Integrated Master in Bioengineering

Exploring wine industry by-products for targeting *Staphylococcus aureus* in diabetic foot infections

Master Dissertation

of

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Abstract

Diabetes Mellitus (DM) is a heterogeneous disease characterized by high blood glucose levels. It is estimated in 2019 that DM affected around 463 million people, representing a cost of USD 760.3 billion in health care worldwide. One of the biggest complications associated with diabetes is the formation of diabetic foot ulcers (DFU) and 1 in 6 people develop diabetic foot infections (DFI). These are characterised by the presence of several microorganisms, namely aerobic Gram-positive, Gram-negative bacteria, and anaerobes, being *Staphylococcus aureus* the most frequent microorganism. This microorganism has several resistance mechanisms and virulence factors, leading to overcome host defences and antibiotic treatment failure. Thus, the need for society to develop new formulations to eradicate these pathogens has increased. In addition, recently, the interest of the scientific community in the development of formulations using natural products is also increasing. Wine production is one of the industries that produces the largest amount of residues representing 5 to 9 million tons of residues per year. For many years, wine waste was not valued as profitable, being discarded, which leads to serious environmental problems. However, the demand of the wine industry for a more sustainable production and application of a circular economy (CE) has led to the search for ways to recover and add value to these wastes. In fact, grape pomace (GP) mainly composed of seeds, skins and stalks concentrate bioactive compounds (BC) of phenolic nature, which may have antimicrobial and antioxidant activities. In this study, firstly BC of phenolic nature were extracted from pomace of *Touriga Nacional*, *Touriga Franca*, *Tinta Moriz* and *Tinta Barroca* red grape varieties, attaining extraction yields in a range of 4 to 30%. The antimicrobial activity and the mode of action of the red grape pomace extracts (RPE) (crude ethanolic extracts (50 and 70%)) were studied by different microbial physiological indices: minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), synergies between the RPE and marketed antibiotics commonly used for the treatment of *S. aureus* in DFI, propidium iodine (PI) absorption, intracellular potassium (K^+) leakage and virulence factors production (total proteases, gelatinases and siderophores). Additionally, the antioxidant potential of the RPE was also tested. The antibacterial activity against *S. aureus* was confirmed once MIC values of 25 mg/mL and 50 mg/mL were obtained for the *S. aureus* strains under study (CECT976 and RN4220). However, the MBC value of 25 mg/mL was obtained only for the *S. aureus* CECT976 strain. Preliminary tests to analyse the synergic potential of RPE with antibiotics showed a potentiation effect in different combinations. An antioxidant effect was detected for the crude ethanolic extract (50%) and not detectable for the crude ethanolic extract (70%). After exposure to the RPE, S. *aureus* showed permeabilization to PI, leakage of intracellular K^+ for RN4220 strain, but not for CECT976. Regarding RPE effect on proteases and gelatinases production, no conclusions were obtained, and for the production of siderophores it was possible to verify an increase when RPE are present. Therefore, it is recognized that it is necessary to carry out future studies to better understand the potentialities of extracts of residues from wine production.

Keywords: Diabetic foot infection, Circular Economy, Red Grape Pomace Extracts, Bioactive Compounds

