In 2007, Kuroda and coworkers showed that the sesquiterpene farnesol was able to reduce the synthesis as well as the activity of β-lactamases (up to 0.4 x MIC) [344]. More recently, we showed that UA and OA interfere with β-lactamase activity (at 0.5 x MIC), but do not act as competitive inhibitors of the soluble enzyme (as previously published [345]), neither as suicide substrates like clavulanic acid. The fact that β-lactamase inhibition is observed only towards living bacteria suggests therefore an indirect effect of UA and OA, possibly related to their insertion in the bacterial membrane, the mechanism of which remains to be established [293].

Concerning the PBPs, it is known that the target of triterpenic compounds is different from that of β-lactams as Kurek et al. showed that none of the *L. monocytogenes* penicillin-binding proteins (PBPs) bind radiolabeled OA and that there is no competition between penicillin and OA for binding to PBPs [334,338]. Moreover, we showed that both UA and OA are capable of delocalizing PBP2 in the membrane of MRSA (at 1 x MIC), which is a critical mechanism for reversing the MRSA phenotype, probably by modification of the membrane properties [293].

As efflux pumps are an important mechanism of resistance against tetracyclines and macrolides, we can hypothesize that they could be a possible target for the triterpenes. Up to now, some proteomic analyses showed an effect of pentacyclic triterpenes (BA, α-amyrin or betulinaldehyde) on genes encoding for ABC transporters [342,343]. However, the effect of these three compounds was never assessed in combination with these antibiotics substrates for efflux. Moreover, Dwivedi and coworkers showed in 2015 that some UA-derivatives (3-O-acetyl-urs-12-en-28 isopropyl ester and 3-O-acetyl-urs-12-en-28- n-butyl ester) significantly inhibited efflux pumps of multidrug-resistant *E. coli* at 0.25 x MIC [346].

In brief, it seems that the primary target for the antimicrobial activity of pentacyclic triterpenoids is the cell membrane, which could also explain some of their other effects and their indirect effects. However, additional studies are still needed to identify their exact molecular target in the bacterial envelope and also to assess the specificity of their activity.

4.5 Conclusion

This review reports 78 pentacyclic triterpenes having a significant antistaphylococcal activity on at least one strain, as well as 8 compounds (UA, OA, BA, α-amyrin, β-boswellic acid, 18β-glycyrrhetinic acid, disodium-3-succinyloxy-β-glycyrrhetinate, and betulinaldehyde) showing synergistic or at least additive effects with antibiotics on different *S. aureus* strains. We also tried to deduce some SAR. However, we have to acknowledge that it is difficult to obtain coherent conclusions on the antistaphylococcal activity of these products because (a) their purity varies from one study to another, (b) few studies compare several products according to the same protocol and on the same strains, (c) generally the number of tested strains is very low, and (d) the absence of cytotoxicity for eukaryotic cells is rarely documented. Nevertheless, some of them are more active than commonly

used antibiotics on some MRSA strains, which are of higher clinical interest compared to MSSA strains.

As compared to the data generally available for conventional antibiotics on the market or in development, more studies are necessary to measure MICs according to a standard method, in comparison with the first therapeutic choices or/and the parent compound on a wide range of strains (in the same study). The results reported in this review for the direct antistaphylococcal activity of pentacyclic triterpenoids have thus to be considered carefully and should stimulate further researches on promising compounds.

Of note, however, these natural products are often found active in extracts where they are present in mixture with several other compounds that can act in synergy, have additive effects, modify their stability or bioavailability, explaining the relatively low efficacy of some of them when purified. It also remains to be established whether selection of resistance is less frequent for these compounds than for conventional antibiotics, but this may depend on their pharmacological target, which is in general unknown. Using them in combination with other antibiotics is another alternative which constitute a quite new topic of research. Up to now, only 8 pentacyclic triterpenes were studied in combination with antibiotics but the positive interactions reported should encourage the search in this field.

RESULTS – PART 1

Preliminary evidences of the direct and indirect antimicrobial activity of 12 plants used in traditional medicine in Africa

In order to identify promising plant extracts to treat staphylococcal infections, we first studied the antistaphylococcal activity of a series of plants used in traditional medicine in Africa. We first selected plants based on literature as well as on ethnopharmacological data. We carefully chose plants already studied for their antistaphylococcal activity but from which no active compounds were previously identified as well as plants never studied in this field but used to treat infections in traditional medicine.

We selected twelve plants namely *Allophylus africanus, Anchomanes difformis, Antocleista schweinfurthii, Cassia sieberiana, Cissampelos mucronata, Cola gigantea, Holarrhena floribunda, Protea elliotii, Schrankia leptocarpa, Spirospermum penduliflorum, Tapinanthus bangwensis* and *Vitellaria paradoxa* which were collected between 2006 and 2012 in Africa.

The results of this screening are detailed in this chapter based on the published paper: Catteau, L., Van Bambeke, F., Quetin-Leclercq, J., 2015. Preliminary evidences of the direct and indirect antimicrobial activity of 12 plants used in traditional medicine in Africa. *Phytochem Rev* 1–17.

Abstract

In a world of increasing resistance to current antibiotics, search of novel therapeutic options is urgently needed. The aim of this work was to screen plant crude extracts for direct or indirect (inhibition of resistance) antimicrobial activity.

Four crude extracts from 12 plants traditionally used in Africa for the treatment of infections were obtained by successive extraction with hexane, dichloromethane, ethyl acetate, and methanol. All extracts were tested against *Staphylococcus aureus* MRSA ATCC33591 (resistant to β-lactams by production of β-lactamases and of a modified PBP target [PBP2a]). Direct antimicrobial activity was tested by determination of Minimal Inhibitory Concentrations (MIC), and indirect activity, by determining interactions between antibiotics and extracts using checkerboard titration and calculation of Fractional Inhibitory Concentration Index (FICI; Synergy: FICI ≤ 0.5; additivity: FICI ≤ 1). Combined antibiotics were ampicillin (sensitive to resistance mediated by β-lactamases and PBP2a) and oxacillin (sensitive to resistance mediated by PBP2a only).

The dichloromethane extract of *Vitellaria paradoxa* leaves, the methanol extracts of *Vitellaria paradoxa*, *Cola gigantea* leaves and twigs, and of *Tapinanthus bangwensis* aerial parts showed direct antimicrobial activity (MIC 250-500 mg/L). The methanol extracts of *Vitellaria paradoxa* and *Cola gigantea* leaves and twigs showed additive or synergistic effects with oxacillin and ampicillin on MRSA ATCC33591 (FICI : 0.28-1), suggesting a possible inhibition of PBP2a. The methanol extract of *Tapinanthus bangwensis* aerial parts and *Anchomanes difformis* roots improved the activity of ampicillin only (FICI 0.38-1), suggesting β-lactamase inhibition. Polyphenols and particularly tannins were shown to be responsible for these last effects, at least partially for *Vitellaria paradoxa*.

These data need further research aiming at identifying the active compounds in these extracts.

1. Introduction

Infections caused by multi-drug resistant bacteria are an increasing problem due to the emergence and propagation of microbial drug resistance and the lack of development of new antimicrobials.

Staphylococcus aureus is a major cause of hospitally-acquired infections, with β-lactam antibiotics often considered as a first line of treatment. However, widespread use of this class of antibiotics has led to an increasing prevalence of bacterial resistance. The main mechanisms of β-lactams resistance consist in (a) the production of β-lactamases inactivating the antibiotics and (b) the production of PBP2a, an additional penicillin-binding protein with decreased affinity for methicillin and most other β-lactam drugs, the production of which is associated to the so-call Methicillin-resistant *S. aureus* (MRSA) phenotype [252]. MRSA is one of the most prominent pathogens known to induce a severe threat to human health [7]. Recent epidemiological studies indicate that the prevalence of MRSA is high over the world posing a significant public health challenge [253,254].

Therapeutic options available for treating MRSA infections have become seriously limited. Indeed, MRSA have also acquired resistance mechanisms to most of the other available antibiotic classes [255,256]. Moreover, the rapid widespread and emergence of resistance to newly introduced antimicrobial agents indicates that new families of antimicrobial agents will have a short life expectancy [347]. Although ceftaroline, telavancin, tedizolid, dalbavancin, and oritavancin have been recently marketed as drugs to treat MRSA infections, the development of new and original strategies to fight them is still needed [257].

A useful alternative approach consists in inhibiting resistance mechanisms, which has already been widely exploited with β-lactamase inhibitors [61]. During the last decade, a few studies reported that plant extracts can contain antimicrobial compounds and/or substances enhancing the *in vitro* activity of certain antibiotics against multi-drug resistant strains of *S. aureus* [101,102,348–352].

In this study, we screened 12 plants traditionally used in Africa to treat diverse infections for their direct antimicrobial activity against a MRSA reference strain. We also determined their indirect antimicrobial activity in combination with two β-lactams (ampicillin and oxacillin). For each plant species, twigs, leaves or roots were extracted with hexane, dichloromethane, ethyl acetate and methanol. Crude extracts were also evaluated for their cytotoxicity on two mammalian cell lines.

2. Materials and methods

2.1 Plant material

Most of the plant materials (leaves and twigs (= aerial parts), and roots) were collected from the South of Benin, especially from Abomey-Calavi (South –West) to the border area with Nigeria (South-East) between September 2006 and August 2012. *Anthocleista schweinfurthii* was collected in Kinshasa (Democratic Republic of the Congo) in August 2007 and *Spirospermum penduliflorum,* in December 2009 in the Eastern Region of Madagascar, taking into account the Nagoya protocol for collections after 2010.. Voucher specimens were identified and deposited at the Herbier National of Abomey-Calavi University in Benin, at the herbarium of the "*Institut Malgache de Recherches Appliquées*" or at the Herbarium of the National Botanic Garden of Belgium, at Meise (**Table 2-1**).

2.2 Preparation of crude plant extracts and tannins free fractions

Dried leaves, twigs, or roots (50-100 g) were extracted using a soxhlet apparatus with four solvents of increasing polarity (hexane, dichloromethane, ethyl acetate and methanol) for eight hours each (20 cycles). The quantity of solvent used for each extraction was 250mL. The extracts obtained were then evaporated to dryness under reduced pressure with a rotary evaporator at a temperature of 30°C. Yields for each extraction are indicated in **Table 2-2**.

Active methanolic extracts were cleared of tannins with a polyamid column (Polyamid CC 6, Macherey – Nagel) according to the method described by Houghton & Raman, 1998 [353]. 20g of polyamid were swollen in 200mL of water during one night. A column was filled with the gel. After one day, the column was conditioned with two column volumes of each solvent used in the elution. 500mg of extract solubilized in a minimum of solvent (mixture MeOH/H_2O 50:50) were deposed on the column. For the elution, we used MeOH, a mixture of MeOH/H_2O 50:50, ethyl acetate and finally dichloromethane.

2.3 Thin Layer Chromatography (TLC) analysis

Methanolic extracts were resolubilized in methanol at a concentration of 10mg/mL. 5µL were then spotted on a TLC Silica gel 60 F_{254} plate (Merck, Darmstadt, Germany). The mobile phase consisted in a mixture of butanol/acetic acid/H_2O (14:5:5). Revelation was done with chlorhydric vanillin prepared according to the method described by Wagner and Bladt (1996) [354].

Table 2- 1. *Plant species under study*

Botanical name	Family	Voucher specimen number	Collecting place	Traditional uses
Allophylus africanus P. Beauv	*Sapindaceae*	AP2131	Benin	To treat headaches, migraines, colic, fever, conjunctivitis and other eye troubles, wounds, infections, diarrhea and malaria [a,b,c,d]
Anchomanes difformis (Blume) Engl.	*Araceae*	AP2132	Benin	As diuretic, to treat diabetes, oral and anal lesions, tuberculosis and malaria[a]
Anthocleista schweinfurthii (Gilg.)	*Gentianaceae*	BR0000009752597	Kinshasa	To treat diabetes, malaria, stomach diseases, ovarian problems, veneral disease, bronchitis, fever, abscesses, malaria and to facilitate wound healing[e,f]
Cassia sieberiana DC	*Caesalpiniaceae*	BR S.P. 848100	Benin	To treat fever, jaundice, stomach ache, gonorrhea, piles, ulcers, abscesses and malaria[g,h]
Cissampelos mucronata A Rich.	*Menispermaceae*	AA 6347/HNB	Benin	To treat wounds, indigestion, schistosomiasis, infections, malaria[i]
Cola gigantea A. Chev. Var. gigantea	*Sterculiaceae*	AP2133	Benin	To treat sores, skin infections and other inflammatory conditions including pains[j]
Holarrhena floribunda (G. Don) Durand & Schinz	*Apocynaceae*	AP2128	Benin	As febrifuge and tonic, to treat dysentery, malaria, female sterility, tripanosomiasis, abdominal pains, nausea, indigestion, diarrhea[k,l,]
Protea elliotii C. H. Wright	*Proteaceae*	AA 6354/HNB	Benin	To treat carious teeth, hemorrhoids, sores, skin diseases[m,n]
Schrankia leptocarpa DC.	*Mimosaceae*	Hougnon 954b	Benin	To treat eruptive fever, hypertension, jaundice, abdominal pains, hiccup and malaria[a]
Spirospermum penduliflorum Thouars	*Menispermaceae*	AML13	Madagascar	To treat malaria, pulmonary tuberculosis, vomiting, colic, liver complains and as cardiac tonic [p][25][19][19]
Vitellaria paradoxa C.F. Gaertn. *ssp. paradoxa*	*Sapotaceae*	AP2130	Benin	To treat hypertension, wound infections, skin infections, dysentery, diarrhea, gastrointestinal tract infections, jaundice, hemorrhoids, fever [m,n,o]
Tapinanthus bangwensis (Engl. & Krause) Danser	*Loranthaceae*	AP2242	Benin	To treat snake bites, disorders in female reproductive system, cancer, arthritis, rheumatism, hypertension, asthma, diabetes, wounds, skin infections [n]

[a] [356], [b] [357], [c] [358], [d] [359], [e] [360], [f] [361], [g] [362], [h] [363], [i] [364], [j] [365], [k] [366], [l] [367], [m] [368], [n] [369], [o] [370], [p] [355]

2.4 Bacteria, cells and media

Crude extracts were tested for their direct and indirect antibacterial activity against *S. aureus* ATCC33591 (MRSA; obtained from the American Type Culture Collection) grown in cation-adjusted Mueller-Hinton broth (caMHB). This reference strain is resistant to β-lactams by two main mechanisms, namely the production of β-lactamases and of targets PBP2a.

Two cell lines were used in parallel, and cultivated in a humidified atmosphere with 5% CO_2 at 37°C. The J774 macrophage-like cell line, derived from BALB/c murine reticulum cell sarcoma, was cultivated in RPMI 1640 medium (Life Technologies, Paisley, UK) containing 2mM L-glutamine supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies) and penicillin-streptomycin (100UI/ml). The human normal fibroblast cell line, WI38, was cultivated in DMEM medium (Life Technologies) containing 4mM L-glutamine, 1mM sodium pyruvate supplemented with 10% heat inactivated fetal bovine serum (Life Technologies) and penicillin-streptomycin (100UI/mL).

2.5 Antibiotics and antibiotic susceptibility testing

Ampicillin (potency 87.99%) and oxacillin (potency 90%) (Sigma-Aldrich, St Louis, MO) were used as tested antibiotics.

Stock solutions of crude extracts were prepared at 20mg/mL in dimethylsulfoxide (DMSO) and then serially diluted in the medium to obtain a final concentration range of 500 – 7.81 mg/L. The highest concentration of solvent to which the bacteria were exposed was 2.5%, which was shown in preliminary experiments to have no effect on the growth of bacteria.

Minimum Inhibitory Concentrations (MIC) and Fractional Inhibitory Concentration Indexes (FICI) were determined by the checkerboard method in caMHB [331,371]. In

a 96-well plate, the β-lactam antibiotic in the combination was serially diluted starting from a final concentration of 2 x MIC along the ordinate. The crude extract was serially diluted along the abscissa using 500mg/L as the highest final concentration. After that, a bacterial suspension (final inoculum 0.5 10^6 to 1 10^6 CFU/ml) was added to all wells.

After 20 h of incubation at 37°C, the MICs of antibiotics and crude extracts were determined as the lowest concentration that completely inhibited the growth of the organism.

Interactions between antibiotics were then evaluated using the FIC indexes, calculated as the sum of the Fractional Inhibitory Concentrations (FICs) as follows: FICI = FIC A + FIC E, where FIC A is MIC of the antibiotic in the combination/MIC of the antibiotic alone and FIC E is MIC of the extract in the combination/MIC of the extract alone[129]. The combination was considered as synergistic for FICI ≤ 0.5, additive for 0.5 < FICI ≤ 1, indifferent for 1 < FICI ≤ 4 and antagonistic for FICI > 4 according to the European Committee for Antimicrobial Susceptibility Testing [333].

To facilitate readings, bacterial growth was detected using resazurin, a blue phenoxazin dye that is reduced by viable bacteria in the pink fluorescent compound resorufin [372]. 30µL of a 0.02% resazurin (Sigma-Aldrich) solution in caMHB was added to each well. Plates were then incubated at 37°C for one hour in the dark. MIC corresponded to the lowest concentration of antibiotic or extract for which the well color did not turn to pink.

2.6 Cytotoxicity assay

The cytotoxicity of the extracts for J774 and WI38 cells was evaluated as described by Stevigny et al. (2002), using tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphényltetrazolium bromide (Sigma-Aldrich) colorimetric method based on the cleavage of the reagent by mitochondrial dehydrogenase in viable cells [374].

Camptothecin (Sigma-Aldrich) was used as a positive cytotoxic reference. Stock solutions of crude extracts at 20mg/mL in DMSO were diluted in medium with a final concentration range of 100 – 0.01 mg/L. The cytotoxicity of solvents was tested in parallel and found to be negligible at the highest concentration used for extracts (0.5%). Each extract was tested in nine serial dilutions in 96-well microtiter plates.

3. Results and discussion

Fifty-six extracts were obtained from the 12 plants under study. *In vitro* direct and indirect antimicrobial and cytotoxic activities of these extracts are summarized in **Table 2-2**.

Concerning the direct antimicrobial activity on MRSA ATCC33591, 6 extracts had an MIC lower or equal to 500mg/L, namely the methanol extracts of *Vitellaria paradoxa* (leaves and twigs), *Cola gigantea* (leaves and twigs), and *Tapinanthus bangwensis* aerial parts as well as the dichloromethane extract from the leaves of *Vitellaria paradoxa*. According to Kuete et al., those crude extracts may be considered as moderately active ($100 < MIC \leq 625$mg/L) and they thus may contain very interesting pure compounds [106].

To determine the indirect antimicrobial activity, we tested the combinations of the crude extracts with two β-lactams selected to allow us to distinguish between a reversion of two mechanisms of resistance. Thus, while both ampicillin and oxacillin are sensitive to PBP2a-mediated resistance, oxacillin resists to the action of β-lactamases, thanks to a steric hindrance on its lateral chain [375,376]. A decrease in the MIC of both antibiotics can thus be interpreted as denoting an influence of the extract on PBP2a-mediated resistance (combined or not with an inhibition of β-

lactamases) while a decrease in the MIC of ampicillin only would be indicative of an inhibition of the β-lactamases activity.

Table 2- 2. *In vitro direct (MIC) and indirect (FICI ranges) antibacterial activity and cytotoxicity of the selected plant extracts (n=3).*

Plants species	Part studied[a]	Extract	Yield (%)	Antibacterial activity against MRSA ATCC33591			Cytotoxicity (IC_{50}, mg/L) average ± standard deviation	
				MIC (mg/L)	FICI AMPI	FICI OXA	WI38	J774
Allophylus africanus	AP	HEX	2.33	> 500	0.63 - 1	1	77.99 ± 8.89	97.81 ± 3.10
		DCM	1.26	> 500	1	1 – 2	60.69 ± 6.18	19.79 ± 2.24
		AcOEt	0.89	> 500	1	1 – 2	>100	62.43 ± 6.27
		MeOH	5.07	> 500	1	1 – 2	>100	>100
Anchomanes difformis	RT	HEX	0.46	> 500	0.18- 1	0.60- 1	>100	76.4 ± 9.3
		DCM	0.56	> 500	1	1	>100	>100
		AcOEt	0.71	> 500	1	1 – 2	>100	>100
		MeOH	8.93	> 500	0.38-1	1	>100	>100
Anthocleista schweinfurthii	RB	HEX	0.53	> 500	0.62 - 1	1 – 2	66.06 ± 7.75	23.17 ± 3.36
		DCM	0.52	> 500	0.75 - 1	1 – 2	67.26 ± 2.16	47.10 ± 2.00
		AcOEt	0.63	> 500	0.75 - 1	1 – 2	>100	91.92 ± 7.57
		MeOH	6.91	> 500	1	1 – 2	>100	>100
Cassia sieberiana	AP	HEX	1.77	> 500	1	1	88.36 ± 0.89	44.54 ± 5.39
		DCM	1.35	> 500	1 - 2	1	63.42 ± 7.29	41.31 ± 8.92
		AcOEt	1.32	> 500	1 - 2	1	>100	>100
		MeOH	14.54	> 500	1	1	>100	>100
Cissampelos mucronata	AP	HEX	1.65	> 500	1	1 – 2	29.17 ± 1.43	31.07 ± 8.64
		DCM	0.82	> 500	1	1 – 2	65.68 ± 6.91	18.32 ± 2.31
		AcOEt	0.78	> 500	1	1 – 2	45.16 ± 3.78	19.24 ± 0.30
		MeOH	7.91	> 500	1	1 – 2	99.57 ± 0.54	35.46 ± 6.15
Cola gigantea	TW	HEX	0.84	> 500	1	1 – 2	42.69 ± 8.49	35.06 ± 0.39
		DCM	0.45	> 500	1	1 – 2	32.85 ± 8.95	31.54 ± 3.32
		AcOEt	0.42	> 500	1	1 – 2	> 100	82.93 ± 3.48
		MeOH	12.34	500	0.5 - 1	0.28- 1	> 100	88.22 ± 6.31
	LF	HEX	3.07	> 500	1	1 – 2	> 100	56.92 ± 6.94
		DCM	1.18	> 500	1	1 – 2	> 100	57.69 ± 2.69
		AcOEt	0.59	> 500	1	1 – 2	> 100	80.48 ± 9.83
		MeOH	12.81	500	0.38- 1	0.31- 1	> 100	> 100
Holarrhena floribunda	AP	HEX	3.96	> 500	1	1 – 2	28.24 ± 0.68	33.04 ± 1.82
		DCM	2.02	> 500	1	1 – 2	28.26 ± 1.64	36.98 ± 5.62
		AcOEt	1.54	> 500	1	1 – 2	25.80 ± 4.65	35.13 ± 1.23
		MeOH	16.56	> 500	0.56- 1	1	16.53 ± 0.43	22.81 ± 0.35
Protea elliotii	AP	HEX	1.25	> 500	0.75- 1	1 – 2	83.16 ± 6.48	27.45 ± 1.52
		DCM	0.89	> 500	1	1 – 2	88.63 ± 3.86	50.61 ± 1.37
		AcOEt	2.7	> 500	0.50- 1	1	> 100	>100
		MeOH	16.56	> 500	0.75- 1	1	> 100	>100
Schrankia leptocarpa	AP	HEX	1.98	> 500	0.62- 1	1	>100	>100
		DCM	0.99	> 500	1	1	>100	51.26 ± 6.75

		AcOEt	0.88	> 500	1	1	>100	>100
		MeOH	14.51	> 500	1	1	>100	>100
Spirospermum penduliflorum	AP	HEX	2.71	> 500	1	1 – 2	61.10 ± 7.38	31.66 ± 3.11
		DCM	1.76	> 500	0.56- 1	1 – 2	10.10 ± 0.20	4.82 ± 0.67
		AcOEt	0.91	> 500	1	1 – 2	75.30 ± 8.45	21.63 ± 4.93
		MeOH	10.18	> 500	1	1 – 2	>100	32.03 ± 6.92
Tapinanthus bangwensis	AP	HEX	2.94	> 500	1	1	>100	71.28 ± 2.93
		DCM	0.68	> 500	0.75-1	1	28.89 ± 1.98	29.63 ± 8.64
		AcOEt	0.87	> 500	1	1	85.1 ± 7.58	63.02 ± 2.02
		MeOH	10.24	500	0.38-1	1	>100	>100
Vitellaria paradoxa	TW	HEX	3.16	> 500	1	1 – 2	86.83 ± 9.23	72.56 ± 1.76
		DCM	0.39	> 500	1	1	25.85 ± 0.92	33.92 ± 0.88
		AcOEt	0.33	> 500	1	1	> 100	>100
		MeOH	7.26	500	0.38-1	0.38-1	95.90 ± 5.80	47.74 ± 1.82
	LF	HEX	4.62	> 500	1	1	53.86 ± 2.89	43.55 ± 2.90
		DCM	1.83	250	0.14-1	0.51-1	7.17 ± 2.93	27.56 ± 0.13
		AcOEt	1.25	> 500	0.63-1	1 – 2	> 100	72.31 ± 0.42
		MeOH	16.06	500	0.50-1	0.50-1	>100	47.74 ± 11.18
Camptothecin				nd	nd	Nd	0.03 ± 0.003	0.4 ± 0.2
Ampicillin				64	nd	Nd	Nd	nd
Oxacillin				32	nd	Nd	Nd	nd

MRSA33491 = Methicillin resistant *Staphylococcus aureus*. J774 = macrophage-like murine cells. WI38 = human normal fibroblast cell. nd = not determined

[a] Plant part used : LF = leaves. TW = twigs. RT = roots. AP = aerial parts (leaves + twigs) RB = root

Globally, 14 extracts, namely the hexane extracts from the aerial parts of *Allophyllus africanus, Anthocleista schweinfurthii, Protea eliotii* and *Schrankia leptocarpa,* the dichloromethane extracts from *Anthocleista schweinfurthii, Spirospermum penduliflorum,* and *Tapinanthus bangwensis,* the ethyl acetate extracts from the leaves of *Protea elliotii, Anthocleista schweinfurthii* and *Vitellaria paradoxa* and the methanol extracts from *Anchomanes difformis* and *Holarrhena floribunda,* showed an additional or synergistic effect in combination with ampicillin only. Those extracts may thus contain potential β-lactamases inhibitors.

On the other hand, 6 extracts were active in combination with ampicillin and also showed a FICI ≤ 1 when combined with oxacillin. Those are the hexane extract from the roots of *Anchomanes difformis*, the methanol extracts from C*ola gigantea* and *Vitellaria paradoxa* leaves and twigs and the dichloromethane extract from *Vitellaria*

paradoxa leaves. This could suggest that these extracts may counteract resistance mediated by PBP2a production (and possibly also by β-lactamases).

In **table 2-3**, we reported available data on phytochemical screenings of all the plants used in this study. Contradictive results were observed but it is well known that the production of secondary metabolites is influenced by numerous factors such as the age of the plant or the leaves, the geographical localization of the plant, the nutrients present in the soil… [377,378]. The influence of such factors on secondary metabolites could be the cause of this discordance, but also the difference of sensitivity of the detection method used. At this stage of the work, it is difficult to predict which class of compounds could be responsible for their direct or indirect antimicrobial activity, each class (alkaloids, anthraquinones, flavonoids, saponins and tannins) containing compounds with antimicrobial activity [98,379–382]. Concerning the indirect antimicrobial activity, the synergy between at least one compound of each class and β-lactams has already been studied [115,117,132,383,384].

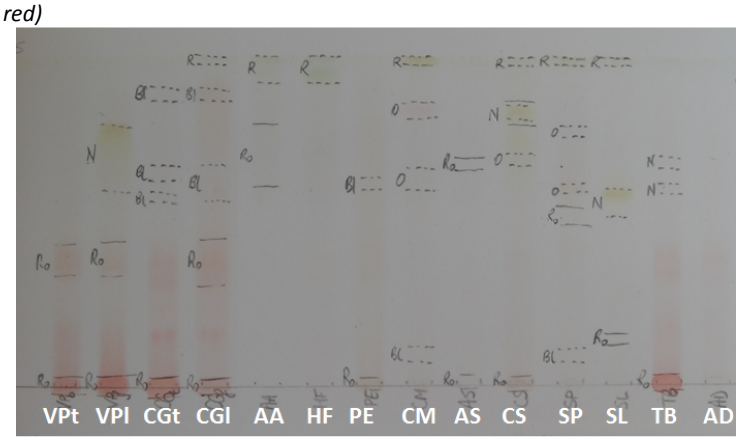
As most active extracts were obtained from methanol and could contain tannins (**table 2-3**) which possess antimicrobial activities, we confirmed their presence by TLC (**figure 2-1**). We then compared the antimicrobial activity of these extracts before and after elimination of tannins on a polyamide column. Results are shown in **Table 2-4**. Except for the extracts from the leaves and twigs of *Vitellaria paradoxa*, elimination of tannins totally suppressed both the direct and indirect antimicrobial activities of the methanolic extracts tested. This is coherent with the fact that tannins have already been reported to be bacteriostatic or bactericidal against *Staphylococcus aureus* [385].

Moreover, different tannins extracted from plants have been reported as resistance modifying agents. Tellimagrandin I and rugosin B extracted from red rose and corilagin extracted from *Arctostaphylos uva-ursi*, markedly reduced the MIC of β-lactam antibiotics against MRSA [116,117,386]. Among them, tellimagrandin and

corilagin have been reported to be active in combination with oxacillin and revert PBP2a-mediated rather than β-lactamase-mediated resistance. [261,387,388]. The presence of compounds of this type may thus explain the *Cola gigantea* and a part of *Vitellaria paradoxa* effects, which showed a synergistic effect with both oxacillin and ampicillin in the present study.

