Bioactives against Superbugs: using phytotherapy to counteract the drug-resistance burden in the 21st century

Paula Parreira, **Teresa Brás**, **Patricia A. Ramos** and **Maria F. Duarte**

Centro de Biotecnologia Agrícola e Agro-Alimentar do Alentejo (CEBAL)/Instituto Politécnico de Beja (IPBeja), 7801-908 Beja, Portugal

Penicillin and other antimicrobials discovery revolutionized medicine but, as a consequence of antibiotic misuse, several bacteria evolved to become resistant to multiple antibiotics classes: "the superbugs". Following the stagnation on the conventional antibiotic-discovery field, scientific attention turned to alternative foundations for the development of novel antibacterial substances, namely natural bio-based compounds. Plants are a rich source of bioactive substances and among the different phytochemicals, terpenes are one of the largest class in nature. This class is structurally diverse and with well described biological potential, making it a strong candidate for discovery of new antimicrobials. Also contributing to the renewed interest in these compounds is the fact that novel techniques for their extraction have been developed. In this chapter a brief overview on multi-drug resistant bacteria will be given, being the main focus onto plant-derived bioactive compounds, in specific terpenes, as well as on their extraction and purification techniques.

Keywords: drug-resistant bacteria; phytotherapy; natural products; terpenes; extraction techniques

1. The rise and fall of antibiotics

1.1 The dawn of the antibiotic era

Bacteria were discovered in the late 19th century, and briefly the link between these microorganisms and infectious diseases, causing high rates of mortality and morbidity, was established. This knowledge boosted the search for preventive and therapeutic regimens against those microscopic agents [1]. Nonetheless, this was only achieved half a century later and, until that breakthrough, hand washing was advocated as the only way of avoiding infection, since no therapy was available [2].

The beginning of the antibiotic era started serendipitously. Returning from holidays in a September morning of 1928, Sir Alexander Flemming, then a Professor of Bacteriology at St. Mary's Hospital in London, noticed in a Petri dish inhibition of *Staphyloccocus* spp. colonies growth around a fungus, which was later identified as a strain of *Penicillium notatum* [3]. This discovery was considered as a turning point in human history, which later allowed saving thousands of lives that in the pre-antibiotic period, the Semmelweis era, would be lost. Contrasting to the lucky events that resulted in the discovery of penicillin by A. Flemming, Selman Waksman introduced in 1940s the once successful discovery platform for new antibiotics. This platform was incredibly simple but yet quite elegant: soil-derived streptomycetes were screened for antimicrobial activity against a susceptible test microorganism, by detecting zones of growth inhibition on an overlay plate [4]. Waksman's application of a systematic screen is what made the difference between luck and a discovery platform, and this breakthrough earned him a Nobel Prize in 1952 [5]. Streptomycetes screening led to the discovery of streptomycin, which was the first effective compound to act against tuberculosis as well as the first aminoglycoside [4]. The 'Waksman platform' was widely adopted by the pharmaceutical industry, allowing to discover many of the antibiotics classes known today, being many of them derived from natural products (Fig. 1 and Table 1) [6].

Antibiotics revolutionized the 20th century, but these "wonder drugs" have been shadowed from the beginning by the appearance of resistant bacterial strains, virtually in parallel to their development/introduction in the market. For instance, sulphonamide resistance was originally reported in the late 1930s and the same mechanisms of resistance operate till these days, more than 85 years later, highlighting the success of bacteria in what concerns drug resistance (Table 1) [2].

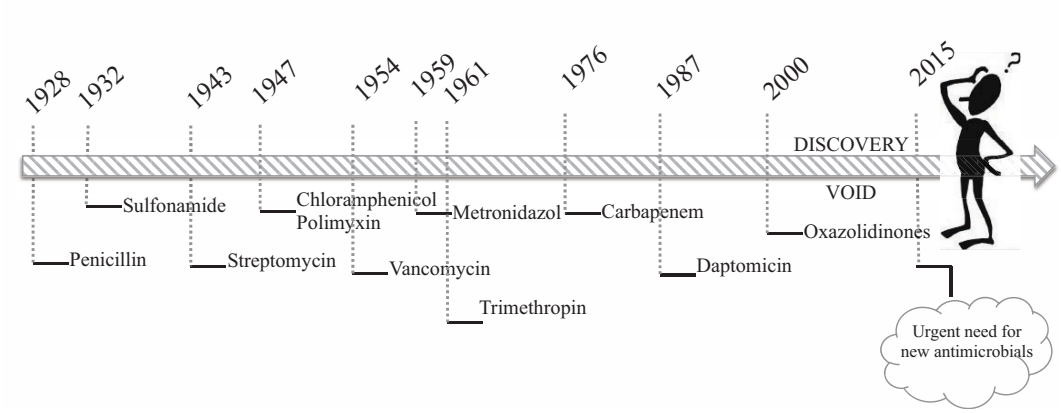

Fig. 1 Timeline of antibiotic discovery.

Table 1 Year of introduction and the year when resistance was observed for some antibiotic classes as well as their activity spectrum and origin (adapted from [4,7]).