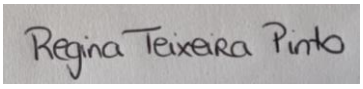
Resumo

Diabetes Mellitus (DM) é uma doença heterógena caracterizada por níveis altos de glicose no sangue. Estima-se que em 2019, DM afetava cerca de 463 milhões de pessoas, representando um custo em cuidados de saúde 760.3 biliões de USD em todo o mundo. Uma das maiores complicações associadas aos diabetes é a formação de úlceras do pé diabético (UPD) e 1 em cada 6 pessoas desenvolve infeções do pé diabético (IPD). Estas são caracterizadas pela presença de diversos microrganismos, nomeadamente, bactérias aeróbias Gram-Positivas e Gram-Negativas e anaeróbios, sendo *Staphylococcus aureus* o microrganismo mais frequente. Este microrganismo tem diversos mecanismos de resistência e fatores de virulência, levando a uma superação das defesas do hospedeiro e a um falhanço no tratamento com antibióticos. Assim, a necessidade da sociedade para desenvolver novas fórmulas de forma a erradicar estes patógenos, tem vindo a aumentar. Para além disso, recentemente, o interesse por parte da comunidade científica no desenvolvimento de novas formulações utilizando produtos naturais também tem aumentado. A produção de vinho é uma das indústrias que produz a maior quantidade de resíduos, gerando cerca de 5 a 9 milhões de toneladas de resíduos por ano. Durante muitos anos, os resíduos do vinho não foram valorizados como rentáveis, sendo descartados, o que levou a sérios problemas ambientais. No entanto, a necessidade de uma indústria vinícola mais sustentável e a aplicação de economia circular (EC), levou à procura de formas de recuperar e adicionar valor a estes resíduos. De facto, o bagaço de uva (BU) é constituído maioritariamente por compostos bioativos (CB) de natureza fenólica, que podem ter atividades antimicrobiana e antioxidante. Neste estudo, primeiramente CB de natureza fenólica foram extraídos de bagaço de uva tinta das castas de Touriga Nacional, Touriga Franca, Tinta Moriz e Tinta Barroca, atingindo rendimentos de extração de 4 a 30%. A atividade antimicrobiana e o modo de ação de extratos de bagaço uva tinta (EUT) (extratos etanólicos 50 e 70%) foram estudados através de diferentes índices fisiológicos microbianos: concentrações mínimas inibitórias (MIC) e concentrações mínimas bactericidas (MBC), sinergias entre EUT e antibióticos comumente utilizados no tratamento de *S. aureus* em IPD disponíveis no mercado, absorção de iodeto de propídio (IP), libertação de potássio (K^+) intracelular e produção de fatores de virulência (total de protéases, gelatinases e sideróforos). Adicionalmente, o potencial efeito antioxidante dos EUT foi também testado. A atividade antibacteriana contra *S. aureus* foi confirmada, uma vez que se obtiveram valores de MIC de 25 mg/mL e 50 mg/mL para as estirpes de *S. aureus* em estudo (CECT976 e RN4220). No entanto, o valor de MBC de 25 mg/mL foi obtido apenas para a estirpe de *S. aureus* CECT976. Testes preliminares para analisar potenciais sinergias entre os EUT com antibióticos mostraram efeito potenciador em diferentes combinações. O efeito antioxidante foi detetado para o extrato etanólico de 50%, mas não para o extrato etanólico de 70%. Após exposição aos EUT, *S. aureus* apresentou permeabilização ao IP, libertação de K^+ intracelular para a estirpe RN4220, mas não para a CECT976. Relativamente ao efeito dos EUT na produção de protéases e gelatinases, não foram obtidas conclusões, e para a produção de sideróforos, foi possível verificar um aumento quando EUT estava presente. Desta forma, é reconhecida a necessidade de realizar estudos futuros para entender melhor as potencialidades dos extratos de resíduos da produção de vinho.

Palavras-Chave: Infeções do pé diabético, Economia circular, Extratos de bagaço de uva tinta, Compostos Bioativos

Declaration

I hereby declare, under word of honour, that this work is original and that all non-original contributions are indicated, and due reference is given to the author and source.