On the other hand, we may suggest that the methanolic extracts from *Anchomanes difformis*, *Holarrhena floribunda*, *Protea eliottii* and *Tapinanthus bangwensis*, which are only active in combination with ampicillin, could contain tannins inhibiting β-lactamases.

***Figure 2- 1** : Thin layer chromatography analysis of methanolic extracts resolubilized in methanol at 10mg/mL. Mobile phase : butanol/acetic acid/H2O (14:5:5). Revelation : chlorhydric vanillin (coloration of tannins in red)*



Tested plants: VP = *Vitellaria paradoxa; CG= Cola gigantea (t= twigs; l= leaves); AA = Allophylus africanus; HF = Holarrhena floribunda; PE = Protea elliotii; CM = Cissampelos mucronata ; AS = Antocleista schweinfurthii; CS = Cassia sieberiana, SP= Spirospermum penduliflorum; SL = Schrankia leptocarpa, TB= Tapinanthus bangwensis; AD= Anchomanes difformis.*

This study being the first one to examine the indirect antimicrobial activity of these plants, it is difficult to compare our results with previously published data. We therefore centered our analysis of literature data on direct antimicrobial activity mainly on *S. aureus* strains studied by the broth dilution method and cytotoxicity. We also report here already identified compounds and their known antibacterial potential

Table 2- 3 *Published phytochemical screenings*

Plants species	Part studied[a]	Extract	Alkaloids	Anthraquinones	Flavonoids	Saponins	Tannins	Reference
Allophylus africanus	**AP**	Ethanolic maceration	-	+	+	+	+	[358]
Anchomanes difformis	**RT**	EtOH/MeOH/H_2O 70 :20 :10 maceration	+	nd	+	+	+	[389,390]
		Hexane soxhlet	-	-	+	-	-	[390]
		Ethanolic soxhlet	+	-	-	-	+	[390]
		Ethanolic maceration	+	-	nd	+	+	[391]
		Entire plant	-	nd	+	-	+	[392]
Antocleista schweinfurthii	**SB**	Ethanolic maceration	+	nd	-	-	-	[393]
		MeOH/DCM 50 :50 maceration	-	nd	+	nd	+	[394]
Cassia sieberiana	**AP**	Ethanolic extract	+	+	+	+	+	[395]
Cissampelos mucronata	**AP**	Ethanolic maceration	-	nd	+	+	+	[396]
Cola gigantea	**TW**	MeOH/H2O 4 :1 maceration	+	nd	+	-	-	[397]
	LF	Ethanolic maceration	+	+	nd	+	+	[398]
Holarrhena floribunda	**TW**	Methanolic maceration	+	-	-	+	+	[367,399]
	LF	Methanolic maceration on a defatted extract	+	nd	nd	+	+	[399]
		Powder	+	nd	+	nd	+	[366]
Protea elliotii	**LF**	80% methanolic maceration	+	-	+	+	+	[400]
Schrankia leptocarpa	**AP**	Entire plant	+	nd	+	nd	+	[401]

Spirospermum penduliflorum	AP	Methanolic maceration	+	nd	nd	nd	nd	[402]
Tapinanthus bangwensis	LF	Methanolic maceration	+	nd	+	+	+	[403,404]
		Methanolic maceration	+	-	+	+	+	[403]
Vitellaria paradoxa	TW	Ethanolic maceration	+	-	nd	+	+	[405,406]
		Methanolic maceration	+	-	+	-	+	[407]
	LF	Methanolic maceration	+	-	-	-	+	[407,408]
		Crude leaves	+	-	-	+	nd	[406]

+ : present ; - : not detected ; nd : not determined

Table 2- 4 *In vitro direct and indirect antibacterial activity on the strain MRSA33591 of the active methanolic extracts before and after polyamide column (n=3).*

Plants species	Part studied	Antibacterial activity against MRSA ATCC33591			
		MIC (mg/L)		FICI AMPI	
		Before	After	Before	After
Anchomanes difformis	AP	>500	>500	0.38-1	1
Cola gigantea	TW	500	>500	0.5-1	1
	LF	500	>500	0.38-1	1
Holarrena floribunda	AP	>500	>500	0.56-1	1
Protea elliotii	AP	>500	>500	0.75-1	1
Vitellaria paradoxa	TW	500	>500	0.38-1	0.56-1
	LF	500	>500	0.5-1	0.63-1
Tapinanthus bangwensis	AP	500	>500	0.38-1	1

3.1 *Allophylus africanus* P. Beauv (Sapindaceae)

Allophylus africanus did not display any antibacterial activity. However, in a study of Sofidiya et al. (2012) [409], a methanolic extract from the leaves showed a direct antimicrobial activity on a strain of *S. aureus* with an MIC of 500mg/L. The difference observed could be explained by the successive extractions made here which could separate plant compounds acting synergistically. Another explanation could be the difference between the bacterial strains used.

Except for pinitol which did notdid not show any antimicrobial activity, none of identified compounds in this plant were tested for this activity [358,410].

Figure 2- 2 *Allophylus africanus [411]*



3.2 *Anchomanes difformis* (Blume) Engl. (Araceae)

Different studies showed an antimicrobial activity of methanolic, ethanolic and aqueous extracts from roots of *Anchomanes difformis* by a disc diffusion method on *S. aureus* at higher concentrations[389,392]. The essential oil of the root part had a MIC of 2000mg/L on a strain of *S. aureus*[412]. Those observations were considered sufficient to justify its use for the treatment of abscesses, inflammation, intertrigo and conjunctivitis. However, in this study, the root extracts from *Anchomanes difformis* did not show any direct antimicrobial activity at the highest concentration tested (500mg/L) on the strain MRSA ATCC33591.

Concerning cytotoxicity, none of the extracts tested on the J774 and WI38 showed any cytotoxicity (IC_{50} > 100mg/L). These results are in contradiction with those reported in the study of Bero et al. (2009) [413]which reported a strong cytotoxicity of dichloromethane, methanol and water extracts from the roots of *Anchomanes difformis* (2.2mg/L < IC_{50} < 26mg/L). However, in this previous study, the extracts were prepared by maceration at room temperature, while we used here a soxhlet apparatus with a heating at the boiling temperature of each solvent. It could explain the absence of toxicity in our extract as it was already reported that the toxicity level decreases when roots were pre-boiled for a long time[414].

Concerning isolated compounds, anchominines A & B, catechin and epicatechin were isolated from the roots of *Anchomanes difformis*[414]. Anchominines A & B were never tested on bacteria while the MIC of both catechin and epicatechin are higher than 256mg/L on *S. aureus* strains [134].

Figure 2- 3 *Anchomanes difformis leaves (left) and roots (right)*



3.3 *Anthocleista schweinfurthii* (Gilg.) (Gentianaceae)

No direct antimicrobial activity was detected for all tested root bark extracts of this plant. This part of plant has never been studied for antimicrobial activity while a successive partition of a crude methanolic macerate from the stem bark between water and hexane, dichloromethane, ethyl acetate, and methanol gave four extracts with MICs between 15.625 and 125mg/L on the strain MRSA ATCC33591 [393].

The hexane, dichloromethane and ethyl acetate extracts showed here an additive effect in combination with ampicillin (FICI 0.62 – 1) with no activity in combination with oxacillin. However, those extracts are quite cytotoxic on the J774 and WI38 cell lines (IC_{50} 23.17 ± 3.36 and 47.10 ± 2.00 respectively), but these activities may not be related.

The root bark was shown to contain schweinfurthiin (steroid); bauerenone and bauerenol (triterpenoids); 1-hydroxy-3,7,8-trimethoxyxanthone and 1,8-dihydroxy-3,7-dimethoxyxanthone (xanthones) with α-glucosidase inhibitory activity but they were never tested for their antimicrobial activity [360].

Figure 2- 4 *Anthocleista schweinfurthii [411]*



3.4 *Cassia sieberiana* DC (Caesalpiniaceae)

No antimicrobial activity was detected for this plant in the present study. However, the traditional use of *Cassia sieberiana* as treatment for abscesses and other infectious diseases was justified based on the fact that concentrations higher than 500mg/L of leaves extracts prepared by other methods previously showed activity against different bacterial strains (*Bacillus subtilis, Shigella dysenteriae, Escherichia coli, S. aureus*) [395,415].

Two polyphenols named l-epicatechol and leucopelargonidol were isolated in the whole plant of *Cassia sieberiana* but their antimicrobial potential is not known [416].

Figure 2- 5 *Cassia sieberiana [417]*



3.5 *Cissampelos mucronata* A Rich. (Menispermaceae)

Cissampelos mucronata did not show any direct or indirect antibacterial activity on the strain MRSA ATCC33591. The ethanolic and dichloromethane extracts from the leaves of this plant showed a direct antimicrobial activity against another strain of *S. aureus* at much higher concentrations (MIC 3125 and 6250mg/L respectively) [364]. While different anti-plasmodially active bisbenzylisoquinoleinic alkaloids were identified in the roots, no compounds have already been identified in the leaves or twigs of *Cissampelos mucronata* [418].

Figure 2- 6 *Cissampelos mucronata [417]*



3.6 *Cola gigantea* A. Chev. Var. *gigantea* (Sterculiaceae)

The soxhlet ethanol extract from the leaves and twigs of *Cola gigantea* were already tested on a methicillin sensitive *S. aureus* (MSSA) reference strain (ATCC25923) and showed MIC of 250 and 125mg/L [365]. We obtained here a bit higher MIC with our methanolic extract on an MRSA strain (500mg/L). Recently, Onyema and Ajiwe identified in the chloroformic fraction from crude antimicrobial methanolic extracts of leaves and twigs of *Cola gigantea* several lipophilic compounds possessing antimicrobial properties on different bacterial strains including a strain of *S. aureus* [397,419]. However, these compounds should only be present, probably in low concentration in our lipophilic extracts (hexane or DCM) and not in the most active methanolic extract whose activity is mostly related to the presence of tannins.

Figure 2- 7 *Cola gigantea [411]*



3.7 *Holarrhena floribunda* (G. Don) Durand & Schinz (Apocynaceae)

Holarrhena floribunda which was shown to be active on different *Bacillus* strains and on *Mycobacterium ulcerans* responsible for the Buruli ulcer [420] appeared to be not directly active at a concentration of 500mg/L on the strain of MRSA used in this study. The tannins, already evidenced in this plant [367], appeared to be responsible for the indirect antimicrobial activity of the methanol extract in combination with ampicillin. However, this extract is quite cytotoxic with IC_{50} of 16.53 ± 0.43 and 22.81 ± 0.35mg/L on WI38 and J774 cell lines respectively.

Holarrhena floribunda contains large amount of identified alkaloids such as conessine, holaphyllidine, holamine,… [366,367,421–423]. Some compounds identified in the plant (conessine, isoquercitroside, progesterone, trichothecolone, and lupeol) were tested for their direct effect on *S. aureus* and for cytotoxicity [317,367,422,424–432]. However, none of them were tested in combination with antibiotics against *S.aureus*.

Our results show that isolated antimicrobial compounds seem to be present at low concentration in our extracts and diluted in non-active compounds although cytotoxic ones may explain, at least in part, the cytotoxicity observed.

Figure 2- 8 *Holarrhena floribunda [411]*



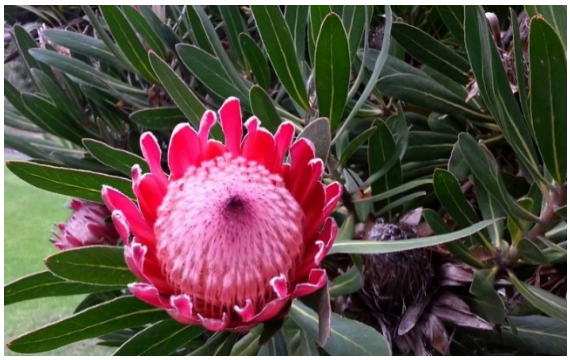
3.8 *Protea elliotii* C. H. Wright (Proeteaceae)

Protea elliotii was mainly studied for its antioxidant properties and its ability to inhibit melanin synthesis [368,433]. The antimicrobial potential of its aerial parts was explored for the first time in this study. The tested extracts did not show any direct antibacterial activity on the strain MRSA ATCC33591. However, the hexane, ethyl acetate and methanol extracts showed an additive effect in combination with ampicillin (FICI 0.5 – 1). Tannins were shown to be responsible for the methanol extract effects.

Except for the hexane extract (IC_{50} of 83.16 ± 6.48 and 27.45 ± 1.52 mg/L on the WI38 and J774 cell lines respectively), none of those active extracts appeared to be cytotoxic (IC_{50} > 100mg/L).

No pure compounds were isolated from the aerial parts of *Protea elliotii*. Three compounds were isolated from the root bark of this plant : oleic acid, β-sitosterol and 2-tridecanone [434]. The MIC of oleic acid is 1000mg/L and that of β-sitosterol is 25mg/mL on *S. aureus* [435,436].

Figure 2- 9 *Protea elliotii [411]*



3.9 *Schrankia leptocarpa* DC. (Mimosaceae)

Different extracts from *Schrankia leptocarpa* are reported to have MICs comprised between 0.625 and 2.5mg/L on a MSSA strain. The diethylether extract was the most active one [401]. Those results are in accordance with the activity we found with the hexane extract, in combination with ampicillin (FICI 0.62-1). Further fractionation of this extract would be of interest because it did not show any cytotoxicity (IC_{50} > 100 mg/L on both cell lines used here).

By a trypanocidal bioassay-guided fractionation of aerial parts of *Schrankia leptocarpa*, different compounds were isolated, namely 1,7-bis-(4-hydroxyphenyl)-3-hepten-5-one;5-hydroxy-1,7-bis(4-hydroxyphenyl)heptan-3-one; methyl-1-chlorogenate; éthyl-1-chlorogenate; caffeic acid; p-coumarylagmatine; manghasline; mauritianin; quercetin 3-neohesperidoside [437]. Amongst them, only caffeic acid was tested for his antimicrobial activity and showed a MIC higher than 50mg/L on *S. aureus* [438].

Figure 2- 10 *Schrankia leptocarpa [439]*



3.10 *Spirospermum penduliflorum* Thouars (Menispermaceae)

Spirospermum penduliflorum was mainly studied for its antiplasmodial alkaloids content but also for its vasorelaxant activity [400,440]. However, this plant was never studied for its antimicrobial activity. We detected here that the dichloromethane extract from the aerial parts of this plant presented an additive effect in combination with ampicillin (FICI 0.56-1). However, this extract showed a quite high cytotoxicity with IC_{50} of 10.10 ± 0.20 and 4.82 ± 0.67mg/L on the WI38 and J774 cell lines. Further studies are needed to determine whether indirect antimicrobial activity and cytotoxicity are due to the same compounds but aporphine alkaloids could be responsible for the observed cytotoxicity [441].

Two vasorelaxant aporphine alkaloids were isolated from the leaves of *Spirospermum penduliflorum* namely dicentrine and néolitsine [400]. Those two compounds are antihelmintic and a dicentrine derivative (dicentrine methane) has a MIC comprised between 50 and 300mg/L on *S. aureus* strains.

Figure 2- 11 *: Spirospermum penduliflorum [442]*



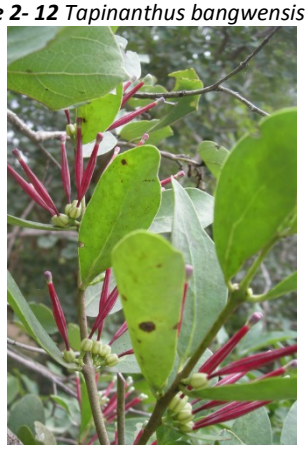
3.11 *Tapinanthus bangwensis* (Engl. & Krause) Danser (Loranthaceae)

The dichloromethane extract from the aerial parts showed an additive effect in combination with ampicillin. However, this extract is cytotoxic at much lower concentrations with IC_{50} of 28.89 ± 1.98 and 29.63 ± 8.64mg/L on WI38 and J774 cell lines respectively.

We showed that tannins were responsible for the direct and indirect antimicrobial activity of the methanolic extract which did not show any cytotoxicity on both cell lines used in this study (IC_{50} >100mg/L on WI38 and J774).

Gallic acid derivatives were identified in the plant, among which methyl syringate possessing a significant antibacterial activity against *S. aureus* [443,444]. In 2005, Shibata et al. also demonstrated that alkyl gallates intensified β-lactam susceptibility in MRSA [136]. Catechin, epicatechin and derivatives were also identified. While the MIC of catechin and epicatechin are higher than 256mg/L, it is known that epicatechin 3-O-gallate has a MIC comprised between 64 and 128mg/L on *S. aureus* strains [134,445,446]. Moreover, catechin derivatives may possess, as epicatechin-3-O-gallate, the capacity to modulate β-lactam resistance in MRSA strains [134]. Other compounds were also identified but were not tested for their antimicrobial properties [445,447,448].

Figure 2- 12 *Tapinanthus bangwensis [411]*

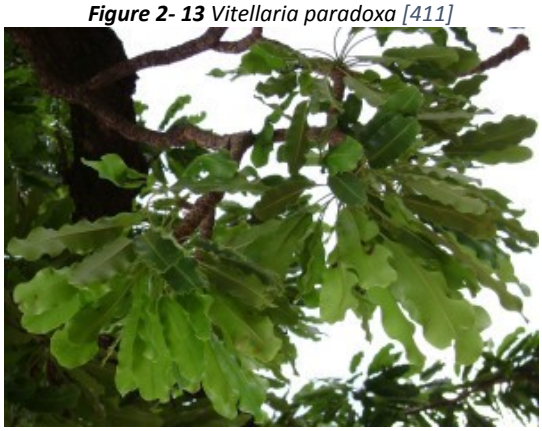


3.12 *Vitellaria paradoxa* C.F. Gaertn. ssp. *paradoxa* (Sapotaceae)

Vitellaria paradoxa is an immensely popular tree because the fat extract from the kernels called shea butter (karité) is widely used in cosmetic industries [449]. The antimicrobial activity of its stem bark and leaves was already studied on strains of *S. aureus* but with an agar well diffusion method at high concentration (50g/L) [370,407]. The dichloromethane extract from the leaves showed the best direct and indirect antimicrobial activities. However, this extract was also cytotoxic with an IC_{50} of 7.17 ± 2.93 and 27.56 ± 0.13mg/L on the WI38 and J774 cell lines respectively. In fact, it has already been described that the prolonged administration of the plant extract at high doses may adversely affect the functions of some vital organs, but compounds responsible for both effects may be different [370].Tannins already identified in this plant [406] were shown to be only responsible for a part of the indirect antimicrobial activity of both twigs and leaves methanol extracts. Indeed, it is the only plant which showed a residual activity after the polyamide separation.

Though many studies focused on shea butter and kernels, only a few identified compounds from the root bark of *Vitellaria paradoxa* among which two antibacterial compounds named 2-O-butyl-1-O-(2'-ethylhexyl) benzene-1, 8-dicarboxylate and 1-phenyl-1, 4-pentanedione [450,451]. However leaves and twigs were not much studied and no compounds were isolated from them.

Figure 2- 13 *Vitellaria paradoxa [411]*



4. Conclusion

In our study, we tested for the first time the direct and indirect antimicrobial activity of 12 plants used in traditional medicine in Africa on a MRSA strain. Several extracts showed direct antimicrobial activity or seem to reverse the resistance of MRSA to β-lactams. Though reported activities may be considered as moderate, they may contain low concentrations of interesting compounds and this may explain at least to some extend their use in traditional medicine. Nevertheless, some extracts showed cytotoxicity which may be related to different compounds than those responsible for the antimicrobial effects, but needs further research to determine their safety in traditional use.

We also showed that tannins were responsible for most antimicrobial activities of methanolic extracts (*Anchomanes difformis, Cola gigantea, Holarrhena floribunda, Protea eliotii, Tapinanthus bangwensis* and *Vitellaria paradoxa*). The last one is the only methanolic extract presenting a residual activity after removal of tannins on a polyamide column.

The best direct and indirect antimicrobial activity was obtained with the dichloromethane extract from the leaves of *Vitellaria paradoxa* which presented a MIC of 250mg/L on MRSA33591 and FICI ranges of 0.14-1 and 0.51-1 in combination with ampicillin and oxacillin respectively. Further research is needed in order to identify active molecules and also determine those responsible for the high cytotoxicity of this extract. The hexane extract from the roots of *Anchomanes difformis* also shows interesting properties, with FICI ranges of 0.18-1 and 0.60-2 in combination with ampicillin and oxacillin respectively, warranting additional studies to identify the active compounds therein.

We also showed here that only a few pure antimicrobial compounds were isolated from the plants studied. It will be thus really interesting to fractionate active extracts.

We have however to acknowledge some limitations of this study, related to the fact that that we studied a single bacterial strain and looked for synergy with two β-lactams only. This design was adopted in order to obtain a first indication about the potential mechanisms of the synergisms observed. In spite of this, and pending for further studies aiming at identifying the active compounds and extending our observation to other bacterial strains or drugs, this work opens the doors to the identification of new resistance modifying agents active on the β-lactams resistance mechanisms from methicillin resistant *Staphylococcus aureus*.

5. Acknowledgements

The authors are grateful to Mr. Agabani (botanist of University of Abomey-Calavi, Cotonou, Benin) , Prof. N. Ngombe (RDC) and Dr M. Rafamantanana (Madagascar) for plant collections and identifications and to Maude Bourlet for her skillful technical assistance. F.V.B. is *Maître de Recherches* of the Belgian *Fonds de la Recherche Scientifique*. The authors gratefully thank the Belgian *Fonds de la Recherche Scientifique* for financial support.

RESULTS – PART 2

Isolation and antistaphylococcal activities of major compounds from the leaf dichloromethane extract of Vitellaria paradoxa

In this second part of the results section, we investigate the composition of the leaf dichloromethane extract from *Vitellaria paradoxa*, the extract showing the best direct and indirect antistaphylococcal activity in the screening described in the previous chapter. LC-MS analysis of this extract allowed us to identify 5 triterpenic acids together with 6 triterpenic esters which were further isolated by preparative fractionation and analyzed by NMR for structure determination. All isolated compounds were tested *in vitro* against three *S. aureus* strains, including one clinical MRSA strain as well as for their cytotoxicity on WI38 human normal fibroblasts.

This chapter is the first part of a paper in preparation: Catteau L., Beaufay C., Girardi C., Hérent M-F., Van Bambeke F., Quetin-Leclercq J. Antistaphylococcal and antiparasitic activities of triterpenic acids and ester derivatives isolated from the leaves of *Vitellaria paradoxa.*

Abstract

Vitellaria paradoxa C.F. Gaertn. ssp. *paradoxa* (Sapotaceae) also called 'Shea butter tree', is used in traditional medicine to treat various symptoms related to infectious diseases including malaria fever, dysentery or skin infections. The dichloromethane extract from its leaves was previously shown to inhibit *Staphylococcus aureus* growth with a MIC of 250 mg/L but being cytotoxic on WI38 human normal fibroblasts with an IC_{50} of 7.17 ± 2.93 mg/L [452].

As cytotoxicity and antimicrobial activity may not be related to the same target and in order to find more active and less cytotoxic compounds, we investigated the composition of this extract. Thanks to LC-MS analyzes and fractionation of the crude extract, 5 pentacyclic triterpenic acids together with 6 ester derivatives were isolated and identified by comparison with standards and/or by analysis of mass and NMR spectra. All isolated compounds were evaluated for their antimicrobial activity on three *S. aureus* strains. While esters showed a poor antistaphylococcal activity (MIC ≥ 128 mg/L), triterpenic acids showed a moderate activity; corosolic acid being the most active with MICs ≤ 8 mg/L. Beside these interesting activities all isolated compounds showed cytotoxicity against the WI38 cell line.

Except for oleanolic and ursolic acids, this is the first report of the isolation of triterpenic compounds in the leaves of *Vitellaria paradoxa*. The results obtained may partially justify the use of *Vitellaria paradoxa* to treat skin infections. The active compounds isolated in this study could be used as lead skeletons for structural modifications in order to improve their activity and decrease their cytotoxicity.

1. Introduction

Antibiotic resistance is a major threat to the global population and new strategies to combat the inexorable rise of multidrug-resistant bacteria are urgently needed. Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major nosocomial pathogen that can cause severe localized and systemic infections [7]. The undisputed public health relevance of this species is related to its multiple-antibiotic resistance and its potential for swift dissemination through healthcare, communities and animal networks [10]. One strategy for fighting this multi-drug resistant bacteria could be the use of natural products [102,453].

The leaves, fruits or stem bark of *Vitellaria paradoxa* are used in traditional medicine to treat several diseases such as skin infections, diarrhoea, stomach ache and ulcers, dysentery, inflammation, malaria fever and breast cancer [369,449]. This tree, also called 'shea-butter tree' grows up to 14m high and is widely studied for its stem bark or kernels, while leaves are understudied. The most valued product from shea tree is shea butter, the edible fat extracted from the kernels, consisting of olein and stearin fractions along with non-saponifiable compounds [454,455]. While shea stearin is used as cocoa butter equivalent, improver or replacer in chocolate manufacture, shea butter is also increasingly popular as component of skin care products and cosmetic product formulations [456,457].

A screening of the antistaphylococcal activity of plants used in traditional medicine in Africa previously exhibited the potential interest of this plant. A crude leaf dichloromethane extract of *Vitellaria paradoxa* possessed an MIC of 250 mg/L against a reference strain of MRSA. However, this extract also showed a relatively high cytotoxicity with an IC_{50} of 7.17 ± 2.93 mg/L against the normal human fibroblast cell line WI38. The aim of the following work was thus to isolate the antistaphylococcal agents in this *Vitellaria paradoxa* extract.

2. Materials and Methods

2.1 Plant material

Leaves of *Vitellaria paradoxa* C.F. Gaertn. (syn. *Butyrospermum parkii*) were collected in Benin (Parakou) in August 2012. A voucher specimen was identified and deposited at the Herbier National of Abomey-Calavi University in Benin, bearing the number AP2130.

2.2 Preparation of extracts

Air dried and powdered leaves (100g) of *Vitellaria paradoxa* were successively extracted using a soxhlet apparatus with 700mL of hexane and dichloromethane (DCM) for 8h each. The extracts obtained (yields = 4.62% and 1.83% respectively) were then evaporated to dryness under reduced pressure with a rotary evaporator at a temperature of 30°C.

2.3 Crude DCM extract HPLC-UV-MS/MS analysis

To analyze the constituents of the leaf DCM crude extract, we used the method previously described to quantify oleanolic (OA) and ursolic acids (UA) with some modifications [293]. We used a LC-UV-MS/MS system consisting in a Thermo Accela pump, autosampler, photodiode array detector and Thermo Scientific LTQ orbitrap XL mass spectrometer. The column used was a Phenomenex Luna C18, 250 x 4.6 mm packed with 5µm particles. The flow rate was 1mL/min using an isocratic binary solvent system: solvent A (20%), H_2O pH6 (CH_3COONH_4 0.02M); solvent B (80%), acetonitrile/methanol 40:35. The DCM extract solution was prepared by dissolving 10mg of extract in 10mL of HPLC grade methanol. The injection volume was 20µL. Peaks were detected at 210nm. High-resolution mass spectra were measured with the APCI source in the negative mode. The following inlet conditions were applied: capillary temperature 250°C, APCI vaporizer temperature 400°C, sheath gas flow

20.00 u.a., auxiliary gas flow 5.00 u.a., sweep gas flow 5.00 u.a. Data acquisition and processing were performed with Xcalibur software.

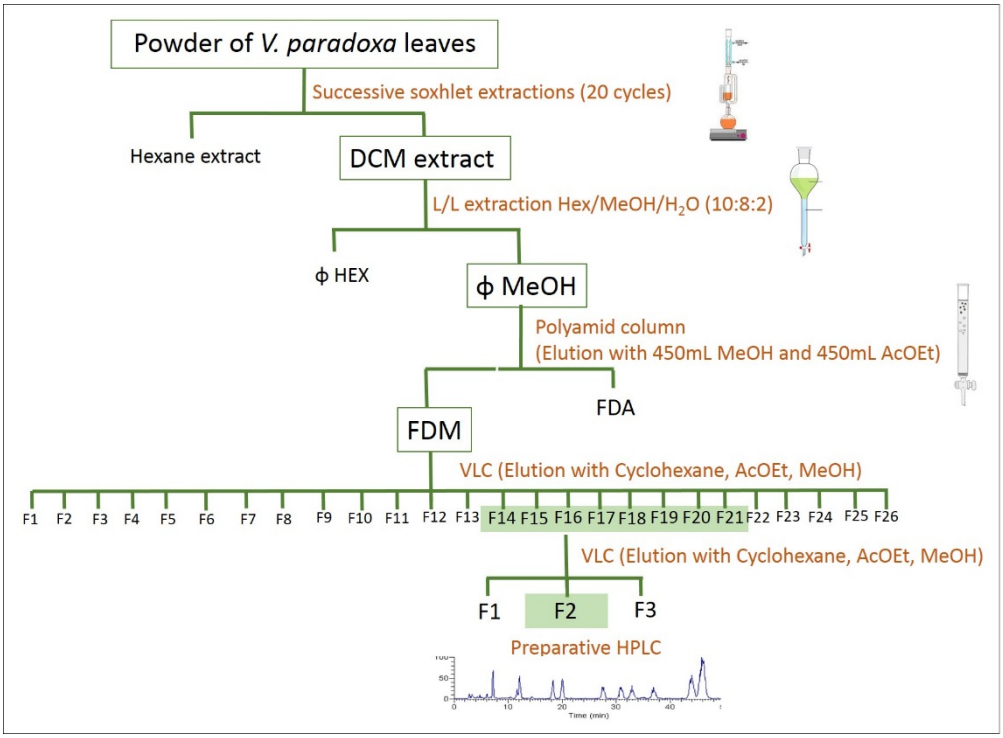
Tormentic (**1**), maslinic (**3**), corosolic (**4**), oleanolic (**9**) and ursolic acids (**10**) were identified from the leaf DCM extract by comparing retention times, mass spectra and fragmentations with standard compounds (AvaChem Scientific, San Antonio, USA).