Antibiotic class/ Example	Yintro	Yres obs.	Activity	Classification
B-lactams/ penicillin	1938	1945	Broad-spectrum	NP-derived
Sulfadrugs/ prontosil	1936	1942	Gram positive	Synthetic
Aminoglycosides/ streptomycin	1946	1946	Broad-spectrum	NP-derived
Chloramphenicol/ chloramphenicol	1948	1950	Broad-spectrum	NP-derived
Macrolides/ erythromycin	1951	1955	Broad-spectrum	NP-derived
Tetracycline/ chlortetracycline	1952	1950	Broad-spectrum	NP-derived
Rifamycin/ rifampicin	1958	1962	Gram-positive	NP-derived
Glycopeptides/ vancomycin	1958	1960	Gram-positive	NP-derived
Quinolones/ ciprofloxacin	1968	1968	Broad-spectrum	Synthetic
Streptogramins/ streptogramin B	1998	1964	Gram-positive	NP-derived
Oxazolidinones/ linezolid	2000	2001	Gram-positive	Synthetic
Lipopetides/ daptomycin	2003	1987	Gram-positive	NP
Diarylquinolines/ bedaquilin	2012	2006	*Mycobacterium tuberculosis*	Synthetic

Yintro: year of introduction; Yres obs: year of resistance observed; NP-derived: Natural product-derived; NP: Natural product

Over the years, indications that new approaches are required to combat the global spread of drug-resistant bacterial pathogens have been accumulating, being among them: i) the increase in death rates from infectious diseases observed since the late 1980's, already withdrawing those linked to AIDS [8,9]; ii) the 40-year innovation gap between the introduction of new molecular classes of antibiotics (fluoroquinolones in 1962 and the oxazolidinone linezolid in 2000) [10]; and iii) the recent trend of several large pharmaceutical companies to leave the antibacterial and antifungal arenas leading to a decrease in scientific expertise in antibacterial-drug discovery [11,12].

1.2 Superbugs- a threat to public health

The development of generations of antibiotic-resistant microorganisms, as well as their wide distribution, is the result of several years of antibiotics underuse, overuse and misuse. This selective pressure created upon bacteria is not a natural process but is instead a man-made situation [2]. Some pathogens evolved to become resistant to multiple classes of antibiotics, being usually known as "superbugs": Multidrug-resistant (MDR) bacteria are subsequent to antibiotic use (either under-, over or misuse) with enhanced morbidity and mortality features due to multiple mutations, providing high levels of resistance to antibiotic classes, specifically recommended for their treatment [2]. Classical examples of MDR include the methicillin-resistant *Staphylococcus aureus* and numerous *Pseudomonas aeruginosa* strains [13].

Nowadays, the antibiotic crisis is epitomized by the spread of MDR "ESKAPE" organisms (*Enterococcus* spp., *Staphylococcus aureus*, *Klebsiella* spp., *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp.) [14]. In some cases, like those related to *Acinetobacter baumannii* infections, a main player in the majority of ventilator-associated pneumonia in intensive care units, the search for novel compounds has reached a level of extreme importance, since virtually none of the available substances is able to fight this MDR pathogen efficiently [15, 16]. ESKAPE organisms gain even more severity when placed in the context of Healthcare-Associated Infections (HAI), also known as nosocomial infections, since they are the ones accountable for them. The Center for Disease Control and Prevention defines HAIs as infections that patients acquire during the course of receiving treatment, for other conditions, or that Healthcare workers acquire while performing their duties within a healthcare setting, including device-associated infections [17]. HAI management brings several concerns regarding treatment costs, and the incredible high prevalence percentages. More than 1.4 million people worldwide suffer from HAI, with rates ranging from 5-25% in developed countries [18], rate which is estimated to be even higher than 40% in Asia, Africa and South America. In 2007, HAI, mainly caused by ESKAPE microorganisms, killed 99,000 patients in the US, being responsible for 37,000 deaths in Europe [18] .

1.3 Targeting the key players

Among the "ESKAPE" microorganisms, *S. aureus* is considered as one of the most successful infectious agents. This Gram-positive bacterium is carried as a nasal commensal in 30% of the population and has long been linked to skin and soft tissue infections [17]. After penicillin discovery, Staph infections seemed to be controllable but soon after resistance arose [2]. In 1959, Methicillin was a landmark discovery being designed to be the first "anti-resistance" antibiotic and was considered as a safe defense-line against the penicillinases (β-lactamases specific for penicillin). However, the appearance of methicillin-resistant *S. aureus* within just 3 years of its introduction, inevitably led to other multi-antibiotic resistant variants and the acronym MRSA, previously used only to Methicillin resistant strains, now stands for multidrug-resistant *S. aureus* [2]. MRSA has been indicated as one of the major causes of nosocomial infections, and its increasing prevalence has been observed in the last decade, making MRSA infections treatment extremely difficult due to the restrict spectra of efficient antibiotics [18]. If one translates infections caused by MRSA into numbers, either regarding their prevalence or concerning in terms of economic impact, the scenario gets even darker. Using as an example the year of 2006, 60-70% of the bacteraemia cases, in the US, were caused by MRSA, and within the same period, the economic costs related to this situation, in the UK, were estimated to be more than £1 billion, without mentioning the personal costs to an individual who survives to a MRSA-infection, related to hospitalization under isolation with the impossibility to have direct contact with family and friends for large periods of time [21]. In 2012, the Portuguese scenario demonstrated that 53.5% of hospital-acquired infections were caused by *S. aureus* being 73.7% characterized as MRSA [22].