(Regina Teixeira Pinto)

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Chapter 1 -Work Outline

1.1 Background and thesis presentation

Diabetes mellitus (DM), commonly known as diabetes, is defined as a heterogeneous group of disorders characterized by high blood glucose levels (Alam et al., 2014). DM brings complications such as neuropathy and ischemia from peripheral vascular disease. The development of diabetic foot ulcers (DFUs) is another critical aspect of diabetes mainly caused by dry skin. Due to the pathophysiological conditions of DFUs, it is estimated that 1 in 6 patients develop diabetic foot infections (DFI), which can lead to amputation. These infections are mainly polymicrobial and include a variety of aerobic Gram-positive bacteria, Gram-negative bacteria, and anaerobes, being methicillin-resistant *Staphylococcus aureus* (MRSA), the most frequent pathogen isolated. In fact, *S. aureus* is present in 30 to 40% of the infection cases and it is usually resistant to several antibiotics, most of the times as result of repeated or prolonged antibiotic usage in DFIs care.

There are several factors that confer resistance to *S. aureus*, including harbouring exfoliatin, EDIN (epidermal cell differentiation inhibitors), PVL (Panton-valentine leucocidin) and TSST-encoding (toxic shock-syndrome toxin) (Dunyach-Remy et al., 2016). They also have numerous surface proteins that mediate the adherence to components of the bone matrix collagen and the capacity to form microbial communities named biofilms. Furthermore, it is known that *S. aureus* expresses more than 50 extracellular virulence factors, including exotoxins, surface proteins and secreted enzymes, such as proteases, nucleases, and lipases, that may interact with the epidermal barrier in DFI.

Multi-drug resistant bacteria are emerging around the world, caused by an abusive and inappropriate use of antibiotics, which leads to a vast personal, social, medical, and economic impact. Health costs associated with DM are evaluated in 760.3 billion USD in 2019, with an expectation to increase to 845.0 billion USD. This way, there has been an increase of awareness of diabetic foot problems leading to the International Diabetes Foundation to declare that there should be a preventable plan for long-term complications of diabetes (Boulton et al., 2005). Besides that, the interest of the society about natural products and the use of extracts from plants as alternative medicine has been also increasing (Silva et al., 2021). Indeed, several studies documented that extracts of medicinal plants can arrest bleeding from fresh wounds, inhibit microbial growth and promote wound healing (Oguntibeju, 2019; Shedoeva et al., 2019).

Wine production is one of the main agriculture activities in the world (Maicas et al., 2020). It is estimated that around 5 to 9 million tons of waste are generated per year, once at least 20% of the fruit weight is discarded as pomace, grapes, and seeds (Ferri et al., 2020; Jimenez-lopez et al., 2020). Although part of the bioactive compounds (BC) is transferred to the wine, during winemaking process, a considerable amount of these compounds still remains in the by-products of its production named grape pomace (GP)

(Melo et al., 2015). GP concentrate BC of phenolic nature such as flavonoids, tannins and lignins that may have antioxidant and antibacterial activities (Altemimi et al., 2017; Dilas et al., 2009; Jimenez-Lopez et al., 2020; Sanzuela et al., 2014).

The concept of circular economy (CE) applied to wine production is firstly related to the possibility of identification, quantification, extraction, and purification of BC, such as phenolic compounds (PC), from the pomace to produce added value products for food, cosmetic and even pharmaceutical industries (Ferri et al., 2020). Besides that, CE concept can also focus on the use of the waste as raw materials, substrate or an additional nutritional supplement for cultivation of microorganisms (Fontana et al., 2017; Maicas et al., 2020).

The foundation for this thesis lays on the urge of *Sogrape Vinhos S.A.* (Portugal) to achieve a world in harmony with nature, celebrate diversity and inclusion, and sustainability and it is a part of POMACE4Wounds project (IJUP2021-SOGRAPE-23). Thus, the positive contributions of these project are:

1) Find natural sources as an alternative or complement for the treatment of *S. aureus* DFI.
2) Add value to the wine waste industry by-products increasing sustainability of wine production by extraction bioactive compounds from Red Grape Pomace (RGP).
3) Study the bioactivity (antimicrobial and antioxidant activity) of the Red Grape Pomace Extracts (RPE) against *S. aureus*.
4) Give an insight of antimicrobial and antipathogenic mode of action of RPE against *S. aureus*.