2.4 Isolation of compounds

A scheme of extraction is presented on **figure 3-1**. The extraction discussed in 2.2 was repeated ten times for a total of 1kg of powdered leaves (mean yield = 1.87%). The DCM extracts were pooled and evaporated to dryness under reduced pressure with a rotary evaporator at a temperature of 30°C yielding 19.3g of crude extract. Two grams of this extract were solubilized in the two phases of a 400mL mixture of hexane-methanol-water (10:8:2) and a liquid-liquid extraction was realized three times with 200mL of methanol-water (8:2) [215]. This was repeated nine times on the rest of the extract. The lower phases (ϕ_{MeOH}) were pooled, evaporated and the residue (10.47g) was submitted to fractionation (5 x 2g) through polyamide (Machery Nagel, SC6 0.05-0.16mm) column (30.0 x 2.3 cm²) first eluted with 450mL of methanol and then with 450mL of ethyl acetate giving two fractions (FDM and FDA, respectively). Vacuum liquid chromatography (VLC) (Si 60 0.040-0.063mm) of the FDM (9g) was performed using a Buchner funnel (5.0 x 113 cm²) and cyclohexane/ethyl acetate (8:2) gradients to obtain 26 fractions (FDM 1-26). FDM 14-21 (2.74g) were pooled and submitted once again to the same VLC fractionation process yielding three fractions F1-F3. F2 was then submitted to preparative HPLC-UV consisting of a Shimadzu LC-20AP pump and a Spd-20AV detector. The column used was a Phenomenex Luna C18, 250 x 30 mm² packed with 5 µm particles. The flow rate was 42mL/min of acetonitrile/methanol/water 45:35:20. Ten peaks were collected using a detection at 210nm and 310nm yielding compounds 1-10.

1D and 2D NMR spectra of (**1**), (**2**), (**3**), (**4**) and (**5-8**) were recorded in deuterated methanol on a Brucker DRX 500 spectrophotometer (^{1}H at 500 MHz and ^{13}C at 125 MHz), with TMS as an internal reference. 2D experiments were performed using standard Bruker microprograms.

***Figure 3- 1** : Scheme of extraction*



2.5 Bacteria, cells and media

The crude extract and isolated compounds were evaluated for their antistaphylococcal activity against one methicillin-sensitive (MSSA ATCC25923, American Type Culture Collection), two methicillin-resistant (MRSA ATCC33591 and the clinical TCH USA300) *S.aureus* strains. Bacteria were cultivated on tryptic soy agar (TSA) (Difco, Richmond, CA, USA) and Minimal Inhibitory Concentrations (MICs) were determined in cation-adjusted Mueller-Hinton broth (caMHB).

The cytotoxicity of isolated compounds was evaluated on human normal fibroblast WI38 cultivated in a humidified atmosphere with 5% CO_2 at 37°C. The cell line, WI-38, was cultivated in DMEM medium (Life Technologies) containing 4mM L-glutamine, 1mM sodium pyruvate supplemented with 10% fetal bovine serum (Sigma) and penicillin-streptomycin (100UI/mL).

2.6 Antimicrobial susceptibility testing

MICs were determined by broth micro-dilution method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [262]. Briefly, bacteria were cultured on TSA (Difco, Richmond, CA, USA), incubated overnight at 37 °C and adjusted to a bacterial density of 10^6 CFU/mL as starting inoculum. Crude extract or isolated compounds were solubilized in dimethylsulfoxide (DMSO) (10 mg/mL) (Sigma, St Louis, MO, USA) and then diluted in the nutrient broth to obtain a final concentration of 500mg/L (extract) or 128 mg/L (pure compound). Serial two-fold dilutions were made to obtain a concentration range from 7.81 to 500 mg/L and 0.25 to 128 mg/L respectively. After overnight incubation, 30 µL of a 0.02% aqueous resazurin (Sigma,St Louis, MO, USA) solution was added to the wells (in order to facilitate the detection of bacterial growth) [452] and the plates were incubated during 1 h at 37 °C in the dark. The MIC corresponds to the lowest concentration of compound for which no metabolization of blue resazurin in pink resorufin was observed by sight. All tests were made in triplicate.

2.7 Cytotoxicity assays

The cytotoxicity of compounds on WI38 cells was evaluated using the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphényltetrazolium bromide (Sigma-Aldrich) colorimetric method as previously described [373]. Camptothecin (Sigma-Aldrich) was used as a positive cytotoxic reference. Stock solutions of crude extracts at 10mg/mL in DMSO were diluted in medium with a final concentration range of

100 – 0.04mg/L (2 wells/concentration). The cytotoxicity of solvents was tested in parallel and found to be negligible at the highest concentration used for extracts (0.5%). Each extract was tested in eight serial dilutions in 96-well microtiter plates at least in triplicate.

3. Results and Discussion

3.1 Crude DCM extract HPLC-UV-MS/MS analysis

An LC-UV-MS analysis of the leaf DCM crude extract allowed the identification of tormentic (**1**), maslinic (**3**) and corosolic acids (**4**) by comparison of retention times, mass spectra and fragmentations with standard compounds. These three triterpenic acids were identified together with oleanolic (**9**) and ursolic acids (**10**) which were previously reported by our team as present in the leaves of *Vitellaria paradoxa* [293]. This method also detected 5 triterpenic esters but the lack of standards did not allow us to identify them directly (**figure 3-2**). The high resolution APCI-MS of **2** and **5**-**8** in the negative ion mode showed molecular peaks at m/z 633.37865 and 617.38561, respectively corresponding to $[M-H]^-$. Thanks to the parameters we selected (presence of only C, H, O atoms) the more plausible molecular formula for **2** was $C_{39}H_{54}O_7$ while the best proposals for **5**, **6**, **7** and **8** were the same, corresponding to $C_{39}H_{54}O_6$. Both MS/MS mass spectra showed a loss of a m/z 164 fragment, forming fragments at m/z 469.34744 and 453.34337 corresponding to $[C_{30}H_{47}O_4, M-164]^-$ and $[C_{30}H_{47}O_3, M-164]^-$ respectively. The loss of the m/z 164 fragment observed in all the MS/MS spectra corresponds to $C_9H_8O_3$ in accordance with a coumaroyl moiety.

Several triterpenoids have already been isolated from the kernels (**figure 3-3**) or the stem bark (**figure 3-4**) of *Vitellaria paradoxa* while no compounds were identified up to now from its leaves, except from oleanolic and ursolic acid [451,454,458–462]. Of note, two isomers of tormentic acid (or 2α, 3β, 19α-trihydroxyurs-12-en-28-oic acid)

namely euscaphic acid (or 2α, 3α, 19α-trihydroxyurs-12-en-28-oic acid) and 2-*epi*-tormentic acid (2β,3β,19α-trihydroxyurs-12-en-28-oic acid) have already been isolated from *Vitellaria paradoxa* kernels [294] and stem bark [461] respectively. Purification of the corresponding peaks was thus necessary to determine or confirm the structure and stereochemistry of the different acids as well as to identify esters present in the extract.

Figure 3- 2 *: LC-UV (A) and LC-MS in negative mode (B) of crude leaf dichloromethane extract and structures of the 11 identified compounds.*

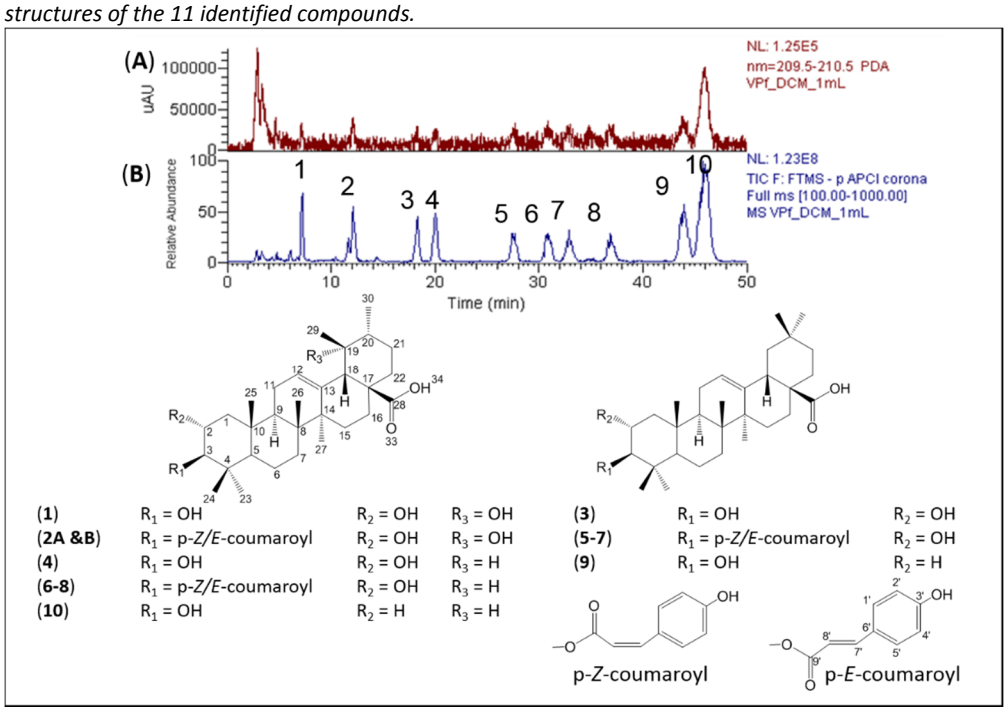


***Figure 3- 3** : Triterpenoids previously reported in shea kernels.[1][463][2][460][3][459][4][464][5][294][6][454]*

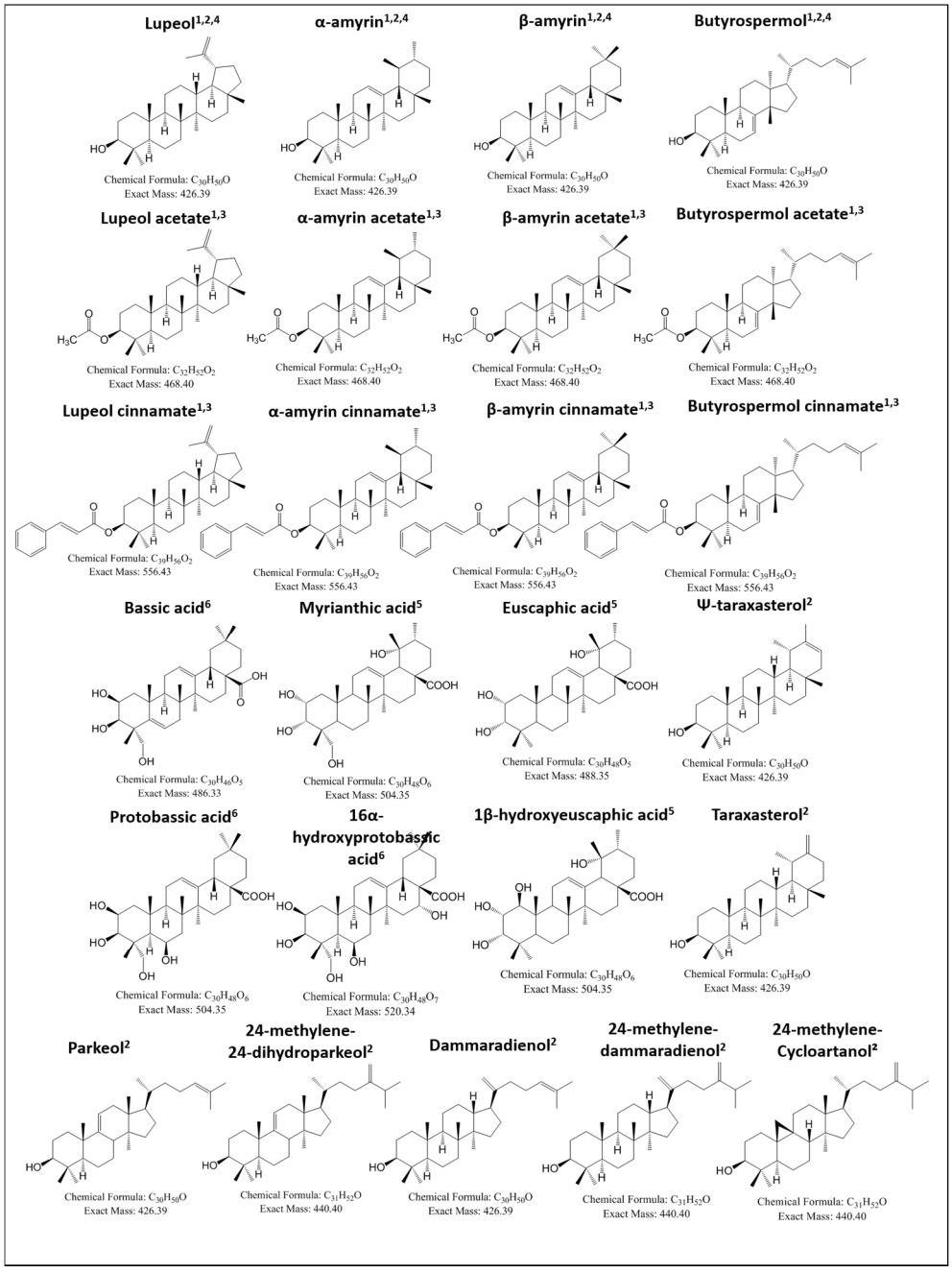
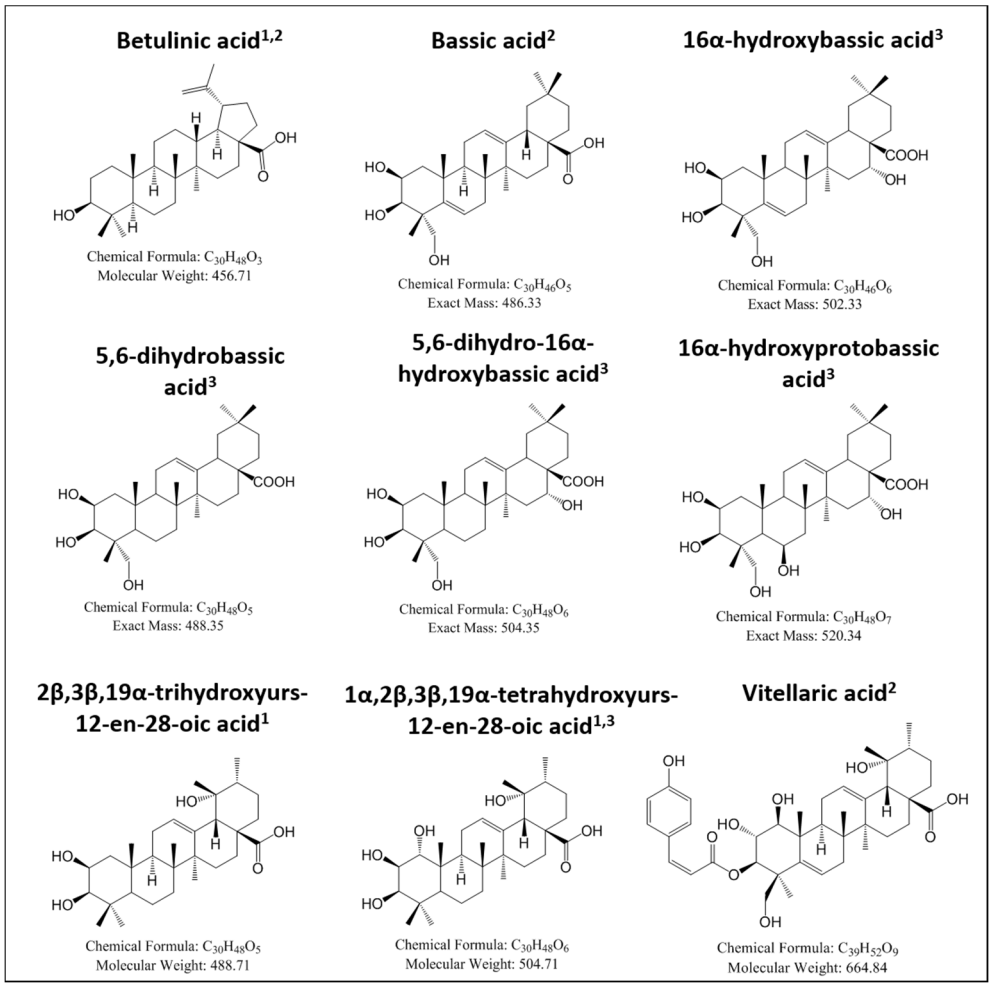


***Figure 3- 4** : Triterpenoids previously reported in Vitellaria paradoxa stem bark [1][461][2][458][3][462]*



3.2 Isolation and structure identification

To allow structure verification of triterpenic acids and determination of the coumaroyl-triterpenic esters (**2**, **5-8**), we purified them as explained in 2.4.

The structure of compound **1** was confirmed as tormentic acid on the basis of its 1D/2D NMR and HRMS analysis and by comparison with the data reported in the literature for this acid [465,466]. The configuration of the hydroxyl groups as 2α-OH and 3β-OH*,* respectively, was deduced from the characteristic coupling constant of 9.8 Hz observed between H-2 and H-3, with H-2 (δ 3.60, ddd, J = 10.8, 9.8, 4.1 Hz) and H-3 (δ 2.90, d, J = 9.8 Hz) [467]. Indeed, euscaphic acid having 2α-OH/3α-OH on the same side of the molecule displays logically a lower coupling constant H-2/H-3 of 2.5 Hz [468] while it was 5 Hz for 2-*epi*-tormentic acid (2β-OH/ 3β-OH*)* [469].
By comparison with NMR spectra of standards, we also confirmed the identification of **3** and **4** as maslinic and corosolic acids.

Surprisingly, inspection of the downfield region of the ^{1}H and ^{13}C NMR spectra of **2** indicates the presence of split signals for all the characteristic positions of a coumaroyl moiety, which appears to be present as a mixture of a *cis* and *trans* forms in equilibrium. Indeed, *cis* or *trans* could not be separated as independent molecule using the developed HPLC method. However, single signals were clearly observed for the ursan core of the aglycon, except position 1 to 4, also split by the influence of the cis or *trans* configuration as the coumaroyl group esterifies the 3 position of the triterpenic core. The characteristic NMR data are reported in **Table 3-1** (**2A** for *trans* and **2B** for *cis*).

During HPLC analyses of isolated compounds **5-8**, we detected a lack of stability of compound **5** which was partially isomerized into **7**. The same was observed for **7** isomerizing into **5**, indicating equilibrium between both structures. An identical

phenomenon was observed for the pair **6-8**. Similarly to compounds **2 A** and **B**, NMR data of both pairs (**5-7** and **6-8**) indicated the presence of a *E*- or *Z*-coumaroyl substructure explaining the equilibrium previously observed, corresponding to a *cis-trans* modification of the double bond geometry which is known to isomerize with light [215,470]. The coumaroyl NMR data (**table 3-1**) correspond to those previously reported [215,471]. The ^{1}H signals of the triterpene residues formed a similar pattern to that of **3** and **4** respectively.

***Table 3- 1** : ^{13}C and ^{1}H NMR chemical shifts of the coumaric acid moieties of the triterpenic esters (**2**), (**5-8**)*

	2 A (*E*)		**2B (*Z*)**		**5 (*E*)**	
	δ_H (mult, J in Hz)	δ_C	δ_H (mult, J in Hz)	δ_C	δ_H (mult, J in Hz)	δ_C
1'; 5'	7.45 (d, J=8.5)	131.2	7.63 (d, J=8.5)	133.7	7.46 (d, J=8.7)	131.1
2'; 4'	6.79 (d, J=8.5)	116.8	6.71 (d, J=8.5)	115.7	6.79 (d, J=8.7)	116.8
3'	-	161.2	-	159.9	-	160.9
6'	-	127.4	-	127.8	-	127.2
7'	7.61 (d, J=15.9)	146.3	6.86 (d, J=12.8)	144.7	7.62 (d, J=15.9)	146.3
8'	6.37 (d, J=15.9)	115.8	5.83 (d, J=12.8)	117.3	6.35 (d, J=15.9)	116.8
9'	-	169.6	-	168.6	-	169.6
	6 (*Z*)		**7 (*E*)**		**8 (*Z*)**	
	δ_H (mult, J in Hz)	δ_C	δ_H (mult, J in Hz)	δ_C	δ_H (mult, J in Hz)	δ_C
1'; 5'	7.64 (d, J=8.7)	133.6	7.45 (d, J=8.5)	130.9	7.63 (d, J=8.5)	133.4
2'; 4'	6.72 (d, J=8.7)	115.7	6.79 (d, J=8.5)	117.3	6.72 (d, J=8.5)	116.2
3'	-	160.1	-	161.0	-	159.8
6'	-	127.8	-	126.9	-	127.7
7'	6,86 (d, J=12,7)	144.7	7.62 (d, J=15,7)	146.1	6.86 (d, J=12.8)	144.5
8'	5.84 (d, J=12.7)	117.4	6.38 (d, J=15.7)	115.6	5.83 (d, J=12.8)	117.1
9'	-	169.5	-	169.4	-	168.4

Analysis of all ^{1}H, ^{13}C, ^{1}H-^{1}H COSY, ^{1}H-^{13}C HMBC and ^{1}H-^{13}C HSQC allowed us to identify the six triterpenoid esters as 3-O-*p*-(*Z/E*)-coumaroyltormentic acid (**2A** and **2B**), 3-O-*p*-(*Z/E*)-coumaroylmaslinic acid (**5-7**) and 3-O-*p*-(*Z/E*)-coumaroylcorosolic acid (**6-8**). The characteristic chemical shifts which allowed us to identify these compounds in comparison to those previously published are reported in table **3-2** [466,472–474]

Compounds **2A** and **B** were earlier reported from various plants, among which *Perilla frutescens* var. acuta (Labiatae) [472] or *Eriobotrya japonica* (Rosaceae) [466]. Compounds (**5-7**) as well as (**6-8**) have been previously reported together in the leaves of *Prinsepia utilis* Royle (Rosaceae), as well as in the fruits of *Zizyphus jujuba* (Rhamnaceae) for the second pair, for example [473,474].

***Table 3- 2** : Main chemical shifts as well as key HMBC correlations observed for (**2**) and (**5-8**)*

(2)		δ (ppm)	HSQC (^{1}H-^{13}C)	HMBC (^{1}H-^{13}C)
2 (*Z*)	CH	67.57	3.84 (m)	
2 (*E*)	CH	67.62	3.77 (m)	
3 (*Z*)	CH	85.66	4.61 (d, J=9.9)	C23, C24, C9', C2, C4
3 (*E*)	CH	85.32	4.58 (d, J=9.9)	C23, C24, C9', C2, C4
12	CH	129.14	5.28 (s.)	C19, C14, C18, C11
13	C	139.8	_	
18	CH	55.59	2.50 (m.)	C28, C19, C14, C12, C11, C13
19	C	73.5	_	
23	CH_3	29.59	1.02 (s.)	
24	CH_3	18.02	0.94 (s.)	
25	CH_3	16.3	0.99 (s.)	
26	CH_3	16.7	0.88 (s.)	
27	CH_3	26.7	1.15 (s.)	
28	C	182.71	_	
29	CH_3	26	0.88	
30	CH_3	16	0.93 (d.)	

(5-7)		δ (ppm)	HSQC (^{1}H-^{13}C)	HMBC (^{1}H-^{13}C)
2 (*Z*)	CH	67.57	3.83 (m)	
2 (*E*)	CH	67.62	3.77 (m)	
3 (*Z*)	CH	85.1	4.59 (d, J=9.9 Hz)	C23, C24, C9', C2, C4
3 (*E*)	CH	85. 5	4.62 (d, J=9.9 Hz)	C23, C24, C9', C2, C4
12	CH	123.3	5.25 (br.t)	C19, C14, C18, C11
13	C	145.4	_	
18	CH	42.5	2.85 (dd, J=4.4; 14.0 Hz)	C28, C19, C14, C12, C11, C13
19	CH_2	47.3	1.14 ; 1.70	
23	CH_3	23.9	0.94 (s.)	
24	CH_3	17.8	0.82 (s.)	
25	CH_3	16.9	1.04 (s.)	
26	CH_3	29.2	0.82 (s.)	
27	CH_3	26.5	1.18 (s.)	
28	C	182.02	_	
29	CH_3	33.4	0.89 (s.)	C20. C21, C19
30	CH_3	18.2	0.93 (s.)	C20, C21, C19
(6-8)		**δ (ppm)**	**HSQC (^{1}H-^{13}C)**	**HMBC (^{1}H-^{13}C)**
2 (Z)	CH	67.5	3.79 (m.)	
2 (E)		67.6	3.84 (m.)	
3 (Z)	CH	84.9	4.59 (d, J=9.9 Hz)	C23, C24,C9', C2, C4
3 (E)		85.2	4.62 (d, J=9.9 Hz)	C23, C24,C9', C2, C4
12	CH	126.3	5.3 (br. t)	C17, C14, C18, C11
13	C	139.6	_	
18	CH	54.6	2.22 (d, J= 11.2Hz)	C29, C28, C27, C17, C14, C19, C13, C12
19	CH	40.3	1.37	
23	CH_3	29.2	0.86 (s.)	
24	CH_3	17.8	0.84 (s.)	
25	CH_3	17	1.05 (s.)	
26	CH_3	29.3	0.86 (s.)	
27	CH_3	24	1.15 (s.)	
28	C	181.9	_	
29	CH_3	17.3	0.88	C20
30	CH_3	21.2	0.95 (d., J= 10.5 Hz)	C20

These coumaroyl esters are reported here for the first time to be present in the leaves of *Vitellaria paradoxa*, while kernels were shown to contain triterpenoid cinnamic esters [459,463], and stem bark one coumaroyl ester, namely vitellaric acid corresponding to (**2**) with two added hydroxyl groups in positions 1 and 23.

3.3 *In vitro* antistaphylococcal activity

The antistaphylococcal activity of isolated compounds is reported in **table 3-3**. According to several authors, the antibacterial activity is considered as significant if MIC ≤ 10 mg/L, moderate if 10 < MIC ≤ 100 mg/L, and low or negligible if MIC > 100 mg/L [105–108]. The antistaphylococcal activity of oleanolic (**9**) and ursolic (**10**) acids, the most common oleanane and ursane class representatives was already extensively studied. However, reported activity of these two acids vary widely amongst publications [108,123,124,177,269–274,277,279,296–299] but **9** and **10** are in general, as here, considered as moderately and significantly active against *S. aureus* strains respectively.

As other isolated compounds correspond to hydroxylated and esterified derivatives of **9** and **10**, we chose to compare their activity to OA (**9**) and UA (**10**) respectively. The literature data concerning the impact of 2α hydroxylation on the antistaphylococcal activity is not clear. Indeed, according to the authors, maslinic acid (**3**) is, more potent ([296] on MSSA ATCC25923) or less potent ([299] on a MSSA strain) than OA (**9**). For corosolic acid (**4**), Ngezahayo and coworkers observed an improved activity compared to UA (**10**) for one strain (MRSA ATCC33591), while they obtained an equal activity on MSSA ATCC6538 and the clinical MRSA isolate C100459, and a lower activity on the MRSA clinical isolate C98506 [278]. Nevertheless, three other authors showed lower MICs for corosolic (**4**) in comparison to UA (**10**) [275,281,284]. In the present work, we confirm the general positive effect of 2α-hydroxylation of OA and UA, the MICs of compounds **3** and **4** being lower than those observed for **9** and **10** respectively.

The negligible activity of tormentic acid (**1**), a di-hydroxylated ursolic acid derivative, is in accordance with Ngezahayo et al., who reported an MIC of 128mg/L and 64mg/L on three MRSA strains and one MSSA strain respectively for the same compound extracted from the aerial parts of *Plastotoma rotundifolium* (Briq.) A. J. Paton [278].

Euscaphic acid, tormentic acid enantiomer, is also reported as inactive (MIC > 250 on MSSA ATCC25922) [294] while 2- *epi*-tormentic acid has never been tested on *S. aureus* strains.

Ester derivatives (**2A** and **2B**, **5-7** and **6-8**) only showed negligible activity (MIC > 128 mg/L) against these *S. aureus* strains. The MIC of **2** extracted from the leaves of *Planchonia careya* (F. Muell.) R. Knuth (*Lecythidaceae*) was 118mg/L against MSSA ATCC25923 [475], which is in accordance with our results, while we tested this compound on MRSA strains for the first time. The two other pairs of esters (**5-7**) and (**6-8**) were tested here for their antistaphylococcal activities for the first time and showed negligible activity. It thus seems that the 3-O-esterification of triterpenic acids by a coumaroyl moiety decreases the activity against *S. aureus* strains.

