Daniela Filipa Firmino Rosa

Graduation in Biochemistry

Bringing the biorefinery concept to the health-care facilities: use of industrial by-products against multi-drug resistant bacteria

Dissertation to obtain the Master degree in Biochemistry

Supervisor: Doctor Paula Cristina Castro Parreira e Guerra, CEBAL

Co-supervisor: Doctor Andreia Patrícia Henriques Ascenso, Faculdade de Farmácia - UL

Jury:

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FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

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Abstract

Multi-drug resistant bacteria (MDRB) are a public health issue worldwide, being alternatives to the conventional failing antibiotherapy required. This fact has turned attention to bioactive compounds with antimicrobial activity features. These bioactives can be extracted from several biomasses sources, namely industrial by-products, within a biorefinery concept.

The main objective of this Thesis was the design of a novel antiseptic formulation (AF), based on natural plant extracts, obtained from forest/agriculture industries by-products.

The antimicrobial activities of three extracts from different sources were studied: *Eucalyptus nitens* total bark (ENTB) extract, *Cynara cardunculus* leaves (CcL) extract and dry olive pomace (DOP) extract. Those that presented better antibacterial activity were chosen to be included in the AF. Studies regarding formulation design and its antibacterial performance were made.

ENTB extract was the one with better anti-MDRB performance, particularly against *Staphylococcus* spp., with minimal inhibitory concentrations between 64 and 2048 µg/mL, being included in the AF. The antibacterial activity of ENTB extract-based-AF presented promising results, achieving a 97±2% bacterial growth inhibition after exposure to 45% of AF.

In conclusion, the developed AF presents potential to be further investigated, namely for hand sanitation within healthcare environment, but additional adjustments should be executed, namely in order to turn it more appellative to users.

Keywords: Multi-drug resistant bacteria, healthcare-associated infections, antimicrobial activity, biorefinery by-products, topical antiseptic formulation.

Resumo

As bactérias multirresistentes a antibióticos (BMRA) são um problema de saúde pública a nível mundial, sendo necessárias alternativas à antibioterapia convencional, cuja eficácia tem decrescido nas últimas décadas. Este facto atraiu a atenção para compostos bioativos com atividade antimicrobiana. Estes bioativos podem ser extraídos a partir de diversas biomassas, nomeadamente subprodutos industriais, dentro do conceito de biorrefinaria.

O principal objetivo da presente Tese foi o desenvolvimento de uma nova formulação antisséptica (FA), com base em extratos naturais de plantas, obtidos a partir de subprodutos da indústria florestal/agrícola.

Para tal, foi estudada a atividade antimicrobiana de três extratos de diferentes origens: extrato da casca total de *Eucalyptus nitens* (ECTEN); extrato de folhas de *Cynara cardunculus* (cardo) e; extrato de bagaço de azeitona seco. Aqueles que apresentaram melhor atividade antimicrobiana foram escolhidos para serem incluídos na FA. Estudos de desenvolvimento da formulação e desempenho antimicrobiano da mesma foram efetuados.

O ECTEN foi o que apresentou melhor desempenho anti-BMRA, particularmente contra *Staphylococcus* spp., com concentrações mínimas inibitórias entre 64 e 2048 µg/mL, sendo incluído na FA. A atividade antibacteriana da FA à base de ECTEN apresentou resultados promissores, alcançando 97±2% de inibição de crescimento bacteriano após exposição a 45% de FA.

Em conclusão, a FA desenvolvida apresenta potencial para prosseguir para investigações futuras, nomeadamente para a higienização das mãos em contexto hospitalar ou unidades de cuidados de saúde, mas ajustes adicionais deverão ser feitos, nomeadamente de forma a tornar a FA mais apelativa para os utilizadores.

Palavras-chave: Bactérias multirresistentes a antibióticos, infeções nosocomiais, atividade antimicrobiana, subprodutos de biorrefinaria, formulação tópica antisséptica.

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List of abbreviations

AF – Antiseptic formulation

BA – Betulinic acid

BOA – Betulonic acid

CcL – *Cynara cardunculus* leaf

CDC – Centers for Diseases Control and Prevention

DMSO – Dimethyl sulfoxide

DNS – 3,5-dinitrosalicylic

DOP – Dry olive pomace

ECDC – European Centre for Disease Prevention and Control

ENTB – *Eucalyptus nitens* total bark

ESKAPE – vancomycin-resistant ***E****nterococcus*, MR**S**A, *β*-lactamase of extended spectrum producer ***K****lebsiella*, imipenem-resistant ***A****cinetobacter*, imipenem-resistant ***P****seudomonas* and third generation cephalosporin-resistant ***E****nterobacter*

GC – Growth control

HAI – Healthcare-associated infections

HPLC-UV/Vis – High Liquid Chromatography with UV/Vis detector

ICU – Intensive care unit

MBC – Minimum bactericidal concentration

MDRB – Multidrug-resistant bacteria

MIC – Minimum inhibitory concentration

MHA – Muller Hinton agar

MHB – Muller Hinton broth

MRSA – Methicillin-resistant *Staphylococcus aureus*

MSSA – Methicillin-sensitive *Staphylococcus aureus*

MTT – (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NI – Nosocomial infections

OA – Oleanolic acid

PBP – Penicillin-biding proteins

PW – Peptone water

RS – Reducing sugars

RT – Room temperature

SC – Solvent control

SNC – Solution control

StC – Sterility control

TAs – Triterpenic acids

UA – Ursolic acid

USA – United States of America

WHO – World Health Organization

1 – Introduction

1.1 – Infectious diseases: the main players and the challenges in the 21st century

Bacteria are one of the main players in what concerns infectious diseases, to which features of high morbidity and mortality are associated. (Zaffiri, Gardner, and Toledo-Pereyra 2012) For centuries, the only available resource to treat infections were plants, known to have, among other health benefits, antimicrobial activity. (Ríos and Recio 2005)

In the early 20th century, respiratory infections, like pneumonia and tuberculosis, were the leading cause of death. (Sabin 1970) In order to minimize infection spreading, public health measures were taken, such as: protection of food and water supplies, improvement of personal hygiene and introduction of a vaccination program. (Lederberg 2000) The revolutionary discovery of antibiotics as new therapeutic agents was the turning point in the treatment of infectious diseases caused by bacteria, allowing to save an uncountable number of lives. (Davies, J, & Davies, D. 2010)

Selman Waksman defined "antibiotic" as *any class of organic molecules able to inhibit or kill bacterial cells through specific interactions, with low toxicity to the mammalian host*. (Davies, J, & Davies, D. 2010) In 1928, Alexander Fleming discovered the first effective antibiotic: penicillin. In 1940s it was introduced in the clinical practice, providing quick and complete treatment of previously incurable bacterial diseases. (Davies, J, & Davies, D. 2010) However, antibiotics efficiency has been shadowed since the beginning by the ability of bacteria to develop resistance, and effectiveness of antimicrobial agents, such as antibiotics, has been decreasing over the years. (Lewis 2013)

According to Instituto Nacional Ricardo Jorge (INSA, Portugal), resistant bacteria are defined *as immunes (not affected) to the bacteriostatic (inhibits cell proliferation) or bactericidal (destruction of the bacterial population) effect of antibacterial agents at its therapeutic dosage*. (Jorge n.d.)

1.1.1 – Bacterial antibiotic resistance: intrinsic vs acquired mechanisms

The resistance mechanism to antimicrobial agents, such as antibiotics, can be either intrinsic or acquired.

1.1.1.1 – Intrinsic mechanisms

In intrinsic resistance mechanism, the microorganism has the innate ability to resist to a class of antimicrobial compounds. A good example of intrinsic resistance is *Pseudomonas aeruginosa*, a Gram-negative bacteria that is naturally resistant to *β*-lactam antibiotics, due to the presence of a multidrug efflux system and the production of enzymes, *β*-lactamases, which have the ability to hydrolyze the *β*-lactamic ring. (Lewis 2013) *β*-lactam antibiotics act on the peptidoglycan cell wall synthesis, which in Gram-negative is more difficult to access, since peptidoglycan is present in the periplasmic space, between the inner and outer lipid membranes, creating a natural

barrier.(Lewis 2013) In Gram-positive bacteria, the lack of an outer membrane, as well as the presence of a thick cell wall, composed by the peptidoglycan layer, (Figure 1.1), makes them more susceptible to antibiotics.(Levy and Marshall 2004)

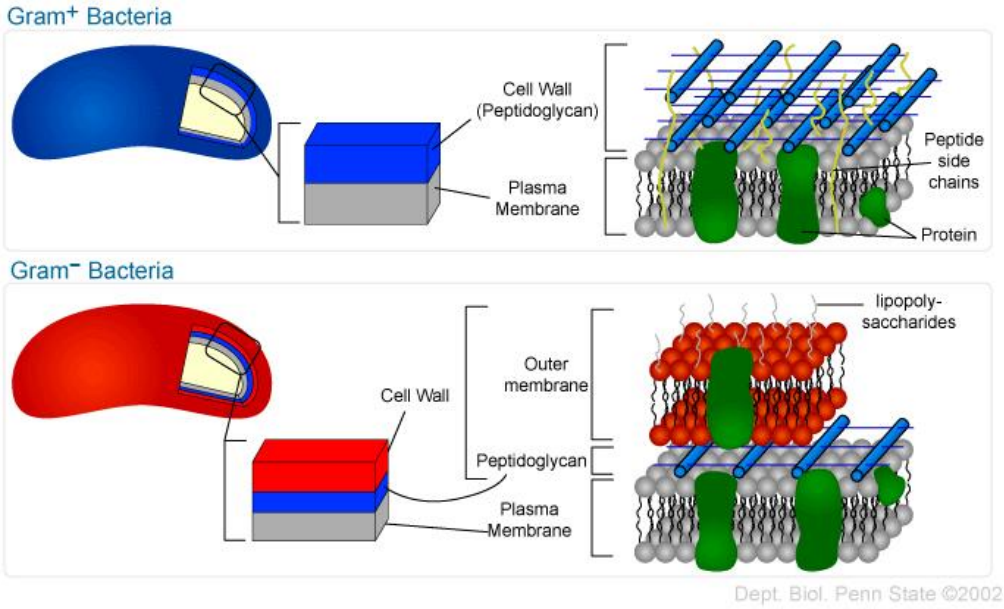

Figure 1.1. Illustration of structural differences in Gram-positive and Gram-negative bacterial cell wall. (State 2002)

1.1.1.2 – Acquired mechanisms

In acquired resistance, the microorganisms develop the ability to resist to the antibiotic's action by genetic material alterations, namely mutations or genetic material transference, through mobile elements, such as plasmids, bacteriophages, transposons, and others. (Levy and Marshall 2004) Antibiotics misuse, which exerts selective pressure upon bacterial populations, contributes to the growing appearance of resistance among bacterial strains. According to the Centers for Disease Control and Prevention (CDC, USA), the misuse of antibiotics is largely caused by the unnecessary or inappropriate antibiotics prescription in 50% of the cases, both in medicine and agriculture fields. ("Health Policy Brief: Antibiotic Resistance" 2015)

1.1.2 – Mechanisms of acquired resistance to antibiotics

There are several pathways by which bacteria may acquire resistance to antibiotics. The transference of genetic material is a common event, leading to a cumulative acquisition of resistance genes to different antibiotics, in a determined bacterial population. (Lowy 2003) For example, methicillin-resistant *Staphylococcus aureus* (MRSA) acquired resistance to *β*-lactam antibiotics through horizontal gene transference of a resistance gene (*mecA*), which is responsible for the synthesis of a class of penicillin-binding proteins (PBP), which are not sensitive to antibiotic inhibition. (Lowy 2003)

Since the discovery of the first effective broad-spectrum antibiotic, able to be used against different bacteria in therapeutic doses, others have been developed and refined, in order to target specific bacteria genre and with defined action mechanisms against bacterial cells. (Davies, J, & Davies, D. 2010) Therefore, antibiotics can be grouped in three major categories, according to their mechanism of action: cell wall synthesis inhibition; protein synthesis inhibition or nucleic acid synthesis inhibition. (Levy and Marshall 2004; McDermott, Walker, and White 2003)

1.1.2.1 – Inhibition of cell wall synthesis

Cell wall is a distinctive bacterial characteristic and, therefore, it is an excellent target for selectively kill or inhibit bacteria in a mammalian organism. The most commonly used inhibitors of cell wall biosynthesis are *β*-lactams (penicillins and cephalosporins) and glycopeptides (vancomycin and teicoplanin). (Tenover 2006)

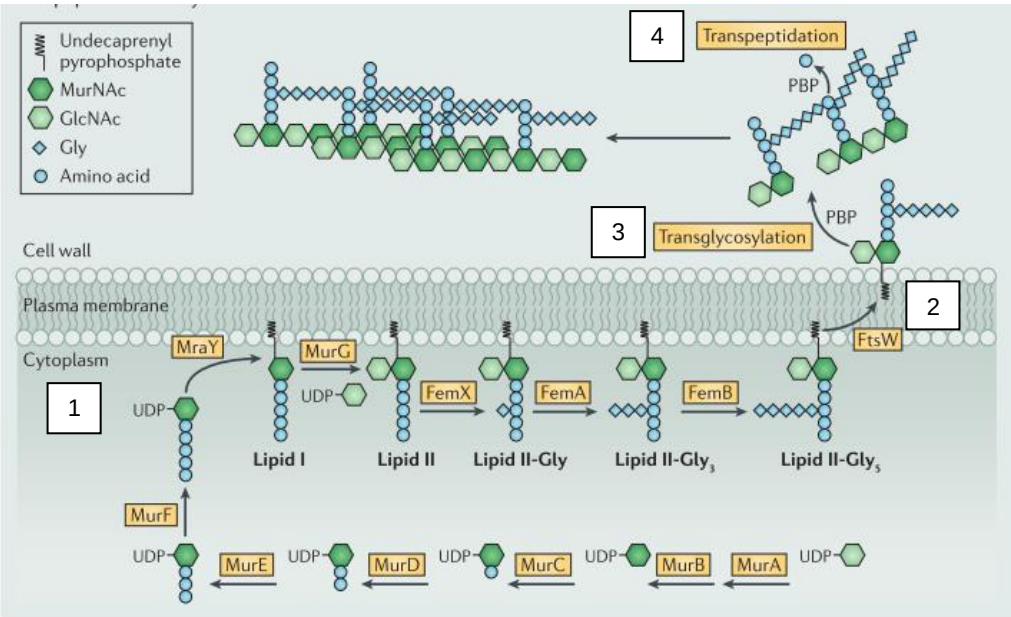

Figure 1.2. Peptidoglycan synthesis: (1) synthesis of precursors in the cytoplasm; (2) transport of lipid-bound precursors across the cytoplasmic membrane; (3) insertion of glycan units into the cell wall; and (4) transpeptidation linking and maturation.(McDermott, Walker, and White 2003) (Adapted from Pinho *et al.*(2013))

β-lactams interact with PBP, the enzymes responsible for the generation of the mature peptidoglycan. On the other hand, glycopeptides bind peptidoglycan side chains, blocking the transglycosylation and transpeptidation reactions necessary to add new subunits to the growing peptidoglycan chain (Figure 1.2). (McDermott, Walker, and White 2003)

The interaction with Gram-negative and Gram-positive bacteria is different: in Gram-positive bacteria, the target is more accessible in the outer layer, while in Gram-negative bacteria the drug needs to be transported through the outer membrane transport system via proteins (porins). (McDermott, Walker, and White 2003) Due to their low permeability, glycopeptides cannot be

transported, therefore having limited spectrum of action against Gram-positive bacteria. (McDermott, Walker, and White 2003)

The resistance mechanisms to *β*-lactams are: i) mutations in the target PBP; ii) acquisition of new PBPs with decreased affinity for the drug; iii) production of one or more *β*-lactamases that inactivate the drug; iv) changes in cell wall porins, which limit the drug movement to the target site; and v) active efflux of the drug out of the cell by energy-dependent pumps. (McDermott, Walker, and White 2003) The resistance mechanism responsible for glycopeptides ineffectiveness is the alteration of the amino acid chain target. (McDermott, Walker, and White 2003)

1.1.2.2 – Protein synthesis

Protein synthesis is a vital process for cell survival and multiplication. Several types of antibacterial agents target the bacterial protein synthesis, by binding to either the 30S or 50S subunits of the ribosomes, (Lewis 2013) which leads to the disruption of the normal cellular metabolism, resulting in death or growth inhibition. Aminoglycosides (streptomycin and gentamicin), chloramphenicol, macrolides (erythromycin) and tetracyclines (tetracycline, doxycycline, minocycline) act at the protein synthesis level. (McDermott, Walker, and White 2003)

The resistance mechanisms associated to these antibiotics are linked to the expression of enzymes able to inactive them, either by phosphorylation, adenylation or acetylation; efflux pumps or target modification, namely at the ribosome level. (Avent et al. 2011) Gram-negative bacteria are intrinsically resistant to macrolides. (McDermott, Walker, and White 2003)

1.1.2.3 – Nucleic acid synthesis

DNA and RNA are the basic keys for the replication of all living forms, including bacteria. Some antibiotics act by binding to nucleotides (sulfonamides) or nucleic acids (quinolones and rifamycins), which are involved in the process of DNA or RNA synthesis, causing interference in the normal cellular processes and, ultimately, compromising bacterial multiplication and survival. (McDermott, Walker, and White 2003) Sulfonamides block the formation of nucleotide precursors, by competing with the active site of the enzyme. (McDermott, Walker, and White 2003) In nucleic acid synthesis, antibiotics usually act through specific binding to RNA polymerase or DNA topoisomerase, like rifamycin and quinolones, respectively. (McDermott, Walker, and White 2003)

The resistance mechanism associated to sulfonamides is the acquisition of an enzyme with low affinity to sulfonamide. (Zaffiri, Gardner, and Toledo-Pereyra 2012) Rifamycin is also enzymatically altered, while quinolones have three different resistance mechanisms: target modification through mutation of topoisomerase genes; decreased permeability of bacterial cell wall or activation of efflux pump. (McDermott, Walker, and White 2003)

In summary, the success of penicillin encouraged the discovery and development of several other molecules with antimicrobial activity, either from natural or synthetic origin, against Gram-

negative, Gram-positive or both, with different action mechanisms, which are summarized in Table 1.1.

Table 1.1. Antibiotics target and resistance mechanisms.

Class (Examples)	**Derivation**	**Spectrum of activity**	**Target**	**Resistance mechanism**	**Ref.**
β-lactams -Penicillins, -Cephalosporins -Carbapenems	N/SS	Broad-spectrum	Cell wall synthesis inhibitors (PBP)	Enzymatic hydrolysis, efflux pump, altered target	(Zervosen *et al*. 2012)
Aminoglycosides -Gentamicin -Streptomycin	N/SS	Broad-spectrum	Protein synthesis inhibitors (30S ribosome)	Phosphorylation, adenylation or acetylation, efflux pump, altered target	(Shi *et al.* 2013)
Glycopeptides -Vancomycin	N/SS	Gram-positive	Cell wall synthesis inhibitors (Peptidoglycan Units)	Enzymatic hydrolysis, efflux pump, altered target	(Butler *et al.* 2014)
Tetracyclines -Tetracycline -Doxycycline	N/SS	Broad-spectrum	Protein synthesis inhibitors (30S ribosome)	Enzymatic hydrolysis, efflux pump, altered target	(Levy and Marshall 2004; McDermott, Walker, and White 2003)
Macrolides -Erythromycin -Azythromycin	N/SS	Broad-spectrum	Protein synthesis inhibitors (50S ribosome)	Hydrolyze, glycosylation, phosphorylation, efflux pump, altered target	(Leclercq 2002; Levy and Marshall 2004)
Phenicols -Chloramphenicol	N/SS	Broad-spectrum	Protein synthesis inhibitors (50S ribosome)	Acetylation, efflux pump, altered target	(McDermott, Walker, and White 2003)
Sulfonamides -Sulfamethoxazole	S	Gram-positive	Nucleic acid synthesis inhibitor (enzyme catalyzes formation of nucleotides precursors)	Efflux pump, altered target	(Zaffiri, Gardner, and Toledo-Pereyra 2012)
Rifamycins -Rifampicin	N/SS	Broad-spectrum	Nucleic acid synthesis inhibitor (RNA polymerase)	ADP-ribosylation, efflux pump, altered target	(McDermott, Walker, and White 2003)

Quinolones -Fluoroquinolone	S	Broad-spectrum	Nucleic acid synthesis inhibitor (DNA topoisomerase)	Acetylation, efflux pump, altered target	(McDermott, Walker, and White 2003)

N – Natural; SS – Semi-Synthetic; S – Synthetic

1.1.3 – Drug-resistant bacteria in health-care facilities

1.1.3.1 – ESKAPE

Multi-drug resistant bacteria (MDRB) are characterized by features of enhanced morbidity and mortality. (McDermott, Walker, and White 2003) The ESKAPE (vancomycin-resistant ***E****nterococcus*, MR**S**A, *β*-lactamase of extended spectrum producer ***K****lebsiella*, imipenem-resistant ***A****cinetobacter*, imipenem-resistant ***P****seudomonas* spp. and third generation cephalosporin-resistant ***E****nterobacter*) are a group of prevalent multidrug-resistant strains, that greatly concerns the scientific and health-care community, since they are increasingly present in health-care facilities, being associated to Nosocomial Infections (NI). (Cars, Hedin, and Heddini 2011) ESKAPE are highly resistant or even non-responsive to first line drugs recommended for their treatment, leading to reduced therapeutic options and to the need of more costly second and third line drugs. (Rice 2008)

In this antibiotic-resistance era, it is a challenging task to the scientific community, as well as to the pharmaceutical industry, to identify and develop new, effective, safe and broad-spectrum antibacterial drugs. (Brown and Wright 2016)

1.1.3.2 – Nosocomial infections (NI)

NI, also known as healthcare-associated infections (HAI), are defined as *those acquired in/or associated with health-care facilities and that were not actively present or in the incubation period when patient admission occurred*. (Breathnach 2005) According to the World Health Organization (WHO), at any given time, the NI prevalence ranges between 5.7% and 19.1%, in low- and middle-income countries.(World Health Organization 2015) NI are more prevalent in intensive care units (ICU), acute surgical and orthopedic wards. (World Health Organization 2015) The core risks associated to NI are intensive-care admission, bone marrow transplantation, blood transfusion and burn unit. (Zarb *et al.* 2012)

NI represent a burden to the 21st century society, reflected in a brutal socio-economical impact. NI lead to longer hospitalization periods, more intensive use of hospital's human resources, increased risk for loss of quality of life and even death. (Zarb *et al.* 2012) The economical impact is not only related to the money spent in the above-mentioned scenarios, but it is also translated in the loss of working days and consequent production within the active population, extended to the patient's relatives/caretakers. (Breathnach 2005; Fiorentino 2014)

As an example, in the USA, approximately 1.7 millions of patients contract a NI, with 100 000 having death as outcome, being the estimated annual costs associated to NI between 28 to 45 billions of USD. (Dick *et al.* 2015) Pneumonia and sepsis, the principal cause of infection related to the presence of external devices, like central lines and ventilators, are the most deadly and costly infections. (Dick *et al.* 2015) A 2012 study from the European Centre for Disease Prevention and Control (ECDC) highlighted that, *per day*, 81 089 patients get a NI, resulting in 3.5 million patients admitted to ICU annually. (Zarb *et al.* 2012) The costs related to these numbers are translated in 7 billion of € in Europe annually, including direct costs, and reflecting 16 million in extra days of hospital stay due to NI. (World Health Organization 2015)

The growing NI negative impact at different socio-economical levels has propelled the introduction of infection control programs. In Europe, in 2004, MRSA was the first bacteria that encouraged the creation of surveillance and control programs, in order to reduce resistant bacteria in hospital environment. (Breathnach 2005)