1.2 Objectives

This project aims to take what usually is considered as waste from wine production, and to extract biocomponents that may have biological interest for the treatment of *S. aureus* DFI. Besides that, a study of the antimicrobial, antipathogenic and antioxidant properties of RPE was performed.

To this end, the project was divided into three main tasks. The first task involved the extraction of the bioactive compounds from the waste of the wine production. This phase, better described on Chapter 3 started with the harvest and transport of the wine waste, samples preparation, more specifically drying and grinding, and then the preparation of the extracts.

The second task involves determining the minimum inhibitory and bactericidal concentrations (MIC and MBC) of RPE singly, and the study of possible synergies between the RPE with commonly used antibiotics to treat DFI in which *S. aureus* is the most common pathogen. Besides that, an evaluation of the antioxidant properties of RPE, which can help wound closure, was performed. In this phase, the microdilution methods in microplates, a modified disc diffusion method and an assay for the antioxidant potential were applied.

Finally, on the third phase, the mode of action and interference of RPE in *S. aureus* virulence was evaluated. First, the assessment of cytoplasmic membrane integrity resorted propidium iodide uptake and potassium leakage assays, and the effect of these extracts on virulence factors production resorting proteases, siderophores and gelatinases production.

1.3 Thesis organization

This thesis is divided in 6 Chapters. The current chapter, Work Outline, intends to describe the scope and the objectives of the work developed.

In Chapter 2, it is possible to find a Literature Review that resumes the most important information related to diabetes problem, the bacteria associated with this disease and what are the alternatives that can complement the antibiotic treatment. Besides that, it is explored the possibility of using waste from the wine industry, in order to extract bioactive compounds, as a sustainable solution, application of the circular economy concept, not only by minimizing the waste from this industry but also the possibility of the production of more sustainable medication.

In Chapter 3, the extraction methods applied to the wine-waste by-products are presented. Chapter 4 describes the activity of the extracted solutions (crude ethanolic extracts (50 and 70%)) by the study of their MIC and MBC and possible synergies with commonly used antibiotics against *S. aureus*. Besides that, the study of the antioxidant properties of the crude ethanolic extracts (50 and 70%) can be found.

The mode of antibacterial action and virulence interference of the RPE was evaluated on Chapter 5. To this purpose, an assessment of membrane integrity due to propidium iodide uptake was performed, as well as the study of intracellular potassium leakage. Besides that, the potential of the extracts to interfere with *S. aureus* extracellular total proteases, gelatinases and siderophores production was determined.

Last but not least, Chapter 6 summarizes the main conclusions of this work and points at the future direction.

Chapter 2- Literature Review

2.1 Diabetes mellitus-An overview

DM, commonly known as diabetes, is defined as a heterogeneous group of disorders characterized by high blood glucose levels (Alam et al., 2014). DM can be classified in two main categories, type I diabetes, and type II diabetes. Type I DM represents around 7-12% of all cases of DM and includes two forms, idiopathic and autoimmune DM (Di et al., 2013). Idiopathic type I DM is an unusual form with unknown cause, with almost complete insulin deficiency and no evidence of autoimmunity. This category of type I DM exhibits a strong hereditary component and lacks immunological evidence for β cell autoimmunity. The disease starts with the appearance of autoantibodies, with consequent pancreatic β cell destruction. It results in insulin insufficiency, and patients develop life-threatening hyperglycaemia. With the disease progression, symptoms such as thirst, weight loss and frequent urination appear, and are more likely to appear in children and adolescents. Almost all patients require daily administration of insulin to regulate the amount of glucose in blood to achieve normoglycemia (Eisenbarth, 1986).

On the other hand, type II DM is the predominant form of DM, representing 87-91% of all cases with predictions to expand. This type of DM is more often present on the older set. However, an increase of type II DM in young people has also been reported and it is mostly related with obesity cases or genetic factors. At a first approach, it is possible to manage the disease with lifestyle changes and adjustments in eating habits (Defronzo, 2015).