***Table 3- 3** : Minimum Inhibitory Concentrations (MICs) (mg/L) and half maximal inhibitory concentrations (IC_{50}) (mg/L) of isolated compounds against three S. aureus strains and the human normal fibroblast WI38 cell line.*

	MSSA ATCC25923	**MRSA ATCC33591**	**MRSA TCH USA300**	**WI38 (IC_{50})**
VPf DCM	250	250	250	7.17 ± 2.93
(1)	128	128	128	85.7 ± 2.13
(2)	>128	>128	>128	21.04 ± 3.24
(3)	32	16	32	20.85 ± 3.97
(4)	4-8	4	4	9.34 ± 2.47
(5-7)	>128	>128	>128	5.89 ± 0.96
(6-8)	>128	128	128	9.04 ± 3.11
Mixture (5),(6),(7),(8)	>128	128	>128	7.90 ± 0.53
(9)	64	32-64	>128	63.26 ± 0.74
(10)	16	8	16	11.14 ± 0.22

In **table 3-3**, we also report the cytotoxicity of the isolated compounds against the human normal fibroblast cell-line WI38. We show that despite their significant to moderate antistaphylococcal activities, maslinic (**3**), corosolic (**4**), and ursolic (**10**) acids are also cytotoxic, while tormentic (**1**) and oleanolic (**9**) acids are less toxic but also less antimicrobial. Except for corosolic acid (**4**), isolated triterpenes show cytotoxicity at lower concentration than their MIC which thus limits their interest as antistaphylococcal compounds. However, we limit our cytotoxicity tests to only one

cell line and different results could be obtained on other models. Moreover, the potential therapeutic interest of this class of compounds is highlighted by the fact that they were evaluated for their anti-inflammatory or anti-diabetic effects in clinical trials [476–480]. Of note, some formulations like liposomal formulations have been used intravenously without showing any toxicity up to doses of 98mg/m²/day for 14 days [478–481]. Furthermore, chemical modifications could be done on these interesting scaffolds in order to decrease their cytotoxicity as well as increase their activity.

4. Conclusions

This is the first report of the presence of tormentic, maslinic, corosolic acids as well as their coumaroyl derivatives in the leaves of *Vitellaria paradoxa*. This can partially justify the traditional use of *Vitellaria paradoxa* leaves to treat skin infections, showing the significant to moderate antistaphylococcal activity of some isolated triterpenic acids. Of interest, these compounds should be tested for their antibacterial properties against other bacterial species implicated in skin infections like *Staphylococcus epidermidis* or *Streptococcus pyogenes* for example. Moreover, we encourage future research in modifying the structure of these compounds in order to improve their selectivity, but showed here that 2α-hydroxylation increases the antistaphylococcal activity but also the cytotoxicity while esterification by coumaric acid in position 3 decreases the antimicrobial activity and increases the cytotoxicity.

RESULTS – PART 3

Synergy between ursolic and oleanolic acids from Vitellaria paradoxa leaf extract and β-lactams against MRSA: in vitro and in vivo activity and underlying mechanisms

After having identified and isolated 11 triterpenic compounds in the dichloromethane extract from the leaves of *Vitellaria paradoxa* as well as studied their antistaphylococcal activities, we quantified the two major components present in this extract, namely ursolic and oleanolic acids. Both compounds were then tested for their potential as β-lactam resistance modifying agents against various Methicillin-Resistant *Staphylococcus aureus* (MRSA) strains. Thanks to a collaboration with Dr Reichmann from ITQB Nova, in Portugal, we also explored their potential mechanism of action. Finally, with the support of a Fulbright grant, we took the opportunity to collaborate with the Pr. Nizet from University of California San Diego, to further assess the combination of the most active compound and a β-lactam in a murine model of *S. aureus* subcutaneous infection.

The results of this work are detailed in this chapter based on a published paper: Catteau L., Reichmann N. T., Olson J., Pinho M. G., Nizet V., Van Bambeke F., Quetin-Leclercq J., 2017. Synergy between ursolic and oleanolic acids from *Vitellaria paradoxa* leaf extract and β-lactams against Methicillin-Resistant *Staphylococcus aureus: in vitro* and *in vivo* activity and underlying mechanisms. *Molecules* (22) 12.

Abstract

Combining antibiotics with resistance reversing agents is a key strategy to overcome bacterial resistance. Upon screening antimicrobial activities of plants used in traditional medicine, we found that a leaf dichloromethane extract from the shea butter tree (*Vitellaria paradoxa*) had antimicrobial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) with further evidence of synergy when combined with β-lactams. Using HPLC-MS, we identified ursolic (UA) and oleanolic acids (OA) in leaves and twigs of this species, and quantified them by HPLC-UV as the major constituents in leaf extracts (21% and 6% respectively). Both pure triterpenic acids showed antimicrobial activity against reference and clinical strains of MRSA, with MICs ranging from 8–16 mg/L for UA to 32–128 mg/L for OA. They were highly synergistic with β-lactams (ampicillin and oxacillin) at subMIC concentrations. Reversion of MRSA phenotype was attributed to their capacity to delocalize PBP2 from the septal division site, as observed by fluorescence microscopy, and to disturb thereby peptidoglycan synthesis. Moreover, both compounds also inhibited β-lactamases activity of living bacteria (as assessed by inhibition of nitrocefin hydrolysis), but not in bacterial lysates, suggesting an indirect mechanism for this inhibition. In a murine model of subcutaneous MRSA infection, local administration of UA was synergistic with nafcillin to reduce lesion size and inflammatory cytokine (IL-1β) production. Thus, these data highlight the potential interest of triterpenic acids as resistance reversing agents in combination with β-lactams against MRSA.

1. Introduction

Vitellaria paradoxa C.F. Gaertn. (Sapotaceae), also called 'shea butter tree', is a tree that grows up to 14m high. This tree is generally protected because of the economic value of shea butter, the fat extracted from the fermented kernels [449]. The leaves, roots, fruits, and stem bark of *Vitellaria paradoxa* (VP) have been used in traditional medicine to treat various infections [368–370]. This time-tested clinical application motivated us to investigate the leaves of this plant for antibacterial compounds and resistance modifying agents, focusing on skin infections caused by *Staphylococcus aureus*.

S. aureus is one of the most prominent human pathogens and a severe threat to public health with β-lactam antibiotics traditionally considered as a first line of treatment [7]. However, over time, *S. aureus* developed two main resistance mechanisms against this class of antibiotics. First, the production of β-lactamases inactivating these drugs, which was circumvented by the development of biopharmaceutical compounds resistant to enzymatic hydrolysis such as oxacillin or nafcillin. Second, exemplified by methicillin-resistant *S. aureus* (MRSA), the production of PBP2a, a cell wall transpeptidase and penicillin-binding protein (PBP), encoded by the *mecA* gene, with a decreased affinity for methicillin and most other β-lactam drugs [252]. This mechanism confers resistance to all β-lactam antibiotics, with the noticeable exception of cephalosporins from the fifth generation like ceftaroline or ceftobiprole recently brought to the market [482]. There thus exists an urgent need to find alternative and original strategies to fight against β-lactam-resistant staphylococci.

In this context, it is of interest to restore the utility of antibiotics made less potent by the development of resistance by combining them with resistance modifying agents. One potential source of such compounds could be plant extracts [102].

In this work, we studied the direct and indirect (in combination with β-lactams) antimicrobial activity of the main components of *VP* leaf extract against *S. aureus* and analyzed their potential mechanism of action.

To date, the antimicrobial activity of VP stem bark and leaf extracts has only been reported on strains of *S. aureus* using an agar diffusion method and high extract concentrations (50 g/L) [370,407]. However, during a preliminary screening of the direct and indirect antimicrobial activity of plants used in traditional medicine in Benin, we found that a VP leaf dichloromethane extract showed the best direct and indirect antimicrobial activities with a MIC = 250 mg/L and fractional inhibitory concentration indices (FICI) = 0.14-1 and 0.51-1 in combination with ampicillin and oxacillin, respectively, against a strain of MRSA [452]. In the present study, we continued our work on the dichloromethane extracts and identified, for the first time in leaves and twigs, oleanolic (OA) and ursolic (UA) acids as bioactive compounds. We quantified these two substances in extracts and studied the direct and indirect antimicrobial activities of these pure compounds in combination with β-lactams (ampicillin, oxacillin or nafcillin) on a panel of *S. aureus* reference strains as well as clinical MRSA isolates. We further explored the potential mechanism of action underlying this synergistic combination focusing on the potential effect of those compounds on the two principal β-lactam resistance mechanism of MRSA: β-lactamases and PBP2a. Finally, we assessed the effectiveness of the combination of UA and nafcillin using an *in vivo* murine skin infection model.

2. Materials and methods

2.1 Plant material

Leaves and twigs of *Vitellaria paradoxa* C.F. Gaertn. (syn. *Butyrospermum parkii*) were collected in Benin (Parakou) in August 2012. A voucher specimen was identified and deposited at the Herbier National of Abomey-Calavi University in Benin, bearing the number AP2130.

2.2 Preparation of extracts

Dried and powdered leaves or twigs (100 g) of *Vitellaria paradoxa* were extracted using a soxhlet apparatus with 700 mL of two solvents of increasing polarity (hexane and dichloromethane) for 8 h each. The extracts obtained were then evaporated to dryness under reduced pressure with a rotary evaporator at a temperature of 30 °C. We obtained 4.62 g and 1.83 g for the hexane and dichloromethane extracts respectively.

2.3 Reference compounds

Ursolic (UA) (purity 91 %) and oleanolic (OA) (purity 98 %) acids were bought from AvaChem, San Antonio, TX. and solubilized in DMSO. Ampicillin (potency 87.99 %), oxacillin (potency 90 %), nafcillin (potency 82 %), gentamicin sulfate (potency 64.40%) and the β-lactamase inhibitor clavulanic acid (potency 50 %) were obtained as microbiological standards from Sigma-Aldrich, St Louis, MO.

2.4 UA and OA identification

The identification of compounds present in the crude extract was performed with a LC MS/MS system consisting in a Thermo Accela pump, autosampler, photodiode array detector and Thermo Scientific LTQ orbitrap XL mass spectrometer (MASSMET platform). The column used was a Merck LiChroCART C18 250 x 4.6 mm packed with 5 µm particles.

The flow rate was 800 µL/min using a binary solvent system: solvent A, HPLC grade water-acetonitrile (9:1) with 0.1 % formic acid and solvent B, acetonitrile with 0.1 % formic acid (0–5 min: 100 % A, 70–85 min: 0 % A, 86–96 min: 100 % A). High-resolution MS was measured with APCI source in the negative mode. The following inlet conditions were applied: capillary temperature 250 °C, APCI vaporizer temperature 400 °C, sheath gas flow 20.00 u.a., auxiliary gas flow 5.00 u.a., sweep gas flow 5.00 u.a. Data acquisition and processing were performed with Xcalibur software.

2.5 UA and OA quantification

The quantification of UA and OA from the crude extracts was performed according to a method adapted from the one described in PHARMEUROPA 2015 for the Common Selfheal fruit-spike [483]. We used an HPLC UV system consisting of a Merck Hitachi pump, autosampler and a Waters UV detector (LambdaMax, model 481). The column used was a Merck LiChroCART C18 250 x 4.6 mm packed with 5µm particles. The flow rate was 800 µL/min using an isocratic binary solvent system: solvent A (20 %), H_2O pH6 ($NH_4H_2PO_4$ 0.04 M); solvent B (80 %), Acetonitrile/Methanol40:35. Peaks were detected at 210nm. A calibration curve was obtained for UA and OA methanolic solutions at concentrations ranging from 0.020 to 0.150 mg/mL (correlation coefficients (r^2) of calibration curves > 0.99). Plant extract solutions were prepared by dissolving 10mg of extract in 10 mL of methanol. The injection volume used was 20 µL. To check the specificity of the method we used an LC-PDA-MS/MS system and the same LC conditions as those used for the quantification except that the pH buffer $NH_4H_2PO_4$ 0.04 M had to be replaced by volatile CH_3COONH_4 0.02 M. After having confirmed that the buffer change did not affect the elution profile with the UV detector, the specificity of the method was checked by comparing the mass and UV spectra at three different zones of the peaks with those of the respective standards.

2.6 Bacterial strains

S. aureus ATCC33591 (MRSA and β-lactamase producer; American Type Culture Collection) was used as a reference strain. Four clinical isolates of MRSA were collected at the Universitair Ziekenhuis Brussel (UZ Brussel) in 2009. The strain VUB2 was isolated from a respiratory infection, VUB10 from a wound infection, and VUB20 and VUB30, from wound infections in diabetic patients. The homogeneous MRSA strain BCBPM073, derived from COL and carrying a single copy of PBP2 with an N-terminal superfast GFP fusion was used for the PBP2 localization study [95]. The strain MRSA USA300 UAMS1182 was used for the murine subcutaneous infection model [484].

2.7 MIC determination

Minimal Inhibitory Concentrations (MICs) were determined by broth micro-dilution method according to the guidelines of the Clinical and Laboratory Standards Institute in cation-adjusted Mueller-Hinton broth (caMHB) [485].

Briefly, bacteria were cultured on tryptic soy agar (TSA) (Difco, Richmond, CA), incubated overnight at 37°C and adjusted to a bacterial density of 10^6 CFU/mL as starting inoculum. Triterpenic acids and crude extracts were solubilized in dimethylsulfoxide (DMSO) (Sigma) and then diluted in the nutrient broth to obtain a final concentration of 500mg/L and 128 mg/L, respectively. Serial two-fold dilutions were made to obtain a concentration range from 0.25 to 128 mg/L and 7.81 to 500 mg/L respectively. After overnight incubation, 30 µL of a 0.02 % resazurin (Sigma) solution was added to the wells (in order to facilitate the detection of bacterial growth) [452] and the plates were incubated during 1 h at 37 °C in the dark to facilitate readings, the MIC corresponding to the lowest concentration of compound for which no metabolization of blue resazurin in pink resorufin was observed by sight. All tests were made in triplicate.

2.8 FICI determination

Fractional Inhibitory Concentration Indexes (FICI) were determined by the checkerboard method in ca-MHB [331]. In a 96-well plate, the β-lactam antibiotic in the combination was serially diluted starting from a final concentration of 2 x MIC along the ordinate. The triterpenic acid or the crude extract were serially diluted along the abscissa using 2 x MIC as the highest final concentration. The bacterial suspension (final inoculum 0.5-1 x 10^6 CFU/mL) was added to the wells.

After 20 h of incubation at 37 °C and addition of resazurin, MICs of antibiotics and triterpenic acids were determined as the lowest concentration that completely inhibited the growth of the organism.

Interactions between antibiotics were then evaluated using the FIC Indexes, calculated as the sum of the Fractional Inhibitory Concentrations (FICs) as follows: FICI = FIC A + FIC C, where FIC A is MIC of the antibiotic in the combination/MIC of the antibiotic alone and FIC C is MIC of the compound in the combination/MIC of the compound alone [129]. The combination was considered as synergistic for FICI ≤ 0.5, additive for 0.5 < FICI ≤ 1, indifferent for 1 < FICI ≤ 4 and antagonistic for FICI > 4 according to the European Committee for Antimicrobial Susceptibility Testing [333]. All tests were made in triplicate.

2.9 Time-kill Assay

Time-kill assay were performed as previously described [486] using ampicillin, oxacillin, UA, OA or the combination of the β-lactam antibiotic and a triterpenic acid, and the reference strain *S. aureus* ATCC33591. Briefly, an initial inoculum of 5 x 10^6 CFU/mL in caMHB was used. Antibiotics were used at subMIC concentrations that did not impair bacterial growth at 24 hours (64 and 16 mg/L for ampicillin and oxacillin respectively) and combined with four concentrations of triterpenic acids corresponding to their respective MIC, MIC/2, MIC/4 and MIC/8. The cultures were incubated at 37°C and aliquots were removed at 0, 4, 8 and 24 h and serially diluted

for colony forming unit (CFU) enumeration on TSA plates. Data were expressed as the change in $\log_{10}$ CFU from the initial inoculum after 24h incubation.

2.10 Effect of compounds on PBP2 localization

Cultures of MRSA strain BCBPM073 carrying the GFP-PBP2 fusion were grown overnight in TSB and diluted 1:200 into 20 ml TSB. Cultures were incubated at 37°C, with shaking (180 rpm), to exponential phase, corresponding to an OD_{600nm} of approximately 0.4. Cultures were divided into 5mL aliquots and incubated a further 30 min with 8 mg/L UA or 16 mg/L OA (concentration equivalent to their MIC). The same volume of solvent DMSO was added to the culture as a negative control, while 256 mg/L oxacillin (1x MIC) was used as a positive control. Cells were pelleted, suspended in Phosphate Buffer Saline (PBS) and observed by fluorescence microscopy on 0.1% PBS agarose pad. A Zeiss Axio Observer inverted microscope equipped with Photometric CoolSNAP HQ2 camera and Zen software was used for conventional microscopy. Structured illumination microscopy was performed using an Elyra PS.1 microscope (Zeiss, Oberkochen, Germany) equipped with a Plan-Apochromat 63x/1.4 oil DIC M27 objective and a Pco.edge 5.5 camera. Images were acquired using five grid rotations, with grating 28 µM period for 488 nm laser (100 mW) and reconstructed using ZEN software (black edition, 2012, version 8.1.0.484) as described previously [487]. Fluorescence ratio (FR) were calculated as (a-c)/(b-c) where “a” corresponds to the fluorescence measured at the septum, “b” to the fluorescence of the peripheral membrane and “c” to the background fluorescence [488] ; these measures were performed on conventional microscopy images using ImageJ software.

2.11 Effect of compounds on PBP2 and PBP2a expression

Cultures of MRSA strain BCBPM073 carrying the GFP-PBP2 fusion were grown overnight in TSB and diluted 1:200 into 200 ml TSB. Cultures were then prepared

following the same conditions as those used for the PBP2 localization experiment. Protein extraction and western blotting were performed as previously described, with minor modifications [489]. Cells were pelleted, suspended in PBS, and lysed using glass beads in a Fast Prep FP120 (Thermo Electro Corporation). Cells were separated from the glass beads by centrifugation for 1 min at 1,657 g, and unbroken cells were removed by centrifugation for 15 min at 16,000 g. Total protein content was quantified using the BCA protein assay kit (Pierce) and 50 mg of protein extract were loaded in a 10 % SDS-PAGE gel. After separation at 80 V for 4 hrs, samples were transferred to a Hybond-P Polyvinylidene difluoride (PVDF) membrane (GE Healthcare) using a semidry transfer cell (Biorad), cut between the 70 kDa and 100 kDa markers (to separate GFP-PBP2 and PBP2a containing regions) and blocked for 1 hr with 5 % milk in PBST (0.5 % Tween 20 in phosphate buffered saline). Membranes were incubated overnight with 1:5000 of polyclonal anti-PBP2 or 1:500 of anti-PBP2a (Slidex MRSA detection, Biomerieux) antibodies, washed with PBST the following day, and incubated for 1hr with 1:50,000 of HRP-conjugated goat anti-mouse or 1:50,000 of HRP-conjugated goat anti-rabbit antibodies, respectively. Bands were visualized using the ECL Plus Western blotting detection kit (Amersham) and Chemidoc XRS+ Imaging System (Biorad).

2.12 Evaluation of β-lactamase activity using the nitrocefin test

Nitrocefin is a chromogenic cephalosporin that can be used as a substrate for β-lactamase activity [490]. This yellow cephalosporin generates a red metabolite upon hydrolysis of its β-lactam ring by β-lactamases, the absorbance of which can be followed overtime. Bacteria were grown overnight in caMHB. A fresh suspension was prepared (OD_{620nm} = 0.1) and incubated for 4 hours at 37 °C and 130 rpm until exponential phase of growth (OD_{620nm} = 0.5). Aliquots of bacteria (2mL) were then incubated for 10 minutes in the presence or absence of UA or OA at appropriate

concentrations after which 180µL of each sample was mixed with 20 µL of a 0.05 % nitrocefin solution (CalBiochem/EMD Millipore, Billerica, MA). The changes of absorbance were recorded at 486 nm during 30 min at 37 °C SpectraMax microtiter plate reader (Molecular Devices LLC, Sunnyvale, CA). Gentamicin sulfate and clavulanic acid were used as negative and positive controls respectively.

In order to distinguish between a direct inhibition of enzymatic activity and an indirect effect on β-lactamase activity dependent on their anchoring in the bacterial membrane of living bacteria, the same experiment was also performed on bacterial lysates. Bacteria in exponential growth (DO_{620nm} = 0.5) were centrifuged 7 min at 1500 g. The supernatant was removed and the bacterial pellet was suspended in 500 µL of a detergent buffer, Bacterial Protein Extraction Reagent (Thermo Fisher Scientific). This lysate was resuspended in the initial volume of medium, added by the tested compounds and then by nitrocefin as described above. All tests were made in triplicate.

2.13 Murine model of subcutaneous infection

MRSA USA300 UAMS1182 was grown to mid-log phase in THB (A_{600} 0.4), washed twice in PBS, and resuspended in PBS. Shaved 7-week-old female C57bl6 mice were anesthetized using isoflurane and injected in the mid-back with 50µL containing approximately 1 x 10^7CFU. A first experiment was made with 5 mice per group and a second one with 8 mice per group. Group one received only the vehicle (Tween/ethanol/PBS 5:5:90), group two received 60 mg/kg of UA, group three received 100 mg/kg for nafcillin and group four received the combination of UA (60mg/kg) and nafcillin (100 mg/kg). Those groups were treated immediately after the inoculation of bacteria and then every 8 hours for three days. All treatments were administered subcutaneously close to the infection site. A fifth group receiving vancomycin (25 mg/kg) twice a day was used as a positive control. Abscesses were photographed after 36 and 52 hour, and the area was quantified using Image J.

Abscess area was defined as the zone of leukocytic infiltration including the contained zone of dermonecrosis. On day 3, abscesses were excised and homogenized in PBS prior to serial dilution and drop plating. Abscess burden was calculated as number of CFU per g of skin. The inflammatory cytokine IL-1β level was quantified in the skin and in the serum by ELISA (R&D Systems) according to the manufacturer's instructions.

Animals were maintained in accordance with the American Association for Accreditation of Laboratory Animal Care Criteria, and the above-described studies were approved by the Animal Care and Use Committee (IACUC) (Protocol S00227M) of the University of California, San Diego.

2.14 Statistical Analysis

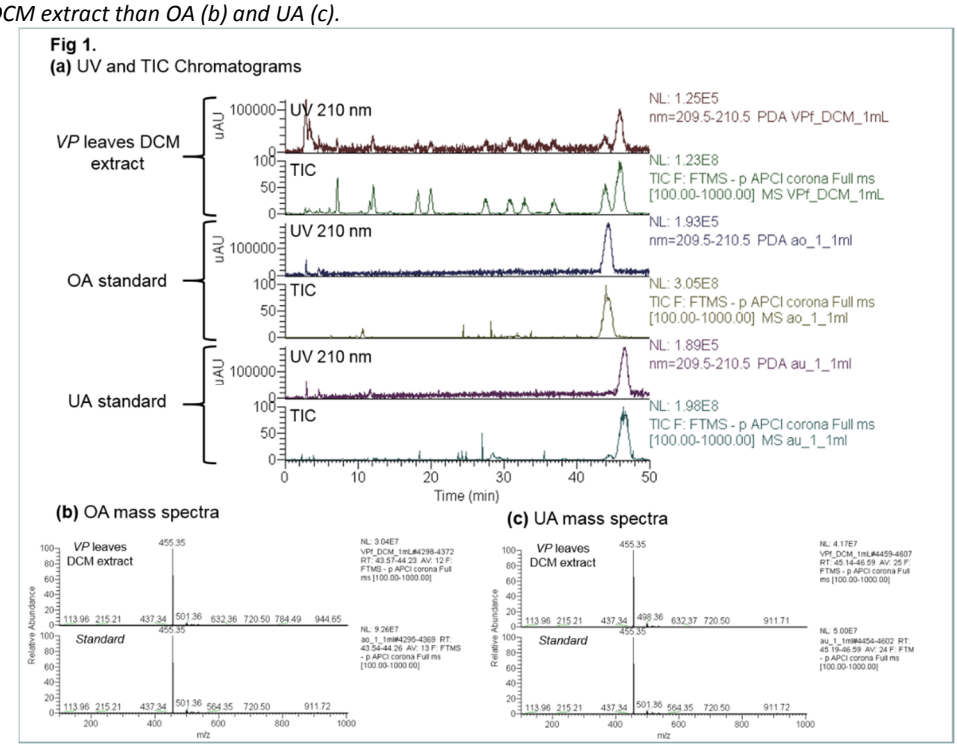
Data were analyzed using GraphPad Prism 6 Software and presented as the mean ± standard error. Differences between the effect of concentrations of triterpenic acids for the time kill assay were analyzed using ANOVA analysis with a Tukey post-hoc test. Comparisons between the effect of the triterpenic acid alone at a given concentration and in combination were performed using Student t-test [***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, $P > 0.05$].Concerning the β-lactamase activity, statistical analyses were performed using two-way ANOVA with Dunnett's post-test comparing each condition to the control « Bacteria » [***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, $P > 0.05$]. Finally, statistical analyses for the in vivo experiment were performed using ANOVA analysis with a Tukey post-hoc test [****, $P < 0.001$; ***, $P < 0.005$; **, $P < 0.01$; *, $P < 0.05$; ns, $P > 0.05$].

3. Results and discussion

3.1 Analysis and quantification of major components of the *Vitellaria paradoxa* crude extract

Building on previous data, [452], we began by identifying the active components of *Vitellaria paradoxa* crude extracts. LC/MS analysis of the crude dichloromethane leaf extract allowed identifying its two major components, UA and OA, by comparison of retention times, mass spectra and fragmentations with standard compounds. Chromatograms of this dichloromethane extract and of OA and UA standards, using the quantification MS compatible method with a 210 nm UV detection (**(a)** UV) or the mass spectrometry detection (**(a)** TIC) are shown in **figure 4-1**. The correspondence between the mass spectra of standards and the corresponding peaks in the extract are shown in **figure 4-1 (b)** and **(c)**. Both triterpenic acids, which are widely distributed in the plant kingdom, are also present in the dichloromethane twig extract but not in hexane extracts. Quantitative analysis revealed that the dichloromethane leaf extract contained 21.18± 0.50% of UA and 6.21 ± 0.12% of OA, vs. 2.06 ± 0.18% and 0.18 ± 0.04% respectively, in the twigs. While many other triterpenic acids have been identified in low amounts in *Vitellaria paradoxa* [451,458], this work is the first to report UA and OA as the most abundant triterpenic acids in the leaves of *Vitellaria paradoxa.*

Figure 4- 1 *: UV (210 nm) and Total Ion Current (TIC) Chromatograms of the Vitellaria paradoxa (VP) leaves dichloromethane (DCM) extract and of ursolic (UA) and oleanolic (OA) acids standards (a). Mass spectra of standards and of the peaks at the same retention times in the chromatogram of VP leaves DCM extract than OA (b) and UA (c).*



3.2 Antimicrobial activity of the VP crude extract major components

Having identified the main components of VP crude extracts, we assessed the direct and indirect antimicrobial activity on MRSA (reference strain ATCC33591 and four clinical isolates) of the pure compounds to determine if they could account for the antimicrobial activity of the leaf extract (MIC 250 mg/L [452]).

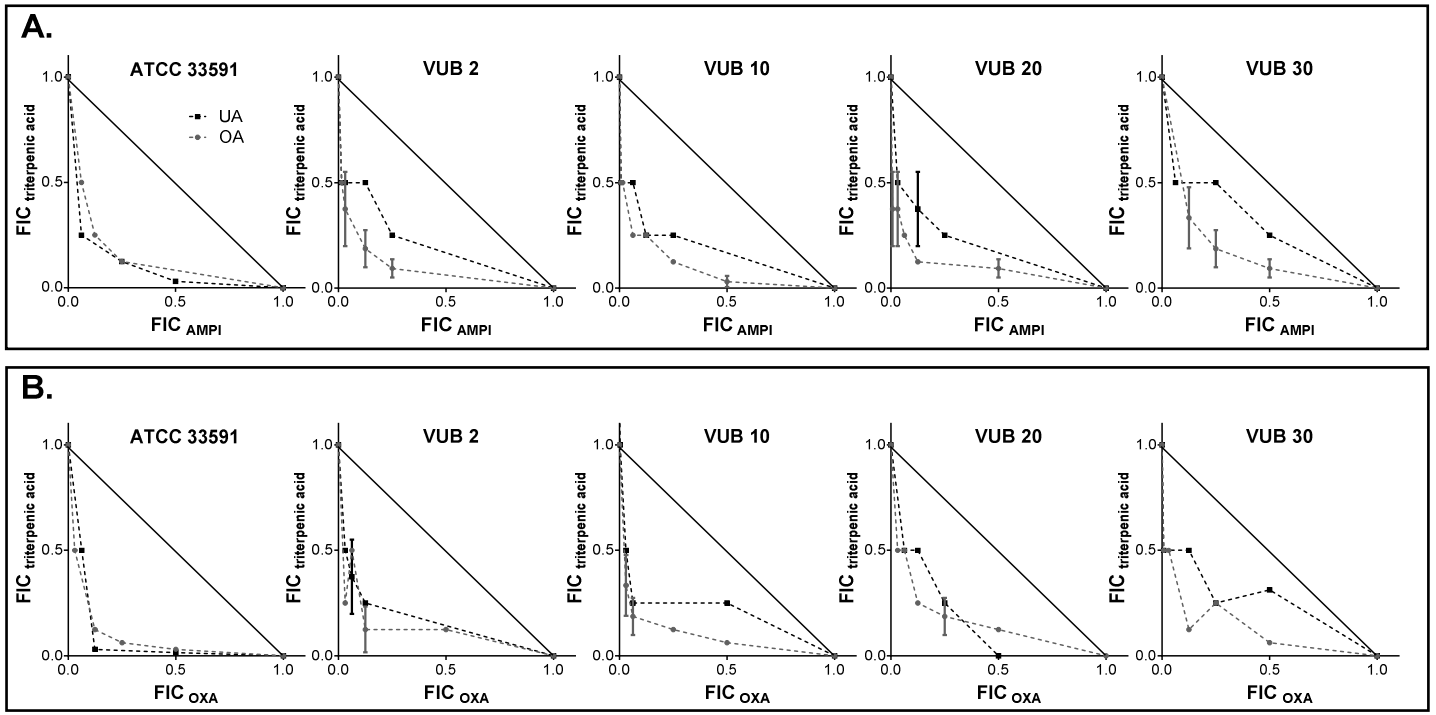
As shown in **Table 4-1**, both triterpenic acids were active against MRSA strains with MICs ranging from 8 - 16 mg/L for UA and 32 - 128 mg/L for OA, respectively. These values, at least for UA, are lower than the MIC of ampicillin or oxacillin against these strains.