Recently, in Portugal, the number of deaths NI-related have increased, from 2973 in 2010 to 4606 in 2013. (Direção-Geral de Saúde 2014) Several control measures have been defined such as equipment, staff and patients disinfection, screening and monitoring of the occurrence of NI, restriction on the use of antibiotics, among others. (Breathnach 2005)

A 2014 study highlighted that the efforts performed by Health Agencies and the Government were able to decrease the incidence of some infections, such as pneumonia-associated with tracheal intubation in ICU, bacteremia associated with central venous catheter and infection associated with colon and rectal surgery. (Direção-Geral de Saúde 2014) However, there was no significant decrease in the number of deaths, with 4500 patients having NI as cause of death. (Direção-Geral de Saúde 2014) Regarding antibiotic consumption, there was a positive evolution, with 27% and 5% less consumption of quinolones in ambulatory and hospitals, respectively. (Direção-Geral de Saúde 2014) Carbapenems use was reduced in 5% in hospitals. (Direção-Geral de Saúde 2014) Resistance rates associated to some MDRB, such as MRSA, *Enterococcus* spp. and *Acinetobacter* spp. have began to decrease. (Direção-Geral de Saúde 2014) Nonetheless, there is still great concern regarding the Gram-negative microorganisms, being quinolones-resistant *E. coli* and carbapenems-resistant *Klebsiella* spp. the main players. (Direção-Geral de Saúde 2014) Over the last couple of years, outbreaks of NI by *K. pneumoniae*, which resulted in dozens of deaths, were reported. (Borja-Santos 2016; Direção-Geral de Saúde 2014) Every day, it is estimated that 12 patients die from NI caused by either *E. coli* or *Klebsiella* spp., a higher mortality rate than that of car crashes. (Madrinha 2016) However, despite all the efforts made, Portugal remains with one of the darkest European scenarios in what concerns NI. (Direção-Geral de Saúde 2014)

Among the several control measures implemented in hospitals, it was determined that the simplest but yet most effective preventive action is hand hygiene, with water and soap or with alcoholic-based solutions, allowing to prevent cross infections. However, adherence to this countermeasure is low among hospital staff for different reasons, such as: skin irritation due to disinfectants agents; forgetfulness; use of gloves; insufficient time for cleaning; lack of knowledge

of this effective measure; among others stated. (Pittet 2001) It is thought that the introduction of new disinfectants agents, with more attractive features, may improve the compliance to this simple but yet highly effective measure for NI prevention. (Pittet *et al.* 2000)

1.2 – Phytotherapy: use of bioactive compounds as therapeutic tools

Plants have been used in folk medicine for centuries, from a trial and error strategy, in an initial form of crude drugs such as tinctures, teas, poultices, powders, infusions, to more recent and advanced formulations, tested with scientific methods. (Balunas and Kinghorn 2005; Gurib-Fakim 2006) Plants produce a wide range of bioactive compounds with diverse physiological and functional roles, such as defense mechanisms, pigments that attract pollinators of flowers, UV protection mechanisms (flavonoid, anthocyanin, etc.) and oxidative stress (phenolic compounds). (Simões, Bennett, and Rosa 2009) In defense mechanisms, a wide range of compounds that exhibit a huge chemical diversity are present, such as glycosteroids, flavonoids, terpenes and isoflavones. (Simões, Bennett, and Rosa 2009) Few infections occur in plants, which may be linked to the presence of a highly effective innate defense mechanism. (Abreu, McBain, and Simões 2012) The scientific advance in the phytotherapy field allowed to further uncover plants biological potential, highlighting its benefits and therapeutic actions associated to their chemical composition (bioactive compounds). (Gurib-Fakim 2006) A bioactive compound is usually a plant's secondary metabolite, able to trigger pharmacological and/or toxicological effects in humans and/or animals, such as: anti-inflammatory; antioxidant; antibacterial; antifungal; antitumor among others. (Simões, Bennett, and Rosa 2009) They can act individually, additively or synergistically and have several biological activities attributed. (Gurib-Fakim 2006)

The decrease of conventional antibiotics effectiveness and the high costs to pharmaceutical industries for the development of new drugs, has contributed to the renewed interest in phytopharmaceuticals. (Abreu, McBain, and Simões 2012) An example of a commercialized drug obtained from natural sources is TAXOL®, and its derivative paclitaxel, anti-cancer and anti-malaria drugs, which are synthesized from the bark of the yew tree (*Taxus brevifolia*). (Expósito *et al.* 2009)

Bioactive compounds antimicrobial performance against bacterial cells has been previously studied, demonstrating activity against a broad array of pathogenic microorganisms, including MDRB. (Simões, Bennett, and Rosa 2009) Despite the antimicrobial potential of bioactive compounds, increased by its diversity and structural complexity, none is currently used as antibiotic. (Gibbons 2004) Nonetheless, there is evidence that bioactive compounds are able to potentiate antibiotic action against MDRB, a promising result for inclusion in the currently available antibiotherapy. (Abreu, McBain, and Simões 2012) Their mechanisms of action are not yet fully understood, which may also be linked to the delay of their complete introduction in the pharmaceutical field, but evidence points that cell wall degradation, damage in cytoplasmic

membrane and membrane proteins, cellular content output, cytoplasm coagulation and depletion of the proton motive force, may be responsible for the antibacterial effect. (Cetin-Karaca and Newman 2015)

From the wide range of bioactive compounds reported in the literature, (Cowan 1999) only those with relevance within the scope of this Master Thesis will be presented in the following sections.

1.2.1 – Phenolic compounds

Phenolic compounds are secondary metabolites derived from pentose phosphate, shikimic acid and phenylpropanoid pathways. (Balasundram, Sundram, and Samman 2006) Structurally, they have an aromatic ring with one or more hydroxyl substituents attached. (Gurib-Fakim 2006) The molecule complexity can go from the most simple to the highly polymerized compounds. (Gurib-Fakim 2006) The most abundant phenolic compounds are conjugated with mono- and polysaccharides, attached to one or more phenolic groups, and they may be functionalized with esters and methyl esters groups. (Balasundram, Sundram, and Samman 2006) All these connections possibilities demonstrate the variety of phenolic compounds classes that may be found in nature. (Gurib-Fakim 2006) They are associated with several biological activities, namely anti-allergic effect, anti-atherogenic, anti-inflammatory, antitumor, antimicrobial, antioxidant, anti-thrombotic, vasodilating and cardioprotective. (Balasundram, Sundram, and Samman 2006; Gurib-Fakim 2006) Phenols can be found in a great variety of fruits, vegetables, nuts, seeds, stems and flowers as well as in teas, wine and honey. (Balasundram, Sundram, and Samman 2006) They also have an important role in plants physiology and morphology, as well as in defense mechanisms and sensorial characteristics. (Balasundram, Sundram, and Samman 2006; Cetin-Karaca and Newman 2015)

Oleuropein, hydroxytyrosol and tyrosol, being the last two degradation products from oleuropein hydrolysis, are phenolic compounds that belong to a more specific group, named secoiridoids. Oleuropein structure consists in three subunits: elenolic acid, glucose and hydroxytyrosol. Hydroxytyrosol and tyrosol structures are based in a phenylethyl alcohol, wherein the hydroxytyrosol has one more hydroxyl group than tyrosol (Figure 1.4). (Omar 2010a; Rodrigues, Pimentel, and Oliveira 2015)

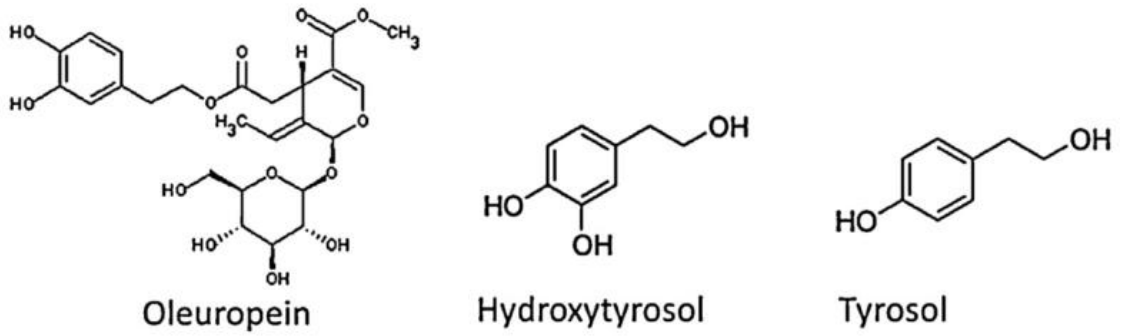

Figure 1.3. Chemical structures of oleuropein, hydroxytyrosol and tyrosol. (Adapted from Rodrigues *et al*. (2015))

Oleuropein, and its hydrolysis products, have been associated to a strong antimicrobial activity against both Gram-negative and Gram-positive bacteria, as well as against *Mycoplasma* spp. (Omar 2010b) It is accepted that the antibacterial effect may be due to damage to the bacterial membrane and/or disruption of the cell peptidoglycan. (Omar 2010b) Other authors suggest that the interference in protein synthesis and stimulation of the phagocytose response of the immune system are possible mechanisms underlying the antibacterial activity. (Omar 2010b)

These three compounds can be found in olive tree (*Olea europaea L.*), mainly in leaves and fruits (peel, pulp and seeds), varying their concentration according to the ripeness state. (Wichers, Soler-rivas, and Espı 2000) Oleuropein is the most abundant, due to its role in defense mechanism, and can reach concentrations of 140 mg/g of green olive dry weight and 60-90 mg/g of leaves dry weight. (Wichers, Soler-rivas, and Espı 2000) In olive, the concentration of oleuropein decreases according with the ripening state, while hydroxytyrosol and tyrosol concentrations increase. (Wichers, Soler-rivas, and Espı 2000)

1.2.2 – Terpenes

Terpenes are natural hydrocarbons with cyclic or acyclic chains isoprene-derived from secondary metabolism. (Cowan 1999) Isoprene units (C_5) are the building blocks in terpenes biosynthesis, forming structures of monomers, dimers or polymers, being its natural precursor dimethylallyl pyrophosphate (DMAPP) and its isomer isopentenyl pyrophosphate (IPP). (Dewick 2002) Hemiterpene (C_5) is the simplest terpene. Adding more isoprene units results in terpenes (C_{10}), diterpenes (C_{20}), triterpenes (C_{30}), tetraterpenes (C_{40}) and sesquiterpenes (C_{15}). (Dewick 2002)

These compounds are commonly associated to plants fragrances, also known as the essential oils fraction. (Cowan 1999) Over the years, their biological activities have been highlighted, such as antimicrobial (antibacterial, antifungal, antiviral and protozoa), anti-inflammatory effect, anti-ulcerogenic, anti-carcinogenic, hepatocellular and cardioprotective

effect. (Cowan 1999) The terpenes of interest in this study are triterpenes and sesquiterpene compounds.

1.2.2.1 – Triterpenes

Triterpenic compounds are constituted by six isoprene units, which can be acyclic or form mono-, bi-, tri-, tetra- or pentacyclic structures. (Cowan 1999) Compounds with relevant biological activity belong to the tetracyclic triterpenes (dammarane and euphane) and pentacyclic triterpenes (oleanane, ursane and lupane) classes. (Dewick 2002) Pentacyclic triterpenes have been widely studied, due to their various pharmacological effects, biological activities (specially antibacterial and antitumor) and low toxicity, which confers high potential for its use as multi-target therapeutic tools. (Dzubak *et al*. 2006; Jäger *et al.* 2009) These compounds are widely distributed and may be isolated from peel of fruits, leaves and bark of the plant stems. (Jäger *et al.* 2009) According to Jäger *et al*. (2009), after the screening of 39 plants, it was possible to identify pentacyclic triterpenes in all but their concentration was higher in the dry extracts of the following plants: flat trunk bark (betulinic acid), olive leaves and pomace, clove flowers and mistletoe shoots (oleanolic acid), any apple pulp (ursolic acid) and equal amount of the three triterpenic acids in rosemary leaves. (Jäger *et al.* 2009)

Betulinic, betulonic, oleanolic and ursolic acids are triterpenic acids (TAs) that belong to the lupane (first two), oleanane and ursane family, with pentacyclic structure (Figure 1.5).

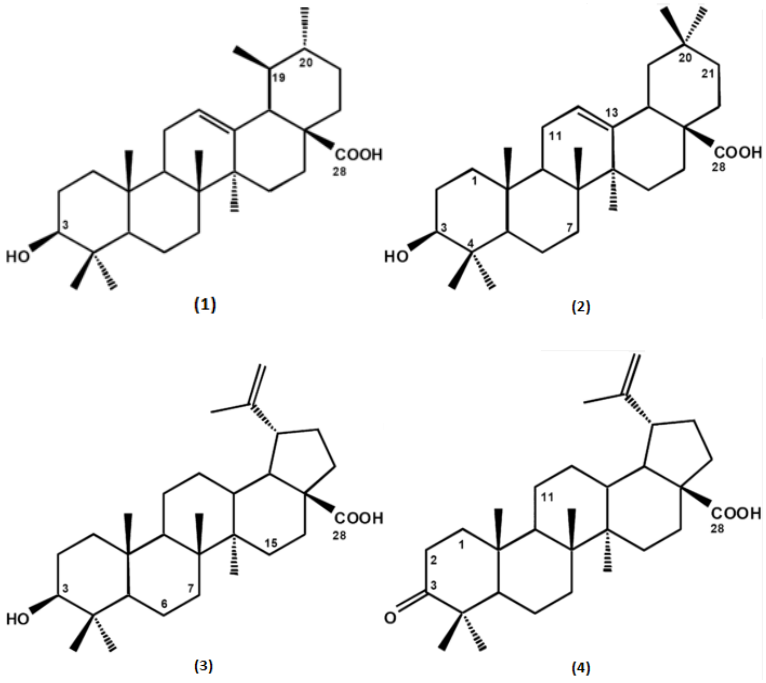

Figure 1.4. Pentacyclic triterpenic acids: (1) ursolic acid; (2) oleanolic acid; (3) betulinic acid and (4) betulonic acid. (Adapted from Muffler, *et al*. (2011))

These TAs have been extensively studied, mainly due to their antimicrobial activity against pathogenic bacteria, such as MDRB, demonstrating promising results in synergistic studies with conventional antibiotics. (Fontanay *et al.* 2008) Ursolic and oleanolic acids exhibit antibacterial effect mainly against Gram-positive bacteria. (Wolska *et al.* 2010) The minimal inhibitory concentrations (MIC) of oleanolic and ursolic acids against *Staphylococcus* spp. range from 8 to 64 µg/mL (Fontanay *et al.* 2008; Gibbons 2004) and between 3 µg/mL and 64 µg/mL (Fontanay *et al.* 2008; Wang *et al.* 2016; Wolska *et al.* 2010), respectively. Moreover, when conjugated with antibiotics, they are capable to influence the susceptibility of multidrug-resistant *S. aureus*, *S. epidermidis* and *L. monocytogenes* to ampicillin and oxacillin. (Kurek *et al.* 2012) It is thought that the primary target of these compounds is the bacterial cell wall, causing autolysis of the cell, while they also may influence the bacterial gene expression responsible for formation and maintenance of biofilms. (Kurek *et al.* 2012; Wolska *et al*. 2010) In a study performed by Chung *et al*. (2011), betulinic acid exhibited antibacterial effect against MRSA with a MIC of 64 µg/mL. (Chung, Navaratnam, and Chung 2011) Furthermore, synergistic studies using betulinic acid plus methicillin and vancomycin individually demonstrated a decrease in bacterial growth, in both combinations. (Chung, Navaratnam, and Chung 2011) Lastly, betulonic acid was described as presenting antibacterial effect against *E. faecalis* and *S. aureus,* diminishing bacterial growth in 74% and 51%, respectively. (Haque *et al*. 2014)

1.2.2.2 – Sesquiterpenes

Sesquiterpenes are formed from three isoprene units (C_5). (Dewick 2002) Due to their long chain and presence of an additional double bond, the number of possible modes of cyclization increases, which is translated in a wide variety of mono-, bi-, and tricyclic structures. Post-synthesis modifications may also occur, such as glycosylation and oxidation, which gives further diversity to the compounds. (Chadwick *et al.* 2013) Sesquiterpene lactones are derived from an oxidation reaction in the C_3 of the side chain, forming a lactone. (Dewick 2002) The more abundant classes of sesquiterpene lactones are: germacranolides; pseudoguaianolides; eudesmanolides and quaianolids, being germacranolides the most important regarding biological activities associated to humans (Figure 1.6).

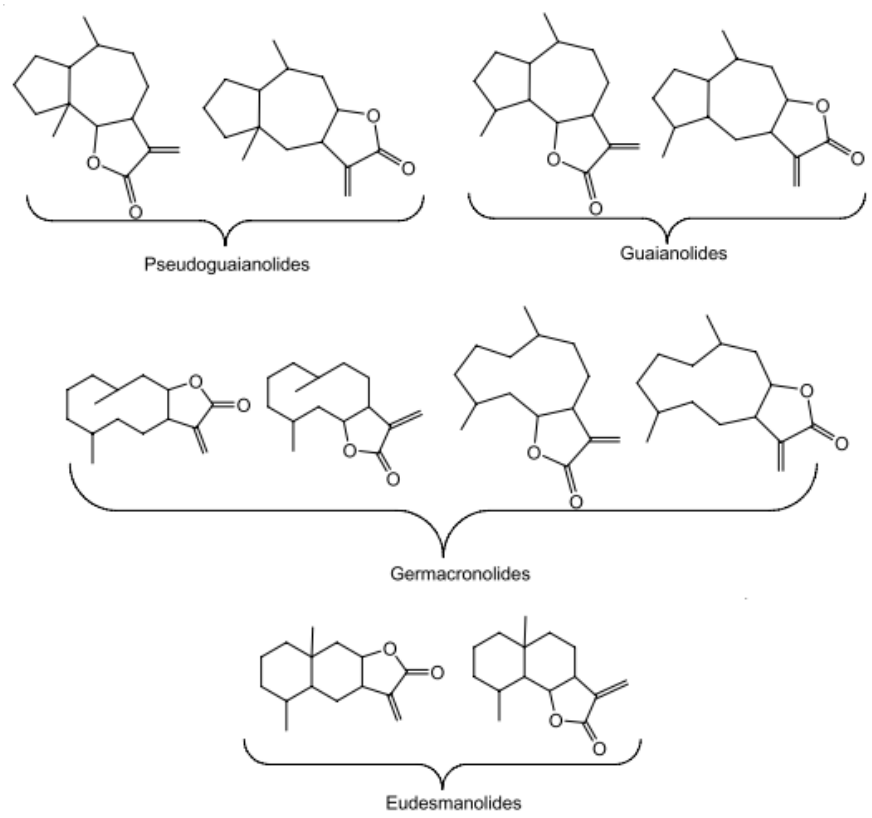

Figure 1.5. Sesquiterpene lactones basic structure. (Adapted from Chaturvedi (2011))

Sesquiterpene lactones have demonstrated benefits within human health improvement, with antitumor, anti-inflammatory, antibacterial, antifungal, antiviral, antiprotozoal, anthelmintics, antiulcer, molluscicide, hepatoprotective and antidepressant effects. (Amorim et al. 2013) In plants, these compounds have an important role in defense mechanisms against stress situations and as predator repellent. (Chadwick *et al.* 2013) Sesquiterpene lactones have inhibitory activity against Gram-positive and Gram-negative bacteria. (Chaturvedi 2011) For instance, vernodalin and vernolide induce higher inhibitory effect in Gram-positive (MRSA), while helenalin act preferably against *Mycobacterium tuberculosis* and *Corynebacterium diptheriae,* among other Gram-negative bacteria. (Chaturvedi 2011) *Inula helenium* is currently used as an antiseptic of the urinary tract. (Chaturvedi 2011) Cynaropicrin revealed bactericidal effect, acting as an inhibitor of the bacterial cell wall formation. (Bachelier, Mayer, and Klein 2006)

Sesquiterpene lactones are present in a diverse family of plants such as *Cactaceae*, *Solanaceae*, *Araceae*, *Euphorbiaceae*, etc., being more abundant in *Asteraceae* family, the more diverse and abundant plants family in the world. (Chadwick *et al*. 2013) They are also present in dietary consumption of fruits and vegetables, such as lettuce and chicory, infusions and as additive in alcoholic drinks, conferring the bitter taste. (Chadwick *et al.* 2013)

The bioactive compounds classes with relevance to this Thesis and respective biological activities are summarized in Table 1.2.

Table 1.2. Bioactive compounds and its biological activities.

Bioactive compounds	Biological activities
Phenolic compounds: -Hydroxytyrosol -Tyrosol -Oleuropein	Anti-allergic, antiatherogenic, anti-inflammatory, antitumor, antimicrobial, antioxidant, anti-thrombotic, vasodilating and cardioprotective. (Balasundram, Sundram, and Samman 2006; Gurib-Fakim 2006)
Triterpenic acids: -Betulinic acid -Betulonic acid -Oleanolic acid -Ursolic acid	Antimicrobial, anti-inflammatory, antiulcerogenic, anticarcinogenic, hepatocellular and cardioprotective. (Cowan 1999)
Sesquiterpene lactone: -Cynaropicrin	Antitumor, anti-inflammatory, antibacterial, antifungal, antiviral, antiprotozoal, anthelmintics, antiulcer, molluscicide, hepatoprotective and antidepressant. (Amorim *et al.* 2013)

1.3 – Biorefinery

Fossil fuels are used worldwide for energy and chemical production, being oil the most used. It is estimated that 84 million barrels of oil are used *per* day only in the transport sector, and this number tends to increase. (Cherubini 2010) Moreover, approximately 4% off the refined oil is widely used for chemical and plastic production. (Cherubini 2010) These processes have been burdening the environment, with air, water and soil pollution, causing climate changes, being recognized that an active intervention and paradigm shift is required. (Fernando *et al.* 2006) Therefore, the search for alternatives to fossil fuels is mandatory but still a huge challenge. The recycling concept is well established in the society, and thus, it can be extended to industries, that while using raw materials of plant origin, generate large amounts of waste (biomass), which can be further reused. (Fernando *et al.* 2006) In this context, the biorefinery concept has emerged, being defined *as the sustainable processing of biomass in a marketable products spectrum and energy*. (Fernando *et al.* 2006) Thus, a biorefinery should consist in a unit (or several) incorporating the equipment and technology required for biomass processing considered as waste (wood, grass, corn, etc.) and its basic components (carbohydrates, proteins, triglycerides,

etc.) into biofuel, electricity and chemicals, in the concept of exhausting its resources completely (Figure 1.7). (Cherubini 2010)

The "power supply" of biorefineries consist in raw carbon-derived materials, supplied from four main sectors: agriculture (crop and waste fields), forestry industries (waste and leftovers processes), housing (solid waste and municipal wastewater) and aquaculture (algae). (Cherubini 2010)

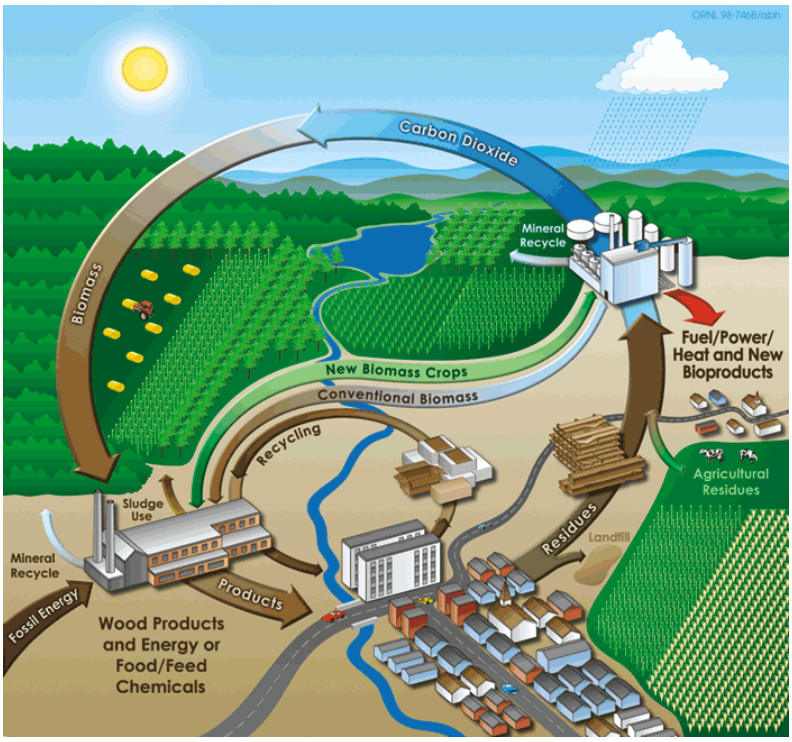

Figure 1.6. Biorefinery concept. (Herrera 2004)

In the present Thesis three biomasses, and respective extracts, from different sources, were used: 1) forestry – *Eucalyptus* spp. total bark, from pulp and paper industry; 2) agriculture – olive pomace, from olive oil production and; 3) *Cynara cardunculus* (cardoon), as an endogenous resource from the Alentejo region, mainly used in artisanal cheese production.