After decades suffering the selective pressure of antibiotics, infections caused by MDR strains of the Gram-negative bacteria *P. aeruginosa* have been increasingly reported worldwide, being this pathogen the main cause of burn-wounds infections and ventilator acquired pneumonia, which are linked to high rates of morbidity and mortality [21]. In Portugal, *P. aeruginosa* is responsible for 27.5% of all HAI [22]. These microorganisms pose additional and bigger challenges that are directly linked to the fact that Gram-negative microorganisms are often more resistant to conventional antimicrobials. For that, several factors play a pivotal role, such as: i) expression of active efflux pumps [19]; ii) release of degrading enzymes and molecular metamorphosis of antibiotic targets; as well as iii) intrinsic nature of the cell wall, and exterior membrane of Gram-negative bacteria [20]. A study in the US, in which *Klebsiella* spp. and *E. coli* were included among other Gram-negative bacteria, reported that patients infected with MDR bacteria had a median total hospital cost of more US $38121 than patients infected with susceptible bacteria (US $ 144414 and 106293, respectively) [26]. The overall impact of infections caused by MDR bacteria in the economy is still largely unknown. However, and taking as an example the US health system, which spends between $21 to $34 US billion dollars, the economical worldwide impact of HAI infections must be massive [27] .

Still, it is undebatable that the loss of effective antibiotics will, in last instance, undermine the ability to fight infectious diseases and manage infections complications, which are even more common in vulnerable patients, such as those undergoing chemotherapy or patients with chronic diseases. In the near future, if no novel antibacterial agents emerge, society will be facing a serious public health threat, with similar conditions to those observed at the Semelweiss era, setting us back to the 1920's in what concerns medical care, where a common infection and minor injuries can be fatal [22].

2. Phytotherapy-use of plant derived compounds

Following the stagnation on the antibiotic-discovery field over the last years, scientific attention has been turning to alternative sources for novel antibacterial substances, namely natural bioactive compounds [4, 23]. These compounds are known to efficiently interact with the cellular membrane, as well as with biological macromolecules (nucleic acids, proteins, lipids, …) to produce a desired outcome, which can be exploited for designing natural-based therapeutic

agents, targeting MDR bacteria [24]. Plants are a rich source of bioactive compounds, most of them produced as a defense mechanism against microorganisms, presenting themselves as promising sources for phytopharmaceuticals development [25]. Supporting this hypothesis is the fact that pentacyclic triterpenes, which have very distinctive structures from those of methicillin or vancomycin (traditional anti-*S. aureus* agents), might be translated into novel mechanisms of action, or target, against *S. aureus* strains [31]. Nonetheless, terpenes mechanism of action is not fully understood, but it is generally assumed that their antimicrobial activity may be due to bacterial membrane disruption by the lipophilic compounds nature, or due to their ability to block cell division by DNA synthesis inhibition [23]. Moreover, and in accordance to World Health Organization (WHO), it is important to emphasize that more than 2/3 of the world population still relies on medicinal plants for their primary pharmaceutical care [26]. Over the last two decades, phytotherapy has gained strength in the scientific community as a new concept in health care, prompted by the need of alternatives to the ineffectiveness of traditional antibiotics [32-36]. Accounting for the renewed and increasing interest in plant-derived bioactive compounds is the fact that novel and efficient extraction techniques have been developed [29–31], which will be reviewed in section 3 of this chapter.

Despite the wide range of plant secondary metabolites that could be extremely interesting for the development of new antimicrobial agents targeting MDR bacteria, in this chapter only terpenes will be discussed, due to their wide spread in nature; abundance in the plant kingdom; great structural diversity; and high number of *in vitro* studies reporting terpenes-derived compounds efficiency against MDR bacteria in the last years, both with plant extracts or isolated pure standards, pointing out a promising future in terms of their exploitation as bactericides and bacteriostatic agents.

2.1 Terpenes

Terpenes agglutinate an entire class derived from two diphosphate esters, namely isopentenyl pyrophosphate (IPP), and its isomer dimethylallyl diphosphate (DMAPP). They carry a vast structural and chemical diversity, being in general classified according to the number of five-carbon building blocks (isoprene units $(C_5)_n$) into: hemi- (C_5), mono- (C_{10}), sesqui- (C_{15}), di- (C_{20}), sester- (C_{25}), tri- (C_{30}), tetraterpenes (C_{40}) and polyterpenes $(C_5)_n$ with $n > 8$ [37].