***Table 4- 1** : Antimicrobial activity of UA, OA and β-lactams on MRSA strains and FICI of their combinations.*

Strain	MIC (mg/L)				FICI			
	UA	OA	AMPI	OXA	AMPI-UA	AMPI-OA	OXA-UA	OXA-OA
ATCC 33591	16	32	64	32	0.38-1	0.31-1	0.25-1	0.16-1
VUB 2	8	64	32	64	0.50-1	0.25-1	0.31-1	0.18-1
VUB 10	8	64	128	128	0.37-1	0.31-1	0.31-1	0.18-1
VUB 20	8	64	64	256	0.37-1	0.25-1	0.50-1	0.37-1
VUB 30	16	128	32	512	0.56-1	0.37-1	0.50-1	0.25-1

Looking for resistance reversing agents, we combined each of the two triterpenic acids with two different β-lactams, namely ampicillin and oxacillin, in order to evaluate their indirect antimicrobial activity. These two antibiotics were selected because they differ in their susceptibility to MRSA resistance mechanisms. While both ampicillin and oxacillin are affected by PBP2a-mediated resistance, oxacillin resists to the action of β-lactamases, thanks to the steric hindrance brought by its voluminous lateral chain [375,376]. A decrease in the MIC of both antibiotics could therefore result from an inhibition of either PBP2a mediated-resistance or of β-lactamase activity, while a decrease in oxacillin MIC would reflect an inhibition of the PBP2a-mediated resistance. As expected, all MRSA strains were resistant to both antibiotics (MICs higher than 32 mg/L), according to the European Committee on Antimicrobial Susceptibility testing (EUCAST) interpretative criteria [333]. UA and OA were synergistic with both ampicillin and oxacillin (FICI ranges including values ≤ 0.5), suggesting that at least PBP2a-mediated resistance was reverted in the presence of triterpenic acids (**Table 4-1**; see also isobolograms constructed from these checkerboard experiments and showing the synergistic interactions between ampicillin or oxacillin and both triterpenic acids in **Figure 4-2**).

Figure 4- 2 : *Isobolograms showing the in vitro interaction between the triterpenic acids (UA ■ or OA ●) and ampicillin (A) or oxacillin (B) on the reference strain ATCC33591 and on the four clinical MRSA strains (VUB2,10,20 & 30). Each point corresponds to the fractional inhibitory concentration (FIC) of the triterpenic acid (Y axis) and the FIC of the antibiotic (X axis). Represented data are the mean ± SD of three individual experiments.*



To further assess the potential of UA and OA to increase the activity of ampicillin or oxacillin, we performed time-kill assays on reference strain MRSA ATCC33591. The change in the $\log_{10}$ CFU/mL from the initial inoculum over time is shown in the supplementary material (**Figures 4-3 and 4-4**), with data obtained after 24 h of incubation reported in **Figure 4-5**. Antibiotics alone at their MIC did not affect the growth of bacteria after 24 h; UA and OA alone reduced bacterial growth only at the highest concentration tested (16 and 32 mg/L, respectively). In combination, both triterpenic acids caused a concentration-dependent increase in antibiotic activity, which became significant for concentrations of 2 and 4 mg/L of UA and OA respectively in combination with ampicillin (**panels a-b**) and of 4 and 8 mg/L, respectively, when combined with oxacillin (**panels c-d**). A synergistic effect (≥2 $\log_{10}$-fold reduction in inoculum as compared to the antibiotic alone) was obtained for concentrations of 4 and 8 mg/L of UA and OA (i.e. ¼ MIC), respectively with both antibiotics. At the highest concentrations of triterpenic acids tested, a 1.5-2 $\log_{10}$ decrease from initial inoculum was observed for both β-lactams.

Figure 4- 3 : *Time-kill curves against MRSA ATCC33591 for the combination of ampicillin (A.) or oxacillin (B.) with four different concentrations of UA corresponding to the MIC, ½ x MIC, ¼ x MIC or 1/8 x MIC. Represented data are the mean ± SD of 3 experiments performed in duplicate.*

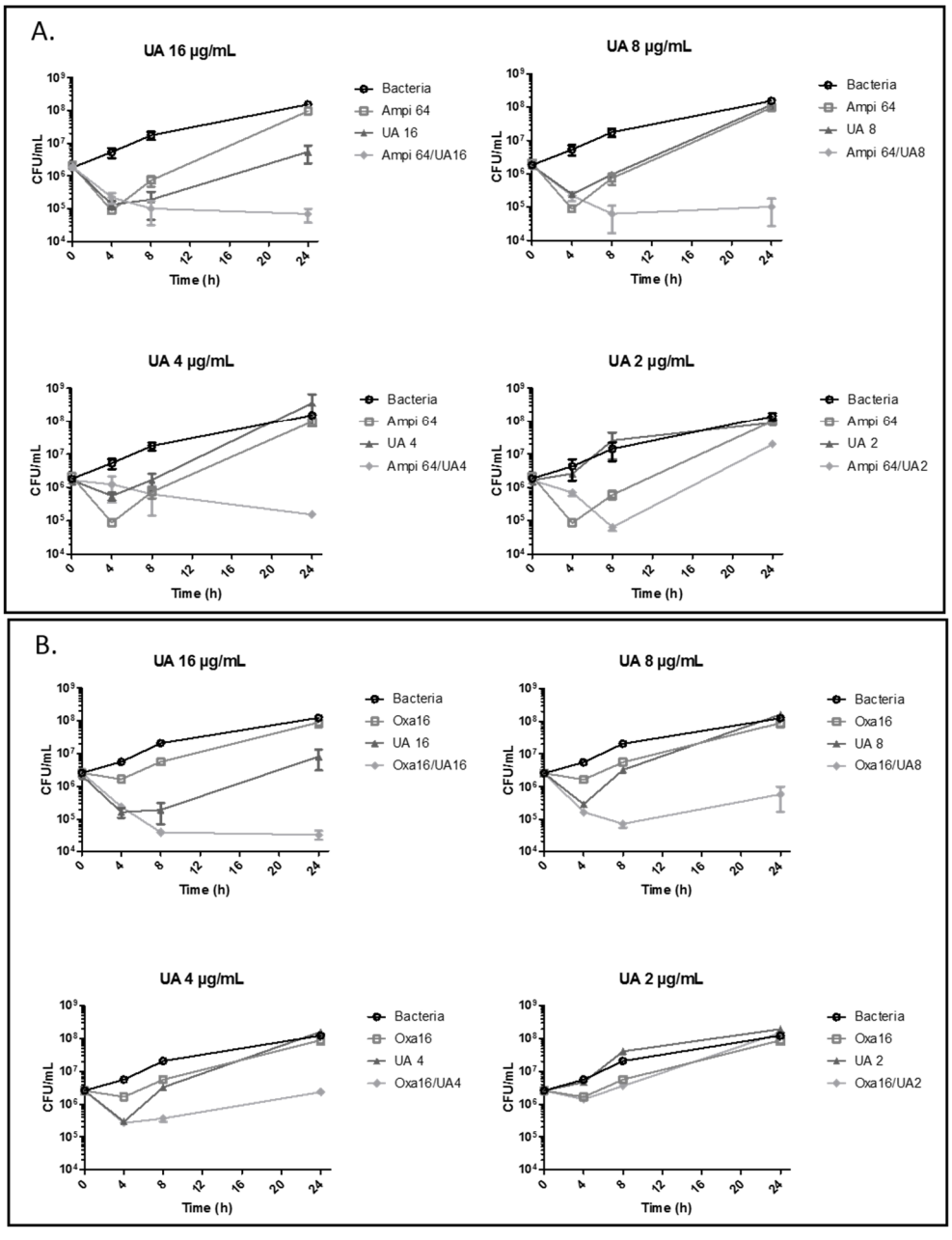


Figure 4- 4 *: Time-kill curves against MRSA ATCC33591 for the combination of ampicillin (A.) or oxacillin (B.) with four different concentrations of OA corresponding to the MIC, ½ x MIC, ¼ x MIC or 1/8 x MIC. Represented data are the mean ± SD of 3 experiments performed in duplicate.*

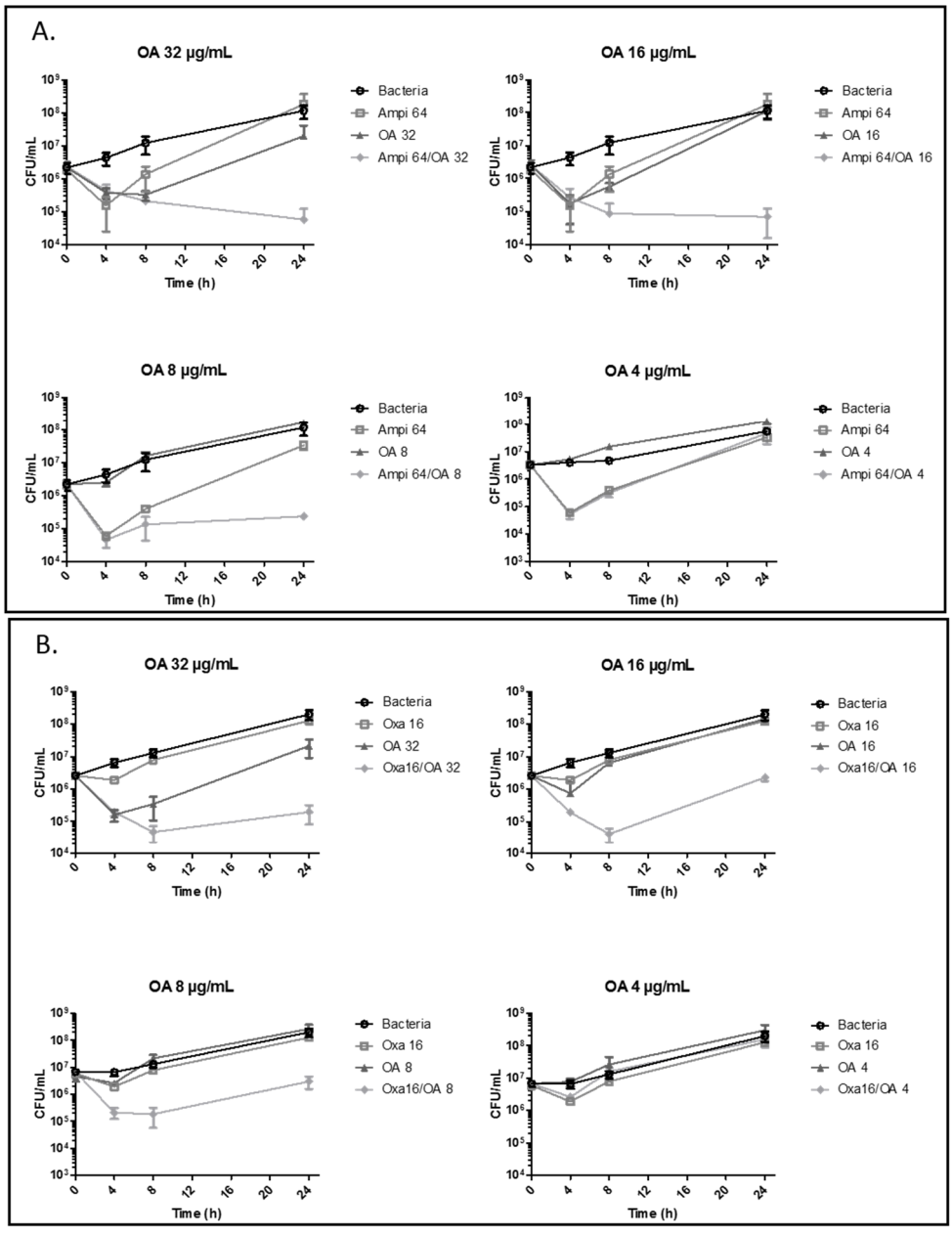
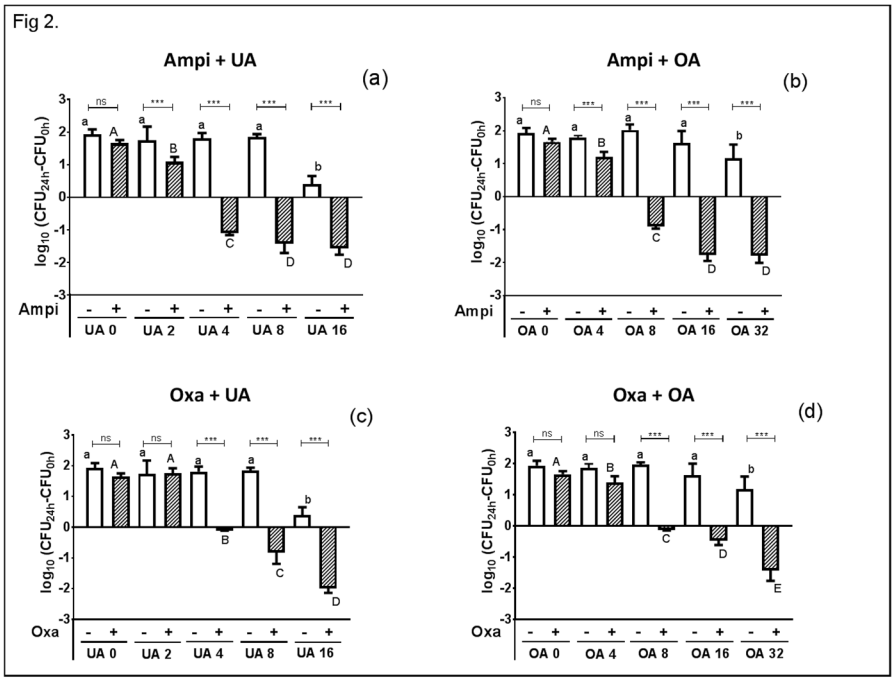


Figure 4- 5 *: Change in bacterial inoculum after 24 h of culture of MRSA ATCC33591 in the presence of ampicillin (top panels) or oxacillin (bottom panels) at their MIC (measured at a lower inoculum (1 x 10^6 CFU/mL) and used alone (open bars) or combined with UA (left panels) or OA (right panels) at the indicated concentrations (mg/L). Data are expressed as changes (in log_{10} CFU/mL) from the initial inoculum. Values are means ± SD of three independent experiments performed in duplicate. Statistical analyses: comparison between different concentrations of triterpenic acid (one-way ANOVA with Tukey post-hoc test): data with different letters are significantly different from one another (P < 0.05; small letters: triterpenic acid alone; capital letters: combination with the β-lactam. Comparison between triterpenic acid alone at a given concentration and in combination (t-test): ***, P < 0.001; **, P < 0.01; *, P < 0.05; ns, P > 0.05.*



These results indicate that the presence of large concentrations of UA and OA in the VP leaf extract could explain the direct and indirect antimicrobial activity we previously described against MRSA exposed to this extract alone or combined with β-lactams [452]. Conversely, the absence of activity for the twig extract could be attributed to its reduced content in these compounds. Previous studies from other groups have also documented antibiotic effects for these triterpenic acids against many bacterial species, their activity being higher against Gram-positive than Gram-negative bacteria and UA being more potent than OA (see [141,229] for recent

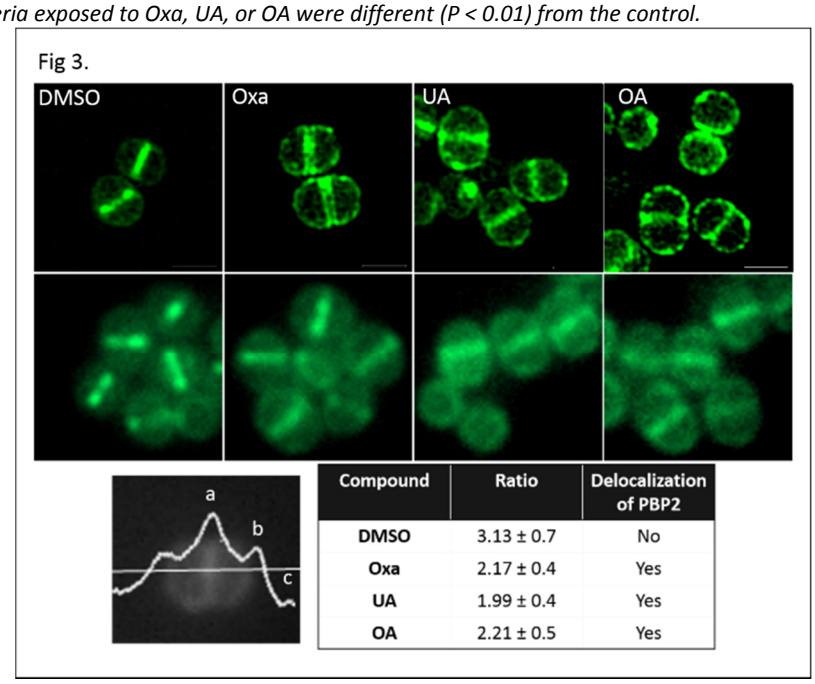
reviews), as also observed here. Yet divergent results have been reported regarding their activity on resistant bacteria, with Fontanay et al. describing no activity on MRSA or vancomycin-resistant enterocci (VRE) and Horiuchi et al. showing that both compounds remained active on both types of strains [248,250]. Their synergism with β-lactams has also been previously established, but only for UA combined with ampicillin against MRSA [249] and for both UA and OA combined with oxacillin against a fully susceptible MSSA [491]. We systemize here these effects, showing that synergy is observed for both compounds combined with either ampicillin or oxacillin against MRSA (including clinical isolates), which are of higher interest for clinical applications than MSSA.

3.3 Effect of OA and UA on PBP2 recruitment to the septum.

The fact that OA and UA synergize with both ampicillin and oxacillin indicates that they may revert resistance mediated by PBP2a. This additional PBP is thought to maintain transpeptidase activity in the presence of β-lactams, and works in concert with the transglycosidase PBP2, which continues to transglycosylate the peptidoglycan. As no MRSA carrying a functional GFP-PBP2a has been engineered, we instead assessed the localization of PBP2 using a GFP-PBP2 fusion in MRSA strain COL by both conventional and structured illumination microscopy (**Figure 4-6**). Similar to previously published data, PBP2 preferentially localized at the septum ($FR_{(septum/peripheral\ membrane)}$ = 3.13), while the addition of oxacillin, used here as a positive control, caused its redistribution over the entire cell membrane, which impairs the synthesis of new peptidoglycan at the cell division site [49]. The addition of both UA and OA also caused a delocalization of PBP2, suggesting that these compounds could also affect cell wall synthesis. Western blot analyses of total protein extracts of similarly treated COL GFP-PBP2 cells did not show consistent

modifications in PBP2 or PBP2a expression when compared to the control, due to difficulties in finding an appropriate internal control (data not shown).

Figure 4- 6 *: Influence of oxacillin and triterpenic acids on PBP2 localization. MRSA BCBPM073 carrying a gfp-pbp2 fusion was grown in TSB and incubated during 30 min without (DMSO) or with 1x MIC of Oxacillin (Oxa), UA or OA and observed by conventional (lower panel) and superresolution structured illumination (upper panel) microscopy. The localization of PBP2 was determined by calculating the fluorescence ratio (FR) (a-c)/(b-c) where "a" corresponds to the fluorescence found at the septum, "b" to the fluorescence of the peripheral membrane and "c" to the background fluorescence [23] , as illustrated in the lower part of the figure. Values are the means ± SD from measurements of fluorescence ratios of 200 cells in each sample. A FR value > 2 indicates an enrichment at the septum [where membranes from two adjacent daughter bacteria are apposed] and values ≤ 2, a localization spread over the entire membrane. Statistical analysis: one-way ANOVA with Dunnett's post hoc test: FR values for bacteria exposed to Oxa, UA, or OA were different (P < 0.01) from the control.*



Compound	Ratio	Delocalization of PBP2
DMSO	3.13 ± 0.7	No
Oxa	2.17 ± 0.4	Yes
UA	1.99 ± 0.4	Yes
OA	2.21 ± 0.5	Yes

3.4 Evaluation of β-lactamase Activity.

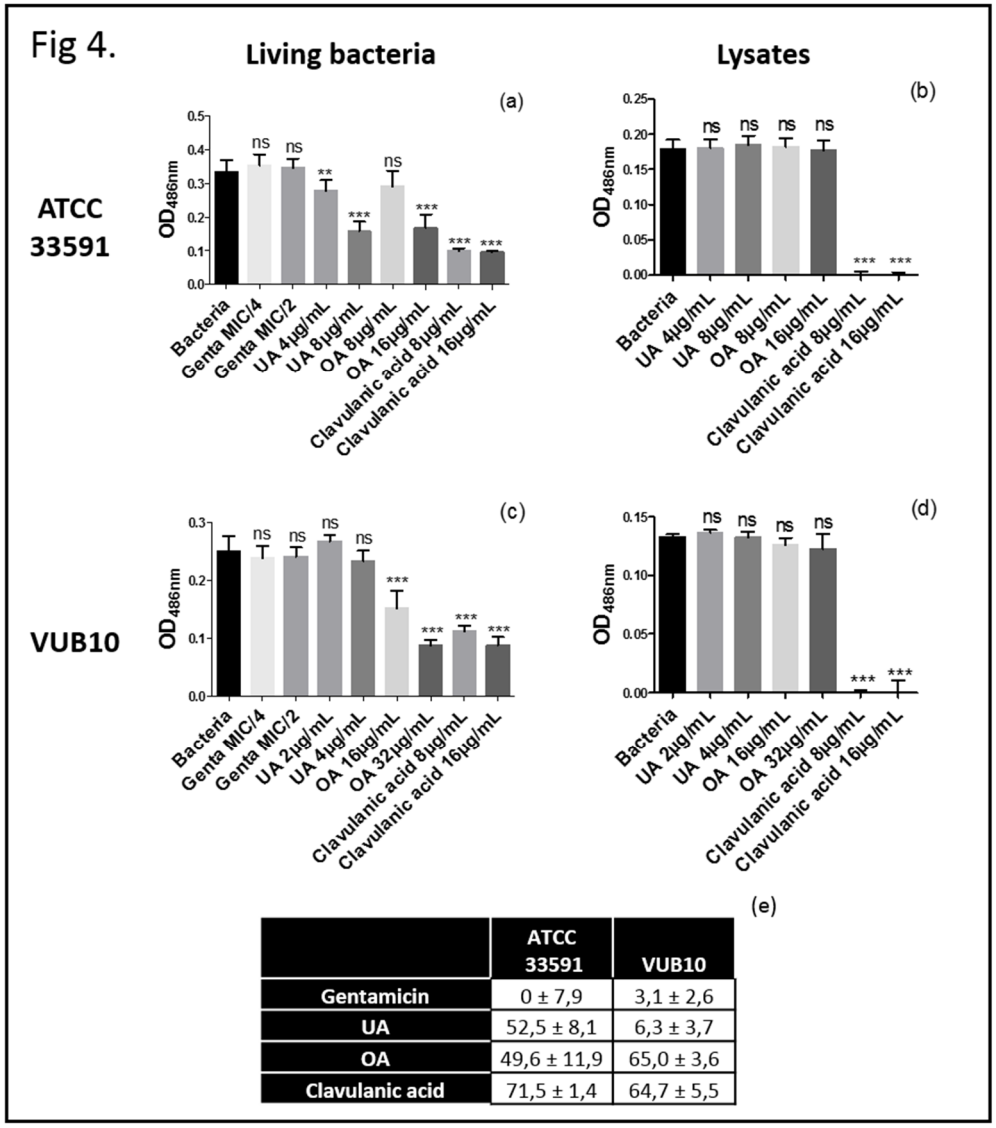
The experiments performed previously did not allow excluding a concomitant reversion of β-lactamase-mediated resistance by triterpenic acids. We therefore examined the capacity of UA and OA to inhibit β-lactamases by following their influence on the rate of hydrolysis of the β-lactamase chromogenic substrate nitrocefin. These experiments were performed using intact bacteria and bacterial

lysates of both the reference strain ATCC33591 and the clinical isolate VUB 10 (**Figure 4-7**).

As expected, gentamicin (protein synthesis inhibitor; MIC = 0.125 mg/L) did not inhibit β-lactamase activity in live bacteria neither in bacterial lysate, while clavulanic acid, a known inhibitor of β-lactamase activity, caused a marked decrease in nitrocefin hydrolysis in live bacteria and fully inhibited its degradation in bacterial lysates at subMIC concentrations. In contrast UA and OA at the highest concentration tested reduced nitrocefin hydrolysis in whole cells (**panel a**), but not in bacterial lysates (**panel b**) from ATCC33591. Similar results were obtained for OA with the clinical isolate VUB10 but not for UA (**panels c,d**), possibly due to the lower concentration tested (higher concentrations would have affected bacterial viability). Thus, these results suggest that these triterpenic acids, as opposed to clavulanic acid, indirectly impair β-lactamase activity, as their effect requires the machinery of a living cell.

Figure 4- 7 *: β-lactamase activity in intact (left panels) or lysed (right panels) MRSA ATCC33591 (upper panels) or VUB10 (lower panels) incubated during 30 minutes in control conditions or in the presence of gentamicin (negative control), clavulanic acid (positive control), UA, or OA at concentrations corresponding to their MIC/2 and MIC/4. Values are means ± SD of three independent experiments performed in duplicate. Statistical analyses were performed (two-way ANOVA with Dunnett's post-test comparing each condition to the control « Bacteria » [***, P < 0.001; **, P < 0.01; *, P < 0.05; ns, P > 0.5]. The Table below the graphs shows the percentages of inhibition of β-lactamase activity in living bacteria for ATCC33591 and VUB10 for agents used at concentrations corresponding to MIC/2 (n=6).*

Fig 4.



(e)

	ATCC 33591	VUB10
Gentamicin	0 ± 7,9	3,1 ± 2,6
UA	52,5 ± 8,1	6,3 ± 3,7
OA	49,6 ± 11,9	65,0 ± 3,6
Clavulanic acid	71,5 ± 1,4	64,7 ± 5,5

Until now, few studies have investigated the mechanism by which UA and OA do exert their antibacterial effects, often suggesting that they target the bacterial membrane or the peptidoglycan [300,491]. Due to their lipophilic character, UA and OA can accumulate in biological membranes, resulting in swelling and increased fluidity, as demonstrated using liposomes reconstituted from bacterial lipids [337]. In the present work, we show that both triterpenic acids revert β-lactam resistance mediated by PBP2a and by β-lactamases.

Although we did not fully establish the underlying mechanisms, we provide some evidence that it probably requires an interaction of the triterpenic acids with the bacterial membrane. First, we show that both UA and OA are capable of delocalizing PBP2 in the membrane. This mechanism is critical for reversing the MRSA phenotype, which depends on the cooperation between PBP2 and PBP2a to carry out peptidoglycan biosynthesis and cross-linking [492]. It has been demonstrated for other adjuvants like the FtsZ inhibitor PC190723 [95], the oxacillin potentiators DNAC-1 and DNAC-2 [493,494] as well as for other natural compounds possessing a lipophilic moiety that allows them to intercalate in the bacterial membrane, like (-)-epicatechin gallate, a flavonoid from green tea [388,495]. Second, we also show that UA and OA interfere with β-lactamase activity, but do not act as competitive inhibitors of the soluble enzyme (as previously published [345]), neither as suicide substrates like clavulanic acid. In this respect, their effect contrasts with that of (-)-epicatechin gallate, which has been described as a direct inhibitor of penicillinase from *S. aureus* [496]. The fact that β-lactamase inhibition is observed only towards living bacteria suggests therefore an indirect effect of UA and OA, possibly related to their insertion in the bacterial membrane, the mechanism of which remains to be established.