1.3.1 – *Eucalyptus* spp. and paper production

Eucalyptus spp. is the main raw material used for pulp and paper production in the paper industry, an economical sector with high economical relevance in the Iberian Peninsula. (Domingues *et al*. 2010) *Eucalyptus urograndis*, *E. grandis*, *E. maidenii*, *E. globulus* and *E. nitens* are the main species (Figure 1.8a). (Domingues *et al.* 2010)

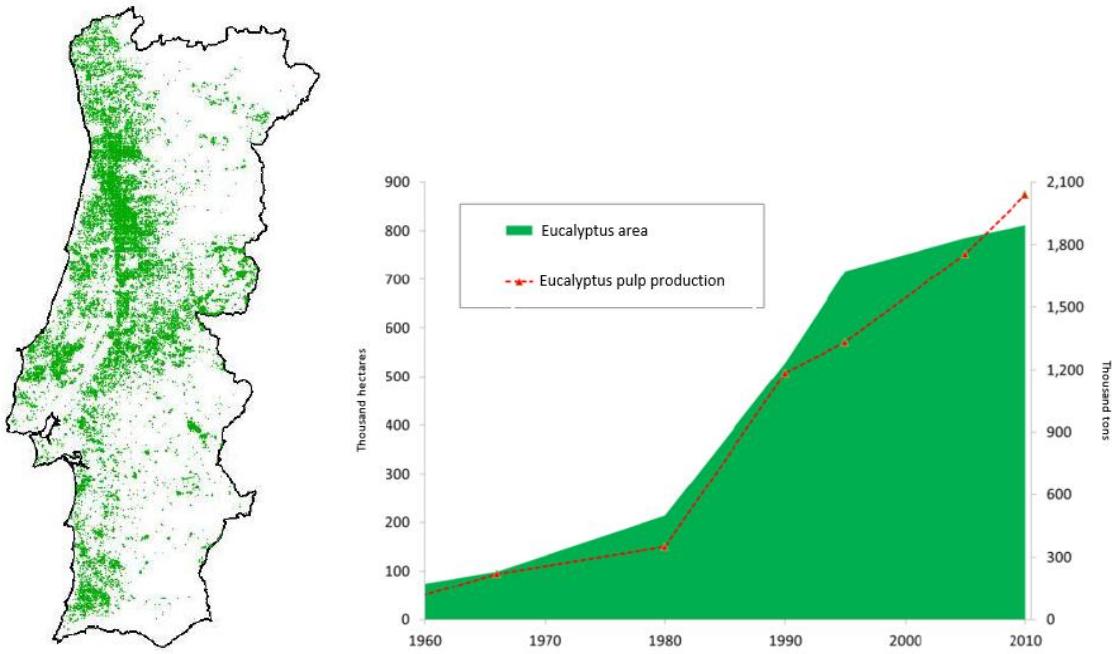

Figure 1.7. *Eucalyptus* spp. in Portugal: a – Geographic distribution of *Eucalyptus* spp. in Portugal; b – Relationship between evolution of *Eucalyptus* spp. area and the *Eucalyptus* spp. pulp production, reaching 812 000 ha in 2010. (Adapted from: Source CELPA and ICNF, I.P.)

This industry generates large quantities of sub-products, approximately 1.0 x 10^5 ton/year, such as bark and wood waste (leaves, branches, fruits, etc.), which are usually left in the forest to nourish the soil or are burned for energy. (Domingues *et al.* 2011; Domingues *et al*. 2012) It is an industry that is constantly increasing its production levels (Figure 1.8b) and, therefore, the implementation of the biorefinery concept would allow to completely reuse the waste (biomass), as well as to increase the economical potential of the sector.(Domingues *et al.* 2011)

The main compounds found in this biomass are phytosterols, such as *β*-sitosterol, lignans and botulin.(Domingues *et al.* 2010) It is described in the literature that the outer bark of *Eucalyptus* spp. is the richer fraction in triterpene compounds, as well as monoterpenes and sesquiterpenes, small amounts of fatty acids and aromatic compounds. (Domingues *et al*. 2010) The main TAs present in *Eucalyptus* spp. are the ursolic, oleanolic, betulinic, betulonic, 3-acetylursolic and 3-acetyloleanolic acids. (Domingues *et al.* 2010) According to Pereira *et al*. (2014) studies, *E. globulus*-derived extracts showed promising results in antimicrobial activity, either isolated or in combination with conventional antibiotics. (Pereira *et al*. 2014)

1.3.2 – Olive and olive oil production

Olive trees (*Olea europaea L.*) are native from the Mediterranean region, but during the last decade, its cultivation worldwide has increased, due to the recognized benefits of olive oil consumption in human health (Figure 1.9a). (Romero-García *et al.* 2014) In 2013, 2.67 million tons of olive oil were produced for human consumption in Europe. (Romero-García *et al*. 2014) In addition to oil, olives are also a product of interest for consumption. (Romero-García *et al.* 2014) In Portugal, between 2000 and 2009, olive oil production increased approximately 63%, especially in the Alentejo region, representing 56% of the total national production. (Ramos *et al.* 2013) In 2013, 999 853 hectoliters of olive oil were produced, having 689 261 hectoliters been produced in the Alentejo region (Figure 1.9b). (Instituto Nacional de Estatísitca (INE) 2014)

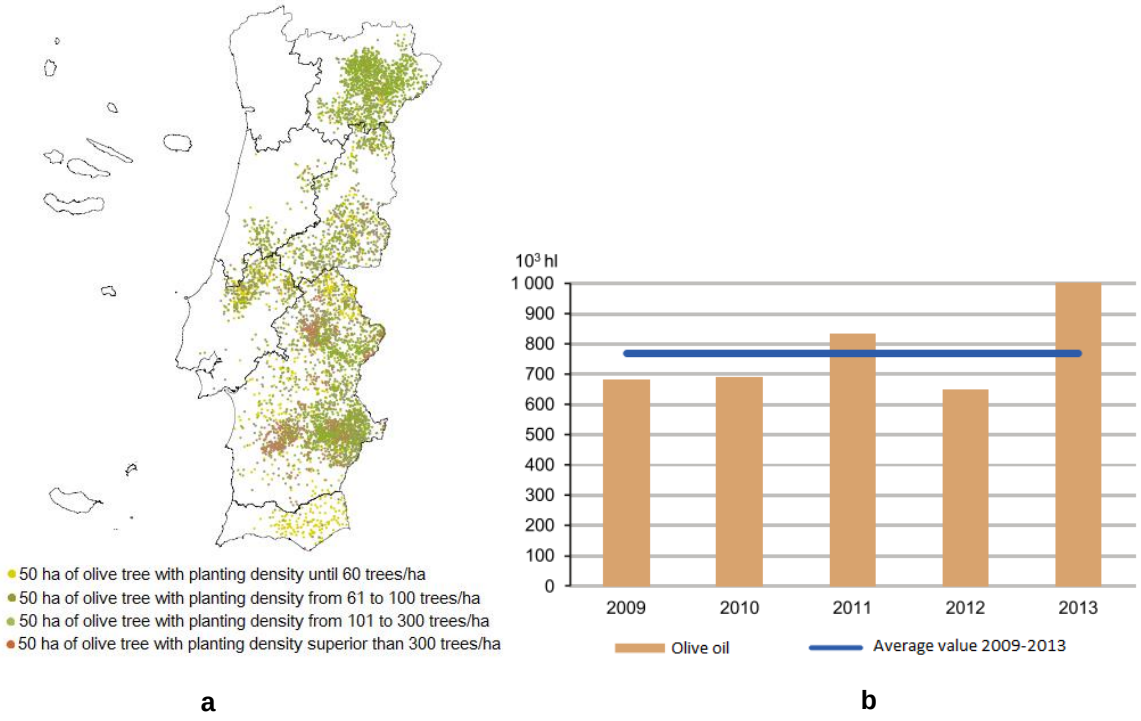

Figure 1.8. Olive tree in Portugal: a – Geographic distribution of olive trees in Portugal. (Adapted from: source INE, RA 2009) b – Olive oil production, between 2009 and 2013, reached an approximated average value of 800 000 hl. (Adapted from: source INE, EA 2013)

In Alentejo, olive oil extraction is made through a centrifugation system in two phases, which generates a large amount of a residue named olive pomace, with high water content and composed mainly of skin, pulp and stone pieces of olive fruit. (Ramos *et al*. 2013) Every year, it is estimated that 400 000 ton of this residue are generated, which is subsequently subjected to drying at high temperatures, resulting in another residue, dry olive pomace that is mainly used for energy production (combustion). (Ramos *et al*. 2013) Phenolic compounds can be extracted from both residues. (Ramos *et al*. 2013) According to Ramos *et al*. (2013), dry olive pomace revealed

higher phenolic content than olive pomace, being hydroxytyrosol the more abundant. *(Ramos et al*. 2013) Other phenolic compounds were identified in dry olive pomace extract, namely HT-1-glucoside, tyrosol, oleuropein aglycone isomers, verbascoside, oleuropein and de(carboxymethyl)oleuropein aglycone isomer in aldehyde form. (Ramos *et al*. 2013)

1.3.3 – *Cynara cardunculus* – artisanal cheese production and other applications

Cynara cardunculus (cardoon) can be found in the Mediterranean region and its distribution in Portugal is represented in Figure 1.10.

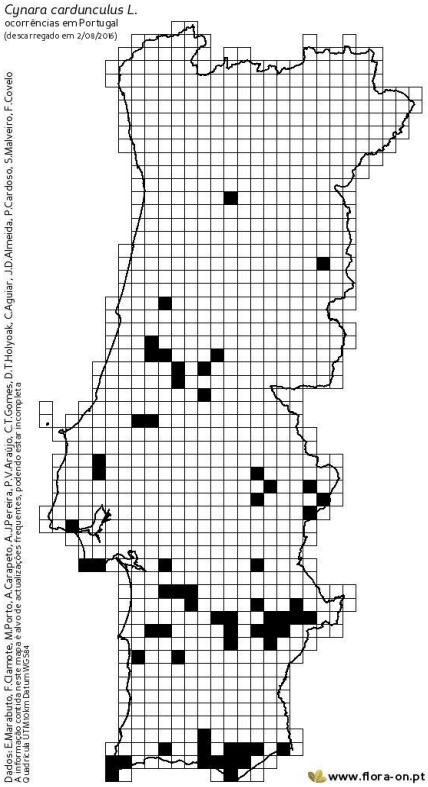

Figure 1.9. Geographic distribution of *Cynara carcunculus* in Portugal. (Marabuto *et al.* 2016)

C. cardunculus is divided in three taxa: two domestic forms, the artichoke (var. *scolymus* L.) and cultivated cardoon (var. *altilis*), and wild cardoon (var. *sylvestris*) (Figure 1.11).

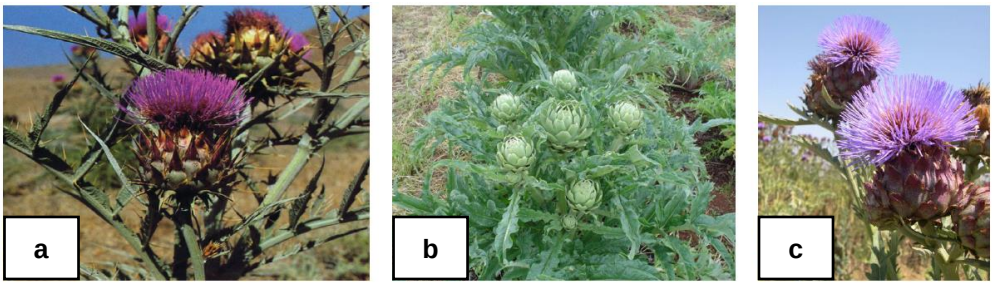

Figure 1.10. Morphological appearance of each variant of *Cynara cardunculus*: (a) var. *sylvestris* , (b), var. *scolymus* L. and (c) var. *altilis*. (Source: CEBAL)

It has rigorous growth conditions, demanding high temperatures, high salinity and low precipitation. (Falleh *et al*. 2008) *C. cardunculus* is associated to a wide variety of applications, such as in the Mediterranean diet in Spain, Italy, France and south of Portugal; in artisanal cheese production and in pulp industry. (Falleh *et al.* 2008; Velez *et al.* 2012) Recently, it has shown promising results for biodiesel production. (Velez *et al.* 2012) The valorization of *C. cardunculus* is mainly linked to the lignocellulosic fraction and the high value compounds that constitute it. (Ramos *et al.* 2013) The major compounds have been identified by Ramos *et al*. (2013), being among them a sesquiterpene lactone (diacylcynaropicrin), four pentacyclic triterpenes (*β*- and *α*-amyrin, lupenyl and ψ- taraxasteryl acetate) and four sterols (stigmasterol, 24-methylenecholesterol, campesterol and Δ^5-avenasterol). (Ramos *et al.* 2013) Cynaropicrin and ψ- taraxasteryl acetate are the sesquiterpene lactone and pentacyclic triterpene, respectively, found in higher concentrations. (Ramos *et al.* 2013) These compounds are responsible for the *C. cardunculus* pharmacological effects, which has led to an increase in its cultivation and biological activity studies. (Velez *et al.* 2012)

1.4 – Pharmaceutical formulation

1.4.1 – Formulation Design

Drugs are usually included in formulated preparations, varying in complexity according to the type and amount of excipients/additives added. (Aulton 2002) These vehicles provide different characteristics to formulations, depending on the final preparation or dosage form desired, such as solubility, thickness, preservation, emulsion, among others. (Aulton 2002)

There is a large variety of dosage forms where the drug can be incorporated, generally chosen according to the more convenient and best administration route and also in order to obtain maximum effectiveness and, consequently, have the maximum therapeutic response. (Aulton 2002) Various administration routes and associated dosage forms are shown in Table 1.3.

Table 1.3. Administration routes and dosage forms associated. (Aulton 2002)

Administration route	**Dosage forms**
Oral	Solutions, syrups, suspensions, emulsions, gels, powders, granules, capsules, tablets
Rectal	Suppositories, ointments, creams, powders, solutions
Topical	Ointments, creams, pastes, lotions, gels, solutions, topical aerosols
Parenteral	Injections (solution, suspension, emulsion forms), implants, irrigation and dialysis solutions
Respiratory	Aerosols (solution, suspension, emulsion, powder forms) - sprays; foams
Nasal	Solutions, inhalators
Eye	Solutions, ointments, creams
Ear	Solutions, suspensions, ointments, creams

Among these dosage forms, solutions for topical administration are those with relevance for this Thesis. According to pharmaceutical terms, solutions are *liquid preparations that contain one or more chemical substances dissolved in a suitable solvent or mixture of mutually miscible solvents*. (Ansel, Popovich, and Allen 1989) Generally, topical solutions are formulated in an aqueous vehicle and it is necessary the addition of co-solvents, among other excipients, to enhance both stability and solubility of the drug. (Ansel, Popovich, and Allen 1989) Liquid dosage forms, as topical solutions, are mainly used for local application and present several advantages, such as ease of administration and faster absorption, since the drug is already dissolved and, therefore, it is easily available for skin absorption. However, solutions can also present disadvantages, such as: the lower chemical stability of the formulation components, as they are more susceptible to hydrolysis; as well as the fact that this may be a suitable medium for microorganisms proliferation. (York 2000)

In a technical way, a pharmaceutical formulation can be defined as *the set of operations that aims to create a physical system which contains the active substance in order to meet the specifications of the formulation and ensure the maintenance of efficacy and safety of the active substance* (Figure 1.12). (Sousa e Silva 2013)

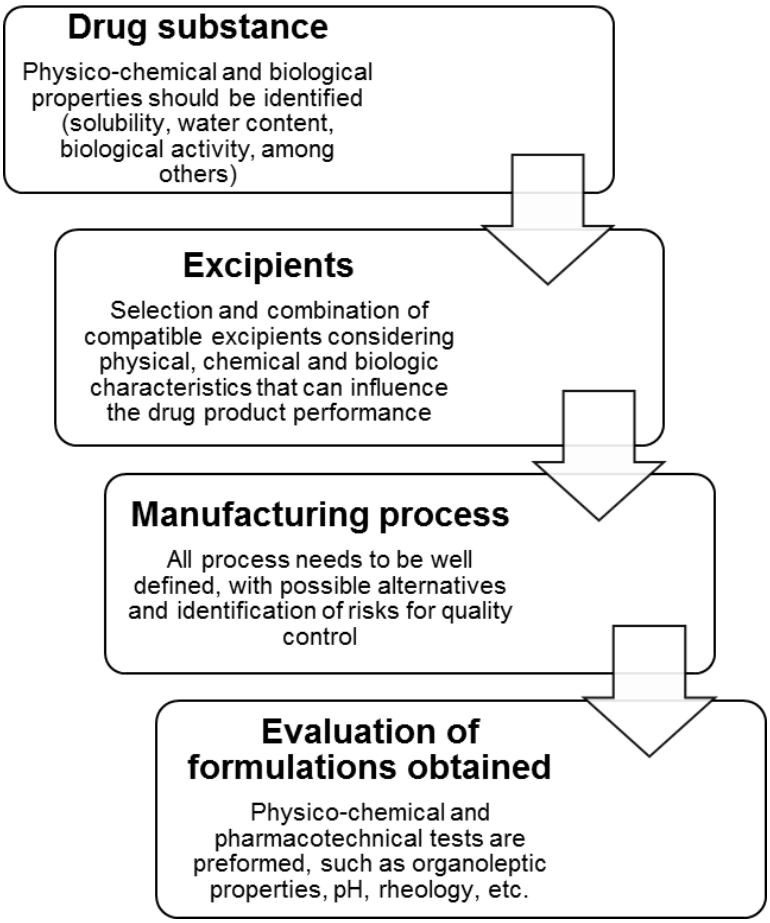

Figure 1.11. Representative scheme of main stages of formulation design.(Sousa e Silva 2013)

This process is performed in order to select, optimize and evaluate the pharmaceutical preparations obtained. (Sousa e Silva 2013) The final product must be stable, efficacious, well-conditioned, attractive, easy to administer, and safe. (Allen 2010)

1.4.2 – Quality control and stability study

The stability of pharmaceutical formulations is defined *as the ability of a formulation, appropriately packed, to maintain the physical, chemical, microbiological, therapeutic and toxicological characteristics in compliance with its specifications*. Environmental conditions such as heat, light and humidity and chemical factors, such as oxidation, reduction, hydrolysis, among others, may play a very important role in stability.

There are official guidelines for quality control that must be followed in order to ensure that pharmaceutical formulations maintain their quality, effectiveness and safety. This quality standard is periodically published in pharmacopeias of major pharmaceutical manufacturing and exporting countries. The U.S. Pharmacopeia, European Pharmacopoeia, International Pharmacopoeia, published by the WHO, and the British Pharmacopoeia are widely used. (WHO 2012)

The main characteristics that should be well established and controlled in a formulation are: identity, purity, drug assay, uniformity of dosage form, and stability (Table 1.4). These characteristics can be affected cumulatively through the several steps of the manufacturing process, including the starting materials, errors in manufacturing process, packaging, transportation and storage conditions. (WHO 2012)

Table 1.4. Examples of quality control parameters of a pharmaceutical formulation. (WHO 2012)

Quality Control	Description
Identity	The identity test should confirm the presence of the active substance.
Purity	The ingredients used should not have potentially harmful contaminants, microorganisms or other products from cross-contamination.
Drug assay	The pharmaceutical formulation should contain the declared amount of the active substance. Most pharmacopoeias specify an average content range of active substance as well as of by-products of degradation that may be harmful.
Uniformity of dosage forms	Consistency, color, shape and size of certain formulation forms should not vary between doses. The lack of uniformity may suggest problems in other quality parameters, and reflect a lack of good manufacturing practices.
Stability	Stability studies allow obtaining information of better conservation conditions and predicting expiration date. They are performed under normal storage conditions, called real-time assays, where physical, chemical and microbiological parameters are evaluated over time.

2 – Objectives

The main objective of this work was to establish the foundations for the design of a novel antiseptic formulation, based on natural extracts obtained from forest/agriculture industry by-products, against multi-drug resistant bacteria (MDRB). Three biomasses, *Eucalyptus* spp., dry olive pomace and *Cynara cardunculus*, described in the Introduction section, were tested and the experimental work was based on four main tasks:

- ✓ Task 1 – The antibacterial activity of industry by-products (in the form of extracts), namely of *E. nitens* total bark, dry olive pomace and *C. cardunculus* leaf -derived extracts, was tested against MDRB, in order to define the best one(s) to be included in the formulation design. For that, minimal inhibitory concentrations (MIC) and minimal bactericidal concentration (MBC) were determined.
- ✓ Task 2 – Time-kill assays: the antibacterial efficiency of the selected biomass(es) extract(s) in decreasing the microbial load over time was studied. Possible synergistic interactions between the extracts and standard antibiotics were also evaluated;
- ✓ Task 3 – Formulation design: various solvents and excipients were tested in order to define the best matrix for dissolving the selected bioactive(s). After achieving the desired formulation, physico-chemical and organoleptic characterizations were performed. Quality control, regarding alterations in the formulation over time, was executed;
- ✓ Task 4 – *In vitro* assays of the designed formulation(s), to infer the antibacterial activity of the designed formulation against a panel of MDRB.

3 – Material & Methods

3.1 – Plant material and extraction method

3.1.1 – *Eucalyptus* spp.

E. nitens total bark lipophilic fraction extract (*E. nitens* total bark (ENTB) extract) was produced at CICECO/University of Aveiro, Aveiro, Portugal, in the framework of the project "NEucBark – New Valorization Strategies for *Eucalyptus* spp. Bark Extracts" (PTDC/AGR-FOR/3187/2012).

Extraction was performed in accordance to Domingues *et al*. (2011). Briefly, Soxhlet method was used with dichloromethane as extraction solvent, since it is fairly specific for lipophilic extractives. Afterwards, solvent was evaporated, dry biomass weighed and results were expressed in percentage of dry bark. The final extract was kept at -20°C until further use. (Domingues *et al.* 2011)

3.1.2 – *C. cardunculus*

C. cardunculus L. var*. altilis* leaves were collected by CEBAL in Experimental Center of Agriculture School of the Instituto Politécnico de Beja, Portugal, in the framework of the project "ValBioTecCynara – Economic valorization of Cardoon (*Cynara cardunculus*): study of natural variability and biotechnological applications" (ALT20-03-0145-FEDER-000038).

The *C. cardunculus* leaves (CcL) lipophilic fraction extraction was performed according to Ramos *et al*. (2013) Leaves, as the other collected samples, were freeze-dried before extraction. Soxhlet extraction method was used with dichloromethane as solvent of extraction. In the end, solvent was evaporated to dryness at low pressure and the extract weighted, being the results expressed as percentage of dry biomass material. Dichloromethane was used as extraction solvent due to its specificity for lipophilic extractives.(Ramos *et al.* 2013) The extract was kept at RT and protected from light until further use.