IPP and DMAPP may be yielded either by the mevalonate pathway, occurring in the nucleus, or by the 1-deoxy-D-xylulose 5-phosphate pathway, located in the plastids [38,39]. The mevalonate pathway contributes for the formation of the most of the sesquiterpenes and triterpenes, whilst the 1-deoxy-D-xylulose 5-phosphate pathway is responsible for the biosynthesis of mono-, di- and tetraterpenes and some sesquiterpenes [39]. In the last years, several naturally occurring terpenes, particularly sesquiterpenic lactones and triterpenic acids, have been identified and isolated, and their broad spectra of pharmacological activities, including anti-inflammatory, antitumoral and antibacterial potentials, have been highlighted throughout the literature [27, 28, 40, 41].

2.1.1 Sesquiterpenes

Sesquiterpenoids are composed by three isoprenoid units (C15) and may have an acyclic or cyclic structure. Farnesyl diphosphate is the biosynthetic precursor of sesquiterpenes, resulting from the addition of an IPP unit to geranyl diphosphate [38]. Among these, sesquiterpene lactones are the most representative sub-group in terms of biological potential, being widely distributed in nature and are particularly present in higher plants. Numerous *in vitro* reports have proved the role of sesquiterpene lactones against MDR bacteria. In the present chapter, xanthatin (Figure 2) is addressed, as an example of sesquiterpene lactone with great effect against *S. aureus*, including MRSA [42]. The minimum inhibitory concentration (MIC) of this compound, isolated from the plant *Xanthium sibiricum* Patr er Widd, was found to range between 7.8-15.6 μg/mL (31.67 - 63.34 μM), against several strains of *S. aureus* and MRSA [42] .

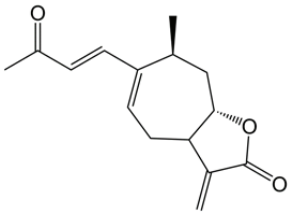

Fig. 2 Structure of xanthatin.

2.1.2 Triterpenes

These compounds are ubiquitously distributed and correspond to acyclic and cyclic 30-carbon precursors. Triterpenes are produced from squalene which is afforded through binding of two molecules of farnesyl pyrophosphate [38]. Within triterpenes, pentacyclic triterpenes, mostly those with lupane, ursane and oleanane backbone structures, have shown promising pharmacological activities. In this chapter, BA, UA and OA (Figure 3) are addressed in particular detail based on its anti-MDR bacteria activity.

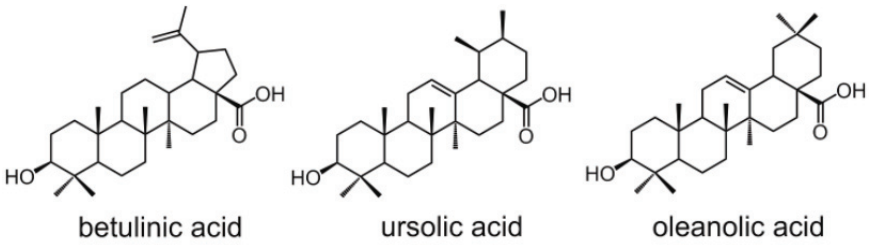

Fig. 3 Structures of pentacyclic triterpenes.

BA showed higher antibacterial effect compared to the antibiotic methicillin on 62.5% of the clinical strains of MRSA, with MICs varying between 4-64 μg/mL (8.8 – 140 μM) [43]. UA, isolated from *Salvia officinalis* [44] and *Baccharis dracunculifolia* [45], was effective against MRSA, with MICs ranging from 8 μg/mL (17.5 μM) [44] to 10 μg/mL (21.9 μM) [45]. OA, isolated from the *Caesalpinia paraguariensis Burk*. [46] and *S. officinalis* [44], presented antibacterial effect on MRSA, with MICs within 16-64 μg/mL (35-140 μM) concentration range [44,46]. In addition to the MRSA antibacterial activity, UA and OA inhibited growth of other MRD bacteria, such as: (i) the penicillin-resistant *Streptococcus pneumoniae*, with MICs of 8 (17.5 μM) and 16 μg/mL (35.0 μM), respectively; and (ii) the vancomycin-resistant enterococci (*Enterococcus faecalis* and *E. faecium*), with MICs of 4 μg/mL (8.8 μM) and 8 μg/mL (17.5 μM), respectively [44] . In fact, these pentacyclic triterpenes were more active than the antibiotic ampicillin against MRSA (MIC=64 μg/mL=183.2 μM), *E. faecalis* (MIC=4 μg/mL=11.4 μM) and *E. faecium* (32-128 μg/mL and 91.6-366.3 μM MICs range) [44].

3. Extraction of bioactive compounds

Extraction plays a crucial role in the qualitative and quantitative recovery of bioactive compounds from vegetable tissues. The scientific advances observed in this area over the last decade, besides contributing to the escalating attentiveness to the phytopharmaceutical research field, may become a propeller force, pushing terpenes one step forward, i.e, into the *in vivo* and clinical trials arena regarding the fight against MDR bacteria. Moreover, the advances in the extraction/purification techniques may help to tailor plant extracts or their derived purified compounds, towards an enhancement of their activity against a particular bacterial agent.