3.5 Murine Model of Subcutaneous Infection.

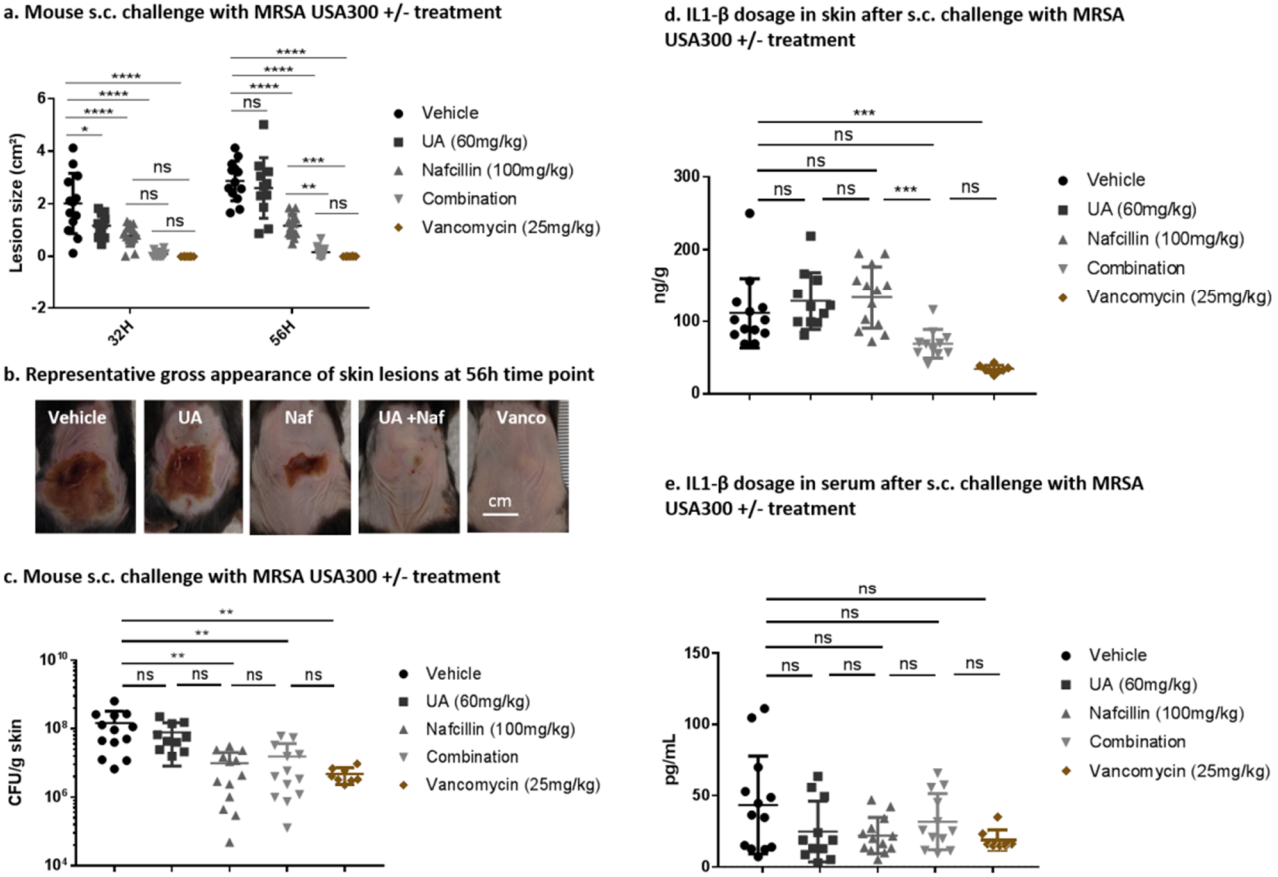
Despite uncertainties regarding the molecular mode of action of UA and OA, we investigated whether the synergistic effect of triterpenic acids with β-lactams was also observed *in vivo*. To this effect, we tested a combination of a triterpenic acid and nafcillin in a murine model of subcutaneous MRSA infection by the strain MRSA USA300 UAMS1182.

Nafcillin was used instead of oxacillin because its activity has been well described before in this model [497] and it is considered as interchangeable with oxacillin based on the comparison of their pharmacological properties [498,499]. UA was selected as triterpenic acid because it was synergistic with oxacillin and ampicillin at lower concentrations than OA (see **Figure 4-5**). Moreover, it was also synergistic when combined with nafcillin against MRSA USA300 UAMS1182, while the combination of OA with nafcillin only led to additive effects (**Table 4-2**). Female C57bl6 mice were infected subcutaneously on their back with for which the model has been established previously [484]. UA, nafcillin, a combination of both, vancomycin (positive control) or a vehicle (negative control) were administered subcutaneously to groups of mice immediately after MRSA infection and then every eight hours for a total of 56 hours (**Figure 4-8**).

***Table 4- 2** : Antimicrobial activity of ursolic acid (UA) , oleanolic acid (OA), oxacillin (OXA) and nafcillin (NAF) and FIC index of their combinations on the MRSA USA300 UAMS1182 strain.*

	MIC (mg/L)	FICI	
		OXA	NAF
UA	64	0.31 – 1	0.28 - 1
OA	> 128	0.50 – 1	0.56 - 1
OXA	32		
NAF	32		

Figure 4- 8 *: Antibacterial effects of UA in combination with nafcillin in a murine model of skin infection. Female c57/bl6 mice were infected with MRSA USA300 UAMS1516, followed by treatment with UA, nafcillin, a combination of both or vancomycin (positive control). (**a**) Lesion size (cm^2) after 32h and 56h and (**b**) representative lesions at 56h are shown. (**c**) CFU/g skin of MRSA recovered from skin lesions harvested from animals at 56h time point. ELISA analysis of IL1-β (ng/g skin) levels recovered from skin lesions (**d**) or from serum (pg/mL) (**e**) harvested from animals after 56h. Statistical analyses were performed by Tukey's multiple comparison test (****, P < 0.001; ***, P < 0.005; **, P < 0.01; *, P < 0.05; ns, P > 0.05).*



Lesion sizes (cm^2) of the resulting necrotic skin lesions were measured after 32 and 56 h and are represented in **panels a, b**. Lesions were significantly smaller in the presence of UA after 32 h, although this effect was not maintained after 56 h. Administration of 100 mg/kg of nafcillin alone every 8 h significantly reduced the lesion size at both time points, and addition of 60 mg/kg of UA with nafcillin significantly improved the beneficial effect of nafcillin observed after 56h. Residual lesions had statistically similar size in the group treated by the combination or by vancomycin. All treatments except UA significantly reduced the lesional MRSA burden (CFU/g skin; **panel c**). However, no statistically significant difference was observed between mice treated by nafcillin alone or combined with UA concerning the bacterial burden. Among the biological activities associated with UA, its anti-inflammatory effect is attributed to its ability to inhibit the immunoregulatory transcription factor NFκB in response to a wide variety of carcinogens and inflammatory agents [500,501]. We therefore measured levels of IL-1β, a key pro-inflammatory cytokine, in the skin and in the serum of infected animals, after 56 h of treatment (**panels d-e**). While nafcillin or UA alone did not affect IL-1β levels recovered from the skin, the nafcillin-UA combination and vancomycin to an even larger extend, caused a significant reduction ($p < 0.05$). This effect did not extend to the serum, where no treatment was effective in significantly altering the IL-1β levels.

We thus demonstrate here that synergy also takes place *in vivo*, using the combination of UA and nafcillin in a model of skin infection. Although UA did not increased the effect of nafcillin on bacterial burden, it improved its effect on the size of the lesion and the production of pro-inflammatory cytokine IL-1β in the skin. These benefits can nevertheless probably be ascribed to a synergy with the antibiotic, as UA alone was ineffective.

These two triterpenic acids have previously been shown to possess several biological activities including, among others, anti-inflammatory, anti-cancerous, or antiparasitic effects; they are thus able to modulate signaling pathways during disease development [502–504]. This lack of specificity and their reported toxicity *in vitro* could be a limitation of this study. However, the potential therapeutic interest of these compounds is highlighted by the fact that they are currently evaluated in a series of clinical trials for their metabolic, anti-inflammatory, or anti-cancer effects [478–481,503,505]. Of interest, published clinical trials (although still limited to Phase I), in which these compounds are administered orally or intravenously using liposomal formulations, did not evidence toxicity,[478–481,505] further encouraging research on their therapeutic potentials in topic formulations to treat skin infections. Our results are also limited to the combination of UA and OA with β-lactams. Combinations with other classes of antibiotics have already been described as synergistic for glycopeptides, tetracyclines or macrolides [249,274] or additive for fluoroquinolones against different *S. aureus* strains [124,279]. The mechanism of these interactions has not been elucidated yet but should implicate other mechanisms than those discovered here, though also possibly related to their interaction with membranes.

In spite of these limitations, our work brings important new pieces of information. We report for the first time the presence of high quantities of UA and OA in the leaves of *Vitellaria paradoxa* and demonstrate their synergistic effects with β-lactams against MRSA. We partially elucidate the mechanism by which they revert resistance mediated by β-lactamases or PBP2a production. Moreover, we confirm the synergistic activity of UA with β-lactams in a murine model of subcutaneous MRSA infection without evidence of toxicity. Additional studies are now needed aiming at identifying their exact molecular target in the bacterial envelope as a way to orient the search of even more potent modulators of resistance from plants or obtain more effective derivatives by hemisynthesis.

4. Acknowledgements

The authors are grateful to Mr. Agabani (botanist of University of Abomey-Calavi, Cotonou, Benin), Dr G. Fernand and S. Hope (*Laboratoire de pharmacognosie et huiles essentielles UFR pharmacie, Faculté des Sciences de la Santé, Université d'Abomey Calavi,* Benin) for plant collections and Dr D. Pierard (Vrije Universiteit Brussel, Brussels, Belgium) for the kind gift of clinical isolates. We wish to thank Jean-Paul Vanheleputte for skillful technical assistance. F.V.B. is *Directeur de Recherches* of the Belgian *Fonds de la Recherche Scientifique*. The authors gratefully thank the Belgian *Fonds de la Recherche Scientifique* (FRFC 2.4555.08, for the MASSMET Platform), and the Faculty of Pharmacy and Biomedical Sciences of UCL for financial support. The authors are grateful to Pr. M. G Pinho and Pr. V. Nizet having hosted L. Catteau in their respective labs.

This work was supported in part by the Belgian *Fonds de la Recherche Scientifique* (FVB), FCT fellowship SFRH/BPD/95031/2013 (NTR), NIH grants AI124316 and HD090259 (VN), European Research Council through grant ERC-2012-StG-310987 (MGP), Project LISBOA-01-0145-FEDER-007660 (ITQB).

A *Fulbright* grant given to L. Catteau supported her work as a visiting graduate student in the Nizet lab.

RESULTS – PART 4

Antiparasitic activities of major compounds isolated from the leaf dichloromethane extract of Vitellaria paradoxa

Given the antiparasitic activity of some pentacyclic triterpenes (cfr introduction), the traditional use of *Vitellaria paradoxa* to treat fevers as those arising in case of parasitic infections, and the interest of our laboratory in this topic, we decided to investigate the *in vitro* antitrypanosomal, antileishmanial as well as antiplasmodial activities of the major triterpenic compounds isolated from the leaf dichloromethane extract from this plant. In this chapter, we compare their antiparasitic activities to their cytotoxicities on the human normal fibroblast cell line WI38 and determine their selectivity indexes.

One of the isolated compounds, showing a significant antitrypanosomal effect as well as a good selectivity index *in vitro*, was further studied in an *in vivo* model for its acute toxicity and antitrypanosomal activity. This *in vivo* study was performed in collaboration with C. Beaufay.

This chapter is the second part of a paper in preparation: Catteau L., Beaufay C., Girardi C., Hérent M-F., Van Bambeke F., Quetin-Leclercq J. Antistaphylococcal and antiparasitic activities of triterpenic acids and ester derivatives isolated from the leaves of *Vitellaria paradoxa.*

Abstract

Vitellaria paradoxa C.F. Gaertn. ssp. *paradoxa* (Sapotaceae) also called 'Shea butter tree', is used in traditional medicine to treat various symptoms related to infectious diseases including malaria fever, dysentery or skin infections. The dichloromethane extract from its leaves was previously investigated for its constituents and 5 pentacyclic triterpenic acids together with 6 ester derivatives were isolated.

As the antiparasitic potential of pentacyclic triterpenoids is already well documented but still mainly limited to triterpenic acids, the antiparasitic and cytotoxic activities of these eleven isolated compounds were evaluated *in vitro* on *Trypanosoma brucei brucei* (strain 427) (*Tbb*), on promastigotes of *Leishmania mexicana mexicana* (MHOM/BZ/84/BEL46) (*Lmm*), on *Plasmodium falciparum* (strain 3D7) (*Pff*) as well as on the human normal fibroblast cell line (WI38) using colorimetric methods.

While the antiplasmodial and antileishmanial activities of individual compounds were always lower than the antitrypanosomal one, one isolated triterpenic ester, 3-O-*p*-coumaroyltormentic acid showed a promising antitrypanosomal activity (IC_{50} = 0.79 mg/L) with a low cytotoxicity (IC_{50} = 21.04 mg/L). Preliminary acute toxicity tests showing that this compound was not toxic up to 100 mg/kg, we investigated its efficacy in an *in vivo* model of *Trypanosoma brucei brucei* agressive infection. It was shown to significantly reduce the parasitemia and improve the survival of infected mice.

1. Introduction

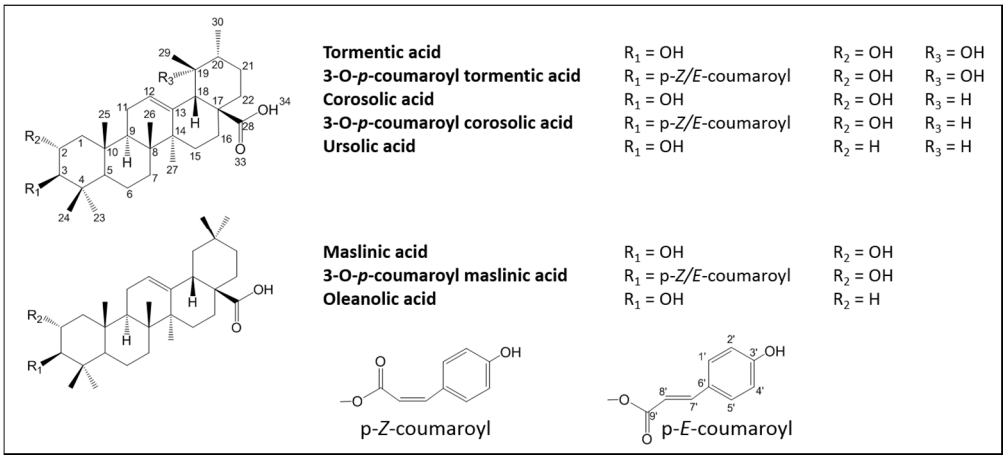
The leaves, fruits or stem bark of *Vitellaria paradoxa* are used in traditional medicine to treat several diseases such as skin infections, diarrhea, stomach ache and ulcers, dysentery, inflammation, malaria and breast cancer [369,449]. This tree, also called 'shea-butter tree' grows up to 14m high and is widely studied for its stem bark or kernels, while leaves are understudied. The most valued product from shea tree is shea butter, the edible fat extracted from the kernels, consisting of olein and stearin fractions along with non-saponifiable compounds [454,455]. While shea stearin is used as cocoa butter equivalent, improver or replacer in chocolate manufacture, shea butter is also increasingly popular as component of skin care products and cosmetic product formulations [456,457].

Parasitic protozoan diseases constitute one of the world's most widely spread human health problem. *Trypanosoma brucei*, *Leishmania mexicana* and *Plasmodium falciparum*, are the causative agents of sleeping sickness, leishmaniasis and malaria respectively, three parasitic diseases related to high morbidity and mortality [208,220,234]. Unfortunately, where drugs are available, their usefulness is being increasingly threatened by parasite drug resistance. The need for new drugs is then high. The vegetal kingdom remains a good source of pharmacologically active compounds. Two years ago, Isah et al. published a review reporting the high activity of 85 pentacyclic triterpenes against different species of *Plasmodium*, *Trypanosoma*, *Leishmania* as well as *Nematoda* highlighting the great interest for this phytochemical group [155].

In a previous work, we isolated 5 triterpenic acids namely tormentic, maslinic, corosolic, ursolic and oleanolic acids and 3 pairs of triterpenic esters: 3-O-*p-(Z/E)*-coumaroyltormentic acid, 3-O-*p-(Z/E)*-coumaroylmaslinic acid and 3-O-*p-(Z/E)*-

coumaroylcorosolic acid from the leaf dichloromethane extract of *Vitellaria paradoxa* (**figure 5-1**).

***Figure 5- 1** : Structure of isolated constituents from Vitellaria paradoxa leaf extract*



The aim of this study was thus to evaluate the in *vitro* antiplasmodial, antitrypanosomal and antileishmanial activities of the leaf dichloromethane extract from *Vitellaria paradoxa* as well as of the 11 isolated triterpenic compounds. The most active compound showing a significant antitrypanosomal activity with a good selectivity index was then tested *in vivo* for its acute toxicity as well as its antiparasitic activity in *Trypanosoma brucei brucei* infected mice and compared to ursolic acid.

2. Materials and Methods

2.1 Plant material

Leaves of *Vitellaria paradoxa* C.F. Gaertn. (syn. *Butyrospermum parkii*) were collected in Benin (Parakou) in August 2012. A voucher specimen was identified and deposited at the Herbier National of Abomey-Calavi University in Benin, bearing the number AP2130.

2.2 Preparation of extracts and compounds isolation

Air dried and powdered leaves (100g) of *Vitellaria paradoxa* were successively extracted using a soxhlet apparatus with 700mL of hexane (yield = 4.62%) then dichloromethane (yield = 1.83%) for 8h each. The extracts obtained were then evaporated to dryness under reduced pressure with a rotary evaporator at a temperature of 30°C. Tormentic, maslinic, corosolic, ursolic and oleanolic acids as well as 3-O-*p-(Z/E)*-coumaroyltormentic, 3-O-*p-(Z/E)*-coumaroylmaslinic and 3-O-*p-(Z/E)*-coumaroylcorosolic acids were isolated as described in part 2 of the results (section 2.4) from the dichloromethane extract.

2.3 Parasites, cells and media

The crude extract and isolated compounds were evaluated for their antiparasitic activities *in vitro* on *Trypanosoma brucei brucei* bloodstream forms (strain 427) (*Tbb* BSF), on promastigotes of *Leishmania mexicana mexicana* (MHOM/BZ/84/BEL46) (*Lmm*) as well as on chloroquine-sensitive *Plasmodium falciparum* (3D7) (*Pff*).

Tbb BSF were cultured *in vitro* at 37°C with 5%CO_2 in HMI9 medium containing 10% heat-inactivated fetal bovine serum, β-mercaptoethanol (20mM) and L-cysteine (150mM).

Lmm were cultivated *in vitro* at 28°C with 5%CO_2 in a semi-defined medium supplemented with 15% heat-inactivated fetal bovine serum and haemin (5 mg/L).

Pff 3D7 asexual erythrocytic stage were cultivated continuously *in vitro* according to the procedure described by Trager and Jensen (1976) at 37°C and under an atmosphere of 5% CO_2, 5%O_2 and 90% N_2 [506]. The host cells were human red blood cells (A or O Rh^+). The culture medium was RPMI 1640 (Gibco) containing 32 mM $NaHCO_3$, 25 mM HEPES and L-glutamine. The medium was supplemented with 1.76 g/l glucose (Sigma-Aldrich), 44 mg/ml hypoxanthin (Sigma-Aldrich), 100 mg/l gentamycin (Gibco) and 10% human pooled serum (A or O Rh+). Parasites were

subcultured every 3–4 days with initial conditions of 0.5% parasitemia and 1% haematocrit.

The cytotoxicity of isolated compounds was evaluated on human normal fibroblasts WI38 cultivated in a humidified atmosphere with 5% CO_2 at 37°C with DMEM medium (Life Technologies) containing 4mM L-glutamine, 1mM sodium pyruvate supplemented with 10% fetal bovine serum (Sigma) and penicillin-streptomycin (100UI/mL).

2.4 *In vitro* antiparasitic activities

In vitro tests were performed as previously reported [507–509]. In brief, the *Lmm* and *Tbb* viability were measured using the AlamarBlue assay while for the *Pff* we used a parasite enzyme activity, the lactate dehydrogenase.

Stock solutions of the crude extract and isolated compounds were prepared at a concentration of 10mg/mL in DMSO. The solutions were further diluted in medium to give 0.1 or 0.5 mg/ml stock solutions. Extracts and compounds were tested in eight serial three-fold dilutions (final concentration range: 50–0.02 mg/L) in 96-well microtiter plates except on *Pff*, in two-fold dilutions (final concentration range: 50-0.39 mg/L). Pentamidine isethionate salt (an antileishmaniasis drug, Sigma-Aldrich, 98% purity), suramine sodium salt (an antitrypanosomal drug, Sigma-Aldrich >99% purity) and artemisinin (an antiplasmodial drug, Sigma-Aldrich 98% purity) were used as positive controls (tested concentration ranges: 10 000-4.6, 5000-2.3 and 100-0.78 µg/L respectively). IC_{50} values, indicating the concentration of the drug needed to obtain 50% of inhibition of parasite growth, were calculated by linear regression on Excel Software based on the sigmoid curves obtained from the mean (2 wells/concentration) of the eight concentrations, and tested at least in triplicate.

2.5 Cytotoxicity assays

The cytotoxicity of compounds on WI38 cells was evaluated using the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphényltetrazolium bromide (Sigma-Aldrich) colorimetric method as previously described [373]. Camptothecin (Sigma-Aldrich) was used as a positive cytotoxic reference. Stock solutions of crude extracts at 10mg/mL in DMSO were diluted in medium with a final concentration range of 100–0.04mg/L (2 wells/concentration). The cytotoxicity of solvents was tested in parallel and found to be negligible at the highest concentration used for extracts (0.5%). Each extract was tested in eight serial dilutions in 96-well microtiter plates at least in triplicate.

2.6 Animals

For *in vivo* tests, NMRI mice (6–8 weeks of age) obtained from Envigo laboratories (The Netherlands) were used. All *in vivo* experiments performed were approved by the Ethical Committee for animals use at the Health Sciences Sector of the Catholic University of Louvain (2017/UCL/MD/017).

2.7 *In vivo* acute toxicity test

The assessment of the highest tolerated dose was based on a DNDi protocol [510] and adapted from [511]. Briefly, 3-O-*p*-coumaroyltormentic acid or UA was given intraperitoneally every 2h to 2 mice using increasing doses: 10-15-25-50 mg/kg from stock solutions of 10 mg/ml in a mixture of water/tween 80/ethanol (90:7:3). Mice were controlled for any health problem symptoms or behavioral changes and monitored for weight and hematocrit after each injection and every day during 48h after administration. Main organs (heart, liver, spleen, lung and kidney) of treated mice were observed and weighed wet during autopsy. Control group received the vehicle, distilled water with 10% of tween 80/ethanol (7:3). The total highest tolerated injected dose was determined and will ensure the non-toxicity of *in vivo* anti-trypanosomal tests.

2.8 *In vivo* antitrypanosomal activity

Mice were randomly divided into 6 mice per group for 3-O-*p*-coumaroyltormentic acid and UA, 4 for positive control (suramine) and 7 for the negative one, and were infested intraperitoneally with 10^4 *Trypanosoma brucei brucei*. All compounds were solubilized in the vehicle (water-tween 80-ethanol (90:7:3)) and administered intraperitoneally. 3-O-*p*-coumaroyltormentic acid or UA were administered at 50 mg/kg at day 3 after infection and then every day until day 7 post-infection. Suramine (0.5 mg/kg) was administered with the same scheme as triterpenic compounds while the vehicle was used as a negative control. From day 3 post-infection, a drop of blood collected each day from mouse-tail was used to count parasitemia by microscopy.

2.9 Statistical analyses

Data were analysed by Graphpad Prism7.01 statistical Software and presented as the mean ± standard error. Differences between independent groups results obtained for *in vivo* assay were analysed by the non-parametric Mann–Whitney test in one-tailed and by the log-rank test to compare survival curves. Statistical significance between treatments was set at p-value < 0.05.

3. Results and Discussion

3.1 *In vitro* antiparasitic activities

The antileishmanial, antiplasmodial and antitrypanosomal activities of *Vitellaria paradoxa* crude leaf dichloromethane extract and isolated compounds are reported in **table 5-1**, together with their cytotoxicity on WI38 cell line.

In line with previous results on plant extracts, antiparasitic activities can be classified as follow: IC_{50} ≤ 15 mg/L: promising activity; IC_{50} = 15-50 mg/L: moderate activity; IC_{50} > 50 mg/L: weak activity and IC_{50} > 100mg/L: inactivity [413,512,513]. In 2010, one paper reported the weak activity of a leaf dichloromethane extract from *Vitellaria paradoxa* collected in Burkina Faso against *Plasmodium falciparum* 3D7 strain with an IC_{50} of 55.45 ± 12.60 mg/L without reporting any selectivity information. In our test, we showed the moderate activity of a similar extract (IC_{50} = 30.03 ± 3.60 mg/L) but observed that cytotoxicity occurs at much lower concentration (SI= 0.2). The observed variations may reflect differences in the phytochemical content in the same plant part collected on two geographically different regions, but also the mode of extraction. Concerning the antitrypanosomal and antileishmanial activities, we report here for the first time the promising potential of this extract (IC_{50} < 15 mg/L), but selectivity indexes were very low. As cytotoxicity and antiparasitic activities may not be related to the same target/compound and in order to find more active and less cytotoxic compounds, we investigated the activities of the constituents of this extract.

In 2005, Pink et al. published in "Nature Reviews", criteria to select a pure compound as a hit for the treatment of parasitic diseases: this compound has to be active *in vitro* against whole protozoa with a IC_{50} ≤ 1 mg/L as well as to be selective (being at least tenfold more active against parasite than against a mammalian cell line) [514]. One year later, Nwaka et al. reported more restrictive criteria, selecting compounds with an IC_{50} ≤ 0.2 mg/L and a selectivity index of 100 [515].

***Table 5- 1** : Antiparasitic activities and cytotoxicities of crude VP DCMe leaf extract and isolated compounds (IC_{50} mg/L (µM)) and selectivity indexes*

	WI38 (IC_{50} mg/L (µM))	***Lmm* (IC_{50} mg/L (µM))**	**SI[a]**	***Pf* (IC_{50} mg/L (µM))**	**SI[a]**	***Tbb* (IC_{50} mg/L (µM))**	**SI[a]**
VPf DCM	7.17 ± 2.93	8.88 ± 0.32	0.8	30.03 ± 3.60	0.2	3.45 ± 0.13	2.1
TA	26.48 ± 0.33 (54.22 ± 0.7)	27.10 ± 1.51 (55.5 ± 3.1)	0.9	>45 (>92)	<0.5	11.12 ± 0.79 (22.7 ± 1.6)	2.4
3-O-*p-(Z/E)*-coumaroyl-TA	21.04 ± 3.24 (44.5 ± 6.9)	11.58 ± 1.53 (18.3 ± 2.4)	1.8	23.61 ± 6.61 (37.2 ± 10.4)	0.9	0.79 ± 0.23 (1.6 ± 0.4)	21.2
MA	20.85 ± 3.97 (44.15 ± 8.4)	9.03 ± 0.41 (19.1 ± 0.9)	2.3	>45 (>95)	<0.5	4.45 ± 0.64 (9.4 ± 1.3)	4.7
CA	9.34 ± 2.47 (19.8 ± 5.2)	3.15 ± 0.19 (6.7 ± 0.4)	3.0	32.45 ± 6.65 (68.6 ± 14.1)	0.3	3.45 ± 0.28 (7.3 ± 0.6)	2.7
3-O-*p-(Z/E)*-coumaroyl-MA	5.89 ± 0.96 (9.5 ± 1.6)	5.22 ± 0.75 (8.44 ± 1.2)	1.1	13.13 ± 2.16 (21.2 ± 3.5)	0.4	2.90 ± 0.37 (4.7 ± 0.6)	2.0
3-O-*p-(Z/E)*-coumaroyl-CA	9.04 ± 3.11 (14.6 ± 5.0)	>50	< 1	37.38 ± 4.44 (60.4 ± 7.2)	0.2	3.39 ± 0.21 (5.4 ± 0.3)	2.7
OA	63.26 ± 0.74 (138.5 ± 1.6)	9.01 ± 0.92 (19.74 ± 2.03)	7.0	27.1* (59.4)		5.8 ± 0.7 (12.7 ± 1.5)	10.9
UA	11.14 ± 0.22 (24.4 ± 0.5)	3.2 ± 0.2 (7.03 ± 0.46)	3.4	14.8* (32.4)		2.3 ±0.2 (5.0 ± 0.4)	4.9
Coumaric acid	>100	-	-	-	-	> 40	-

IC_{50} of standard drugs (mg/L): camptothecin (WI38) 0.04±0.01, pentamidine (*Lmm*) 0.11±0.06, artemisinin (*Pf*) 0.009±0.003, suramine (*Tbb*) 0.03±0.00. [a] Selectivity indexes are calculated by SI = $IC_{50(WI38)}$/ $IC_{50(Tbb,\ Lmm\ or\ Pff\ respectively)}$.*[215]

Oleanolic (OA) and ursolic (UA) acids, oleanane and ursane classes representatives, are the most studied triterpenoid compounds amongst the constituents we tested here. However, this is the first report of their activity against *Leishmania mexicana mexicana* species. We show here that UA seems more active than OA which confirms the tendency observed by several authors on other *Leishmania* species like *L. major*, *L. amazoniensis* or *L. dovani* [237,238,240]. In 2014, Sifaoui et al. studied for the first time the antileishmanial activity of maslinic acid (MA). They report a higher activity for MA (IC_{50} = 9.32 ± 1.65 and 12.46 ± 1.25 mg/L) than for OA (IC_{50} = 17.25 ± 0.65 and 20.62 ± 0.75 mg/L) against *L. infantum* and *L. amazoniensis* respectively [516]. They also highlighted a possible synergy between OA and MA, as a mixture of both exhibited a higher antileishmanial activity than each pure compound tested alone [516]. Here we observe a similar activity for both compounds. Another study reported that corosolic acid (CA) (IC_{50} = 19.0 mg/L) was less active than UA (IC_{50} = 3.7 mg/L) against *L. amazoniensis* [212] while we observed a similar activity. Further studies are thus needed to understand the impact of a 2α-hydroxylation on the antileishmanial activity of ursane and oleanane acids.