3.1.3 – Dry olive pomace

Dry olive pomace (DOP) was provided by Mariano Lopes & Filhos, Lda. (União de Cooperativas Agrícolas do Sul - UCASUL), in the framework of the project "RefinOlea – An integrated valorization strategy for by-products of olive oil extraction industry" (FCOMP-01-0202-FEDER-005450), being kept at RT until further use.

3.1.3.1 – DOP phenolic extraction

The extraction protocol was performed according to Ramos *et al*. (2013). Briefly, about 8 g of DOP were mixed with 120 mL of distilled water under constant stirring, protected from light, at RT, for 40 min. The mixture was then centrifuged at 9 000 x *g,* for 10 min, at RT (Centrifuge

Hermle Z323K, Hermle Labor Technik). The supernatant was collected and protected from light; while the leftover solid residue was extracted five times more. The extraction conditions were similar, except for the stirring time, which was successively shorter (30, 15, 10 and 2x 5 min). All the collected supernatants were filtered through a 0.22 µm PES filter (Pall Life Sciences). Finally, water was removed by freeze-drying (Coolsafe TM, Scanvac) and solid extracts were kept at −20°C, protected from light, until further analysis. (Ramos *et al.* 2013)

3.1.3.1.1 – Determination of total phenolic content

The quantification of total phenolic content was performed using Folin-Ciocalteau method, according to Falleh *et al*.(2008). Briefly, in a 96-well plate, 10 µL of extract were added at a concentration of 1mg/mL (extract dissolved in distilled water) and 150 µL of Folin-Ciocalteau reagent 10% (v/v). The plate was vortexed and kept in the dark for 5 min, at RT. Subsequently, 150 µL of sodium carbonate solution 60 g/L were added, the plate vortexed and kept in the dark for 60 min. Finally, optical density (OD) was read against a blank composed by distilled water, instead of extract, in a plate reader (Multiskan FC, Thermo Scientific) at λ= 750 nm. A calibration curve was performed, using Gallic acid as standard phenolic compound, in a concentration range of 0.04 – 0.250 mg/mL. Triplicates for each extract sample and standard concentration were made.

3.1.3.1.2 – Quantification of hydroxytyrosol, tyrosol and oleuropein by high-performance liquid chromatography-UV/Vis (HPLC-UV/Vis)

Quantification of the phenolic compounds hydroxytyrosol, tyrosol and oleuropein were performed with a Merck Hitachi HPLC system (Tokyo, Japan). A LiChrospher® RP-18 column 5µm particle size (Merk Millipore) was used at 30°C, with a flow rate of 1.5 mL/min and a gradient elution. The mobile phases consisted in water (HPLC grade) with 0.5% of acetic acid (Sigma-Aldrich) (A) and acetonitrile (Fisher Scientific) (B). The following multistep linear gradient (% v/v) was applied: 0 min, 5%B; 10 min, 30%B; 12 min, 33%B; 17 min, 38%B; 20 min, 50%B; 23 min, 95%B, 27 min, 5%B until the end of the run (37 min). The injection volume of the sample was 5 µL and the UV/Vis detection was performed at λ= 280 nm. Before HPLC injection, extract samples were dissolved in water (HPLC grade) and then filtered through a 0.22 µm syringe filter (VWR). Calibration curves were constructed using standard solutions of each phenolic compound, using the following concentrations: hydroxytyrosol 50, 100, 200, 300, 500 µg/mL; tyrosol 50, 75, 150, 300, 500 µg/mL; oleuropein 50, 100, 200, 300, 450, 600 µg/mL.

3.2 – Bacterial strains and growth

A panel of MDRB was used (Table 3.1). These bacteria were chosen for being the main players in NI.

Table 3.1. MDRB panel used.

Bacteria	
Gram-negative	*Escherichia coli* ATCC 25922
	Escherichia coli OXA
	Escherichia coli TEM 180
	*Klebsiella pneumoniae**
	*Citrobacter freundii**
	*Salmonella enterica**
	Pseudomonas aeruginosa PAOI
Gram-positive	*Staphylococcus saprophyticus**
	*Staphylococcus epidermidis**
	Staphylococcus aureus ATCC 43300
	Staphylococcus aureus ATCC 6538

* clinical isolates

Bacterial cultures were kept at -80°C in freezing medium, composed of Brucella broth (Fluka Analytical) with 20% (v/v) of glycerol (Liofilchem)). To start the cultures, bacteria were defrosted at RT, being afterwards incubated in Muller Hinton Broth (MHB, Liofilchem), with agitation (200 rpm) in an orbital shaker (SI-300/300R/600/600R, Lab companion), at 37°C, for 6 hours. After incubation, cultures were plated in Mueller Hinton Agar (MHA, Liofilchem) and Tryptic Soy Agar (TSA, Liofilchem), using the quadrant streak method. Plates were incubated overnight at 37°C.

All bacterial assays were performed in a sterile environment, in a biosafety level 2 laminar flow chamber (TOPSAFE 1.2, EuroClone).

3.3 – Antibacterial activity assays

3.3.1 – Antibiotics antibacterial activity assay

In this study, 4 antibiotics were used: tetracycline (Duchefa Biochemie, Alfagene), ampicillin (Applichem, VWR), rifampicin (Duchefa Biochemie, Alfagene) and gentamicin (Duchefa Biochemie, Alfagene) as representative of broad-spectrum antibiotics.

3.3.1.1 – Minimal inhibitory concentrations (MIC)

MIC is defined as *the minimum concentration of antibacterial agent that can inhibit visible bacterial growth* and is mostly used to test bacterial susceptibility to antibacterial agents. (Wiegand, Hilpert, and Hancock 2008) MICs were determined by the microbroth dilution method as described elsewhere with small changes (Figure 3.1). (Wiegand, Hilpert, and Hancock 2008)

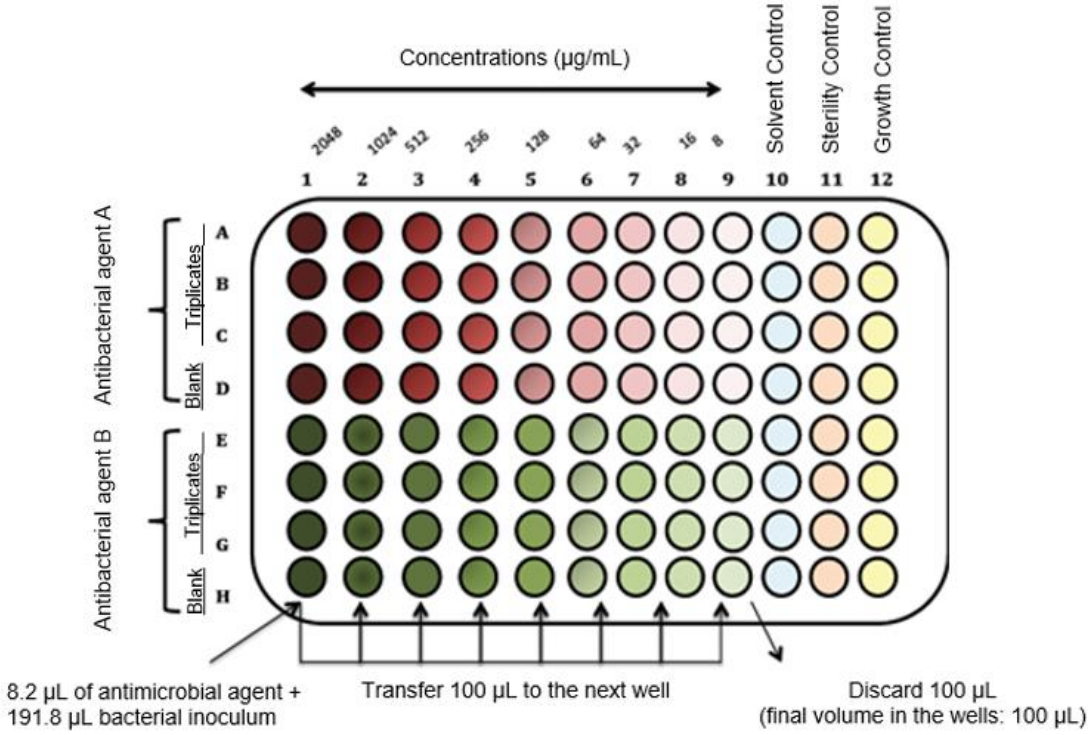

Figure 3.1. Microbroth dilution method.

In this method, a defined number of bacterial cells are incubated with the antimicrobial agent in test in a defined concentration range, by performing 1:2 serial dilutions. After the incubation period, bacterial growth, if occurring, is visible to the naked eye through turbidity of solution. However, in this study, both qualitative and quantitative methods were required to improve the results assessment, since the tested extracts added turbidity to the solution *per se*. Therefore, a qualitative method (MTT assay) was performed, in order to be possible to observe results with

naked eye. To add further precision to the assay, OD measurements (λ= 600 nm) were performed (quantitative method).

Assays were performed with bacterial cultures grown in MHA plates. Cultures were harvested from the plates with 1 mL of MHB medium, centrifuged twice at 2700 rpm, for 5 min. OD was determined at λ= 600 nm and adjusted to 0.04 (approximately 10^5 cfu/mL, previously determined by our group) in MHB medium. Bacterial incubation was done at 37ºC, 200 rpm, until exponential growth phase was reached and then, the OD was again adjusted to 0.04. In 96-well plates (Sarstedt), serial dilutions of the antibiotics were added to the previously calibrated inoculum (10^5 cfu/mL), starting with the highest concentration (2048 µg/mL) and performing serial dilutions until 8 µg/mL (Figure 3.1). Growth control (GC), sterility control (StC) and solvent control (SC) were also prepared: GC, the positive control, ensures that bacterial growth is occurring, being used to compare with bacterial growth in the presence of the antibiotics; StC, the negative control, allows to exclude contaminations in the assay; SC is used to assess if the solvent, at the maximum percentage used in the assay, influences bacterial growth. Plates were sealed with parafilm to avoid evaporation during overnight incubation (~16h) at 37°C. After, MICs were determined qualitatively through (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and quantitatively by OD measurement (λ= 600 nm) (Multiskan FC, Thermo Scientific).

MTT (Merck) is widely used to test cell metabolic activity. Metabolically active cells react with MTT, resulting in a purple colored formazam product that can be observed with unaided eye.(Riss, Niles, and Minor 2004)

The MIC determination protocol with MTT was designed and performed based on Eloff (1998). Shortly, an MTT stock solution, in a concentration of 0.2 mg/mL in BPW, sterilized with a 0.22 µm filter (VWR), was kept at -20ºC. For each assay, the stock solution was defrosted at RT and, afterwards, 40µL of the MTT solution were added to the each well in the 96-well plate. Incubation was done at 37ºC, 40 min. Assays were performed in triplicate and in three independent experiments. The lowest concentration where did not occur color change, in the three wells (triplicates of the assay), where defined as MIC of the antibacterial agent in test, in this case of the antibiotics.

3.3.2 – TAs and ENTB extract for antibacterial activity assays

For antibacterial activity assays, betulinic acid (BA) (Molekula), betulonic acid (BOA) (CHEMOS GmbH), oleanolic acid (OA) (Molekula) and ursolic acid (UA) (Molekula), were used as pure compounds, representative of the main TAs found on the ENTB extract composition.

TAs and ENTB extract stock solutions, 50 mg/mL, were prepared in absolute ethanol (99.8%; VWR Prolab), aliquoted and kept at -20°C. Before use, TAs and ENTB stock solutions were defrosted at RT. MICs were determined using the same procedure as previously described for antibiotics antibacterial activity assay (3.3.1.1).

3.3.2.1 – Minimal bactericidal concentration (MBC) determination

MBC is defined as *the lowest concentration of antimicrobial agent required to kill 99.9% of the final inoculum after incubation for 24 h.* (Balouiri, Sadiki, and Ibnsouda 2016) MBCs were determined using the spreading technique in MHA plates. MICs, previously determined, were serially diluted (10^{-1} to 10^{-4}) in buffered peptone water (BPW; Liofilchem) and 10 µL of each, as well as a non-diluted MIC (10^{0}), were plated. MHA plates were incubated at 37°C, for 24 hours, being observed afterwards if there was bacterial growth. If bacterial growth occurred, then the number of colony forming units (CFUs) was determined. MBC was subjectively defined as the lowest concentration, at which 99.9% of the final inoculum were killed.

3.3.2.2 – Time-kill assay

Time-kill assay is used to observe the evolution of the inhibitory/bactericidal effect of an antimicrobial agent over time, being based on the MIC determination (3.3.1.1). For each incubation time, a 96-well plate was prepared corresponding to the different incubation times (2h, 6h, 24h and 48h). The MIC was qualitatively determined through MTT assay (3.3.1.1) and quantitatively by OD measurement at λ= 600 nm.

3.3.2.3 – Synergistic assays (ENTB extract plus antibiotics)

In this study, four antibiotics were used: tetracycline (Duchefa Biochemie, Alfagene), ampicillin (Applichem, VWR), rifampicin (Duchefa Biochemie, Alfagene) and gentamicin (Duchefa Biochemie, Alfagene) together with ENTB extract. Microbroth dilution method was used (3.3.1.1) in a concentration range of 2 µg/mL up to 2048 µg/mL. GC and StC were also prepared. Results were obtained through MTT assay (3.3.1.1) and by OD measurement at λ= 600 nm on a plate reader (Multiskan FC, Thermo Scientific). Factorial inhibitory concentration index (FICI) was calculated to classify interaction between ENTB extract and antibiotics.(Pereira et al. 2014) Each of the combinations was calculated according to the following equation (Equation 1):

$$FICI = \frac{MIC\ (Antibiotic+ENTB\ extract)}{MIC\ (Antibiotic)} \quad (1)$$

Results were interpreted as follows: FICI ≤ 0.5 synergistic, 0.5 < FICI < 1 partially synergistic, FICI = 1 additive, 1 < FICI ≤ 4 indifferent and FICI > 4 antagonistic.

3.3.3 – CcL extract antibacterial activity assay

For the antibacterial activity assay, the pure compound cynaropicrin (Extrassynthese) was used as control of the main bioactive compound found in CcL extract.

Stock solutions of cynaropicrin and CcL extract were prepared at a concentration of 50mg/mL in DMSO (99.5%, AppliChem), aliquoted and kept at -20°C. MICs were determined using the same procedure as previously described for antibiotics antibacterial activity assay (3.3.1.1).

3.3.4 – DOP extract antibacterial activity assay

For DOP extract antibacterial activity assay, pure compounds of phenolic compounds hydroxytyrosol (H), tyrosol (T) and oleuropein (O) (Sigma-Aldrich) were used as controls of the main bioactive compounds detected in DOP extract.

Stock solutions of phenolic compounds and DOP extract were prepared at a concentration of 50 mg/mL in absolute ethanol (99.8%; VWR Prolab). Aliquots were kept at -20°C. MICs were determined as previously described (3.3.1.1).

3.4 – Quantification of reducing sugars content

3.4.1 – ENTB extract

Reducing sugars were determined by the 3,5-dinitrosalicylic (DNS) colorimetric method, according to Miller (1959). Briefly, when the DNS alkaline solution reacts with reducing sugars, it is converted into 3-amino-5-nitrosalicylic acid (orange color), being the OD of the solutions measured at λ= 540 nm.

The reaction mixture was composed of 500 µL of ENTB extract, 50 mg/mL in absolute ethanol (99.8%; VWR chemicals) and 3 mL of DNS reagent. The mixture was homogenized in a vortex (Velp Scientific) and incubated for 5 min, at 100°C in a thermic bath (WND29, Memmert). After cooling in ice, 15 mL of distilled water were added and again left to cool at RT for 15 minutes. The supernatant absorbance was measured at λ=540 nm (Helios Alpha, Thermo Scientific), against a blank composed of water and DNS reagent. Calibration curves were done using standard solutions of xylose and glycose, with concentrations between 1 - 8 mg/mL, following the same procedure previously described. Triplicates for each extract sample and standard concentration were performed.

3.4.2 – CcL extract

Concentrations of reducing sugars were determined as described for ENTB extract (3.4.1) CcL extract was dissolved in DMSO (99.5%, AppliChem) to a final concentration of 50 mg/mL.

3.4.3 – DOP extract

Reducing sugars concentration was determined as previously described for ENTB extract (3.4.1). DOP extract was dissolved in distilled water to a final concentration of 50 mg/mL.

3.5 – Formulation

3.5.1 – Formulation design

Formulations design and characterization were performed at Faculdade de Farmácia of Universidade de Lisboa (FFUL), Lisboa, Portugal. Further adjustments in the formulation composition were executed at CEBAL, according to the obtained antimicrobial activity results.

Preformulation tests started by dissolving one of the extracts, the ENTB extract, in absolute ethanol (99.8%, VWR Prolab), and then different excipients were added under stirring, in order to confer physical stability to the formulation. The excipients tested were: Emulcin® (Bial), propylene glycol (Merck) and TAGAT®CH40 (Evonik). Ascorbic acid was added to prevent oxidation and potassium sorbate as a preservative (Table 3.2). Finally, purified water obtained by reverse osmosis (Millipore, Elix 3) was added Qs 100%.

After preparation, the solution was stored in dark flasks at 4ºC.

Table 3.2. Ingredients of the initial formulation.

Ingredients	Concentration (% w/w)
ENTB extract	0.1
Ethanol	4
TAGAT®CH40	5
Ascorbic acid	0.2
Potassium sorbate	0.1
Purified water	Qs 100

3.5.2 – Formulation characterization

Formulation characterization was assessed in different parameters. Stability tests were performed at both RT (approximately 25°C) and 4°C. The more relevant parameters were evaluated at predetermined time points.

3.5.2.1 – Organoleptic characteristics

The operator performed macroscopic appearance and sensorial analysis.

3.5.2.2 – Identification and quantification of the active substances

TAs quantification (BA, BOA, UA and OA) was performed with a Dionex UHPLC UltiMate 3000 system. A LiChrospher® RP-18 column 5 µm particle size (Merk Millipore) was used at 20°C with a flow rate of 1 mL/min using an isocratic elution. The mobile phase was composed 20% by water (HPLC grade): 80% acetonitrile (%v/v) (Fisher Scientific, HPLC grade). The injection volume of the sample was 50 µL and the UV/Vis detection was performed at λ= 204 nm. Calibration curves were obtained using TAs standard solutions, between 0.2 and 1 mg/mL. According to the results obtained (4.4.1.1), the quantification was performed in Formulation C (0% of TAGAT®CH40) samples containing higher concentration (0.7%) than those in formulation (0.1%), due to the equipment limit of detection.

3.5.2.3 – pH determination

The formulation pH was evaluated by potentiometry (Metrohm pH Meter 744 with a glass electrode; Herisau, Switzerland).

3.5.2.4 – Rheology

The rheology study was performed using a controlled shear-strain rheometer (Malvern Kinexus lab+, England) connected to a refrigeration circuit with controlled temperature. Flow curves were generated by ramping the controlled shear rate from 10 and 200 s^{-1}. All tests were carried out on, approximately, 5 mL samples, at 20ºC using a 2°/60 mm cone.(Dias *et al*. 2016) Determinations were done in triplicates. The obtained flow curves were evaluated according to Newton's Law (Equation 2):

$$\tau = \mu \times \dot{\gamma} \qquad (2)$$

Were τ refers to shear stress (Pa), μ refers to dynamic viscosity (Pa.s) and $\dot{\gamma}$ shear rate (s^{-1}).

3.5.2.5 – Microbiological assay

The microbiological assay was performed through the spread plate technique. Briefly, 10 µL of serial dilutions of the formulation (10^{-1} to 10^{-4}), as well as a non-diluted sample (10^{0}), were plated in TSA plates. Plates were incubated at 37°C for 24h, being the results observed afterwards.

3.5.3 – Antimicrobial activity of the formulations

The antimicrobial activity of the formulation(s) was evaluated according to the microbroth dilution method, previously described (3.3.1.1), using a formulation % range from 5.6 % up to 90

%. SC, GC and StC were also performed. Results were determined through OD measurement, at λ= 600 nm, on a plate reader (Multiskan FC, Thermo Scientific).

3.6 – Statistical analysis

All parameters measured were performed at least in duplicate and analyzed using the PROC GLM option of SAS (SAS Institute Inc., Cary, NC, USA). Values of $p < 0.05$ were considered statistically significant. Least square means and standard deviation are presented in tables, except for ENTB and CcL extracts antibacterial effect, which least square means and standard deviations are presented in figures (Figure 4.2 and Figure 4.5).

4 – Results & Discussion

4.1 – Plant material extraction

DOP extraction was performed in triplicate, being the total phenolic content in the extract determined for each replica. Gallic acid was used as standard and results are expressed as milligram of Gallic acid per gram of dry weight of extract and biomass (mg GAE/g DW). Results concerning the extraction yield and the total phenolic content, in extract and biomass, are presented in Table 4.1.

Table 4.1. DOP extraction yield (%) and total phenolic content expressed as milligrams of Gallic acid (GAE) equivalents per gram of dry weight (DW). Values are expressed as mean ± standard deviation of triplicates.

	Total phenolic content (mg GAE/g DW)	
Extraction yield (%)	Extract	Biomass
17.32 ± 3.04	87.91 ± 7.74	17.55 ± 2.06

In comparison to the results obtained by Ramos *et al*. (2013), the present extraction yield was 1.62 times lower. However, the total phenolic content in the present extraction was similar, as expected. (Ramos *et al.* 2013)

Phenolic content in hydroxytyrosol, tyrosol and oleuropein, the ones of interest in the present work, was determined for each extract replica and in threefold. This quantification was performed to determine if the quantities of hydroxytyrosol, tyrosol and oleuropein present in the extract are sufficient to confer significant anti-MDRB activity to the extract. Results are presented in Table 4.2.

Table 4.2. Extract phenolic content in hydroxytyrosol, tyrosol and oleuropein (DW) and its respective retention times. Values are expressed as the mean ± standard deviation of triplicates

	Phenolic content (mg/g DW)		Retention time (min)
	Extract	Biomass	
Hydroxytyrosol	25.39 ± 1.08	6.93 ± 0.81	5.62
Tyrosol	1.93 ± 0.28	0.60 ± 0.06	6.87
Oleuropein	3.35 ± 0.28	0.58 ± 0.11	10.61

DOP extract presented higher content in hydroxytyrosol, followed by tyrosol and oleuropein. These phenolic compounds represent 24.11, 1.83 and 2.23 %, respectively, of the total phenolic content in DOP extract; and 43.59, 3.80 and 3.68 %, respectively in biomass.

In the present work, hydroxytyrosol, tyrosol and oleuropein concentrations are similar to those reported by Ramos *et al*. (2013), both in extract and biomass, as expected, since the same methodology was applied.

4.2 – Antibacterial activity assays

4.2.1 – Antibiotics

The antibiotics MICs were determined by microbroth dilution method, in a concentration range between 8 and 2048 µg/mL. Microbroth dilution method was chosen because it is more accurate and easier to perform than other methods described for antibacterial activity assays, such as agar dilution and macrobroth dilution methods. (Balouiri, Sadiki, and Ibnsouda 2016) In agar dilution method, an impregnated disc with the antibacterial agent is used, being then the agent diffused to the media, where bacteria were previously plated and, after incubation, the diameter of the inhibition halo is measured in mm. (Balouiri, Sadiki, and Ibnsouda 2016) This method is simple and cheap, however it is not the most accurate for MICs determination, since it is not possible to quantify the amount of the antimicrobial agent diffused into the agar medium. (Balouiri, Sadiki, and Ibnsouda 2016) The main advantages of microbroth dilution compared to the macrobroth dilution method are the lower volumes of antibacterial agents and reagents required to perform the assay, as well as the higher reproducibility and the possibility to perform several replicates or test more than one antibacterial agent at the same time, making this method easier to perform, economical and less time consuming. (Balouiri, Sadiki, and Ibnsouda 2016)

MICs for gentamicin, rifampicin, ampicillin and tetracycline against MDRB are presented in Table 4.3.