Weight loss, polyurea, polydipsia, polyphagia, constipation, lethargy, cramps, blurred vision, and candidiasis are some of the symptoms of DM. Patients with type I diabetes for a long time are more likely to develop microvascular issues as well as macrovascular disease (coronary artery, heart, and peripheral vascular disease). Peripheral vascular disease can also lead to an end-organ damage, which include DFU. On the other hand, cardiovascular problems and end-stage renal disease are the leading causes of death in people with type II diabetes (Baynes, 2015).

The risk of a diabetic person to develop DFU can reach 25% and it is estimated that 20% of these patients require hospitalization due to this condition (Bandyk, 2018; Clayton et al., 2009). The increased propensity of developing foot ulcerations is related to persistent and poorly controlled hyperglycaemia that lead to neuropathic and vascular abnormalities. A systematic review and meta-analysis of the global prevalence of DFUs showed that the global prevalence of DFUs was 6.3% higher in males than in females, and higher in type II than in type I diabetic patients (Ramirez-Acuna et al, 2019).

The hyperglycaemic state increases the action of the enzyme aldose reductase (AR) and sorbitol dehydrogenase (SDH) resulting in the conversion of intracellular glucose to sorbitol and fructose (Clayton et al., 2009). The reduction of glucose into fructose is due at the expense of NADPH, which is essential for generation of intracellular antioxidant (GSH). The depletion of NADPH by AR may impair intracellular

antioxidant defence. After that, sorbitol is converted into fructose by SDH, causing a production of NADH, which leads to an increase of reactive oxygen species (ROS). The higher levels of GSH and lower levels of ROS cause oxidative stress and cell destruction, leading to retinopathy, neuropathy, and nephropathy complications (Figure 1) (Apelqvist, 2012; Tang et al., 2020).

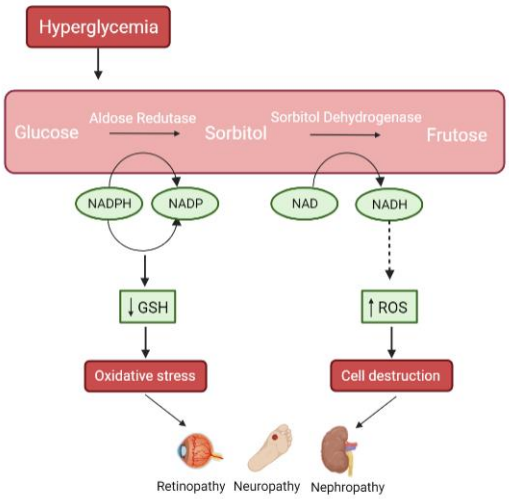

Figure 1- Representative schematic diagram of the connection of the polyol pathway with oxidative stress and other pathologies associated to diabetes

Added to neuropathy and ischemia from peripheral vascular disease complications mentioned above, the development of DFUs result from other factors like foot deformity, callus formation and elevated skin pressure (Clayton et al., 2009; Frykberg, 2002). Besides that, the reduced skin perfusion due to peripheral artery occlusive disease, repetitive trauma and ill-fitting shoes, that can produce friction in the skin, also contribute to lesions occurrence (Bandyk, 2018; Frykberg, 2002). Another critical aspect of diabetics is the fact that usually the skin becomes dry and more likely to develop fissures, which decreases the effectiveness barrier against microorganism invasion (Bandyk, 2018). This way and due to pathophysiological conditions of DFUs, skin ulcers become often colonized by microorganisms, leading inevitably to DFIs development. Most of the patients with DFIs do not present fever or chills as symptoms. However, the ones who do have systemic signs usually indicate a more severe and deeper infection (Barder, 2008). It is estimated that amongst of the 5 to 25% individuals that develop foot ulceration during their lifetime, 1 in 6 patients contract infections, which usually respond poorly to treatment options and require amputation (Vu et al., 2014).