Tormentic acid (TA) corresponding to 19α-hydroxylated-CA is less active than CA itself, and also than UA. While it is the first report of the antileishmanial activity of TA against *L. mexicana mexicana*, it was reported to exhibit a much higher IC_{50} than UA (95 and 5 mg/L respectively) on *L. amazoniensis* [238], in accordance with our results. A third hydroxylation of the E ring seems thus deleterious for the antileishmanial activity. We also tested three pairs of 3-O-*p*-*Z*/*E*-coumaroyl derivatives of TA, MA and CA respectively. According to the skeleton, the addition of the coumaroyl moiety enhances (TA and MA) or suppresses (CA) the antileishmanial activity. These compounds are tested here for the first time against *Leishmania*. In the literature, only a few papers report the antileishmanial activity of OA or UA C-3 esters. The 3-O-acetylation of UA seems to reduce its activity

against *L. amazoniensis* promastigotes (from 9.41 to > 11.4 mg/L) as well as on another strain of the same species (from 360.3 to 406.5 µM) while the same structural modification increased the activity of OA on the same strain (from > 11.4 to 2.28 mg/L) [239,517]. Of note these modifications did not induce any change in the antileishmanial activity against another *Leishmania* strain (*L. infantum*) [239]. Peixoto et al. also showed that the 3-O-esterification of OA with saturated fatty acids (C12 and C18) enhanced the antileishmanial activity against *L. amazoniensis* [517].

Nevertheless, none of the *Vitellaria paradoxa* major constituents exhibit an $IC_{50} \leq 1$ mg/L with a good selectivity. Even if some pentacyclic triterpenoids seem to be promising antileishmanial compounds, assays on a larger number of UA and OA derivatives are necessary in order to determine structure-activity relationships as well as find a potential hit compound.

The antiplasmodial activity of UA and OA have been extensively studied on various *Plasmodium* strains with a general better activity for UA than OA [210,211,214,216]. Hydroxylations (2α and/or 19α) seem to decrease the antiplasmodial activity and we can classify the activity of derivatives as follows: MA < OA and TA < CA < UA. While no paper compares the activity of MA to OA, our results are in accordance with Filho et al. who reported an IC_{50} of 3.2 mg/L for CA higher than the one of UA (1mg/L) against the chloroquine-sensitive *Plasmodium falciparum* D6 strain [212]. While TA seems to be not active against the tested *Pf* strain 3D7, it was previously reported to have an IC_{50} of 15.2 mg/L against the chloroquine-resistant W2 strain [518].

Concerning antiplasmodial activity, the 3-O-esterification with a coumaroyl moiety increased the activity of TA and MA while it did not induce any significant effect for CA derivatives. However, the selectivity indexes of these derivatives stay really low. It has been shown in the literature that the 3-O-esterification with a short alkyl

chain improved the antiplasmodial activity [214,239,519]. Moreover, 3-caffeoyl betulinic acid was isolated from the leaves and twigs of *Diospyros quaesita* THW. (Ebenaceae) and showed antiplasmodial activity of 0.9 and 0.6 mg/L respectively against D6 and W2 strains with selectivity indexes of 2.9 and 4.0 (compared to KB cells) [520]. Of note, some 27-O-coumaroyl triterpenic esters showed promising antiplasmodial activities. Messagenic acids (A and B) , the 27-*O-p-(Z)*- and 27-*O-p-(E)*-coumarate esters of betulinic acid, isolated from *Gardenia saxatilis* Geddes (Rubiaceae) exhibited IC_{50} values of 1.5 and 3.8 mg/L on a multidrug resistant strain of *Plasmodium falciparum* (K1) [521]. A mixture of uncarinic acid E (27-*O-p-(E)*-coumaroyloxyoleanolic acid) and 27-*O-p-(*E*)*-coumaroyloxyursolic acid was also isolated from the same plant and had an IC_{50} of 2.9 mg/L on K1. A mixture of the *cis* and *trans* forms of 27-O-p-coumaroyloxyursolic and oxyoleanolic acids extracted from the twigs of *Keetia leucantha* (K. Krause) Bridson showed an IC_{50} of 2.35 mg/L against *Plasmodium falciparum* 3D7 [215]. These authors showed that the *p*-coumarate moieties at the 27-position contributed to the antiplasmodial activity [215,521]. Even if the IC_{50} obtained for 3-O-coumaroyl derivatives are higher than those obtained for 27-O coumaroyl derivatives, it is difficult to attribute the decrease of activity to the ester position as our derivatives are hydroxylated in position 2α or/and 19α and we previously showed the negative impact of these hydroxylations on the antiplasmodial activity.

Concerning antitrypanosomal activities, our results are in accordance with previously published ones on the same strain, UA being again more active than OA [227,228]. No previous study reported the *in vitro* activity of MA or CA against *T. brucei brucei*. Of note, in 2006, Cunha et al., reported that a mixture of MA + CA was less active than a mixture of OA + UA against one strain of *Trypanosoma cruzi* (IC_{50} = 48.5 and 5.4µM respectively) [522]. In our study, we did not observe any clear effect of the 2α-hydroxylation, derivatives showing similar antitrypanosomal

effects. This is the first report concerning the antitrypanosomal activity of TA, which is here less active than CA indicating that hydroxylation of the E ring also seems deleterious for the antitrypanosomal activity. Concerning the ester derivatives, only a few literature data are available. It was shown that a single C-3 acetylation did not improve activity or decreased it [523,524]. However, a tri-esterified oleanane derivative, 3β-acetoxy-27-trans-caffeoyloxyolean-12-en-28-oic acid methyl ester, displayed high selective antitrypanosomal activity on *Trypanosoma brucei brucei* without significant activity on *rhodesiense* subspecies (IC_{50}= 1.5 ± 0.17 (SI=7.2) and 26.8 mg/L respectively) [525] suggesting the importance of substituents nature and orientation. In general, in the present study, the 3-O esterification seems to enhance the activity against *T. brucei brucei* but is also accompanied by a slight increase in the cytotoxicity against human normal fibroblast WI-38. Noteworthy, the mixture of 3-O-*p-(Z/E)*-coumaroyltormentic acids are the only compounds corresponding to the hit selection criteria proposed by Pink et al., with a $IC_{50} \leq$ 1mg/L and a SI > 20 [514]. By testing coumaric acid (IC_{50} > 40 mg/L), we showed that this activity was not due to this part of the molecule. We thus decided to further study the *in vivo* acute toxicity of this promising compound as well as its activity against *T. brucei brucei* infected mice.

3.2 *In vivo* acute toxicity

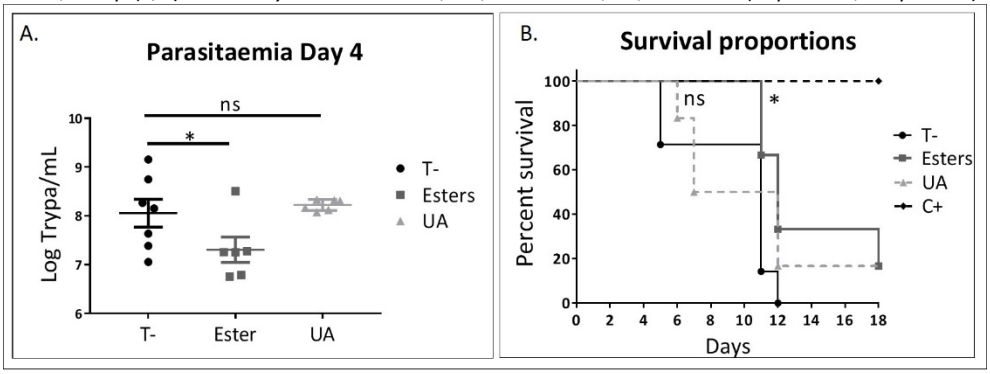
In order to avoid any side effects during the treatment and to estimate the therapeutic window, it is crucial to evaluate the *in vivo* toxicity of a pure active compound, starting by an acute model. Indeed, pentacyclic triterpenes are often associated with toxicity due to their cytostatic properties [137]. In the present case, no acute toxic symptom was observed in each group (UA and 3-O-*p-(Z/E)*-coumaroyltormentic acid) after the repeated injections of the treatments did not impact neither weights nor haematocrits. As autopsy of treated mice did not reveal any macroscopic signs of toxicity and organs weights were normal, the total

cumulative highest tolerated doses were evaluated as 100 mg/kg for both compounds. According to the Drug for Neglected Diseases initiative (DNDi) protocol, the treatment regimen for efficacy tests can be done at 50% of the highest tolerated dose without any risk of toxicity, we thus used the dose of 50mg/kg/day in our *in vivo* antitrypanosomal assays [510]. Interestingly, this dose is two times lower than the one usually used to assess the efficacy of antiparasitic compounds [514].

3.3 *In vivo* antitrypanosomal activity

The mixture of 3-O-*p-(Z/E)*-coumaroyl tormentic acids isolated from the leaves of *Vitellaria paradoxa* exhibit a significant decrease of the parasitemia on day 4 post-infection when administered intraperitoneally at 50 mg/kg/day (**figure 5-2 A**). This compound was more active than UA which did not show any effect on the parasitemia count. Concerning survival analyses, the esters treatment significantly improved the survival of infected mice in comparison to the untreated group, contrarily to UA for which no significant difference was observed at day 19 post-infection (**figure 5-2 B**). Positive control mice survive during all the experiment period while all mice died after 12 days in the negative control group and in both treated groups only one mouse survived till the end of the experiment. Remaining mice were euthanized on day 19 post-infection. Of note, on day 12 post-infection, a survival increase of 16.7% and 33.3% were observed for UA and esters treated mice respectively.

Figure 5- 2 *: **A.** In vivo parasitemia (logarithm of the trypanosoma number/mL) on day 4 post infection in mice infected by Trypanosoma brucei (*: p < 0.05; ns: p> 0.05). **B.** Survival. T-, vehicle treated mice; Esters, 3-O-p-(Z/E)-coumaroyltormentic acids; UA, ursolic acid; C+, suramine (*: p < 0.05; ns: p> 0.05).*



Up to now, only three papers reported the *in vivo* antitrypanosomal activity of pentacyclic triterpenes limited to *Trypanosoma cruzi* infected mice [226,229,526]. In 2006, Cunha et al showed that daily intraperitoneal doses of 2 mg/kg of UA, OA or the potassium salt of UA were efficient against the lethal Y strain of *T. cruzi* infected mice, with a more marked reduced parasite load for UA and the salt (75.7 and 70.4%, respectively) [226]. In another study, oral treatment with 20 mg/kg/day of OA and UA resulted in a 40 and 60% of parasitemia reduction respectively at the parasitemic peak of mice infected with a Bolivian strain of *T. cruzi* [229]. Three years later, the same team demonstrated that the effectiveness of UA and OA treatment in *T. cruzi* infected mice was dependent on the bioavailability as well as the potential immunosuppressive activity of the compounds. They observed that oral administration of the compounds (50 mg/kg/day) resulted in 79 and 76% decrease in parasitemia for UA and OA respectively, while administration of the same concentrations via the intraperitoneal route was not effective. They hypothesized that the intraperitoneal route achieved higher effective concentration of the compounds, which could have modulatory effects on pro and anti-inflammatory cytokines that resulted in an observed immunosuppression [526]. Since the immune system participates in the parasitic clearance, the immunosuppressive

effect observed at high doses of UA and OA could explain the lack of efficiency observed in controlling the infection [526]. This hypothesis could perhaps explain the fact that UA did not showed any antitrypanosomal activity in *Tbb* infected mice in the present study as we used the intraperitoneal route and the same dose of 50mg/kg/day. Moreover, these findings are also in agreement with the previous results obtained by Cunha et al. who used the intraperitoneal route but with a dose 25 times lower [226]. Hence, at low concentrations (achieved via the oral route due to low oral bioavailability of UA and OA or low intraperitoneal dose), these compounds were able to decrease the parasitemia. Further research are thus necessary to determine if the *in vivo* antitrypanosomal activity of the esters tested in the present study is due to a direct antitrypanosomal effect or an effect mediated by the immune system, as their hydrolysis gives TA which is less effective in *vitro* but also shown to be devoid of immunosuppressive activity [527].

4. Conclusions

Vitellaria paradoxa (VP) is used in traditional medicine to treat several ailments including malaria fever. Testing a leaf dichloromethane extract of VP on three parasites *(Leishmania, Plasmodium* and *Trypanosoma)* showed a more promising activity against *T. brucei* than against other parasite species but with a low selectivity index. By testing the antiparasitic activities of 11 pentacyclic triterpenic constituents of this extract, we showed that, compared to UA and OA, hydroxylations at 2α and 19 does not have a positive effect on antiparasitic activities, while esterifications at 3-OH by coumaric acid may have positive effects. Among all tested compounds, only one mixture of ester isomers, namely 3-O-*p-(Z/E)*-coumaroyltormentic acid, exhibited a really good *in vitro* antitrypanosomal activity ($IC_{50} \leq 1$mg/L) with a high selectivity index (SI > 20). After having verified the safety of this mixture and of UA in an acute model, we showed the effectiveness and the superiority of this compound (vs UA) to significantly reduce the parasitemia as well as improve the survival of mice infected by *T. brucei brucei*. This is the first report of the *in vitro* as well as *in vivo* antitrypanosomal activity of this potential hit compound. Our future work will aim at studying its bioavailability, pharmacokinetics as well as its mechanism of action.

GENERAL DISCUSSION
&
PERSPECTIVES

Staphylococcus aureus is a major cause of community- and hospital- acquired infections, with β-lactam antibiotics often considered as a the first line of treatment [7]. However, since their emergence, methicillin-resistant *S. aureus* (MRSA) strains have evolved resistance to multiple classes of antibiotics, compromising clinical outcomes. Novel antimicrobials and treatment regimens are urgently needed to improve the management of *S. aureus* infections and restore outcomes to the levels seen for methicillin-susceptible *S. aureus* (MSSA).

A potential source of such novel antimicrobials may lie in plant extracts. Indeed, plants are a rich source of secondary metabolites, as quinones, tannins, terpenoids, alkaloids, flavonoids, and polyphenols that are used by plants themselves as important defense mechanisms against predation by microorganisms, insects and herbivores. Moreover, over the past few years, research interest on medicinal plants has been amplified, not only to find new antimicrobials, but also to discover new agents that are able to attenuate bacterial antimicrobial resistance (resistance-modifying agents) [101,102,104,528,529].

The present study was therefore designed to obtain information on the potential of selected plant species that have been used in traditional medicine in Africa to treat various infections, as well as to discover novel natural compounds as antibacterials or as β-lactam resistance modifying agents against *S. aureus* strains.

Most of the data presented in the four results chapters have been discussed in their respective discussion and conclusion parts. Nevertheless, at the end of this work, several points deserve some additional discussion and open the doors to numerous additional perspectives, due to the multidisciplinarity of this work. Several of them are given below in different areas.

1. Traditional use of selected plant species

1.1 Undetected activity

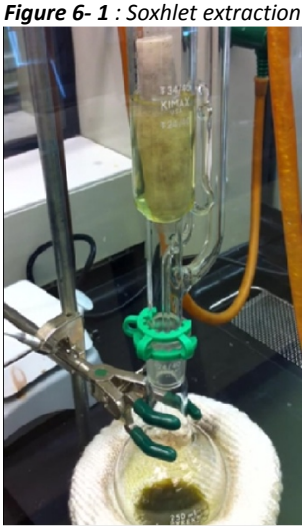
During our studies, we did not detect activity of various plants used in traditional medicine. However, our work has several limitations.

The first one could be the **difference between our extraction process and the mode of preparation used by traditional healers**. Even if recipes are often kept secret, they most often use macerations or decoctions (in water, oil, alcohol, sometimes with additives (salts, …)) with or without filtration before use.

To prepare plant extracts, we used a soxhlet apparatus (**Figure 6-1**). This mode of extraction was chosen to spare solvent and time, and improve reproducibility compared to the maceration method, most popular but time- and solvent consuming [530]. Moreover, the successive extraction using four solvents of increasing polarity seemed to us really interesting to allow a preliminary fractionation. However, some active compounds could have been partially **thermally degraded** during the extraction.

Furthermore, the preliminary fractionation using the different solvents could have separated different compounds having additive or synergistic activity against pathogens when together in the plant.

***Figure 6- 1** : Soxhlet extraction*



We also detected a growth inhibitory activity of *S.aureus* for some extracts but their **MICs were higher than the maximum tested concentration** (500mg/L). Of note, in traditional medicine, plants are often used as cataplasm, with a direct contact between the plant and microorganisms which increases the active local concentration.

Another limitation concerning plants used in traditional medicine by the oral route, could be the **metabolism necessary to activate some molecules**, thereby enhancing or activating otherwise inert compounds *in vitro* into active compounds *in vivo*. Indeed, some polar molecules like saponosides or other heterosides, may be present as prodrugs which are metabolized into more active nonpolar molecules.

In order to select the most active plant extract(s) as potential MRSA therapeutics, we utilized **only one MRSA strain**. However, considerable heterogeneity in susceptibility can be seen between different strains, as shown in our introduction. Even if most plants were selected for their traditional use in skin infections, abscesses or wound treatments, for some cases the infection for which the plant is used was not specified.

By limiting our screening to only one bacterial strain, we may have missed antimicrobial activity of some extracts against other Gram-positive or Gram-negative bacteria, including other *S. aureus* strains. Furthermore, we only used one method and one culture medium for activity determination, so it is also possible that some active compounds from extracts interact with this medium; it could then be interesting to use complementary methods to assess the activity. Moreover, **some plants could act on providing symptomatic relief of the infection via anti-inflammatory activity.**

1.2 Extracts containing tannins

Methanolic extracts from the leaves and twigs of *Cola gigantea*, from the aerial parts of *Tapinanthus bangwensis* and from the leaves and twigs of *Vitellaria paradoxa* were detected as active against *S. aureus.* **We attributed this activity to their contents in tannins**, which is in accordance with previously published data [397,398,405–408].

Tannins are plant secondary metabolites known since ancient times for their ability to tan or convert animal skin into leather [143]. These compounds are classified as being water soluble phenolic compounds, with a molar mass comprised between 300 and 3000, which possess the ability to precipitate alkaloids, gelatins and other proteins [143]. **Many biological activities** have been attributed to this class of phytochemicals such as antioxidant [531], antiviral [532], antimutagenic [533,534], antidiabetic [535,536], cardioprotective [537], as well as antibacterial [538], **including bacteriostatic and bactericidal activities against *S. aureus*** [385]. As the activities of tannins were mainly studied for mixtures or for enriched plant extracts, it is difficult to attribute the observed activities to one specific molecule. Moreover, tannins are **instable molecules**. The tannin content in food or plant extracts can be affected by storage, thermal processes, or oxidative processes [539].

Furthermore, due to their **poor bioavailability**, mainly related to their high molecular weight, it is believed that tannins may exert their biological effects in two different ways: (a) as an **unabsorbable complex structure** which may produce **local effects in the gastrointestinal tract or on skin lesions** or (b) as absorbable tannins (probably low-molecular weight) and **absorbable metabolites** from colonic fermentation of tannins which may produce **systemic effects** in various organs [539]. Given their poor absorption, tannins have generally been considered as **low toxic**. However, **detrimental effects** may be observed in the form of **reduced nutrient absorption** due to formation of insoluble complexes with tannins, as well as the possibility of **irritation of the gastrointestinal mucosa** at high concentrations [540,541].

Given their tannins contents exerting an anti-staphylococcal activity and their low or non-toxicity on the human normal fibroblast cell line WI38 (IC_{50} from 95.9 to >100 mg/L); **we confirm the potential interest of *Cola gigantea*, *Tapinanthus bangwensis* and *Vitellaria paradoxa* in the local treatment of wound infections**. In this context, given their **antimicrobial** properties but also their **anti-inflammatory** and **wound healing effects, tannins are really interesting compounds that can be used in healing wound infections** [542–544]. Interestingly, several recent patents have been issued for pharmaceutical compositions containing tannins that are applicable to cutaneous lesions [545,546]. These plants could thus be considered as good candidates for ITM (improved traditional medicines) and allow therapy accessibility at lower cost in developing countries.

Perspectives

In order to discover more new antimicrobials, belonging to other phytochemical classes, it could be interesting to use higher concentrations of our extracts, test them against other bacterial strains, or use other extraction techniques more

close than those used by the traditional healers like decoctions or macerations, or using local solvents.

The activity of methanolic extracts was attributed to tannins, but no active molecule has been isolated yet. Even if isolation and structure identification of tannins is challenging due to their lack of stability, elucidating the tannins compositions of active methanolic extracts could be relevant (e.g. for standardization purposes).
Moreover, to further validate the traditional use of these active plants, a lot of work still has to be done, including for example clinical trials.

2. Rationale to combine plant extracts with common antibiotics (e.g. ampicillin and oxacillin)

In 2002, Taylor et al. suggested that the use of **agents that do not kill pathogenic bacteria** but **modify them to produce a phenotype that is susceptible to an antibiotic**, could be an **alternative approach for the treatment of infectious diseases** [547]. Such agents could restore the susceptibility of the pathogen to a previously ineffective antibiotic, and because the modifying agent applies little or no direct selective pressure, this concept could **slow down or prevent the emergence of resistant genotypes**.

As **plant extracts** could be a **great source of such agents** [101,102,528,548], we **combined all selected plant extracts with two β-lactams** (ampicillin and oxacillin) to distinguish between a reversion of the two main resistance mechanisms developed by *S. aureus* against β-lactams. This design allowed us not only **to have a rapid idea of the indirect antistaphyloccal activity** of plant extracts, but also to **propose a first hypothesis of their mechanism of action**. Given the complexity of

crude extracts, the probability that only one mechanism of action is involved in the observed effect is quite low. However, we observed a **total effect of extracts** which need to be **further fractionated to clarify the mechanism of action of isolated compound**. It is what we achieved with the most active extract, for which the fractionation led to pure compounds, acting on both PBP2a and β-lactamase resistance mechanisms, in accordance with the results obtained in the screening. On another hand, the use of **crude extracts** is interesting to study, as they could **contain mixture of compounds acting in synergy**.

Perspectives

As we limited our study to only one bacterial species and strain, as well as one class of antibiotics with only two different β-lactams, perspectives can take numerous directions. We could try combinations of the selected plant extracts with different antibiotic classes on different bacterial strains or select other plant species.

It is for example known that plants are an abundant source of efflux pumps inhibitors, another important resistance mechanism developed by many microorganisms against antibiotics [528,529]. The quinolone antibiotics have been proposed as a possible alternative to parenteral vancomycin *S. aureus* therapy on the basis of several *in vitro* [549] and *in vivo* animal model data [550]. However, the bacteria rapidly developed resistance against this class of antibiotics mainly through the production of an efflux pump called NorA (see introduction) [551]. Another design could thus be adopted in order to find new natural inhibitors of NorA.

Interestingly, in some cases, plant crude extracts can contain pure compounds acting in synergy. The problem with the use of crude extracts is the lack of information about their phytochemical content. After having isolated pure

compounds, it could be thus really interesting to assess the potential of 'standardized mixtures' in various models.

Regarding pentacyclic triterpenes, only few papers studied their effects as standardized mixtures. It is for example reported that addition of α-amyrin improves the activity of betulinic acid against MRSA and MSSA strains [286]. In the context of their antiparasitic activities, it is reported that a mixture of OA and UA had a higher activity than each single compound against a strain of *Trypanosoma cruzi* as well as against *Leishmania* strains [226,517].The same phenomenon was also observed for a combination of OA and its 2α-hydroxylated derivative (MA) [516]. However, the concentration of each compound present in the mixture is rarely reported. We are thus convinced that the activity of standardized triterpenes mixtures could be enhanced in comparison to individual compounds, potentially without any increased of cytotoxicity.

3. Interest of UA or OA as β-lactams adjuvants

3.1 General advantages and limitations

3.1.1 Widespread occurrence

UA and OA are **widespread in plants** and were reported in more than 120 plant species. Amongst plant matrices with a high content of these compounds, we can mention apple fruit peel (*Malus domestica*), marjoram leaves (*Origanum marjorana*), oregano leaves (*Origanum vulgare*), rosemary leaves (*Rosmarinus officinalis*), black elder leaves and bark (*Sambucus nigra*), hawthorn leaves and flowers (*Crataegus* spp.), … OA or UA highest reported concentrations can reach **up to 2-3% of dry matter in olive leaves and in rosemary leaves** respectively [142].

In this work, we reported the presence of **high amounts** of UA and OA **in the leaves of *Vitellaria paradoxa***, corresponding to **0.4 and 0.1% of dry matter**, respectively.

Given their prevalence and their ease of access, UA and OA could be an **easy source of new drugs**.

3.1.2 Toxicity

As mentioned in the introduction, one interest of triterpenic acids is their **plethora of biological activities** which can be useful in therapy. However, this **lack of specificity**, and thus the **risk of toxicity** is one of the major drawbacks of using triterpenoids. In this context, several **discrepancies** are found in the literature: the absence of OA/UA cytotoxic effects is described in several reports (for example [552,553]) while their cytotoxicity is the major subject of other studies [248,554].
In our work, the **MICs** of UA (8-16 mg/L) and OA (64 mg/L) against ***S. aureus*** strains are **in the same range as their IC_{50}** obtained against the human normal fibroblast cell line **WI38** (about 11 mg/L for UA and 63 mg/L for OA). The obtained **selectivity indexes** are thus **really low**. However, it is **difficult to compare** the antimicrobial activity to the cytotoxicity as the **assay conditions are different**. First, while we determine MIC against bacteria, we measure a concentration killing only 50% of cells. Second, we incubated **bacteria for 24h** with the compounds whereas **cells were maintained for 72h** in contact of triterpenic acids. Finally, we limited our cytotoxicity assay to **only one cell line** which is not representative of the whole organism. Moreover, synergies between triterpenic acids and conventional β-lactams, were observed at sub-MICs of triterpenes. These cytotoxicities should thus be considered as preliminary and should not stop investigations.

Indeed, **regarding the literature data, UA and OA seem relatively safe**. OA is used as a **dietary supplement** and an **over-the-counter Chinese medicine** for the treatment of liver disorders [555]. On another hand, **topical cosmetic preparations containing UA** have been patented in Japan for the prevention of skin cancer [556]. These utilizations are maybe precipitated as we **lack information regarding their**

benefit/risk balance. However, several **clinical trials are currently conducted** to assess the real therapeutic benefit of these triterpenic acids. Currently, **UA liposomes** have been studied successfully and have been **approved by the State Food and Drug Administration of China** to enter clinical trials [478]. A phase 1 clinical trial showed that consecutive 14-day intravenous infusion every 21 days at doses of 56, 74, and 98 mg/m² was safe and has the potential to improve cancer remission rates [478]. Another clinical study showed that the daily oral administration of 150 mg of UA for 12 weeks achieved transient remission of the metabolic syndrome in half of enrolled patients [481]. Bang et al also showed that an 8-week resistance-training program with UA supplementation (450mg, 3 times a day) can decrease body fat percentage and increase IGF-1, irisin, and skeletal muscle strength without affecting muscle mass in men [557].

In the 16th century, Paracelsus pointed out that "**All substances are poisons; there is none which is not poison. The right dose differentiates a poison from a remedy**". Like many herbal ingredients, the selection of the appropriate dose of these triterpenoids is critical. For example, low-doses of OA are hepatoprotective, while high-doses could produce cholestasis and hepatotoxicity [558]. In future studies, we have to consider that the dose determines the mode of action. As we observed a beneficial effect for the addition of UA to nafcillin in the murine model of MRSA subcutaneous infection without any sign of toxicity, we feel that further studies in this field are pertinent.

3.1.3 Bioavailability

Given their **high lipophilicity** and their **poor aqueous solubility**, the **predicted bioavailability of OA and UA is very low**. However, different **strategies** can be used **to enhance their bioavailability** as for example, the use of **prodrugs, nanoformulations or liposomes** (see [258] for review). For example, Yang et al

developed a self-microemulsifying drug delivery system to enhance the solubility and bioavailability of OA, and compared its pharmacokinetic parameters to conventional tablet in rats. This drug delivery system increased the bioavailability of OA to **507%** [559]. Regarding UA, an interesting result it the one achieved by Zhi-Qiang Ge et al. who reported that the oral bioavailability of UA nanoparticles prepared using d-α-tocopheryl polyethylene glycol 1000 succinate was **27.5-fold higher** than that of the UA free compound [560].

Beside these strategies, another possibility to avoid the bioavailability issues is the **topical application of UA or OA on the skin lesion**. Indeed, topical application has many potential advantages like the concentration of antimicrobial at the site of infection, the limitation of amount of antimicrobial needed as well as the limitation of potential systemic absorption and toxicity [561]. In specific circumstances, topical antimicrobial therapy, although not currently advisable for most clinically chronic wounds, does have a role; for example in the case of burn wounds in which blood vessels to the skin are often destroyed [562].

3.1.4 Multiplicity of biological activities

As already mentioned, pentacyclic triterpenes and more precisely UA and OA are known for a **plethora of biological activities** including anti-tumor [148], anti-inflammatory [149], antioxidant [150], cardio- [563] and hepato-protective[152], anti-diabetic [153] , anti-viral [564], as well as antiparasitic [155] and antibacterial [156].

In the context of *S. aureus* infections, the **combination of their anti-inflammatory together with their antimicrobial activities** could be of interest. Indeed, as a protective response, the goals of inflammation are to clear the body of the inciting organisms and begin tissue repair. Unfortunately, in some circumstances, the inflammatory response is deleterious. With diabetic foot ulcer infections, *S aureus*

infections with Panton-Valentine leucocidin production and toxic shock syndrome, the persistence of tissue damage or pathogens may perpetuate the inflammatory response. As a result, inflammation may be the source of ongoing tissue damage [565].

Previous studies have demonstrated the **beneficial effects of UA on several experimental models of inflammation-related diseases**, such as asthma [566], arthritis [170], colitis [501] or for exemple, in prostate cancer [567]. In link with their anti-inflammatory and antioxidant properties, their **wound healing potential** is of great interest in our study.