Table 4.3. MIC of antibiotics against MDRB

Bacteria	**MIC (µg/mL)**			
	Gentamicin	Rifampicin	Ampicillin	Tetracycline
Escherichia coli ATCC 25922	< 8	32	1024	< 8
Escherichia coli OXA	512	128	>2048	16
Escherichia coli TEM 180	< 8	32	>2048	< 8
Klebsiella pneumoniae *	256	512	>2048	< 8
Citrobacter freundii *	< 8	64	512	< 8
Salmonella enterica *	< 8	< 8	64	< 8
Pseudomonas aeruginosa PAO1	< 8	64	>2048	32
Staphylococcus saprophyticus *	< 8	< 8	>2048	< 8
Staphylococcus epidermidis *	< 8	< 8	256	16
Staphylococcus aureus ATCC 43300	32	< 8	256	< 8
Staphylococcus aureus ATCC 6538	32	< 8	256	< 8

* clinical isolates

Tetracycline was the antibiotic to which higher susceptibility was demonstrated, with 8/11 (72%) of the tested bacteria presenting a MIC inferior to 8 µg/mL. To gentamicin and rifampicin, 7/11 (64%) and 5/11 (45%), respectively, were susceptible to concentrations lower than 8 µg/mL. Ampicillin was the less effective antibiotic, with 6/11 (55%) presenting MIC values between 64

and 1024 µg/mL. *Salmonella enterica* was the most sensitive to the antibiotics assayed, with maximum MICs of 64 µg/mL.

Ampicillin, a molecule considered to be one of the most successful *β*-lactamic, since it has multiple PBP targets,(Lewis 2013) showed less effectiveness against the tested bacteria, due to the intrinsic resistant mechanisms of the Gram-negative bacteria.(Lewis 2013) *Pseudomonas aeruginosa* had a MIC to this therapeutic agent higher than 2048 µg/mL, as expected, since this Gram-negative bacterium, in addition to the expression of active efflux pumps, is capable of producing *β*-lactamases that hydrolyze ampicillin. (Tenover 2006) *Staphylococcus* spp. are known to acquire resistance mechanisms through staphylococcal cassette chromosome *mec* (SCC*mec*), most commonly *S. aureus*. (Higashide et al. 2008) However, although rare, it has been reported antimicrobial resistance to ampicillin in *S. saprophyticus*. (Higashide et al. 2008) This might explain the result obtained for this strain (MIC >2048 µg/mL), which probably acquired this resistance mechanism, as well as the other *Staphylococcus* strains, to which ampicillin exhibited less efficacy (MIC= 256 µg/mL). This observation corroborates that, like other antibiotics, there is a tendency to lose efficacy over time, due to selective pressure exerted onto MDRB, translated in expression of more/novel resistance mechanisms. (Rossolini et al. 2014)

Gentamicin, a bactericidal antibiotic from the aminoglycosides class, has been used over the past 20 years, being recommended for short-term and prolonged therapy, in most cases for serious Gram-negative infections. (Avent et al. 2011) However, the number of gentamicin-resistant strains isolated have been increasing over the past 10 years, such as MDR *P. aeruginosa*, *K. pneumoniae*, *E. coli* and *Acinectrobacter* spp.. This might be explained by gentamicin constant misuse over the years.(Direção-Geral de Saúde 2014) In the present study is possible to verify less effectiveness of gentamicin against *E. coli* and *K. pneumonia*. This has already been reported by Magalhães *et al.* (2005). in *E. coli* and by Johnson *et al.* (1994) in *K. pneumoniae*, both associated with the enzymes 3-*N*-aminoglycoside acetyltransferases (AAC(3)) action of inactivate gentamicin. (Johnson, A.P., Burns, L., Woodford, N., Threlfall, E. J., Naidoo, J., Cooke, E. M. and George, R.C. 1994; Magalhães and Blanchard 2005) For *S. aureus* strains it was also observed less effectiveness of gentamicin, with MICs of 32 µg/mL, a higher value than the ones reported for MRSA, in the range of 16 and 24 µg/mL. (Vázquez et al. 2016) For *S. aureus* ATCC 6538 this value is higher than expected, probably because of features at cellular level, such as denser peptidoglycan layers.

Tetracycline mechanism of action is similar to gentamicin, but has improved characteristics that enable its penetration in Gram-negative bacteria, making it more effective, as observed in the present results. (Chopra and Roberts 2001) It is also one of the most common alternative antibiotic used for penicillin-allergic patients. (Rubio-López et al. 2012) *P. aeruginosa* presents the higher MIC for tetracycline, which can be explained by the intrinsic mechanism of efflux pumps that actively pumps out this antibiotic. (Li, Livermore, and Nikaido 1994)

Rifampicin is described to be one of the most broad-spectrum antibiotics, used as first-line therapy for *Mycobacteria tuberculosis*. (Alifano *et al*. 2015; Kohanski, Dwyer, and Collins 2010) As also being reported, rifampicin is considered more effective against Gram-positive bacteria,

achieving bactericidal effect in contract to the bacteriostatic action against Gram-negative bacteria. This difference is observed in the present results, being explained by the lower uptake of the molecule. (Kohanski, Dwyer, and Collins 2010) Considering the obtained results, rifampicin has demonstrated considerably MICs against Gram-negative bacteria. The emergence of resistance to Gram-negative bacteria has already been reported, (Goldstein 2014) and it constitutes a problem since it is a specific antibiotic for tuberculosis treatment. This translates the misuse of rifampicin for other therapies.

After defining the antibiotics MIC, the antibacterial activity for each extract, and its representative bioactive compound(s), were tested against MDRB, in the same concentration range.

4.2.2 – ENTB extract

4.2.2.1 – MIC determination

MICs were determined for ENTB extract and for TAs standard compounds against MDRB. Results are presented in Table 4.4.

Table 4.4. MICs of ENTB extract and standard compounds of TAs: betulinic acid (BA), betulonic acid (BOA), oleanolic acid (OA) and ursolic acid (UA), against MDRB

	MIC (µg/mL)				
Bacteria	**ENTB extract**	**BA**	**BOA**	**OA**	**UA**
Escherichia coli ATCC 25922	>2048	>2048	>2048	>2048	1024
Escherichia coli OXA	>2048	>2048	>2048	>2048	1024
Escherichia coli TEM 180	>2048	>2048	>2048	>2048	1024
*Klebsiella pneumoniae**	>2048	1024	>2048	1024	512
*Citrobacter freundii**	>2048	1024	>2048	1024	512
*Salmonella enterica**	>2048	1024	>2048	1024	512
Pseudomonas aeruginosa PAO1	>2048	1024	>2048	1024	1024
*Staphylococcus saprophyticus**	>2048	1024	>2048	1024	512
*Staphylococcus epidermidis**	128	512	>2048	1024	256
Staphylococcus aureus ATCC 43300	64	512	>2048	512	128
Staphylococcus aureus ATCC 6538	64	512	>2048	1024	256

* clinical isolates

Regarding TAs antibacterial activity, most bacteria were susceptible to BA, OA and UA, with MIC ranging from 512 and 1024 µg /mL for BA and OA, and 128 and 1024 µg/mL for UA. BOA did not presented antibacterial activity against this bacterial panel and in the range of concentrations tested. Previous studies regarding anti-MDRB activity of BA, UA and OA can be found in literature. A study performed by Fontanay *et al*.(Fontanay et al. 2008), using approximately the same conditions as the ones of the present work, studied the antibacterial

activity of UA, OA and BA against ATCC strains and clinical isolates. (Fontanay *et al.* 2008) Results showed that UA and OA presented better antibacterial performance against Gram-positive bacteria, exhibiting MIC against *S. aureus* ATCC 29213 of 8 µg/mL and 32 µg/mL and against *S. aureus* ATCC 25923 of 8 µg/mL and 64 µg/mL, respectively. However they were not effective against clinical isolates, at the concentration range tested, which was to a maximum of 256 µg/mL, while BA was devoid of any antibacterial activity against ATCC strains and clinical isolates. (Fontanay *et al*. 2008) Results obtained in the present work can overcome these results, where the concentrations range was higher, making it possible to determine the MIC for at least eight bacteria. Although MICs obtained were higher than the ones reported for UA and OA, these can be explained by the fact that *Staphylococcus* strains tested were different and so its characteristics, which may be linked to less susceptibility for these compounds. Nonetheless, the results obtained describe a good antibacterial activity, but the possible cytotoxicity factor should be kept in mind.

Other studies have reported the extraction and isolation of these TAs from plant material and respective evaluation of their antibacterial activity in comparison to the commercially available ones. Differences between commercial (synthetized) and natural compounds (directly isolated from the plant) were observed, wherein isolated BA, UA and OA had higher antibacterial effect, with lower MIC, against ATCC strains and clinical isolates, than the commercially available forms. According to Nascimento *et al*.(2014), UA has higher effect against *E. coli* ATCC 25922 and *S. aureus* ATCC 6538, both also used in the present work, showing MIC of 64 µg/mL and 32 µg/mL, respectively. These results suggest that compounds extracted directly from plants have better performance against bacteria, probably due to the synthesis of these compounds as defense mechanisms of plants, which can be an explanation for the higher TAs MICs values obtained in the present work. BOA antibacterial activity is rarely reported in literature, showing inhibitory effect against *E. faecalis* (74% of inhibition) and *S. aureus* (51% of inhibition), which agrees with the obtained data.(Haque *et al.* 2014)

The screening highlighted three Gram-positive bacteria as susceptible to ENTB extract: *S. epidermidis*, *S. aureus* ATCC 43300 and *S. aureus* ATCC 6538, with MIC of 128, 64 and 64µg/mL, respectively (Figure 4.1). For the last two strains, the results were considered as promising, since the MIC is very close to the one determined for the broad-spectrum antibiotics used in clinical field (MIC=32 µg/mL).

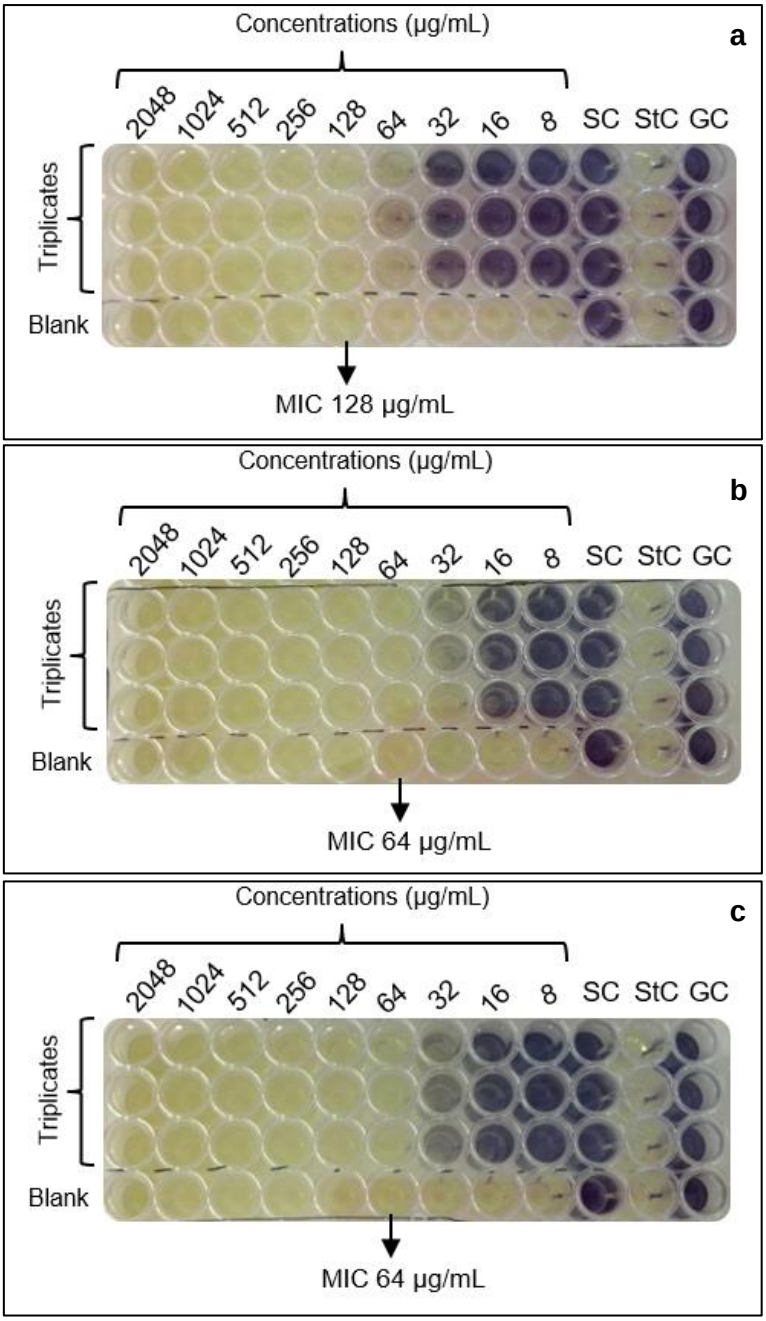

Figure 4.1. MTT assay results for ENTB extract against *Staphylococcus* spp.: a – *S. epidermidis* with MIC of 128 µg/mL; b – *S. aureus* ATCC 43300 with MIC of 64 µg/mL; c – *S. aureus* ATCC 6538 wth MIC of 64 µg/mL.

ENTB extract effect is higher than that of TAs alone, which may be explained by synergistic or additive effect of the compounds in the extract. Different bioactive compounds in a mixture can interact to provide a combined effect, which is similar to the sum of the effects of the individual components (additive) or, the combination of bioactive compounds can exert a higher effect than the sum of the individual components (synergistic). (Ginsburg and Deharo 2011) In the present extract, TAs content was assessed and results showed that 350.68 mg of TAs are present *per* g of ENTB extract, more specifically 87.14 ± 13.15 mg of BOA/g of extract, 40.37 ± 8.42 mg of BA/g of extract, 116.17 ± 10.03 mg of OA/g of extract and 107.01 ± 9.99 mg of UA/g of extract.

Regarding these results and the MICs obtained, it is possible to verify that 112.22 µg/mL of TAs are linked to *S. aureus* ATCC 43300 and *S. aureus* ATCC 6538 growth inhibition (specifically, 27.88 µg /mL of BOA, 12.92 µg /mL of BA, 37.17 µg /mL of OA and 34.24 µg /mL of UA) and 224.44 µg/mL for *S. epidermidis* growth inhibition (specifically, 55.77 µg /mL of BOA, 25.84 µg /mL of BA, 74.35 µg /mL of OA and 68.49 µg /mL of UA).

The effect of ENTB extract in bacterial cell growth was assessed and results are represented in Figure 4.2.

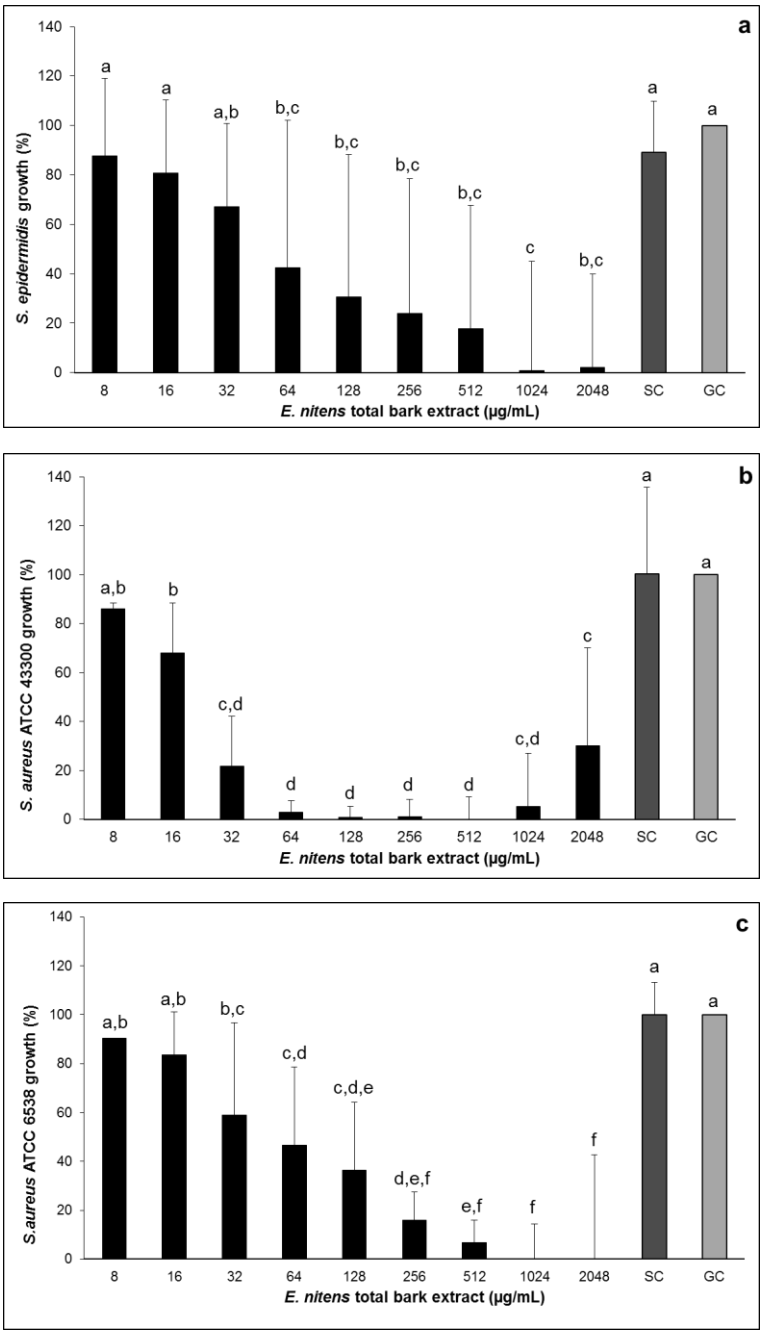

Figure 4.2. ENTB extract antibacterial effect in bacteria cell growth, in comparison to growth control (GC): a – *S. epidermidis*; b – *S. aureus* ATCC 43300; c – *S. aureus* ATCC 6538. Each value is expressed as mean ± standard deviation of triplicates. In each column, different letters mean significant differences ($p < 0.05$).

S. epidermidis and *S. aureus* ATCC 6538 presented a gradual susceptibility to ENTB extract, *i.e*. as they were exposed to higher extract concentration, bacterial cell growth decreased. For *S. epidermidis*, ENTB extract exerted a higher statistically significant effect when compared to GC, starting at 64 µg/mL. For *S. aureus* ATCC 43300, the antibacterial effect is statistically significant from concentrations as low as 32 µg/mL, when compared to GC.

ENTB extract presented a gradual antimicrobial effect against *E. coli* ATCC 25922, between 512 and 2048 µg/mL (statistically significant different from GC), reaching maximum effect at 2048 µg/mL, with 72% of growth inhibition. For *S. enterica*, similar behavior was observed, with 38% of inhibition achieved at 512 µg/mL and the maximum at 2048 µg/mL (78% of growth inhibition).

The remaining MDRB tested did not present statistically significant differences after exposure to ENTB extract when compared to GC bacteria (without exposure to bioactive(s)).

Regarding these results, ENTB extract was chosen to be included in the formulation.

In what concerns other antibacterial activity studies described in literature and, to the best of our knowledge at the present time, there are no studies using ENTB extract. Outer bark extract of *E. nitens* was studied regarding its antibacterial activity in our research group, and results showed MIC ranging from 64 µg/mL for *S. aureus* strains to 512 µg/mL for *E. coli* and *P. aeruginosa*. (Parreira *et al.* 2015) These results show that the *E. nitens* outer bark and the total bark extracts possess similar antimicrobial activity against MRSA. However, the *E. nitens* outer bark extract has better performance against Gram-negative bacteria, which might be explained by the higher content in TAs. (Parreira *et al.* 2015)

TAs antibacterial mechanism of action is not yet fully disclosed, but one hypothesis is that the antibacterial performance might be linked to the compounds lipophilic nature. (Barreto *et al.* 2014) Due to this, TAs interaction with the hydrophobic sites on the plasma membrane and on the outer membrane of Gram-negative bacteria is facilitated, causing changes in the bacterial membrane namely: increase of permeability; loss of integrity; loss of cytoplasmic content; dissipation of the proton-motive force; lysis and cell death. (Barreto *et al.* 2014) Also, it is thought that OA and UA might have PBP as target, which is involved in the peptidoglycan synthesis, due to the higher susceptibility of Gram-positive bacteria to these compounds, when compared with Gram-negative bacteria. (Kurek *et al.* 2012)

In order to assess if the ENTB extract MIC and/or the concentration immediately above had bacteriostatic or bactericidal effect, MBCs were determined.

4.2.2.2 – MBC determination

ENTB extract presented MIC for the following MDRB: *S. epidermidis*; *S. aureus* ATCC 43300 and *S. aureus* ATCC 6538 and therefore, MBCs were determined for these bacteria. Results are presented in Table 4.5.

Table 4.5. MBC of ENTB extract against MDRB

Bacteria	**MBC (µg/mL)**
*S. epidermidis**	>2048
S. aureus ATCC 43300	256
S. aureus ATCC 6538	>2048

* clinical isolates

In the present assay, it was possible to assess if ENTB extract exerted bactericidal or bacteriostatic effect at the MIC. Results showed that ENTB extract has bacteriostatic effect against *S. epidermidis* and *S. aureus* ATCC 6538 at the MIC., which means that ENTB extract inhibits bacterial growth of these strains, keeping them in the stationary phase of growth. Bactericidal effect was observed for *S. aureus* ATCC 43300, at 256 µg/mL. This result means that ENTB extract exerts bacteriostatic effect at the MIC but is also capable of killing at concentrations above.

Regarding the study of Parreira *et al*.(2015), referenced above, using *E. nitens* outer bark extract, the MBC obtained in the present work for *S. aureus* ATCC 43300 is higher, which suggests less bactericidal effect of ENTB extract. This result can be explained by the higher content of TAs in the outer bark extract, translated in higher antibacterial effectiveness. (Parreira et al. 2015)

After MICs and MBCs determination, time-kill assay was performed.

4.2.2.3 – Time-kill assay

Time-kill assay was performed only for susceptible bacteria to ENTB extract: *S. epidermidis*, *S. aureus* ATCC 43300 and *S. aureus* ATCC 6538 (Figure 4.3). The assay was performed at 2, 6, 24 and 48 hours of exposure to ENTB extract, in the same range of concentrations used for MIC determination (8 µg/mL - 2048 µg/mL). MICs for ENTB extract were again assessed for each bacteria and, while performing this assay, changes to the previously determined MICs were observed for *S. epidermidis* and *S. aureus* ATCC 6538, 2048 µg/mL to 128 µg/mL and 1024 µg/mL to 64 µg/mL, respectively. A possible explanation for this observation might be related to TAs degradation in the ENTB extract during storage, which would explain the decrease in antibacterial activity. However, and because for one of the tested bacteria the same MIC was determined and concordant to the previously obtained, changes regarding the bacterial strains might also have occurred in what concerns susceptibility. Therefore, sub-MIC ENTB extract

concentrations of 1024 µg/mL, 32 µg/mL and 512 µg/mL were chosen to evaluate bacteriostatic/bactericidal effect against *S. epidermidis*, *S. aureus* ATCC 43300 and *S. aureus* ATCC 6538, respectively.

Assays were performed in triplicates. Results for sub-MIC were used to evaluate behavior along the time (Figure 4.3).