Due to the vast personal, social, medical, and economic impact of this problem, there has been an increase of awareness of diabetic foot problems leading to the International Diabetes Foundation to declare that there should be a preventable plan for long-term complications of diabetes (Boulton et al., 2005). It was estimated that in 2019, 463 million persons had diabetes, which resulted in approximately 760.3 billion

USD in health costs around the world. Future predictions do not seem positive once these numbers are expected to grow to 700.2 million persons and 845.0 billion USD in 2045 (Alzaid et al., 2021).

2.2 Diabetic foot infections: bacterial microbiota

DFIs are mainly polymicrobial and include a variety of aerobic Gram-positive bacteria (*Staphylococcus aureus* (MRSA) and *Streptococcus* β-homolytic), Gram-negative bacteria (*Pseudomonas aeruginosa*, *Proteus spp*.), and anaerobes (*Peptostreptococcus spp*., *Bacteroides spp*., *Prevotella spp*., *Clostridium spp*.), being methicillin-resistant *Staphylococcus aureus* (MRSA), the most frequent pathogen isolated followed by *Pseudomonas aeruginosa* (Banu et al., 2015; Dunyach-Remy et al., 2016; Frykberg, 2002; Ramirez-Acuña et al., 2019). These microorganisms can spread to subcutaneous tissues such as tendons, joints, fascia, muscle, and bone, worsening the clinical condition of infected patients (Dunyach-Remy et al., 2016).

The DFIs can be characterized in acute infection or chronic infection. In acute infections, there is a rare complication called fasciitis, which results in necrosis. It is mostly caused by gram-positive cocci, *S. aureus*, and *Streptococci* β-homolytic. The affected area is characterized by being dusky, presenting purple patches over the inflammation (Alpelqvist, 2012; Fisher et al., 2010).

On the other hand, chronic infections usually are associated with a deeper infections and wounds with necrotic tissue in which a polymicrobial flora is most common. These last ones are a combination of Gram-negative, anaerobic, and Gram-positive organisms and have a tendency for biofilm formation (Alpelqvist, 2012).

S. aureus is present in 30 to 40% of the infection cases leading to an increase of amputation risk since this bacterial strain is usually resistant to several antibiotics, most of the times as result of repeated or prolonged antibiotic usage in DFIs care (Bandyk, 2018). There are a lot of characteristics that make *S. aureus* the most commonly isolated pathogen in DFIs, which are going to be described afterwards (Vu et al., 2014). Since microorganisms are present in almost every skin wound (including DFUs), the variability of bacterial virulence factors and the level of host resistance must be considered when purulence or inflammation are present (Dunyach-Remy et al., 2016; Frykberg, 2002).

2.2.1 The particular case of *S. aureus*

S. aureus is a Gram-positive bacterium that colonizes approximately 30% of the population, being both a parasite bacterium and a human pathogen, causing a wide range of clinical infections. It is characterised for having high adaptation for soft tissues and bone infections (Dunyach-Remy et al., 2016; Tentolouris et al., 1999).

Two strains of *S. aureus* have been distinguished in DFIs, non-toxicogenic and toxicogenic. Non-toxicogenic strains are mainly localized in deep structures and bone involving diabetic foot osteomyelitis. On the other hand, the toxicogenic *S. aureus* strains include harbouring exfoliatin, EDIN (epidermal cell differentiation inhibitors), PVL (Panton-valentine leucocidin) and TSST-encoding (toxic shock-syndrome

toxin) genes and are often present in infections with a more severe grade and systemic impact (Dunyach-Remy et al., 2016).

One of the main characteristics of *S. aureus* that makes it so relevant in DFI is the capability of attachment to the ulcer surface. This propensity in turn depends on numerous surface proteins that mediate the adherence to components of the bone matrix collagen. Adhesins and microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) facilitate bacterial adhesion to skin tissue and are essential for intracellular bone invasion, affecting osteoblasts, fibroblasts, and endothelial cells. Besides that, toxicogenic *S. aureus* strains are able to survive preserving the integrity of the host cell in a metabolically inactive state by forming small-colony variants (SCVs), which are particularly resistant and difficult to eradicate with antibiotic therapy (Dunyach-Remy et al., 2016).