Concerning topical application, Moura-Letts and colleagues reported that OA (at 40mg/kg) accelerated wound closure and improved the tensile strength of healing tissue [568]. Lee et al showed that the stimulation of the differentiation of epidermal keratinocytes as well as the increased ceramide production enhanced by OA could be related to its activation of PPAR-α [569]. They also showed that UA had less effect than OA in their model [569]. It was recently reported that daily application of UA at 0.2% or 0.25% in a topic formulation enhances excellent wound-healing in several wound models in rats [570,571]. Though the exact mechanism of wound healing activity is not fully understood, it is suggested that UA can either (i) directly inhibit the release of some inflammatory mediators along with the expression of PGE_2, TNF-α, IL-1β and IL-6; (ii) help in collagen deposition, epithelization, wound contraction, maturation and remodeling [571].

Of note, in January 2016, The European Medicines Agency approved the commercialization of **Episalvan®**, a **birch bark triterpene extract**, mainly constituted of betulin, betulinic acid and OA, as a gel, for its **positive effect on skin wounds** [572].

However, **in a context of infection, *in vivo* studies using UA or OA are limited**. It was shown that UA may have a potential in the prevention and treatment of

obesity, linked with its **impact on intestinal microbiota** [573]. Intra-gastric administration of UA solution (0.25 and 1 %) for 45 days decreased the microbial diversity in the proximal intestine while having an opposite effect in the distal intestine. UA also inhibited colonization by energy harvest-related microbes and might be able to enhance intestinal health by inhibiting colonization by *Proteobacteria* [573]. Hu et al. conducted research on **sepsis** induced by cæcal ligation and puncture. They found that one intraperitoneal injection of UA (10mg/kg) after the surgery was able to significantly **improve survival and attenuate lung injury of infected rats** [574].

More studies are thus needed to take advantage of the pleiotropic activities of triterpenic acids. All effects are not observed at the same dose but given the huge number of studies in many different pathologic situations, using various models, it is challenging to link a specific biological activity to a precise dosage.

3.2 Potential in antistaphylococcal therapy

3.2.1 Antistaphylococcal target

As mentioned in the introduction (**table 1-19**), published data attribute the **antibacterial activity pentacyclic triterpenes** to an **hydrophobic interaction and accumulation in the bacterial membrane**.

According to our *in vitro* observations we can also hypothesize that the **antistaphylococcal** activity of OA and UA, as well as **the synergy observed with β-lactams**, may be **linked to the interaction of these triterpenic acids with the bacterial membrane**. We suggest that the PBP2 delocalization as well as the fact that β-lactamase inhibition is observed only towards living bacteria could be due to an indirect effect of UA and OA, possibly related to their insertion in the bacterial

membrane and an eventual **destabilization of functional membrane microdomains** (FMM).

Indeed, Fernandez et al., recently characterized FMM in MRSA. They showed that the scaffold protein flotillin (FloA) preferentially binds isoprenoid saccharolipids derived from the carotenoid staphyloxantin and oligomerizes in staphyloxanthin-rich membrane microdomains (*see section 1.1.2 in this introduction*). They demonstrated that FloA scaffold activity promotes efficient PBP2a oligomerization, and that perturbation of FMM architecture affects this phenomenon and reduces MRSA proliferative capacity in the presence of β-lactam antibiotics [27].

To date, **studies of the effect of UA and OA on membrane models are limited** (**table 6-1**). In general, model used are not representative of the real complexity of the bacterial membrane and investigators often used **phospholipids extracted from *E. coli*,** a Gram - bacterium, UA being inactive against this strain. Moreover, while OA and UA have been shown to **disturb the organization of cardiolipin-rich domains**, the existence of such domains in *S. aureus* strains have not been described yet [575]. Up to now, **no literature data reports any specific interaction of UA or OA with phospholipid bilayers mimicking *S. aureus* membrane** or any real discrimination between eukaryotic or bacterial membrane models.

***Table 6- 1** : Reported effects of UA and OA on membrane models*

Lipid composition	Tested compound (concentration)	Technique	Result	Difference OA and UA	Ref
DPPC	UA/OA (incorporation of 10, 20 or 30% mol/mol%)	Fluorescence anisotropy DPH	Decrease fluidity in liquid crystalline phase	UA = OA	[576]
		Calcein leakage	Decrease permeability	UA = OA	
E. coli inner membrane model (POPE/ECCL or POPE/PG; 75/25 mol/mol%)	Surface ratio UA/phospholipid: 1/4, 1/2, 1/1, 2/1, 4/1	Langmuir monolayers	Disorganizing effects UA incorporation can lead to disintegration of CL-rich domains	-	[577]
ECCL	Surface ratio UA or OA/phospholipid: 1/4, 1/2, 1/1, 2/1, 4/1	Langmuir monolayers	Destructive effect for CL-rich domains No selectivity between used species	UA > OA	[575]
BHCL					
TOCL					
ECPG			Selective condensation of bacterial PG without any effect on eukaryotic PG		
DLPG					
DOPG					
POPC	10, 30 or 50% of UA	Langmuir monolayers	Preferential interaction with POPC whereas incorporation in POPE films in energetically unfavorable	-	[578]
POPE	10, 30 or 50% of UA				
POPC/POPE/CL (47.05/35.30/17.65 (mol/mol %))	5, 15 or 30% of UA		Destructive effect (even at 5%) Hypothesis: in presence of POPE (H bond donor), UA organizes at the surface air/water interface with its COOH (H bond acceptor) in the subphase. This organization cannot be maintained during compression		

			which causes disruption of the monolayer and phase separation		
POPC	UA	Molecular dynamics simulation	UA is located in parallel of the membrane near the surface in vicinity of phospholipids headgroups	-	[579]
POPC/POPE/POPG/PSM/Chol (64/16/16/16/16)			UA is located perpendicular to the membrane near the surface in vicinity of phospholipids headgroups. Interaction with POPE, PSM and Chol, excluding POPG → could lead to phase separation or domain formation		

CL: cardiolipin (ECCL = *E. coli* CL; BHCL = beef heart CL and TOCL = tetraoleoyl CL)
DPPC: dipalmitoyl phosphatidylcholine
PG: phosphatidylglycerol (ECPG = *E. coli* PG; DLPG = dilinoleoyl PG and DOPG = dioleoyl PG)
POPC: palmitoyl-oleoyl phosphocholine
POPE: palmitoyl-oleoyl phosphoethanolamine
POPG: palmitoyl-oleoyl phosphoglycerol
PSM: palmitoyl-sphingosyl phosphorylcholine

Perspectives

First, it is necessary to characterize more precisely the interaction of UA and OA with *S. aureus* membrane and its components. Study the effect of UA and OA on the permeability and fluidity of liposomes mimicking *S. aureus* membranes could be realized. Calcein release experiments can be used to assess the liposome permeability, and Laurdan (a polarity sensitive probe [580]) experiments to assess the fluidity/hydration changes. By modifying the ratio of each phospholipid, the possible greater affinity of both triterpenic acids for any of the used phospholipids could be assessed. Moreover, the microscale thermophoresis technique (MST) can be used in order to analyze specific interaction between UA or OA and defined phospholipids [581]. Another perspective could aim at discriminating the affinity of UA and OA for bacterial or mammalian membranes using the same technique. As the existence of FMM in *S. aureus* is a quite new topic, studying the effect of UA and OA on their organization would be challenging but could provide really interesting information about their mechanism of action.

3.2.2 In vitro synergy

Using checkerboard and time kill assays, we showed the potential of UA and OA to synergize with both ampicillin and oxacillin at up to ¼ their MICs against various MRSA strains. The **synergism of UA and OA with β-lactams** had been previously established by several authors, but was shown to vary widely between strains belonging to the same species (see **table 1-18** in the introduction) [249,279,491]. While these studies often use only one *S. aureus* strain, we here give more data to allow a generalization of their effects, showing that **synergy is observed for both compounds combined with either ampicillin or oxacillin against several MRSA**

(including clinical isolates), which are of higher interest for clinical applications than MSSA.

If we **compare UA with epicatechin gallate** (ECG), a flavonoid extracted from green tea, extensively studied in combination with β-lactams [117,134,135,388,496,582,583], **we observe a lower synergistic effect** (4 dilutions decrease in oxacillin MIC for UA vs 9 dilutions for ECG), **but at lower dose** (2mg/L for UA vs 25mg/L for ECG) [134] . Even if the intensity of the synergy varies widely among the strains, our observations were not as pronounced with pentacyclic triterpenes. **ECG thus seems to be a more potent β-lactam modifying agent than OA or UA**. However, the direct antistaphylococcal activity of UA (MIC = 8mg/L) is higher than the one of ECG (MIC = 64 mg/L).

Many parameters have to be taken into account to compare these compounds as potential β-lactams adjuvants, including their **toxicity,** their **stability**, **bioavailability**, **pharmacokinetics parameters**… Of note, concerning the cytotoxicity, ECG seems to be safer than UA (IC_{50} > 100µM vs 24.4 µM against WI38 cell line [584]). At this stage, even if the *in vitro* data seem favorable for ECG, more comparison studies are needed to conclude for its highest potential than UA.

Perspectives

First, we showed that the lack of selectivity of pentacyclic triterpenes is a major drawback. However, cytotoxicity and antistaphylococcal or resistance modifying activity may be related to different targets. UA and OA are thus interesting leads for the synthesis of derivatives with lower toxicity and higher therapeutic potential. We showed for example in the second part of the results of this thesis that 3-O-esterification with a coumaroyl-moiety decreases the antistaphylococcal activity while it enhances the cytotoxicity; both activities are thus not completely linked. In the literature, special interest is already paid to the synthesis of UA or OA derivatives with less toxicity [585].

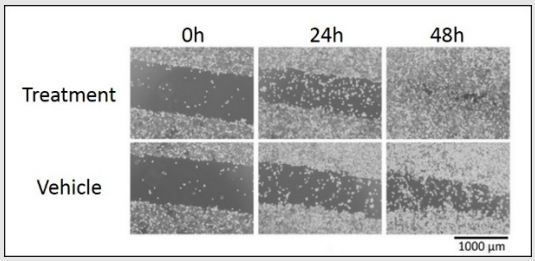
Second, we limited our *in vitro* tests to combinations between UA or OA and only one antibiotic class. However, these compounds had already been shown to synergize with other antibiotic classes (see **table 1-18** in the introduction). Other combinations should thus be studied and compared with our results obtained with β-lactams. In a context of *S. aureus* skin infections, to avoid issues of poor bioavailability or systemic toxicity, it could be interesting to combine these triterpenic acids with existing topical antibiotics already marketed. Mupirocin, commonly used in a 2% ointment, or neomycin usually formulated as 20% neomycin sulfate in petrolatum could be assessed. Polymyxin B formulated in a petrolatum base, usually exclusively used against Gram-negative pathogens should also be tested. Indeed, while pentacyclic triterpenes have never been assessed in combination with the two first topic antibiotics, 18β-glycyrrhetinic acid was shown to improve the activity of polymyxin B (16-64 fold) against MRSA [335]. Testing combination of isolated triterpenic compounds with these three topical antibiotics could be interesting in reducing either the emergence of resistance or risk of systemic toxicity by decreasing the doses of each compound.

Moreover, we also limited our studies to planktonic bacteria, meaning bacteria floating as single cells in the medium. Interestingly, OA and UA has been shown to be potent inhibitors of the *S. aureus* biofilm formation [491,586,587]. While UA showed a high MIC against *S. aureus* strain NCTC 8325 of 250 mg/L, at ¾ its MIC it decreases the biofilm adherence up to 70% [586]. Moreover, Kurek et al. reported synergistic interaction between OA and UA with ampicillin (FICI = 0.369 and 0.250 respectively) as well as OA with oxacillin (FICI = 0.292) against *S. aureus* biofilms. Up to now, the effects of OA an UA on *S. aureus* biofilms are limited to only a few studies. Their anti-biofilm mechanism of action is still poorly understood but triterpenes are believed to influence the fatty acid composition of the cell membrane, and thus cell hydrophobicity which could prevent

aggregation of bacteria and consequently biofilm formation [588]. Further exploration of the activities of OA and UA against *S. aureus* biofilms could be a relevant perspective.

Furthermore, as the wound healing properties of triterpenes could be interesting in a context of *S. aureus* infections, future work could be performed in this context. To begin such studies, the easiest model would be the *in vitro* scratch assay which is an easy, low-cost and well-validated method to measure cell migration *in vitro* [589].

***Figure 6- 2** : Representative results of a scratch assay*



This test could help to obtain structure–activity relationships to identify more effective healing agents. Furthermore, it would be of interest to assess the activity of triterpenic acids in a scratch assay involving the presence of *S. aureus* (or other bacterial strains).

1.1.1 In vivo synergy

We administered **subcutaneously UA, nafcillin, or a combination of both** to groups of mice immediately **after MRSA infection** and then every 8 hours for a total of 56 hours. In order to assess the effect of the treatment, we then measured surface area (cm^2) of the resulting necrotic skin lesions after 32 and 56 h and assessed the bacterial inoculum (CFU/g tissue).

Ideally, **the route of drug administration** in the animal experimental model should be the same as route intended for human. Using this model, we wanted **to mimic a local application in order to avoid low bioavailability or toxicity issues.** Due to the lack of time to develop a topic formulation, we chose to use subcutaneous injection near the infection site. We used this treatment scheme as a first experiment to test the effect of a combinational treatment.

Surprisingly, **UA did not increase the effect of nafcillin on bacterial burden** after 56 hours of treatment, but it **reduced lesion surface size and the production of pro-inflammatory cytokine IL-1β in the skin**. As UA alone was ineffective, even in reducing the inflammation, these benefits can probably be ascribed to a **synergy with the antibiotic**.

To explain this effect, **other potential mechanisms of UA** should be discussed. It is reported that **sub-inhibitory concentrations of β-lactams can induce the production of exotoxins** like α-hemolysins (α-*hla*), PVL or TSST [590,591]. Moreover, **α-*hla*** is known to play a **key role in the pathogenesis of *S. aureus* skin infections**. It has been shown that rabbits infected with a USA300 MRSA mutant deficient in the expression of α-*hla* developed attenuated dermonecrotic skin lesions as compared to an isogenic wild-type MRSA or complementation restored MRSA *α-hla* mutant [592]. Interestingly, it has been shown that **UA inhibited the effect of α-hla produced by *E. coli*** strains as observed when plating bacteria on sheep-blood agar plates [593]. In 2014, Kurek et al. reported that **UA and OA inhibit hemolysis caused by listeriolysin** without affecting its secretion by *Listeria monocytogenes* [594]. The inhibition of virulence factors by pentacyclic triterpenes could perhaps explain a part of the *in vivo* observed synergy between UA and nafcillin. Indeed, **UA could act as a virulence inhibitor and counteract β-lactam induction of exotoxin production.**

Perspectives

Concerning the administration route, we could imagine a topical formulation containing UA and nafcillin as an alternative when treating infected wound. However, it is advised to avoid using topical antibiotics that are available for systemic therapy when treating these lesions, because they can favor superinfection and select for resistant pathogens [595]. Moreover, in the clinical practice, such wounds usually require systemic antibiotic therapy. In the future, testing a systemic treatment of infected wounds with nafcillin combined with a topical therapy of UA could be relevant, being careful on the vehicle choice. Indeed, it has been shown that a petrolatum vehicle ointment promoted wound healing but initially increased bacterial burden in a murine model of MRSA-infected skin wounds while a polyethylene glycol vehicle ointment did not show any effect [596].

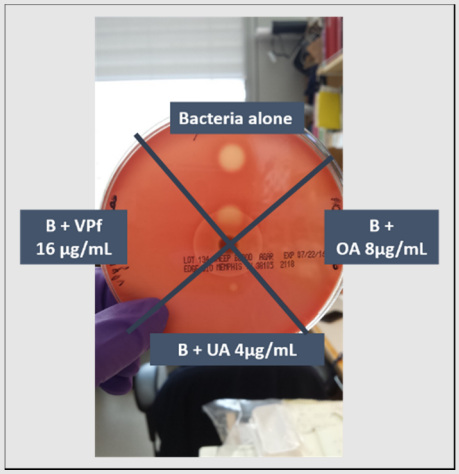
Even if no effect has been seen using the combination on the bacterial burden *in vivo*, our results obtained *in vitro* could justify a test using a combination of UA and nafcillin in a systemic *S. aureus* infection model. Indeed, as we mentioned above, intravenous or oral administration of UA is safe [478,481] and its bioavailability can be enhanced using cyclodextrins [597,598], nanoparticles [560,599] or liposomes (in clinical trial in China [478]).

Furthermore, in order to understand the observed *in vivo* synergy, the effects of UA and OA on the synthesis or secretion of α-hla by *S. aureus* strains could be measured using different *in vitro* tests.

We performed a preliminary test by plating the supernatant of MRSA USA300 incubated with sub-inhibitory concentrations of the *Vitellaria paradoxa* leaf dichloromethane extract, UA or OA on sheep-blood agar. Interestingly, no or a

negligible hemolytic activity was observed (**figure 6-3**), further encouraging research in this direction.

***Figure 6- 3** : Preliminary hemolysis assay*



Moreover, in order to check the *in vivo* mechanism of action of the observed synergy, running an experiment using the same model but with an α-hla deficient strain, could allow us to see if the beneficial effect of UA disappears in order to confirm its mode of action.

4. Potential of 3-O-*p-Z/E*-coumaroyltormentic acids in anti-trypanosomal therapy

The **mixture of 3-O-*p-Z/E*-coumaroyltormentic acids** exhibited a **really good *in vitro* antitrypanosomal activity** ($IC_{50} \leq 1mg/L$) with a **high selectivity index** ($SI > 20$), corresponding to the criteria of Pink et al, to select a pure compound as a hit for the treatment of parasitic diseases [514]. We then showed during a preliminary anti-trypanosomal *in vivo* study that the mixture was able to significantly **reduce the parasitemia and increase mice survival when administered at 50mg/kg/day using the intraperitoneal route**.

This route was used in order to obtain first efficacy data and to limit pharmacokinetics influence. However, the **main selection criteria for new anti-trypanosomal drugs** is the use of the **oral route**. Given its **high molecular weight** (634 g/mol) and its **unfavorable logP** (clogP = 8.23 according to ChemDraw software), it is **unlikely** that this pentacyclic triterpene derivative will be **absorbable in the gastrointestinal tract** in high quantity. Moreover, gastrointestinal microorganisms will probably **hydrolyze the ester link** in the gut [258]. **Specific formulations** such as **liposomes or nanoparticules** will be necessary to increase the oral bioavailability. Of note, it is known that high **fat meals** enhance triterpenes absorption while **fruit juices rich in ester linkages** can inhibit esterase activity [258,600].

At this stage, we **don't have any information** about the **antitrypanosomal mechanism of action** of 3-O-*p-Z/E*-coumaroyltormentic acids. In order to obtain preliminary information**, following the cytokines level** during the *in vivo* infection and treatment could have provided interesting data. Indeed, it is known that the control of trypanosomal infection is critically dependent on cytokines that mediate *in vitro* macrophage activation to promote intracellular parasite killing [601–604].

It has been shown that INF-γ activates macrophages for intracellular trypanosomal killing while IL-10 can inhibit INF-γ induced trypanocidal effects [601,604,605]. Interestingly, da Silva Ferreira et al observed reduced INF-γ as well as increased IL-10 plasma levels at the parasitemic peak of *Trypanosoma cruzi* infected mice treated intraperitoneally with 20 mg/kg/day of UA and OA [526]. They thus hypothesized that the absence of antiparasitic activity observed using these conditions was due to the immunomodulatory effects caused by both triterpenic acids which interfere with host parasitemia control [526]. Moreover, they observed a significant reduction in parasitemia using the same infection model in orally-treated mice. They attributed these effects to the poor oral bioavailability of OA and UA which could lead to a reduced immune alteration associated with a better control of parasite number [526]. More studies on the **balance of effects on immune system and parasitemia** are thus necessary.

According to the literature, triterpenes can also **target specific trypanosomial enzymes**. Having no functional Krebs cycle nor mitochondrial respiratory chain, bloodstream stage of trypanosomes use glycolysis as their only source of ATP [606]. **Inhibition of glycolysis** thus leads to rapid death of these parasite stages. If an increased activity of a compound is observed against the bloodstream form, the potential target of this compound could be the glycolytic pathway, responsible for the breakdown of glucose into pyruvate, with a concomitant production of ATP [607]. Moreover, despite the fact that glycolysis is a relatively well conserved pathway, important structural and/or mechanistic differences have been identified in most trypanosomal enzymes compared to their human homologues [607].
Betulinic acid (BA) has already previously been shown to be a competitive **inhibitor of the *T. brucei* GAPDH**, a specific glycolytic enzyme which catalyzes the phosphorylation of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate in the presence of NAD^+ and inorganic phosphate [232]. Moreover, our team showed in

2013 that **UA, OA and BA** were able to **inhibit the *T. brucei* GAPDH** at 30.4, 72.6 and 30.6 % respectively at 20mg/L [228]. However, this mechanism of action can only explain a part of their antitrypanosomal activities as concentrations higher than their IC_{50} against *Tbb* only inhibit 30 % of the activity.

Finally, unlike UA and OA which are widespread in plants, 3-O-*p*-*Z*/*E*-coumaroyl-tormentic acid has **only been isolated in a few plant species**. Moreover, the **time-consuming purification process** resulted in a **low yield** of pure compound (35 mg of 3-O-*p*-coumaroyl-tormentic acid) obtained from *Vitellaria paradoxa* leaves (1kg). In order to further assess the potential of this compound, a **more efficient extraction or production process will be needed**.

Perspectives

Stability, bioavailability, pharmacokinetics

Much work is still necessary to characterize pharmacokinetics and bioavailability of this esters mixture. Some preliminary studies could be conducted *in vitro* at different physiological pH to study the stability of the esters. We could also perform liver microsome assays to measure *in vitro* intrinsic clearance or to identify the metabolites formed. Preliminary permeation studies could also be done using Caco-2 monolayer assay [608]. If the pharmacokinetic or bioavailability parameters are not good, the use of nanotechnologies could be another strategy to improve them [609].

Mechanism of action

Before performing a new *in vivo* experiment aiming at identifying the immunomodulatory effects of the esters mixture during a trypanosomial infection, which require big quantities of pure compounds, several trypanosomial targets could be assessed using *in vitro* models.

A first step could be a comparison of its *in vitro* activity against the bloodstream and the procyclic forms of *T. brucei brucei* in order to detect a potential activity against the trypanosomial GAPDH [228].

If the GAPDH inhibitory activity cannot explain totally the activity observed *in vitro*, this compound could be tested on other purified trypanosomial glycolytic enzymes (hexokinase, phosphofructokinase,…).

As mentioned in the introduction, pentacyclic triterpenes are widely studied for their cytostatic properties. In this context, they are known to inhibit human topoisomerases I and II. Another potential target of 3-O-*p*-coumaroyl-tormentic acids could thus be the trypanosomial topoisomerases. Indeed, substantial differences between trypanosome topoisomerase I and their homologues in mammals have been detected and they were thus identified as interesting antitrypanosomal targets [610].

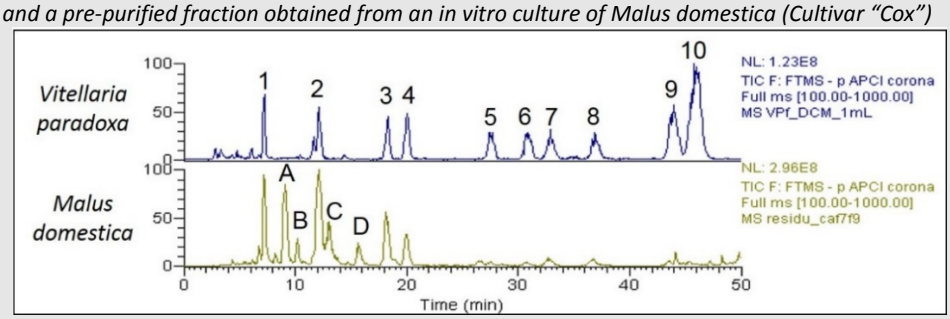
In order to obtain other information on possible targets, a metabolomic analysis of trypanosomes in the presence or not of the compound could be conducted, analyzing modifications of contents of metabolites using LC-MS and ^{1}H NMR [611].

Alternative efficient production process

A collaboration was established with Dr. Christelle André, from the Luxembourg Institute of Science and Technology (LIST) who used *in vitro* production of plant cells (*Malus domestica* Bork, cultivar 'Cox') to obtain higher yields of triterpenes with up to 83.5 mg of triterpene per gram of dry cell weight. The LC-MS fingerprint of the pre-purified triterpenes fraction they obtained is compared in **figure 6-4** with our characterized leaf DCM extract from *Vitellaria paradoxa*. We can see that except from four compounds (A, B, C and D), the main metabolites identified in this apple extract correspond to those extracted from *Vitellaria paradoxa*, namely tormentic acid (**1**), 3-O-*p-Z/E*-coumaroyl-tormentic acid (**2**),

maslinic acid (**3**) and corosolic acid (**4**) as well as traces of 3-O-*p*-*Z*/*E*-coumaroyl-maslinic and -corosolic acid (**5-8**). A preliminary quantification revealed a content of about 25 % of 3-O-*p*-coumaroyltormentic acid. This process could thus be a useful alternative to obtain high quantities of the ester.

Figure 6- 4 *: HPLC-MS (APCI-) profile of the leaf dichloromethane extract from Vitellaria paradoxa and a pre-purified fraction obtained from an in vitro culture of Malus domestica (Cultivar "Cox")*



5. Short term perspectives

In front of this multitude of perspectives, we decided to select some of them as short term perspectives.

i) Towards new information to support the traditional use of plants analyzed in the screening…

To validate or not the possible efficacy of plants analyzed in our screening, it would be interesting to test extracts against other bacterial strains, with other methods or culture medium, or obtained by other extraction techniques more close than those used by the traditional healers as water or local solvents decoctions, macerations,...

ii) Towards new resistance modifying agents…

Plant extracts used in the screening presented in this thesis could be combined with other antibiotic classes in order to find other resistance modifying agents, focusing i.e. on efflux pump inhibitors.

iii) Towards an enhanced antistaphylococcal activity of triterpenic compounds…

To analyze the potential of triterpenic derivatives, it would be interesting to test the direct and indirect antistaphylococcal activities of standardized mixture of UA and OA as well as of other isolated/(semi)-synthetic triterpenic compounds. As their antistaphylococcal and cytotoxicity targets seems to be different (selectivity indices vary a lot between compounds), it is expected to obtain better antimicrobial activity with decreased cytotoxicity.

iv) Towards a better understanding of UA and OA antistaphylococcal mechanism of action…

As no literature data report any interaction between UA or OA with phospholipids mimicking *S. aureus* membranes, it would be important to study the effects of UA and OA on such membrane models. The first goal will be to detect any specific affinity of those pentacyclic triterpenes for any of the phospholipid present in the

bacterial membrane or for staphyloxanthin, known to be the major constituent of FMM in *S. aureus* membrane.

6. Conclusion

The main aim of this work was to obtain information on the antistaphylococcal potential of selected plant species used in traditional medicine in Africa to treat various infections, as well as to discover novel natural compounds as antibacterials or as β-lactam resistance modifying agents against *S. aureus* strains.

A preliminary screening of selected plant species allowed us to measure their *in vitro* antistaphylococcal activities. We showed that the most efficient extracts were those containing tannins, emphasizing their potential use as local anti-infectious as well as wound healing extracts.

Combining these plant extracts with two different β-lactams against MRSA, we identified plants which could contain potential β-lactams resistance modifying agents by targeting β-lactamases and/or PBP2a. Working on the most active extract, we further confirmed the medicinal utility of the leaves of the shea butter tree (*Vitellaria paradoxa*), but other plants, among which the hexane extract from the roots of *Anchomanes difformis* for example, should also be further studied.

We also isolated, identified and assessed the direct anti-staphylococcal activity of eleven triterpenoid components. This class of phytochemicals characterized by their widespread occurrence have been extensively studied for multiple biological activities but only four compounds (ursolic, oleanolic, maslinic and corosolic acids), although possessing some cytotoxicity *in vitro* against a human normal cell line, showed moderate antistaphylococcal activity.

We further showed the *in vitro* potential of sub-MIC concentrations of ursolic and oleanolic acids to restore the activity of β-lactams against various MRSA strains,

including clinical isolates which are of higher clinical interest. We partially elucidated the mechanism by which they revert β-lactam resistance mediated by β-lactamases or PBP2a production, suggesting an interaction with the bacterial membrane. Moreover, we demonstrated the additional benefit of ursolic acid added to a β-lactam in a murine model of subcutaneous MRSA infection, without evidence of toxicity. Further investigations are needed in order to completely understand its mechanism of action (possibly linked to an anti-virulence effect which could counteract β-lactam induction of *S. aureus* exotoxin production), to determine its balance benefit/risk and its potential as therapeutic adjuvant to treat skin infections.

Finally, to further valorize isolated compounds, we showed the promising *in vitro* and *in vivo* anti-trypanosomal activity of a mixture of two triterpenic esters which could open the doors for future research aiming at identifying their mechanism of action and studying their pharmacokinetics.

All our research findings show the interest of the leaves of *Vitellaria paradoxa* as a novel source of pentacyclic triterpenes with therapeutic potential as adjuvants in antimicrobial therapy or as antiparasitic compounds. We also proved that slight structural modifications as hydroxylations or coumaroyl-esterifications, were able to induce high changes in the activity.

Although the pathway of clinical drug discovery is long and arduous, we believe that the collaboration between pharmacognosy, analytical and organic chemistry, pharmacology and galenics will ultimately give to these compounds the opportunity to assist clinicians toward improving patients care.

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