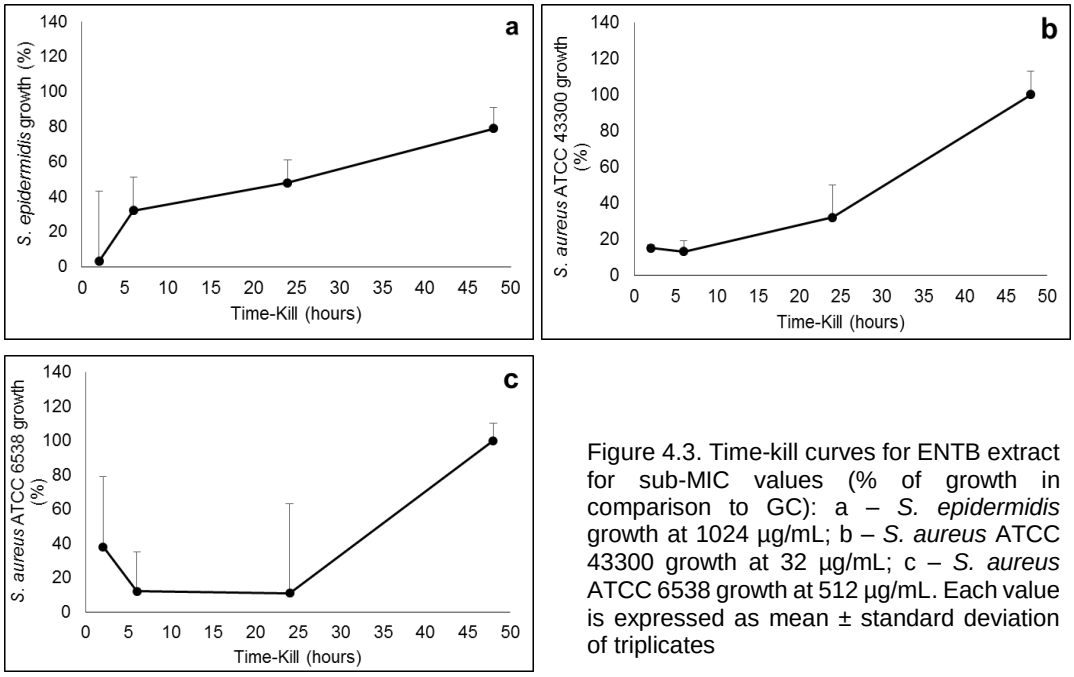

Figure 4.3. Time-kill curves for ENTB extract for sub-MIC values (% of growth in comparison to GC): a – *S. epidermidis* growth at 1024 µg/mL; b – *S. aureus* ATCC 43300 growth at 32 µg/mL; c – *S. aureus* ATCC 6538 growth at 512 µg/mL. Each value is expressed as mean ± standard deviation of triplicates

The 3 tested *Staphylococcus* spp. strains showed different behavior. *S. epidermidis* cell growth gradually increased over time, starting with 3% until 79%, at 48h, which means that ENTB extract exerts higher effect up to 2h of exposure, decreasing after that. Inhibition of *S. aureus* ATCC 43300 was higher until 6h of exposure, with only 13% of bacterial growth, but afterwards the antibacterial effect was gradually lost, being residual after 48h. Lastly, ENTB extract inhibitory effect against *S. aureus* ATCC 6538 was more pronounced between 6h and 24h of exposure, with only 11% of bacterial growth, but an accentuated increase in bacterial growth occurred next, being this trend visible up to 48h.

These results were expected since ENTB extract exerts bacteriostatic effects against *S. aureus* ATCC 6538 and *S. epidermidis*, as determined by the MBC assay, meaning that bacterial growth is only temporarily inhibited by the extract and cell death is not induced. Nonetheless, the bacteriostatic effect of ENTB extract is maintained until 24h, being then required a new exposition to ENTB, in order to maintain bacterial growth at minimum levels.

Although ENTB extract had bactericidal action against *S. aureus* ATCC 43300 at 256 µg/mL, at a sub-MIC concentration (32 µg/mL), this effect was not observed. However, when evaluating MIC and MBC over time for this *Staphylococcus* strain, it was possible to observe that the inhibitory and bactericidal effect was maintained until 48h of exposure, highlighting ENTB potential against *S. aureus* ATCC 43300. This *S. aureus* strain is the most used in antibacterial

studies as reference strain to MRSA. On the other hand, *S. aureus* ATCC 6538 is used as strain reference for Methicillin-sensitive *Staphylococcus aureus* (MSSA), so it was expected that this strain would be more susceptible to ENTB extract than *S. aureus* ATCC 43300, which was not observed. This difference could be related to the TAs mechanism of action at the cellular level against *S. aureus* strains.

Studies related with effectiveness of antibacterial agents over time are widely reported in literature. However, exposure time to the antibacterial agents generally does not exceed 24h, being the ones using 48h rare. In the present study, the exposure time was longer, in order to further evaluate the effectiveness and potential of ENTB extract. In what concerns TAs antibacterial effectiveness over time, it is reported that OA and UA lose their antibacterial effect after 2h of exposure, against both *S. aureus* and *S. epidermidis*, using sub-MIC concentrations. (Kurek *et al.* 2012) A study by Wang *et al*. (2016) demonstrated antibacterial effectiveness of UA over time, at sub-MIC concentration against MSSA (MIC of 16 µg/mL) and MRSA (MIC of 64 µg/mL), showing the results that the antibacterial effect is lost after 6h of exposure, for both bacteria, which is in agreement with data obtained in the time-kill assay. (Wang *et al.* 2016)

4.2.2.4 – Synergistic activity (antibiotic plus extract)

Synergistic activity between ENTB extract and antibiotics was tested to assess if the ENTB extract might be used as a coadjuvant to broad-spectrum antibiotics, maximizing their effect. Rifampicin, gentamicin, ampicillin and tetracycline, were tested against *S. epidermidis*, *S. aureus* ATCC 43300 and *S. aureus* ATCC 6538. MICs for antibiotics were again determined. Factorial inhibitory concentration index (FICI) was determined to classify interaction between ENTB extract and antibiotics. Results are shown in Table 4.6.

Table 4.6. MIC (expressed in µg/mL) of antibiotic, ENTB extract (MIC) and ENTB extract together with antibiotics (MIC[a]) against *Staphylococcus* spp. Factorial inhibitory concentration index (FICI) is shown for all the mixtures. Interactions are expressed as synergistic (S), partially synergistic (PS), additive (Ad), indifferent (I) or antagonist (A).

		Bacteria		
		*S. epidermidis**	*S. aureus* ATCC 43300	*S. aureus* ATCC 6538
ENTB extract	MIC	2048	64	1024
Rifampicin	MIC	32	16	64
	MIC[a]	8	<2	64
	FICI	0.25 (S)	<0.125 (S)	1 (Ad)
Gentamicin	MIC	512	>256	32
	MIC[a]	256	16	16
	FICI	0.5 (S)	<0.06 (S)	0.5 (S)
Ampicillin	MIC	>2048	128	256
	MIC[a]	>2048	64	>2048
	FICI	>1 (I/A)	0.5 (S)	>8 (A)
Tetracycline	MIC	32	>256	16
	MIC[a]	16	16	16
	FICI	0.5 (S)	<0.06 (S)	1 (Ad)

* clinical isolate

Regarding FICI values, calculations were performed according to Equation 1 (3.3.2.3) and interpreted as: synergistic, if FICI ≤ 0.5; partially synergistic, if 0.5 < FICI < 1; additive, if FICI = 1; indifferent, if 1 < FICI ≤ 4 and antagonistic, if FICI > 4.

Synergy was observed for the majority of antibiotics when in combination with ENTB extract against MDRB, and a particularly high synergistic effect was observed against *S. aureus* ATCC 43300. *S. epidermidis* also presented synergistic effect when using ENTB extract in combination with rifampicin, gentamicin and tetracycline. For ampicillin+ENTB extract against *S. epidermidis*, results are inconclusive, since the MIC is >2048 µg/mL. Finally, for *S. aureus* ATCC 6538 only gentamicin+ENTB extract presented synergistic effect, while the remaining mixtures presented antagonistic effect. Since TAs structures are quite different from those of antibiotics, the mechanism of action and/or target may be different and, therefore, other pathways/targets might be involved in bacterial inhibition.

As already described, natural compounds are good candidates to be used in drug combinations, achieving favorable results such as enhanced efficacy, decrease dosage and delayed development of drug resistance. (Ginsburg and Deharo 2011) Previous studies reported excellent outcomes when using TAs in combinations with antibiotics. A synergistic study, performed by Wang *et al*.(2016), showed that UA and OA had a synergistic effect when combined

with ampicillin and tetracycline against MRSA and MSSA (Wang et al. 2016). A study performed by Chung *et al*.(2011) reported synergistic effect between BA and methicillin against *S. aureus* ATCC 43300. (Chung, Navaratnam, and Chung 2011) The present study suggests that ENTB extract might be used as coadjuvant or as part of drug combinations, due to the synergistic effect observed. These are promising results and in the future, it would be interesting to assess effectiveness over time (time-kill assay) of the combinations used.

4.2.3 – *Cynara cardunculus* leaf (CcL) extract

MICs were determined for CcL extract against MDRB and results are represented in Table 4.7.

Table 4.7. CcL extract MIC against MDRB.

Bacteria	**MIC (µg/mL)**
Escherichia coli ATCC 25922	>2048
Escherichia coli OXA	>2048
Escherichia coli TEM 180	>2048
*Klebsiella pneumoniae**	>2048
*Citrobacter freundii**	>2048
*Salmonella enterica**	2048
Pseudomonas aeruginosa PAO1	>2048
*Staphylococcus saprophyticus**	>2048
*Staphylococcus epidermidis**	>2048
Staphylococcus aureus ATCC 43300	2048
Staphylococcus aureus ATCC 6538	>2048

* clinical isolates

CcL extract only presented inhibitory effect against *S. aureus* ATCC 43300 and *S. enterica*, with MIC of 2048 µg/mL, for both (Figure 4.4). As previously determined, the major bioactive compound in CcL extract, cynaropicrin, represents 455.2 mg/g of extract and has a MIC against *S. aureus* ATCC 43300 of 128 µg/mL. (Patrícia Alexandra Bogango Ramos 2015) At the MIC, 182.1 µg of cynaropicrin are present, and, therefore, a better antibacterial performance was expected, since the MIC obtained, 2048 µg/mL, is 16 times higher. A possible explanation for this might be the fact that cynaropicrin antibacterial activity may be partially or completely inhibited by other extract compounds, in an antagonistic effect behavior.

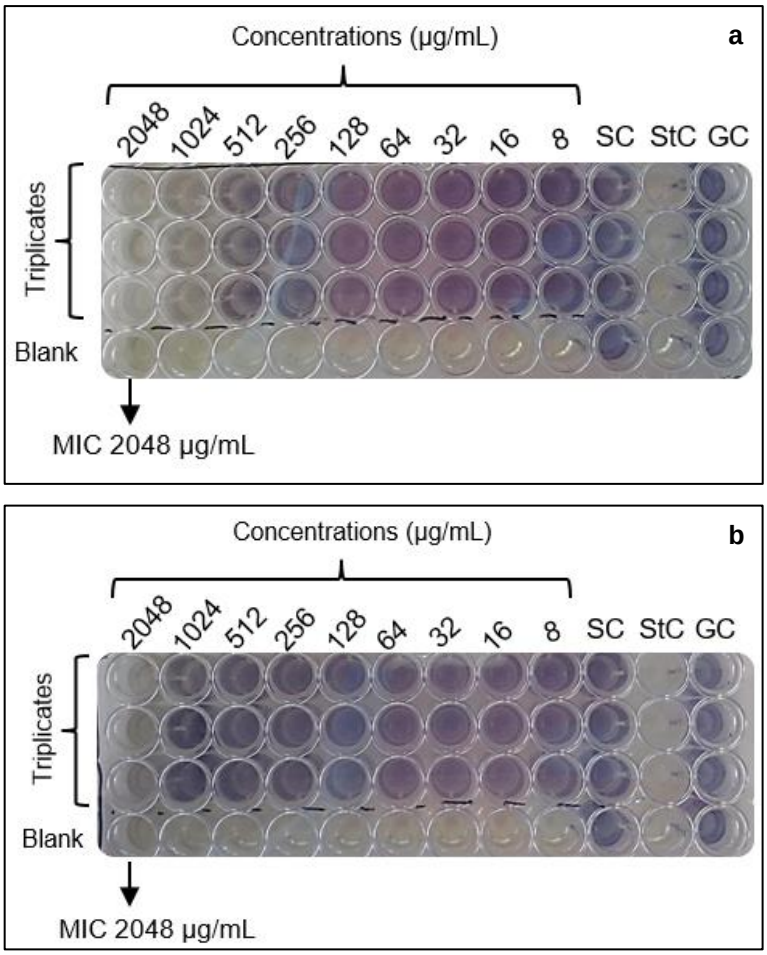

Figure 4.4. MTT assay results for CcL extract: a – *S. aureus* ATCC 43300 with MIC of 2048 µg/mL; c – *S. enterica* with MIC of 2048 µg/mL.

The inhibitory effect of CcL extract against *S. aureus* ATCC 43300 is represented in Figure 4.5.

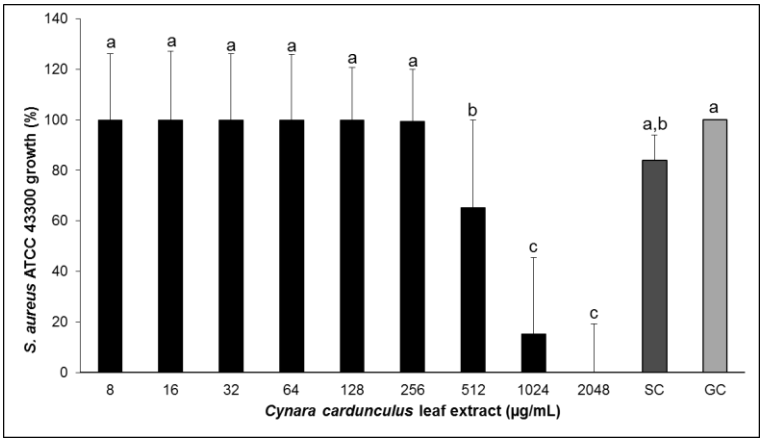

Figure 4.5. CcL extract antibacterial effect against *S. aureus* ATCC 43300. Results are expressed in comparison to growth control (GC). Each value is expressed as mean ± standard deviation of triplicates. In each column, different letters mean significant differences ($p < 0.05$) between different samples (Tuckey's test).

CcL extract presented a gradual inhibitory effect, beginning this effect to be statistically different from GC at 256 µg/mL, but the maximum inhibitory effect was obtained at 2048 µg/mL.

No inhibitory effect of CcL extract against the remaining tested bacteria was observed, exception made for *S. enterica* (Table 4.8).

Studies focusing on the use of CcL extract and its antibacterial activity, using the microbroth dilution method, are rare in the literature. In a recent study, the CcL extract antibacterial activity against *P. aeruginosa* and MRSA was determined, being determined that it was effective against MRSA at 1024 µg/mL, a MIC lower than the one obtained in the present work. (Patrícia Alexandra Bogango Ramos 2015) This result may be related to differences in strains characteristics, which confers to *S. aureus* ATCC 43300 less susceptibility.

Given that just two bacteria were susceptible to CcL extract and with a MIC of 2048 µg/mL, a relatively high value, CcL extract was not chosen to undergo further studies regarding the formulation design.

4.2.4 – DOP extract

MICs were determined both for DOP extract and for the standard phenolic compounds against MDRB. Results are presented in Table 4.8.

Table 4.8. MIC for DOP extract and for standard compounds of phenolic compounds hydroxytyrosol (H), tyrosol (T) and oleuropein (O).

Bacteria	**MIC (µg/mL)**			
	DOP extract	**O**	**HT**	**T**
Escherichia coli ATCC 25922	>2048	>2048	>2048	>2048
Escherichia coli OXA	>2048	>2048	>2048	>2048
Escherichia coli TEM 180	>2048	>2048	1024	>2048
Klebsiella pneumoniae *	>2048	>2048	1024	>2048
*Citrobacter freundii**	>2048	>2048	>2048	2048
*Salmonella enterica**	>2048	>2048	n.d.	>2048
Pseudomonas aeruginosa PAO1	>2048	>2048	n.d.	>2048
*Staphylococcus saprophyticus**	>2048	>2048	n.d.	2048
*Staphylococcus epidermidis**	>2048	>2048	1024	2048
Staphylococcus aureus ATCC 43300	>2048	>2048	1024	>2048
Staphylococcus aureus ATCC 6538	>2048	n.d.	1024	>2048

* clinical isolates; n.d. – not determined.

In what concerns antibacterial activity, given the amount of phenolic compounds obtained (4.1.1) and in accordance to what is described in the literature (Wichers, Soler-rivas, and Espı 2000), better DOP antibacterial performance was expected, since the MICs ranged between 1024 and 2048 µg/mL. Oleuropein did not show MIC against the MRDB panel tested, while tyrosol only presented inhibitory effect against 3/11 (27%) of the MDRB, with MIC of 2048 µg/mL. Hydroxytyrosol showed a slightly better inhibitory effect than the other two compounds, with MIC

of 1024 µg/mL for 5/11 (45%) of MDRB. Concerning what is described in the literature regarding the antibacterial activity of phenolic compounds, hydroxytyrosol is the one with better antibacterial efficacy, being reported in a recent study a MIC of 400 µg/mL against *S. aureus* ATCC 25923 and 800 µg/mL against *E.coli* ATCC 25922. (Lim et al. 2016) In the same study, oleuropein antibacterial performance was also assessed, exhibiting less antibacterial activity, with MIC of 3200 µg/mL for *E. coli* ATCC 25922 and 800 µg/mL for *S. aureus* ATCC 25923. (Lim et al. 2016) Oleuropein lower antibacterial activity is explained by the lack of ability to penetrate the cell or reach the target site due to the presence of a glycosidic group. (Lim et al. 2016) In other different studies, tyrosol antibacterial activity was assessed and results showed MIC higher than 5 mg/mL. (Aissa et al. 2012; Chakroun et al. 2013) This difference between hydroxytyrosol and oleuropein antibacterial performance is mainly attributed to the lack of an o-diphenol structure, which confers the antibacterial activity to the molecules. (Tuck, Tuck, and Hayball 2015) In the present study, it was possible to assess that *Staphylococcus* strains tested were less susceptible to hydroxytyrosol than *S.aureus* ATCC 25923 with MIC of 1024 µg/mL. However *E. coli* ATCC 25922, also used in the study mentioned, was not susceptible at the concentration reported as MIC. The explanation for these inconsistent results is not clear, but it is possible that interferences occurred with the MTT assay. Obtained results for oleuropein are concordant with what is described, since *S. aureus* ATCC 25923 is more susceptible than the other strains tested. (Lim et al. 2016) For tyrosol, the MIC against *Citrobacter freundii*, *S. saprophyticus* and *S. epidermidis* was established at 2048 µg/mL.

The MICs that were not determined were due to a detected limitation for the use of the MTT assay, since the purple color appeared in higher concentrations of the tested compound but not on the lowest. This observation might be related to the fact that MTT reacts with antioxidant compounds, resulting the purple colored formazam product. (Bruggisser et al. 2002) In future work, Alamar blue bioassay should be considered as an alternative to the MTT assay. (Rampersad 2012)

Interestingly, a closer observation of the obtained results for DOP bacterial growth inhibition, in comparison to GC, showed an unexpected behavior. As the DOP extract concentration increased, the bacterial growth also increased, whereas it was expected to observe growth reduction. These results led to the question of whether the sugars present in the extract would be interfering with the antibacterial activity, being used as Carbon source for bacterial growth. Therefore, the reducing sugars were quantified and results are presented in section 3.4.3.

There are few studies that report the antimicrobial activity of olive pomace and, those available, are mainly focused on ecological and agronomic applications. Studies using DOP extract as an anti-MDRB agent were not found in literature, being the present work, to the best of the knowledge, the first to report that DOP phenolic extract has no anti-MDRB activity up to 2048 µg/mL.

Given these results, DOP extract was not chosen to pursue further tests regarding formulation design.

4.3 – Reducing sugars quantification in the extract

Reducing sugars were determined to assess if this parameter could be interfering with the extracts antibacterial activity. Triplicates were made for each extract. Xylose and glycose were used as standards compounds, being results expressed in milligram of each reducing sugar per gram of extract (Table 4.9).

Table 4.9. Reducing sugars (RS) content in xylose (Xyl) and glycose (Gly) in ENTB, DOP and CcL extracts. Each value is expressed as mean ± standard deviation of triplicates. In each column different letters mean significant differences ($p < 0.05$) between different samples (Tuckey's test).

Extract	Total reducing sugars content (mg RS/g of extract)	
	Xyl	Gly
ENTB	45.26 ± 2.68 c	45.30 ± 3.06 c
DOP	70.88 ± 1.78 a	74.59 ± 2.04 a
CcL	60.20 ± 2.17 b	62.38 ± 2.48 b

DOP extract was the one with higher content in reducing sugars, xylose and glycose, being ENTB extract the one with lower concentration of both sugars. Results presented statistically significant differences between the three tested extracts.

A mixture of xylose and glycose was tested against *Staphylococcus* spp. to observe if the growth profile was similar to the one previously obtained with DOP extract, which was confirmed. Therefore, the DOP extract, despite its composition in bioactives with recognized antibacterial potential, is not a suitable candidate as an anti-MDRB option, since bacteria are able to use this extract as Carbon source for growth.

4.4 – Formulation Design

4.4.1 – Preformulation and characterization studies

During preformulation studies, several excipients were tested, aiming to achieve an ENTB extract aqueous solution. Ethanol was used to solubilize ENTB extract according to previous studies performed by our research group. Regarding the surfactants, Emulcin® and propylene glycol, were unable to stabilize the formulation, occurring the extract precipitation and, therefore, were discarded as options. TAGAT®CH40 contributed to obtain a clear and limpid formulation and thus, it was selected as surfactant in this first phase. Three formulations were prepared with equal excipients, only changing the TAGAT®CH40 percentage. The composition of these formulations is presented in Table 4.10.(Rowe, Sheskey, and Quinn 2009)

Table 4.10. Qualitative and quantitative composition of ENTB formulations.

Ingredients (w/w %)	**Functions**	**Formulations**		
		A	B	C
ENTB extract	Active substance	0.1	0.1	0.1
Ethanol	Cosolvent	4	4	4
TAGAT®CH40	Surfactant	5	2	0
Ascorbic acid	Antioxidant	0.1	0.1	0.1
Potassium sorbate	Antimicrobial preservative	0.2	0.2	0.2
Purified water	Solvent	90.6	93.6	95.6

After preparation, the formulations were characterized regarding the main quality control parameters (Table 4.11).

Table 4.11. Quality control of ENTB formulations. Each value of viscosity is expressed as mean ± standard deviation of duplicates. Different letters mean significant differences ($p < 0.05$) between samples (Tuckey's test).