The efficiency of extraction depends not only on the extraction method, but also on solubility of the analytes into solvent. Terpenes chemical family owns polycyclic structures and present very low solubility in water and hydrophilic solvents.

Traditional extraction techniques of plant materials are mostly based on the use of heat and/or agitation to increase the rate of mass transfer to suitable leachant. Solid liquid extraction (SLE) is the most common and traditional method for isoprenoids extraction, due to its simple mode of operation and the need of inexpensive equipment. Also traditional, but requiring more energy costs, appears heating assisted extraction (HAE) and Soxhlet extraction (SE). All those methodologies are often low efficient, time consuming and require large volumes of organic solvents, which result on additional costs, and environmental hazards. Besides that, many natural products are thermally unstable and may degrade with high extraction temperatures, which compromise their microbiological activity.

Alternative extraction methodologies such as: ultrasound and microwave assisted extraction, pressurized liquid, accelerated solvent and supercritical fluid extractions will be discussed within the present chapter. Novel terpenes extraction techniques will be explored and results compared with the traditional ones, according to results present in literature regarding anti-microbiological potential enhancement.

3.1 Extraction methodologies

3.1.1 Ultrasound Assisted Extraction (UAE)

UAE presents several advantages in comparison with conventional solid-liquid extraction, reducing solvents, time and temperature, which represents an important factor when extracting thermolabile compounds, potentiating the biological activity of the natural-based extractables. This is the simplest and most economical technique is also easy to scale up to industrial level.

3.1.2 Microwave Assisted Extraction (MAE)

This methodology implies the heating of water molecules in plant cells, absorbing microwave energy, and quickly transferring it into solvent. The inappropriate use of MAE can result in drastic conditions, which can lead to chemical changes of some compounds. Irradiation time and power should be optimized towards different biomass, in order to obtain an extract with the greatest terpenes yield extraction.

3.1.3 Supercritical fluid extraction (SC-CO_2)

SC- CO_2 uses supercritical CO_2, being capable of extracting a wide range of diverse compounds, including more polar substances, by combining CO_2 with a cosolvent, such as methanol, ethanol, acetone, among others [35]. Yet, ethanol may be the better choice in SC-CO_2 of nutraceuticals and food ingredients because of its lower toxicity. Due to its polarity, SC-CO_2 has shown a moderate capacity to dissolve terpenes, although an appropriate combination of pressure and ethanol (EtOH) concentration, as cosolvent, may increase the yield of terpenes extraction [47,48] .

3.1.4 Accelerated Solvent Extraction (ASE)

This extraction method enhances the extraction by temperature and pressure [35]. ASE is a form of pressurized solvent extraction, similar to SFE. Extraction conditions including temperature, flush volume and extraction cycle are important parameters affecting the extraction efficiency [49].

3.1.5 Pressurized liquid extraction (PLE)

PLE uses high pressures in order to retain solvents in the liquid state beyond their normal boiling point. The combination of high pressures and high temperatures enhances mass transfer, facilitating the extraction process. However, the lack of industrial scale pressurized liquid extraction equipment, leads to a moderate application of this technique [48].

Several studies have been described in terms of evaluation of terpenes extraction methods efficiency. Although this research data is not, most of the times, focused on terpenes extraction as a class, but in some specific terpenes, some conclusions can be taken. In 2012, Wójciak-Kosior *et al*.[50], studied the effect of traditional extraction techniques such as: SLE, SE, and HAE, with recent methodologies such as: UAE and MAE, for the extraction of two pentacyclic triterpenes from *Lamii albi flos*. Although, classic techniques (SLE, SE and HAE) are still frequently used, for active components isolation from plant material, the extraction efficiency of two triterpenic acids, OA and UA appeared to be poor, and extraction time relatively long. MAE, in closed system, was the most efficient technique, presenting as advantage, over classic techniques, the low time-consumption. Although, with not so good results as MAE, UAE due to its simplicity, inexpensive equipment and relatively good extraction efficiency appeared as a good alternative method for OA and UA extraction. Pinilla *et al.* [48] investigated the recovery of a pentacyclic triterpenes, BA [49], from plane tree *Platanus acerifolia* L., where SLE, UAE and PLE were carried out using EtOH and ethyl acetate, and produced a BA recovery in the range of 10-15 mg/g bark, with concentrations between 25-35% mass (UAE>SLE>PLE). In this study, the comparison of traditional assays was made with supercritical fluid extraction, without the use of cosolvent, which produced low BA recoveries (0.5-8 mg/g bark) with respect to liquid extraction. The addition of EtOH as cosolvent lead to a significant improvement in BA recovery. In comparison with SLE, UAE and PLE, 20% EtOH cosolvent resulted in high yield (4.34%), BA higher concentration in the extract (18.30% mass), and almost one third of EtOH consumption. Rhourri-Frih *et al.* [49] isolated three pentacyclic triterpenes from *Manilkara bidentata* resin, with different extraction methods, UAE, SE, HAE and ASE with EtOH and EtOAc as solvents, being the extraction efficiency higher for UAE, followed by SE and HAE. These results are in agreement with previous works, where UAE demonstrated best extraction efficiency for some species, and have also shown that ASE yield was low for some compounds.

In what concerns to novel technologies of extraction, each vegetable species needs to be studied as a different biomass. Although studies performed highlight that novel techniques offer best kinetic rates, low time consumption and best yields of extraction, those methodologies need to be further studied for each biomass.

3.2 Purification/isolation of terpenes: pentacyclic triterpenes and sequisterpene lactones

Although the comparison of terpenes extraction methodologies is made in most part of the studies as a class, there are several studies searching for terpenes purification after microextraction (ME) or SLE. Not requiring temperature or pressure, microextraction is characterized by a sequential of extractions, normally using solvents with low polarity, where the biomass remains in contact with the solvent, during a certain period of time, followed by centrifugation, and filtration, being the extraction repeated several times until biomass exhaustion. Silica gel and Sephadex LH-20 columns are nowadays the more used methods for terpenes purification, using different solvents, in order to obtain different and purified fractions.

Yang *et al*. [51] extracted two new terpenes from *Liquimbar formosana*, with a prior extraction with petroleum spirit, followed by a silica gel chromatography. With the same method of purification, Mbaze *et al*. [52] found two new terpenes present in *Fagara tessmani*, using hexane and ethyl acetate (7:3), during the TLC. Using Sephadex LH-20 to obtain purified fractions, Katerere *et al.* [53] isolated four pentacyclic triterpenes from *Combretum imberbe* leaves, being two of them, novel glycosidic derivatives of 1alpha,3beta,23-trihydroxyolean-12-en-29-oic acid (hydroxyimberbic acid). In this study, the biomass was first defatted with *n*-hexane to remove highly nonpolar components, and then extracted with dichloromethane by SLE. Montoya-Peláez *et al*. [54] investigated the chemical composition of a pentacyclic triterpene fraction from *Cecropia telenitida* roots. The extraction was made by SLE, where

roots were cleaned with *n*-hexane and then extracted with ethyl acetate. The extract was concentrated, diluted in methanol and purified by Sephadex LH-20 using medium pressure liquid chromatography to eliminate molecules with a high molecular weight, such as polymers. A novel compound was found: yarumic acid.

In what concerns liquid extraction, four sesquisterpenes lactones (hirsutinolides and glaucolides) from *Vermonia scorpioides* were extracted and fractionated using ME with a sequential extraction, with increasing solvent polarities [55].

Liquid extraction and solid-phase extraction are used as purification and isolation techniques, being solid-phase extraction recommended based on low organic solvents used, selectivity and a large number of commercially available solid-phase extraction sorbents. New methodologies can also be employed for purification and compounds isolation. Already applied with other compounds [56–58], membrane separation processes are known as a green technology, which could be employed for extract purification and compound isolation, in order to enhance the desired biological activity.

4. Concluding remarks

Highly effective antimicrobials with the ability to fight, not only but mostly the ESKAPE pathogens are required. Plant-derived compounds, such as terpenes, which are widely available in nature, seem a logical path in the quest for novel drugs against Superbugs.

The significant advances observed over the last years regarding compounds extraction and purification technology has provided the basic skills to make this a reality closer to the present than to the far future. Highlighted is the need for scaling-up extraction/purification techniques, in order to tailor the desired chemical composition towards the required antimicrobial activity.