		Organoleptic properties			**Density (g/mL)**	**pH**	**η (Pa.s) at 20ºC**
		Appearance	Color	Odor			
Formulation	A	Limpid	Light green	Extract characteristic	1.048	5	*
	B	Lightly limpid	Light green	Extract characteristic	0.997	5	1.72×10^{-3} a ($\pm8.63\times10^{-5}$)
	C	Opaque	White	Extract characteristic	0.993	5	1.28×10^{-3} b ($\pm 8.21\times10^{-5}$)

η - Viscosity; * - not determined;

All formulations presented the ENTB extract characteristic odor (eucalyptus). The appearance and color of formulations A, B and C depended on TAGAT®CH40 amount: formulation A, the one with a higher percentage of this excipient, was the more limpid and the one that presented an intensified color (Figure 4.6). Considering the appearance of formulation C, probably the ENTB extract was suspended in this vehicle, since it turned to an opaque white colored formulation. Density was directly dependent on TAGAT® CH40 amount as well. pH value of the three formulations was 5, which is ideal for topical delivery products (4.5-7.5) and for the potassium sorbate preservative activity against yeasts and molds. (Daudt *et al.* 2015; Rowe, Sheskey, and Quinn 2009)

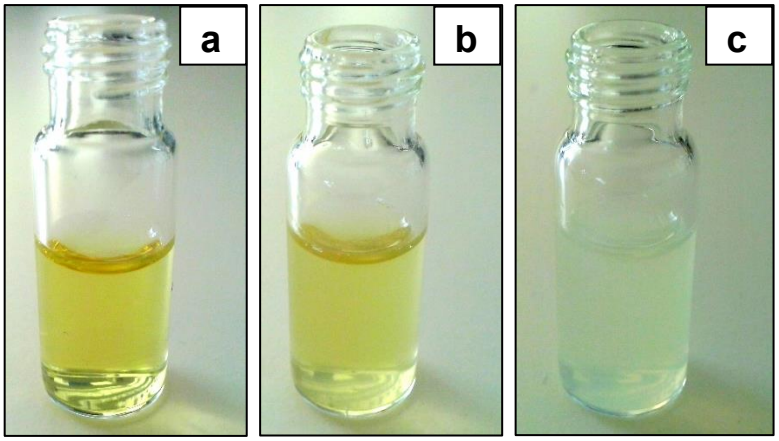

Figure 4.6. Visual appearance of the three formulations designed: a – Formulation A (5% of TAGAT®CH40); b – Formulation B (2% of TAGAT®CH40); c – Formulation C (without (0%) TAGAT®CH40).

Formulations B and C presented statistically significant differences of viscosity, confirming that TAGAT®CH40 had an important effect on physical properties of the tested formulations.

The flow curves of these formulations are represented in Figure 4.7.

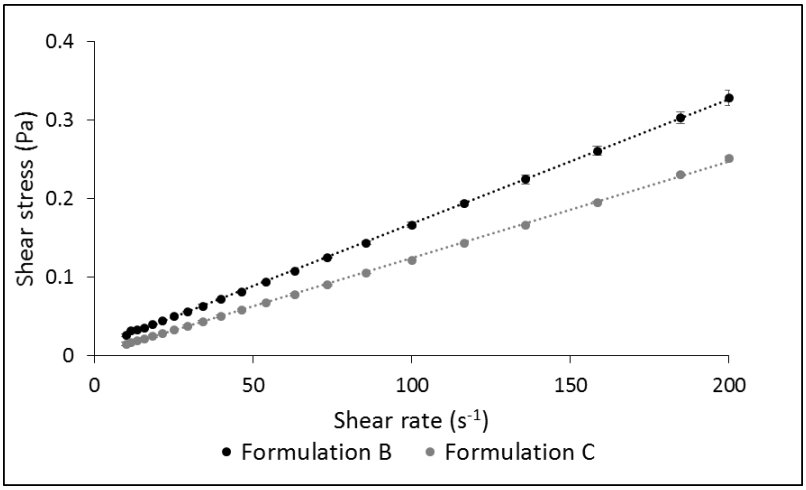

Figure 4.7. Flow curves of formulations B and C at different shear rates from 10 to 200s^{-1} at 20°C. Each value is expressed as mean ± standard deviation of duplicates.

According to Figure 4.7, both formulations presented a linear tendency, correspondent to a Newtonian fluid, as theoretically expected for aqueous solutions. (Banker and Rhodes 2002)

4.4.2 – Antibacterial activity of formulations A, B and C

Antibacterial activity was performed for all formulations against *S. aureus* ATCC 43300, at 5.6 and 90% range. Solution control (SNC), *i.e*. the vehicle without active substance and antimicrobial agent, was a very important parameter to be assessed in order to ensure that antibacterial activity observed was merely attributed to ENTB extract. Results are showed in Figure 4.8.

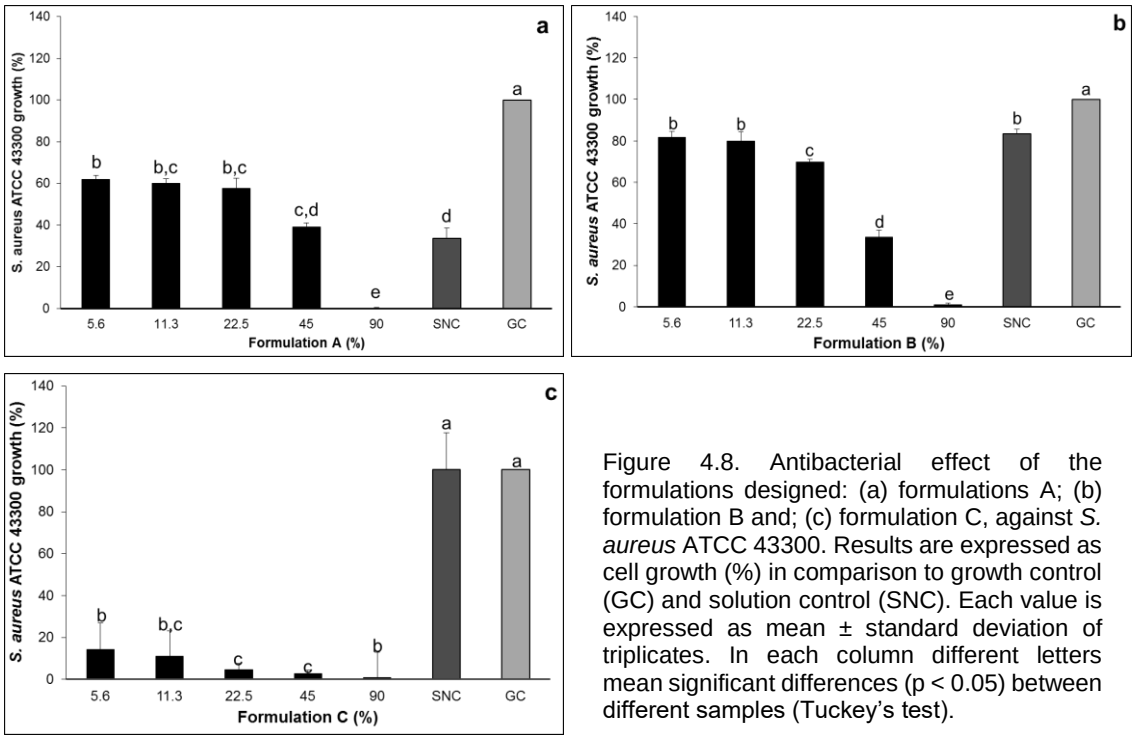

Figure 4.8. Antibacterial effect of the formulations designed: (a) formulations A; (b) formulation B and; (c) formulation C, against *S. aureus* ATCC 43300. Results are expressed as cell growth (%) in comparison to growth control (GC) and solution control (SNC). Each value is expressed as mean ± standard deviation of triplicates. In each column different letters mean significant differences ($p < 0.05$) between different samples (Tuckey's test).

Results highlighted that SNC of formulations A and B (composed by ethanol, TAGAT®CH40 and purified water) exerted antibacterial effect, translated in only 34% and 84% of growth (66 and 16% of growth inhibition, respectively), with statistically significant differences when compared to GC. On the contrary, the SNC of formulation C, composed only by ethanol and purified water, did not present antibacterial effect, being statistically equal to GC. This meant that the formulation effect observed against bacteria was only due to ENTB extract. In particular, all the percentages tested showed an antibacterial effect with statistically significant differences in comparison to GC. Regarding each percentage, it is possible to observe that 22.5 and 45% presented a higher inhibitory effect against *S. aureus* ATCC 43300, statistically significant different from the others. These results might be related to the bioavailability of the formulation in aqueous media and its interaction with bacteria in the interface zone.

Results highlighted formulation C as the one with better antimicrobial activity and without interference of vehicle in the final antibacterial performance. The antimicrobial effect of the vehicle in other formulations might be explained by the fact that nonionic surfactants, like TAGAT®CH40,

eventually have toxic effect against bacteria following two mechanisms: disruption of the cellular membrane and/or reactions of the surfactant with enzymes essential to cell functioning. (Song and Bielefeldt 2012) Given these results, only Formulation C was chosen to proceed to further studies.

4.4.3 – Stability studies

Stability studies of the Formulation C were performed at different temperatures (room temperature (RT) approximately 25°C and 4°C) evaluating selected properties (organoleptic characteristics, pH, microbiologic assay, viscosity and identification and quantification of active substances) (Table 4.12).

Table 4.12. Stability study of formulation C at different temperatures: room temperature (RT) and 4°C.

Month	Storage	Organoleptic characteristics			pH	Microbiologic assay
		Appearance	Color	Odor		
1	RT	Opaque	W	EC	5	NG
	4°C	Opaque	W	EC	5	NG
2	RT	Opaque	W	EC	5	NG
	4°C	Opaque	W	EC	5	NG
3	RT	Opaque	W	EC	5	NG
	4°C	Opaque	W	EC	5	NG
4	RT	Opaque	W	EC	5	NG
	4°C	Opaque	W	EC	5	NG
5	RT	Opaque	LY	EC	5	NG
	4°C	Opaque	W	EC	5	NG

Color: LY – light yellow/ W – white; Odor: EC – Extract characteristic odor; Microbiologic assay: NG – No growth

Formulation C maintained its appearance, pH and microbiologic assay without alterations over the course of the stability studies (5 months). This formulation only suffered color alterations at RT and during the last month, being therefore considered more stable when kept at 4° C. Unfortunately, rheological behavior was not monitored due to limitations of formulation amount.

Considering the identification and quantification of active substances, initial concentrations of TAs assessed by HPLC and conversion to actual percentage in formulation (0.1%) are shown in

Table 4.13. ENTB extract concentrations over time were determined, and results are shown as ratio concentration : initial concentration (Figure 4.9).

Table 4.13. Initial concentration (C_0) of TAs: betulinic acid (BA), betulonic acid (BOA), oleanolic acid (OA) and ursolic acid (UA) at 0.7% used for HPLC analysis, and 0.1% used in the formulation. Each value of viscosity is expressed as mean ± standard deviation of triplicates. Different letters mean significant differences ($p < 0.05$) between different samples (Tuckey's test).

ENTB extract (%)	C_0 (µg/mL)			
	BOA	BA	OA	UA
0.7	207.38 ab (± 63.42)	176.75 b (± 20.28)	289.68 a (± 13.81)	231.85 ab (± 20.96)
0.1	29.63 ab (± 9.06)	25.11 b (± 2.90)	41.24 a (± 1.97)	32.98 ab (± 2.99)

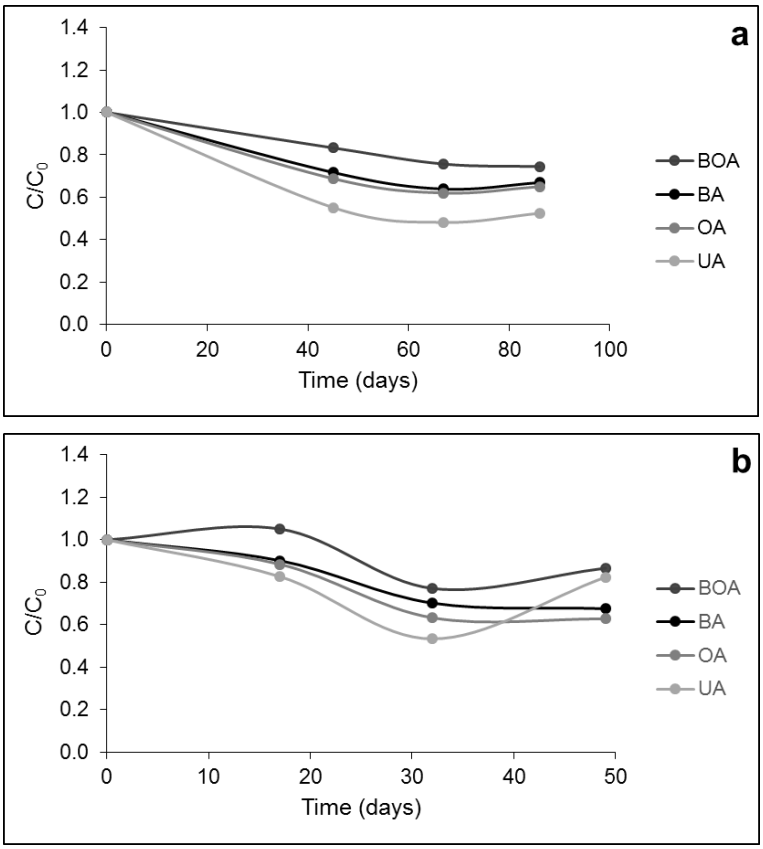

Figure 4.9. Quantification of TAs in formulation C over time represented as ratio concentration : initial concentration (C/C_0): a – Formulation stored at RT; b – Formulation stored at 4°C. Each value is expressed as mean of triplicates.

Results showed a decrease in TAs concentration, approximately after 20 days of storage, at both temperatures. This decrease is translated in a reduction of 70 and 66% in the TAs average, both at RT and 4°C, respectively (specifically, 83% of BOA, 72% of BA, 69% of OA and 55% of

UA at RT, and 77% of BOA, 70% of BA, 63% of OA and 53% of UA at 4°C). These results are not according to the Portuguese Pharmacopeia, in which a formulation is considered chemically stable when the percentage of the active substance remains up until 90% of initial concentration. (Farmacopeia Portuguesa 2005) Therefore, Formulation C is not considered chemically stable at both RT and 4°C. Moreover, it is likely that the antimicrobial activity of the formulation will also decrease, due to the TAs concentration fluctuation. The occurrence of chemical reactions in compounds that constitute ENTB extract might be an explanation to the decrease observed, to which the color changes in the formulation may also be related.

Overall, formulation C stored at 4°C seems to be the best option regarding all parameters studied. However, some aspects need to be further explored, such as solubility and how to enhance the stability of these bioactive compounds at RT, since ideally the formulation should be stable at RT, concerning its ease of use in healthcare facilities.

4.4.4 – Sensorial evaluation

Sensorial evaluation is considered a quality guarantee since this analysis integrates multidimensional measures. (Daudt *et al.* 2015) The advantage of this analysis is the ability to evaluate how many tasters like and dislike, and more importantly, allows to refine sensorial characteristics of the formulation and detect failures or aspects that consumers identify that need improvement. (Daudt *et al.* 2015)

The present analysis involved both male and female participants with different ages. Different parameters (general appreciation, touch, spread, feeling and final appreciation) were evaluated and classified using a scale from 1 to 5, where 1 represents the lower level of appreciation and 5 the maximum (Appendix 1). Regarding the consistency, the scale refers to fluidity, where 1 represents the maximum level and 5 the lower level. Sensorial evaluation is represented in Figure 4.10.

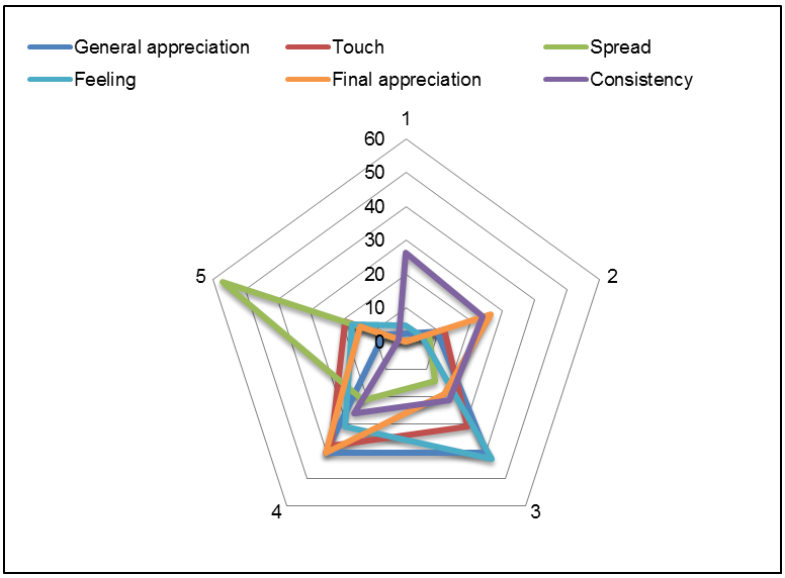

Figure 4.10. Sensorial evaluation of Formulation C (n=42). Five parameters were evaluated, being classified from 1 (not like at all) to 5 (really like). Results are expressed in percentage, considering the total number of participants in each evaluation.

Formulation C presented diverged opinions regarding the initial analysis, where 40% of tasters liked (3) and 40% liked much (4). The next four parameters were evaluated, where 38% of tasters liked much (4) the touch, 57% thought it was really easy (5) to spread, 26% found the formulation very fluid (1) and 43% liked (3) the feeling on the skin. 26% of tasters assigned level 4 of appreciation regarding consistency. This high difference between levels might be associated to the scale of consistency, which was different from the others, and some misunderstandings might have occurred. Lastly, 40% of tasters liked much formulation C in the final general appreciation. Interestingly, formulation C had a median level of appreciation on the initial evaluation, but at the end it obtained a good evaluation. This result demonstrates that appearance is a very significant parameter for consumers.

Suggestions and comments were quite welcome during this evaluation, and the most common were related to: improvement of consistency; color (turn to colorless); more oily formulations and maintaining the eucalyptus smell in the hands to give the “fresh-feeling”. Regarding the improvement of consistency, it should be noticed that the preferential rheological behavior for topical formulations is pseudoplastic, *i.e*., consistency must be sufficient to be well spread but not too liquid, which is not observed for Formulation C. Moreover, the formulation rheological behavior must also be adjusted to the packaging material of the pharmaceutical product and all of these features must be taken in account when refining the consistency characteristic. An alternative dosage form to the hydroalcoholic solution, like in formulation C, might be gels or extemporaneous formulation. Gels are usually more appreciated by tasters/consumers in what concerns consistency and rheological behavior, while

extemporaneous formulation possesses advantages in what concerns physico-chemical stability and storage conditions.

5 – Conclusion & Future perspectives

The main objective of this work was to establish the foundations for the development of a novel antiseptic formulation based on natural extracts. For that purpose, the antibacterial activities of three industry by-products (biomasses) were studied and ENTB extract was selected as the one with best antibacterial performance, with MIC concentrations against *Staphylococcus* spp. between 128 and 64 µg/mL. DOP extract and CcL extract anti-MDRB activity did not meet the requirements to proceed to preformulation tests. Moreover, ENTB extract also showed to be bactericidal against *S. aureus* ATCC 43300 at a concentration of 256 µg/mL, being this effect maintained until, at least, 48h of exposure. Promising results were obtained when ENTB extract was tested in combination with antibiotics, demonstrating synergistic activity, particularly against *Staphylococcus* spp., which highlighted its usefulness as a possible coadjuvant to the conventional available antibiotherapy. Thus, ENTB extract was formulated with different vehicles in preformulation studies to further evaluate its potential as an active substance of an anti-MDRB formulation. Preformulation design allowed to assess the ENTB extract potential when included in a hydro-alcoholic solution without non-ionic tensioactive TAGAT®CH40 (Formulation C). Antibacterial activity assay of Formulation C, tested against *Staphylococcus* spp., showed promising results, with growth inhibition of 97%, approximately, when exposed to 45% of formulation. Overall, the main purpose of this work was accomplished.

As future perspectives, several items still require to be investigated to further pursue this phytopharmaceutical research line, which yielded promising results. The fact that there was a decrease in TAs concentrations and changes in the final formulation color over time, indicated a lack of ENTB extract chemical stability in aqueous solution, which suggests that other dosage forms, such as gels or an extemporaneous formulation, may present advantages, mainly regarding stability issues and storage conditions. Furthermore, the formulation design (composition, rheology and stability studies) could be more explored since the vehicle of initial solutions was modified according to microbiological results. Also, cytotoxicity assays with epithelial cell lines and establishing the ENTB mechanism of action are also required. Further aspects that may be explored are other possible uses of an ENTB-based-formulation, namely its use as an antifungal agent or as a detergent for surfaces sanitation within health-care facilities.

6 – References

Abreu, Ana Cristina, Andrew J. McBain, and Manuel Simões. 2012. “Plants as Sources of New Antimicrobials and Resistance-Modifying Agents.” *Natural product reports* 29(9): 1007–21. http://www.ncbi.nlm.nih.gov/pubmed/22786554.

Aissa, Imen et al. 2012. “Synthesis of Lipophilic Tyrosyl Esters Derivatives and Assessment of Their Antimicrobial and Antileishmania Activities.” *Lipids in Health and Disease* 11(1): 13. http://www.lipidworld.com/content/11/1/13.

Alifano, Pietro, Carla Palumbo, Daniela Pasanisi, and Adelfia Talà. 2015. “Rifampicin-Resistance, rpoB Polymorphism and RNA Polymerase Genetic Engineering.” *Journal of Biotechnology* 202: 60–77. http://dx.doi.org/10.1016/j.jbiotec.2014.11.024.

Allen, Loyd V. 2010. “Dosage Form Design: Pharmaceutical and Formulation Considerations.” *Ansel’s Pharmaceutical Dosage Forms and Drug Delivery Systems*: 91–141.

Amorim, M Helena R, Rui M Gil da Costa, Carlos Lopes, and Margarida M S M Bastos. 2013. “Sesquiterpene Lactones: Adverse Health Effects and Toxicity Mechanisms.” *Critical Reviews in Toxicology* 43(7): 559–79. http://www.ncbi.nlm.nih.gov/pubmed/23875764\nhttp://informahealthcare.com/doi/abs/10.3109/10408444.2013.813905.

Ansel, Howard C., Nicholas G. Popovich, and Loyd V. Allen. 1989. “Ansel’s Pharmaceutical Dosage Forms and Drug Delivery Systems.” *Journal of Chemical Information and Modeling* 9: 160.

Aulton, Michael E. 2002. *Pharmaceutics: The Science of Dosage Form Design*. Churchill livingstone. BOOK.

Avent, M. L., B. A. Rogers, A. C. Cheng, and D. L. Paterson. 2011. “Current Use of Aminoglycosides: Indications, Pharmacokinetics and Monitoring for Toxicity.” *Internal Medicine Journal* 41(6): 441–49.

Bachelier, Anke, Ralf Mayer, and Christian D. Klein. 2006. “Sesquiterpene Lactones Are Potent and Irreversible Inhibitors of the Antibacterial Target Enzyme MurA.” *Bioorganic and Medicinal Chemistry Letters* 16(21): 5605–9.

Balasundram, Nagendran, Kalyana Sundram, and Samir Samman. 2006. “Phenolic Compounds in Plants and Agri-Industrial by-Products: Antioxidant Activity, Occurrence, and Potential Uses.” *Food Chemistry* 99(1): 191–203.

Balouiri, Mounyr, Moulay Sadiki, and Saad Koraichi Ibnsouda. 2016. “Methods for in Vitro Evaluating Antimicrobial Activity: A Review.” *Journal of Pharmaceutical Analysis* 6(2): 71–79. http://dx.doi.org/10.1016/j.jpha.2015.11.005.

Balunas, Marcy J., and A. Douglas Kinghorn. 2005. “Drug Discovery from Medicinal Plants.” *Life Sciences* 78(5): 431–41.

Banker, Gilbert Banker S, and Christopher Thomas Rhodes. 2002. *Modern Pharmaceutics,*. http://books.google.com/books?hl=fr&lr=&id=s1-BerNQAtsC&pgis=1.

Barreto, Humberto M. et al. 2014. “Chemical Composition and Possible Use as Adjuvant of the Antibiotic Therapy of the Essential Oil of Rosmarinus Officinalis L.” *Industrial Crops and Products* 59: 290–94. http://linkinghub.elsevier.com/retrieve/pii/S0926669014003057 (July

31, 2014).

Borja-Santos, Romana. 2016. “Centro Hospitalar de Coimbra Confirma Três Mortos Com a Mesma Bactéria Do Surto de Gaia.” *Público*: 1. https://www.publico.pt/sociedade/noticia/ha-24-doentes-em-coimbra-com-a-mesma-bacteria-que-causou-surto-em-gaia-1722220.