References

[1] Sneader W. Drug Discovery: A History. Wiley; 2006. p.1-468.
[2] Davies J, Davies D. Origins and evolution of antibiotic resistance. Microbiol Mol Biol Rev. 2010;74:417–33.
[3] Singh SB, Barrett JF. Empirical antibacterial drug discovery-foundation in natural products. Biochem Pharmacol. 2006;71(7):1006–15.
[4] Lewis K. Platforms for antibiotic discovery. Nat Rev Drug Discov. 2013;12(5):371–87.
[5] Woodruff HB. Selman A. Waksman, winner of the 1952 nobel prize for physiology or medicine. Appl Environ Microbiol. 2014;80(1):2–8.
[6] Drews J. Drug Discovery: A Historical Perspective. Science. 2000;287(5460):1960-4.
[7] Butler MS, Buss AD. Natural products - The future scaffolds for novel antibiotics?. Biochem Pharmacol. 2006; 71(7): 919-929.
[8] Armstrong GL, Conn LA, Pinner RW. Trends in infectious disease mortality in the United States during the 20th century. JAMA. 1999;281:61–6.
[9] Cohen ML. Changing patterns of infectious disease. Nature. 2000;406:762–7.
[10] Walsh C. Where will new antibiotics come from? Nat Rev Microbiol. 2003;1:65–70.
[11] Projan SJ. Why is big Pharma getting out of antibacterial drug discovery? Curr Opinion Microbiol. 2003; 6(5): 427–30.
[12] Shlaes DM. The abandonment of antibacterials: Why and wherefore? Curr Opinion Pharmacol. 2003; 3(5):470–3.
[13] Sydnor ERM, Perl TM. Hospital epidemiology and infection control in acute-care settings. Clin Microbiol Rev. 2011;24:141–73.
[14] Boucher HW, Talbot GH, Bradley JS, Edwards JE, Gilbert D, Rice LB, et al. Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. Clin Infect Dis. 2009;48(1):1–12.
[15] Al-Anazi KA, Al-Jasser AM. Infections caused by *Acinetobacter baumannii* in recipients of hematopoietic stem cell transplantation . Frontiers in Oncology . 2014; 4-186.
[16] Peleg AY, Hooper DC. Hospital-Acquired Infections Due to Gram-Negative Bacteria. N Engl J Med. 2010; 362(19):1804–13.
[17] Sehulster L, Chinn RYW. Guidelines for environmental infection control in health-care facilities. Recommendations of CDC and the Healthcare Infection Control Practices Advisory Committee (HICPAC). MMWR Recomm Rep. 2003;52(RR-10):1–42.
[18] World Health Organization. Report on the Burden of Endemic Health-Care Associated Infection Worldwide–A Systematic Review of the Literature. World Health Organization Geneva. 2011.
[19] David MZ, Daum RS. Community-associated methicillin-resistant *Staphylococcus aureus*: Epidemiology and clinical consequences of an emerging epidemic. Clin Microbiol Rev. 2010; 23(3): 616–87.
[20] Chambers HF. The changing epidemiology of *Staphylococcus aureus*? Emerg Infect Dis. 2001;7:178–82.
[21] Gould IM. Costs of hospital-acquired methicillin-resistant *Staphylococcus aureus* (MRSA) and its control. International Journal of Antimicrobial Agents. 2006; 28(5):379–84.
[22] Pina E, Nogueira PJ. Prevalência De Infeção Adquirida No Hospital E Do Hospitais Portugueses Inquérito 2012. Direcção Geral de Saúde. 2013.
[23] Nordmann P, Naas T, Fortineau N, Poirel L. Superbugs in the coming new decade; multidrug resistance and prospects for treatment of *Staphylococcus aureus*, *Enterococcus* spp. and *Pseudomonas aeruginosa* in 2010. Curr Opin Microbiol. 2007; 10(5):436–40.
[24] Piddock LJ V. Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. Clinical Microbiology Reviews. 2006; 19(2):382–402.
[25] Tumah HN. Bacterial biocide resistance. J Chemother. 2009;21:5–15.