Breathnach, a S. 2005. “Nosocomial Infections.” *Medicine* 33(3): 22–26.

Brown, Eric D., and Gerard D. Wright. 2016. “Antibacterial Drug Discovery in the Resistance Era.” *Nature* 529(7586): 336–43. http://www.nature.com/doifinder/10.1038/nature17042.

Bruggisser, Regina et al. 2002. “Interference of Plant Extracts, Phytoestrogens and Antioxidants with the MTT Tetrazolium Assay.” *Planta medica* 68(5): 445–48.

Butler, Mark S et al. 2014. “Glycopeptide Antibiotics: Back to the Future.” *J Antibiot* 67(9): 631–44. http://dx.doi.org/10.1038/ja.2014.111.

Cars, Otto, Anna Hedin, and Andreas Heddini. 2011. “The Global Need for Effective Antibiotics - Moving towards Concerted Action.” *Drug Resistance Updates* 14(2): 68–69. http://dx.doi.org/10.1016/j.drup.2011.02.006.

Cetin-Karaca, Hayriye, and Melissa C. Newman. 2015. “Antimicrobial Efficacy of Plant Phenolic Compounds against Salmonella and Escherichia Coli.” *Food Bioscience* 11: 8–15. http://dx.doi.org/10.1016/j.fbio.2015.03.002.

Chadwick, Martin, Harriet Trewin, Frances Gawthrop, and Carol Wagstaff. 2013. “Sesquiterpenoids Lactones: Benefits to Plants and People.” *International Journal of Molecular Sciences* 14(6): 12780–805.

Chakroun, Hanen et al. 2013. “Enzymatic Transformation of Tyrosol by Trametes Trogii Laccases: Identification of the Product and Study of Its Biological Activities.” *Journal of Molecular Catalysis B: Enzymatic* 87: 11–17. http://dx.doi.org/10.1016/j.molcatb.2012.10.006.

Chaturvedi, Devdutt. 2011. “Sesquiterpene Lactones : Structural Diversity and Their Biological Activities.” *Opportunity, Challenge and Scope of Natural Products in Medicinal Chemistry* 661(2): 313–34.

Cherubini, Francesco. 2010. “The Biorefinery Concept: Using Biomass instead of Oil for Producing Energy and Chemicals.” *Energy Conversion and Management* 51(7): 1412–21. http://dx.doi.org/10.1016/j.enconman.2010.01.015.

Chopra, Ian, and Marilyn Roberts. 2001. “Tetracycline Antibiotics : Mode of Action , Applications , Molecular Biology , and Epidemiology of Bacterial Resistance Tetracycline Antibiotics : Mode of Action , Applications , Molecular Biology , and Epidemiology of Bacterial Resistance.” *Microbiology and Molecular Biology Reviews* 65(2): 232–260. http://www.facm.ucl.ac.be/Full-texts-FACM/Vanbambeke-2006-4.pdf.

Chung, Pooi Yin, Parasakthi Navaratnam, and Lip Yong Chung. 2011. “Synergistic Antimicrobial Activity between Pentacyclic Triterpenoids and Antibiotics against Staphylococcus Aureus Strains.” *Annals of clinical microbiology and antimicrobials* 10(1): 25. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3127748&tool=pmcentrez&rendertype=abstract.

Cowan, Marjorie Murphy. 1999. “Plant Products as Antimicrobial Agents.” 12(4).

Daudt, Renata Moschini et al. 2015. “Pinhão Starch and Coat Extract as New Natural Cosmetic Ingredients: Topical Formulation Stability and Sensory Analysis.” *Carbohydrate Polymers* 134: 573–80. http://www.sciencedirect.com/science/article/pii/S0144861715007791.

Davies, J, & Davies, D. 2010. “Origins and Evolution of Antibiotic Resistance.” *Microbiology and Molecular Biology Reviews* 74(3): 417–33.

Dewick, Paul M. 2002. *Medicinal Natural Products: A Biosynthetic Approach*. John Wiley & Sons. BOOK.

Dias, J M et al. 2016. “Impact of Olive Oil Usage on Physical Properties of Chocolate Fillings.” *Grasas y Aceites* 67(3): e145.

Dick, Andrew W. et al. 2015. “A Decade of Investment in Infection Prevention: A Cost-Effectiveness Analysis.” *American Journal of Infection Control* 43(1): 4–9. http://dx.doi.org/10.1016/j.ajic.2014.07.014.

Direção-Geral de Saúde. 2014. *Prevenção E Controlo de Infeções E de Resistência Aos Antimicrobianos Em Números – 2014*.

Domingues, R. M a et al. 2010. “Eucalyptus Globulus Biomass Residues from Pulping Industry as a Source of High Value Triterpenic Compounds.” *Industrial Crops and Products* 31(1): 65–70. http://linkinghub.elsevier.com/retrieve/pii/S0926669009001368 (August 8, 2014).

Domingues, Rma et al. 2011. “Eucalyptus Biomass Residues from Agro-Forest and Pulping Industries as Sources of High-Value Triterpenic Compounds.” *Cellulose CHEMISTRY AND TECHNOLOGY* 45: 475–81. http://www.cellulosechemtechnol.ro/pdf/CCT45,7-8(2011)/p.475-481.pdf.

Domingues, Rui M a et al. 2012. “Supercritical Fluid Extraction of Eucalyptus Globulus Bark-A Promising Approach for Triterpenoid Production.” *International journal of molecular sciences* 13(6): 7648–62. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3397551&tool=pmcentrez&rendertype=abstract (August 8, 2014).

Dzubak, Petr et al. 2006. “Pharmacological Activities of Natural Triterpenoids and Their Therapeutic Implications.” *Natural product reports* 23(3): 394–411.

Eloff, J N. 1998. “A Sensitive and Quick Microplate Method to Determine the Minimal Inhibitory Concentration of Plant Extracts for Bacteria.” *Planta medica* 64(8): 711–13.

Expósito, O et al. 2009. “Biotechnological Production of Taxol and Related Taxoids: Current State and Prospects.” *Anti-Cancer Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Cancer Agents)* 9(1): 109–21.

Falleh, Hanen et al. 2008. “Phenolic Composition of Cynara Cardunculus L. Organs, and Their Biological Activities.” *Comptes Rendus - Biologies* 331(5): 372–79. http://www.ncbi.nlm.nih.gov/pubmed/18472083 (August 8, 2014).

Farmacopeia Portuguesa, VIII. 2005. “INFARMED.” *Lisboa, Portugal*.

Fernando, S, S Adhikari, C Chandrapal, and N Murali. 2006. “Biorefineries: Current Status, Challenges, and Future Direction.” *Energy Fuels* 20(4): 1727–37. http://dx.doi.org/10.1021/ef060097w.

Fiorentino, Francesca. 2014. “Hospital-Acquired Infections: A Cost Estimation for BSI in Portugal.” http://www3.eeg.uminho.pt/economia/nipe/PEJ2014/Francesca Fiorentino.pdf.

Fontanay, Stéphane et al. 2008. “Ursolic, Oleanolic and Betulinic Acids: Antibacterial Spectra and Selectivity Indexes.” *Journal of Ethnopharmacology* 120(2): 272–76. http://www.ncbi.nlm.nih.gov/pubmed/18835348 (August 8, 2014).

Gibbons, Simon. 2004. “Anti-Staphylococcal Plant Natural Products.” *Natural product reports* 21(2): 263–77.

Ginsburg, Hagai, and Eric Deharo. 2011. “A Call for Using Natural Compounds in the Development of New Antimalarial Treatments – an Introduction.” *Malaria Journal* 10(Suppl 1): S1. http://www.malariajournal.com/content/10/S1/S1.

Goldstein, Beth P. 2014. “Resistance to Rifampicin: A Review.” *The Journal of antibiotics* 67(9): 625–30. http://www.ncbi.nlm.nih.gov/pubmed/25118103.

Gurib-Fakim, Ameenah. 2006. “Medicinal Plants: Traditions of Yesterday and Drugs of Tomorrow.” *Molecular Aspects of Medicine* 27(1): 1–93.

Haque, Shafiul et al. 2014. “Screening and Characterisation of Antimicrobial Properties of Semisynthetic Betulin Derivatives.” *PLoS ONE* 9(7).

“Health Policy Brief: Antibiotic Resistance.” 2015. *Health Affairs*.

Herrera, Stephan. 2004. “Industrial Biotechnology—a Chance at Redemption.” *Nature Biotechnology* 22(6): 671–75.

Higashide, Masato et al. 2008. “Methicillin-Resistant Staphylococcus Saprophyticus Isolates Carrying Staphylococcal Cassette Chromosome Mec Have Emerged in Urogenital Tract Infections.” *Antimicrobial Agents and Chemotherapy* 52(6): 2061–68.

Instituto Nacional de Estatísitca (INE). 2014. *I.P., Estatísticas Agrícolas 2014, Ed 2015, Lisboa – Portugal.* https://www.ine.pt/xportal/xmain?xpid=INE&xpgid=ine_publicacoes&PUBLICACOESpub_boui=210756829&PUBLICACOESmodo=2.

Jäger, Sebastian et al. 2009. “Pentacyclic Triterpene Distribution in Various Plants - Rich Sources for a New Group of Multi-Potent Plant Extracts.” *Molecules (Basel, Switzerland)* 14(6): 2016–31. http://www.ncbi.nlm.nih.gov/pubmed/19513002 (July 31, 2014).

Johnson Burns, L., Woodford, N., Threlfall, E. J., Naidoo, J., Cooke, E. M. &amp, A P, and R C George. 1994. “Gentamicin Resistance in Clinical Isolates of Escherichia Coli by Genes of Veterinary Origin.” *J. Med. Microbiol.* 40(1994): 221–26.

Jorge, Instituto Nacional Ricardo. “Vigilância Epidemiológica Das Resistências Aos Antimicrobianos.” http://www.insa.pt/sites/INSA/Portugues/Paginas/AntibioticosResi.aspx#oque (October 16, 2016).

Kohanski, Michael A, Daniel J Dwyer, and James J Collins. 2010. “How Antibiotics Kill Bacteria: From Targets to Networks.” *Nature reviews. Microbiology* 8(6): 423–35. http://www.ncbi.nlm.nih.gov/pubmed/20440275\nhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC2896384.

Kurek, Anna, Paulina Nadkowska, Sylwia Pliszka, and Krystyna I Wolska. 2012. “Modulation of Antibiotic Resistance in Bacterial Pathogens by Oleanolic Acid and Ursolic Acid.” *Phytomedicine : international journal of phytotherapy and phytopharmacology* 19(6): 515–19. http://www.sciencedirect.com/science/article/pii/S0944711312000037 (August 8, 2014).

Leclercq, R. 2002. “Mechanisms of Resistance to Macrolides and Lincosamides: Nature of the Resistance Elements and Their Clinical Implications.” *Clin.Infect.Dis.* 34(1537–6591 (Electronic)): 482–92. C:\Karsten\PDFs\Antibiotika-PDFs\Anti-2002\Leclercq-Mechanisms of resistance to macrolides and lincosamides.pdf.

Lederberg, Joshua. 2000. “Infectious History.” *SCIENCE* 288: 288–93.

Levy, S B, and B Marshall. 2004. “Antibacterial Resistance Worldwide: Causes, Challenges and Responses.” *Nat.Med.* 10(1078–8956 (Print)): S122–29. C:\KARSTEN\PDFs\Antibiotika-PDFs\Anti-2004\Levy - Marshall-Antibacterial resistance worldwide- causes challenges and responses.pdf.

Lewis, Kim. 2013. “Platforms for Antibiotic Discovery.” *Nature reviews. Drug discovery* 12(5): 371–87. http://www.ncbi.nlm.nih.gov/pubmed/23629505.

Li, X. Z., D. M. Livermore, and H. Nikaido. 1994. “Role of Efflux Pump(s) in Intrinsic Resistance of Pseudomonas Aeruginosa: Resistance to Tetracycline, Chloramphenicol, and Norfloxacin.” *Antimicrobial Agents and Chemotherapy* 38(8): 1732–41.

Lim, Anxy et al. 2016. “Plant Phenols as Antibiotic Boosters: In Vitro Interaction of Olive Leaf Phenols with Ampicillin.” *Phytotherapy Research* 30(3): 503–9.

Lowy, Fd. 2003. “Antimicrobial Resistance: The Example of *Staphylococcus Aureus*.” *Journal of Clinical Investigation* 111(9): 1265–73. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=154455&tool=pmcentrez&rendertype=abstract\nhttp://www.jci.org/cgi/content/abstract/111/9/1265.

Madrinha, Mariana. 2016. “Relatório. Infeções Hospitalares Matam Mais Do Que Acidentes de Viação.” *Jornal i*: 1. http://ionline.sapo.pt/artigo/500625 (October 2, 2016).

Magalhães, Maria L., and John S. Blanchard. 2005. “The Kinetic Mechanism of AAC(3)-IV Aminoglycoside Acetyltransferase from Escherichia Coli.” *Biochemistry* 44(49): 16275–83.

Marabuto, E. et al. 2016. *Cynara Cardunculus L. - Mapa de Distribuição*. http://www.flora-on.pt/#wCynara+cardunculus.

McDermott, Patrick F, Robert D Walker, and David G White. 2003. “Antimicrobials: Modes of Action and Mechanisms of Resistance.” *International journal of toxicology* 22(2): 135–43.

Miller, G L. 1959. “Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar.” *Analytical Chemistry* 31(3): 426–28. http://dx.doi.org/10.1021/ac60147a030.

Nascimento, Patrícia G G et al. 2014. “Antibacterial and Antioxidant Activities of Ursolic Acid.” *Molecules* 19(Mic): 1317–27.

Omar, Syed Haris. 2010a. “Cardioprotective and Neuroprotective Roles of Oleuropein in Olive.” *Saudi Pharmaceutical Journal* 18(3): 111–21. http://dx.doi.org/10.1016/j.jsps.2010.05.005.

———. 2010b. “Oleuropein in Olive and Its Pharmacological Effects.” *Scientia Pharmaceutica* 78(2): 133–54.

Parreira, P et al. 2015. “EUCALYPTUS SPP. OUTER BARK EXTRACTS AGAINST MULTIDRUG RESISTANT BACTERIA.” *BOOK OF*: 79.

Pereira, V. et al. 2014. “Antibacterial Activity and Synergistic Effects between Eucalyptus Globulus Leaf Residues (Essential Oils and Extracts) and Antibiotics against Several Isolates of Respiratory Tract Infections (Pseudomonas Aeruginosa).” *Industrial Crops and Products* 52: 1–7. http://linkinghub.elsevier.com/retrieve/pii/S0926669013005438 (August 8, 2014).

Pittet, D et al. 2000. “Effectiveness of a Hospital-Wide Programme to Improve Compliance with Hand Hygiene. Infection Control Programme.” *Lancet* 356(9238): 1307–12.

Pittet, D. 2001. “Compliance with Hand Disinfection and Its Impact on Hospital-Acquired Infections.” *Journal of Hospital Infection* 48(SUPPL. A).

Ramos, Patrícia et al. 2013. “Valorization of Olive Mill Residues: Antioxidant and Breast Cancer Antiproliferative Activities of Hydroxytyrosol-Rich Extracts Derived from Olive Oil by-Products.” *Industrial Crops and Products* 46: 359–68. http://dx.doi.org/10.1016/j.indcrop.2013.02.020.

Ramos, Patrícia A B et al. 2013. “Lipophilic Extracts of Cynara Cardunculus L. Var. Altilis (DC): A Source of Valuable Bioactive Terpenic Compounds.” *Journal of Agricultural and Food Chemistry* 61(35): 8420–29.

Ramos, Patrícia Alexandra Bogango. 2015. “Chemical Characterization and Evaluation of Biological Activity of Cynara Cardunculus Extractable Compounds.”

Rampersad, Sephra N. 2012. “Multiple Applications of Alamar Blue as an Indicator of Metabolic Function and Cellular Health in Cell Viability Bioassays.” *Sensors (Switzerland)* 12(9): 12347–60.

Rice, Louis B. 2008. “Federal Funding for the Study of Antimicrobial Resistance in Nosocomial Pathogens: No ESKAPE.” *The Journal of Infectious Diseases* 197(8): 1079–81. http://jid.oxfordjournals.org/lookup/doi/10.1086/533452.

Ríos, J. L., and M. C. Recio. 2005. “Medicinal Plants and Antimicrobial Activity.” *Journal of Ethnopharmacology* 100(1–2): 80–84.

Riss, Terry L, Andrew L Niles, and Lisa Minor. 2004. “Cell Viability Assays Assay Guidance Manual.” *Assay Guidance Manual*: 1–23.

Rodrigues, Francisca, Filipa B. Pimentel, and M. Beatriz P P Oliveira. 2015. “Olive by-Products: Challenge Application in Cosmetic Industry.” *Industrial Crops and Products* 70: 116–24. http://dx.doi.org/10.1016/j.indcrop.2015.03.027.

Romero-García, J. M. et al. 2014. “Biorefinery Based on Olive Biomass. State of the Art and Future Trends.” *Bioresource Technology* 159: 421–32.

Rossolini, Gian Maria, Fabio Arena, Patrizia Pecile, and Simona Pollini. 2014. “Update on the Antibiotic Resistance Crisis.” *Current Opinion in Pharmacology* 18: 56–60. http://dx.doi.org/10.1016/j.coph.2014.09.006.

Rowe, R, P Sheskey, and M Quinn. 2009. “Handbook of Pharmaceutical Excipients.” *Handbook of pharmaceutical excipients, Sixth edition*: 549–53.

Rubio-López, Virginia et al. 2012. “Molecular Epidemiology, Antimicrobial Susceptibilities and Resistance Mechanisms of Streptococcus Pyogenes Isolates Resistant to Erythromycin and Tetracycline in Spain (1994-2006).” *BMC microbiology* 12: 215. http://www.scopus.com/inward/record.url?eid=2-s2.0-84868521381&partnerID=tZOtx3y1.

Sabin, A B. 1970. “Control of Infectious Diseases.” *The Journal of infectious diseases* 121(1): 91–94.

Shi, Kun, Shane J Caldwell, Desiree H Fong, and Albert M Berghuis. 2013. “Prospects for Circumventing Aminoglycoside Kinase Mediated Antibiotic Resistance.” *Frontiers in cellular and infection microbiology* 3(June): 22. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3691515&tool=pmcentrez&rendertype=abstract.

Simões, Manuel, Richard N Bennett, and Eduardo a S Rosa. 2009. “Understanding Antimicrobial Activities of Phytochemicals against Multidrug Resistant Bacteria and Biofilms.” *Natural product reports* 26: 746–57.

Song, Myoungsuk, and Angela R. Bielefeldt. 2012. “Toxicity and Inhibition of Bacterial Growth by Series of Alkylphenol Polyethoxylate Nonionic Surfactants.” *Journal of Hazardous Materials* 219–220: 127–32. http://dx.doi.org/10.1016/j.jhazmat.2012.03.063.

Sousa e Silva, JP. 2013. “Pharmaceutical Formulation.” *Pharmaceutica Analytica Acta* 4(1): 4. http://dx.doi.org/10.4172/2153-2435.1000e145.

State, Penn. 2002. “Prokaryotes II -Structure and Function.” : 1. https://online.science.psu.edu/biol110_sandbox_8862/node/8971.

Tenover, Fred C. 2006. “Mechanisms of Antimicrobial Resistance in Bacteria.” *American Journal of Infection Control* 34(5 SUPPL.).

Tuck, Kellie L, Kellie L Tuck, and Peter J Hayball. 2015. “Major Phenolic Compounds in Olive Oil : Metabolism and Health Effects.” 2863(DECEMBER 2002): 636–44.

Vázquez, Nicolás M., Graciela Fiorilli, Paulo A. Cáceres Guido, and Silvia Moreno. 2016. “Carnosic Acid Acts Synergistically with Gentamicin in Killing Methicillin-Resistant Staphylococcus Aureus Clinical Isolates.” *Phytomedicine* 23(12): 1337–43.

Velez, Zélia et al. 2012. “Biological Characterization of Cynara Cardunculus L. Methanolic Extracts: Antioxidant, Anti-Proliferative, Anti-Migratory and Anti-Angiogenic Activities.” *Agriculture* 2(4): 472–92. http://www.mdpi.com/2077-0472/2/4/472/htm.

Wang, Chao Min et al. 2016. “Antibacterial and Synergistic Activity of Pentacyclic Triterpenoids Isolated from Alstonia Scholaris.” *Molecules* 21(2): 1–11.

WHO. 2012. “Quality Assurance for Pharmaceuticals.” *Managemente sciences for health* 10(3): 3–23.

Wichers, Harry J, Cristina Soler-rivas, and Juan Carlos Espı. 2000. “Review Oleuropein and Related Compounds.” *Journal of the Science of Food and Agriculture* 80(November 1999): 1013–23.

Wiegand, Irith, Kai Hilpert, and Robert E W Hancock. 2008. “Agar and Broth Dilution Methods to Determine the Minimal Inhibitory Concentration (MIC) of Antimicrobial Substances.” *Nature*

protocols 3(2): 163–75.

Wolska, Krystyna I. et al. 2010. “Antibacterial Activity of Oleanolic and Ursolic Acids and Their Derivatives.” *Central European Journal of Biology* 5(5): 543–53.

World Health Organization. 2015. “Health Care-Associated Infections Fact Sheet.” *World health Organization*: 4. http://www.who.int/gpsc/country_work/gpsc_ccisc_fact_sheet_en.pdf.

York, Peter. 2000. “The Design of Dosage Forms.” (1999): 2000.

Zaffiri, L, J Gardner, and L H Toledo-Pereyra. 2012. “History of Antibiotics. From Salvarsan to Cephalosporins.” *Journal of investigative surgery : the official journal of the Academy of Surgical Research* 25(2): 67–77. http://www.ncbi.nlm.nih.gov/pubmed/22439833.

Zarb, P. et al. 2012. 17 Eurosurveillance *The European Centre for Disease Prevention and Control (ECDC) Pilot Point Prevalence Survey of Healthcare-Associated Infections and Antimicrobial Use*.

Zervosen, Astrid et al. 2012. “Development of New Drugs for an Old Target - The Penicillin Binding Proteins.” *Molecules* 17(11): 12478–505.

7 – Appendix

Appendix 1 – Sensorial evaluation questionnaire for formulation C

Análise sensorial de uma formulação tópica

Nome_____

Data: _____/_____/_____ Idade:_____

Descrição da formulação: Solução hidroalcoólica tendo como princípio ativo extrato natural da casca de *Eucalyptus nitens*.
Este estudo tem como objetivo saber qual a sua opinião e preferência em relação à formulação fornecida. Peço que siga as instruções de aplicação do produto e responda ao questionário.
Muito obrigada pela colaboração!

Aplicação do produto

1. Aplicar a formulação nas mãos e espalhar
2. Massajar até a formulação desaparecer
3. Responder ao questionário

Questionário

O questionário tem 6 perguntas às quais é atribuída uma escala de 1 a 5. Deve assinalar com um ☒ qual corresponde a sua opinião.

1. **Antes de aplicar, qual a sua opinião geral sobre a formulação (Ex: Cor, cheiro, aparência)?**

1	2	3	4	5

(1: Não gosto nada → 5: Gosto muito)

2. **Qual a sensação ao tocar na formulação?**

1	2	3	4	5

(1: Nada agradável → 5: Muito agradável)

3. **Como considera o espalhamento da formulação?**

1	2	3	4	5

(1: Muito difícil → 5: Muito fácil)

4. **Classifique a consistência.**

1	2	3	4	5

(1: Muito líquido → 5: Muito viscoso)

5. **Qual a sensação que o produto lhe causou após ser aplicada?**

1	2	3	4	5

(1: Desagradável → 5: Muito agradável)

6. **Após aplicação, qual a sua opinião geral sobre a formulação (Ex: cheiro na pele)?**

1	2	3	4	5

(1: Não gosto nada → 5: Gosto muito)

7. **Sugestões (Facultativo)**