[26] Mauldin PD, Salgado CD, Hansen IS, Durup DT, Bosso JA. Attributable hospital cost and length of stay associated with health care-associated infections caused by antibiotic-resistant gram-negative bacteria. Antimicrob Agents Chemother. 2010;54(1):109–15.
[27] Antimicrobial resistance-Global Report of Surveilance. World Health Organization Geneva. 2014.
[28] Butler CC. Antibiotics: Responding to a Global Challenge. Antibiotics. 2012;1(1):14–6.
[29] Cowan MM. Plant Products as Antimicrobial Agents Plant Products as Antimicrobial Agents. 1999;12(4):564-582.
[30] Ajikumar PK, Tyo K, Carlsen S, Mucha O, Phon TH, Stephanopoulos G. Terpenoids: Opportunities for biosynthesis of natural product drugs using engineered microorganisms. Molecular Pharmaceutics. 2008;5(2):167–90.
[31] Chung PY, Navaratnam P, Chung LY. Synergistic antimicrobial activity between pentacyclic triterpenoids and antibiotics against Staphylococcus aureus strains. Ann Clin Microbiol Antimicrob. 2011;10(25):1-6.
[32] McChesney JD, Venkataraman SK, Henri JT. Plant natural products: back to the future or into extinction? Phytochemistry. 2007;68(14):2015–22.
[33] Cowan MM. Plant Products as Antimicrobial Agents Plant Products as Antimicrobial Agents. 1999;12(4):564-582.
[34] Wang G, Tang W, Bidigare RR. Terpenoids as therapeutic drugs and pharmaceutical agents. in Natural Products: Drug Discovery and Therapeutic Medicine. 2005. p. 197–227.
[35] Wang L, Weller CL. Recent advances in extraction of nutraceuticals from plants. Trends Food Sci Tech. 2006;17(6):300–12.
[36] Domingues RMA., Sousa GDA., Freire CSR, Silvestre AJD, Neto CP. *Eucalyptus globulus* biomass residues from pulping industry as a source of high value triterpenic compounds. Ind Crops Prod. 2010;31(1):65–70.
[37] Breitmaier E. Terpenes: flavors, fragrances, pharmaca, pheromones. Wiley-VCH; 2006. p. 1-4.
[38] Dewick PM. Medicinal Natural Products: A biosynthetic approach. 2nd ed. John Wiley & Sons, Ltd.; 2002. p. 167-290.
[39] Humphrey AJ, Beale MH. Terpenes. In: Crozier A, Clifford MN, Ashihara H, editors. Plant Secondary Metabolites: Occurrence, Structure and Role in the Human Diet. 1st ed. Oxford, UK: Blackwell Publishing Ltd.; 2006. p. 47–136.
[40] Jäger S, Trojan H, Kopp T, Laszczyk MN, Scheffler A. Pentacyclic triterpene distribution in various plants - rich sources for a new group of multi-potent plant extracts. Mol Basel Switz. 2009;14:2016–31.
[41] Domingues RMA, Guerra A., Duarte M, Freire CSR, Neto CP, Silva CM., et al. Bioactive triterpenic acids: From agroforestry biomass residues, to promising therapeutic tools. Mini Rev Org Chem. 2014;11:382-389.
[42] Sato Y, Oketani H, Yamada T, Singyouchi K-I, Ohtsubo T, Kjhara M, et al. A xanthanolide with potent antibacterial activity against methicillin-resistant *Staphylococcus aureus*. J Pharm Pharmacol. 1997;49:1042–4.
[43] Chung PY, Chung LY, Navaratnam P. Potential targets by pentacyclic triterpenoids from *Callicarpa farinosa* against methicillin-resistant and sensitive *Staphylococcus aureus.* Fitoterapia. 2014;94(0):48–54.
[44] Horiuchi K, Shiota S, Hatano T, Yoshida T, Kuroda T, Tsuchiya T. Antimicrobial activity of oleanolic acid from Salvia officinalis and related compounds on vancomycin-resistant enterococci (VRE). Biol Pharm Bull. 2007;30(6):1147–9.
[45] Filho AA da S, Sousa JPB de, Soares S, Furtado NAJC, Andrade e Silva ML, Cunha WR, et al. Antimicrobial activity of the extract and isolated compounds from *Baccharis dracunculifolia* D. C. (Asteraceae). Z Naturforsch. 2008;63(1-2):40–6.
[46] Woldemichael GM, Singh MP, Maiese WM, Timmermann BN. Constituents of antibacterial extract of Caesalpinia paraguariensis Burk. Z Naturforsch. 2003;58(1-2):70–5.
[47] De Melo M, Domingues R, Sova M, E L, H S, Lang J, et al. Scale-up studies of the supercritical fluid extraction of triterpenic acids from *Eucalyptus globulus* bark. J Supercrit Fluids. 2014;95:44–50.
[48] Fornari T, et al. Recovery of betulinic acid from plane tree (*Platanus acerifolia* L.). J Supercrit Fluids. 2014;545:541–5.
[49] Rhourri-Frih B, Renimel I, Chaimbault P, André P, Herbette G, Lafosse M. Pentacyclic triterpenes from Manilkara bidentata resin. Isolation, identification and biological properties. Fitoterapia. 2013;88:101–8.
[50] Wójciak-Kosior M, Sowa I, Kocjan R, Nowak R. Effect of different extraction techniques on quantification of oleanolic and ursolic acid in *Lamii albi* flos. Ind Crops Prod. 2013;44:373–7.
[51] Yang NY, Chen JH, Zhou GS, Tang YP, Duan JA, Tian LJ, et al. Pentacyclic triterpenes from the resin of Liquidambar formosana. Fitoterapia. 2011;82(6):927–31.
[52] Mbaze LMA, Poumale HMP, Wansi JD, Lado JA, Khan SN, Iqbal MC, et al. α-Glucosidase inhibitory pentacyclic triterpenes from the stem bark of *Fagara tessmannii* (Rutaceae). Phytochemistry. 2007;68(5):591–5.
[53] Katerere DR, Gray AI, Nash RJ, Waigh RD. Antimicrobial activity of pentacyclic triterpenes isolated from African Combretaceae. Phytochemistry. 2003;63(1):81–8.
[54] Montoya Peláez GL, Sierra JA, Alzate F, Holzgrabe U, Ramirez-Pineda JR. Pentacyclic triterpenes from *Cecropia telenitida* with immunomodulatory activity on dendritic cells. Brazilian J Pharmacogn. 2013;23(5):754–61.
[55] Buskuhl H, De Oliveira FL, Blind LZ, De Freitas RA, Barison A, Campos FR, et al. Sesquiterpene lactones from *Vernonia scorpioides* and their *in vitro* cytotoxicity. Phytochemistry. 2010;71(13):1539–44.
[56] Brás T, Guerreiro O, Duarte MF, Neves L. Impact of extraction parameters and concentration by nanofiltration on the recovery of phenolic compounds from *Cynara cardunculus* var. *altilis*: Assessment of antioxidant activity. Ind Crop Prod. 2015;67(0):137–42.
[57] Conidi C, Cassano A, Drioli E. Recovery of phenolic compounds from orange press liquor by nanofiltration. Food Bioprod Process. 2012;90(4):867–74.
[58] Mello BCBS, Petrus JCC, Hubinger MD. Concentration of flavonoids and phenolic compounds in aqueous and ethanolic propolis extracts through nanofiltration. J Food Eng. 2010;96(4):533–9.