The synthesis and secretion of glycocalyx and the toxins produced by *S. aureus* also play a role in the colonization, persistence, evasion of the immune system and dissemination. Within the toxins produced, pore-forming toxins (PFTs), exfoliative toxins (ETs), superantigen exotoxins (SAgs) and EDIN toxins can stand out (Dunyach-Remy et al., 2016).

The PFTs have the ability to lyse host cells. Inside this group, there are alfa-toxin that targets red blood cells and leukocytes (except neutrophils), phenol soluble modulins that are membrane-injuring toxins and Bi-Component Leukotoxins. There are a lot of types of Bi-component leukotoxins that confer cytotoxicity on neutrophils and monocytes-macrophages. These facts lead to high virulence, contribute to neutrophil killing, promotes the survival of *S. aureus* in human whole blood, restricts neutrophil-mediated killing and promotes CA-MRSA (community-acquired MRSA) (Dunyach-Remy et al., 2016).

The ETs are linked to human infection, once they act as molecular scissors facilitating skin invasion (Dunyach-Remy et al., 2016).

SAgs proteins have critical virulence factors as they promote colonization and overall development of chronic DFU wounds. It is suggested that therapies that neutralize or reduce SAgs production may be one of the keys to manage infections in patients with DFI (Dunyach-Remy et al., 2016; Vu et al., 2014).

The EDIN factor might promote bacterial dissemination in tissues and, when working together with the arsenal of *S. aureus* virulence factors, it can give the bacteria a higher potential for systemic infection (Dunyach-Remy et al., 2016).

Besides that, *S. aureus* have also the capacity to form sessile microbial communities named biofilms. The biofilm matrix creates a unique environment for bacteria by resulting in a spatially and temporally varied bacterial community. Extracellular polymeric substances (EPS) immobilize biofilm cells, keeping them in proximity for long periods of time allowing strong interactions such as cell-to-cell communication, horizontal gene transfer, and the creation of synergistic microconsortia (Flemming et al., 2010; Davis et al, 2006). Biofilm formation is an important virulence factor once it establishes a protective environment for the organisms in many ways. The close cell-cell contact in the biofilm allows nutrients to enter and circulate, the synthesis of proteins, as well as the accumulation of waste and toxins to exit. It also

permits for the bacteria a more effective transfer of plasmids which can contain multidrug resistance genes (Banu et al., 2015; Mottola et al., 2016). Interestingly, the formation of biofilm by *S. aureus* begins with the production of MSCRAMM. Altogether, these components mediate the adherence to host tissues, gathering thus, the necessary conditions for the process of biofilm formation start (Mottola et al., 2016). It is of highlight that biofilms are recognized as the main responsible for the DFIs non-healing and consequent persistence/chronicity.

2.3 Current antibiotic regimens and therapeutic strategies for diabetic foot infections treatment

The main goal in treating DFIs is to obtain wound closure. For that, there must be an evaluation and classification of its severity and vascularity, and the presence of infection (Frykberg, 2002).

According to the Infectious Diseases Society of America/International Working Group on the Diabetic Foot (IDSA/IWGDF) system, DFIs can be classified in three different categories (mild, moderate, and severe). When the infection presents symptoms such as local swelling or induration, erythema between 0.5-2 cm around the ulcer, local tenderness, or pain and/or local warmth or purulent discharge, the infection is considered mild. For local infections with erythema superior to 2 cm around the ulcer, or involving structures deeper than skin and subcutaneous tissues, such as bones, joints, tendons or muscles, and no systemic signs or symptoms, the infections are classified as moderate on the infection severity. Finally, when the local infection has more than 2 cm along with fever, heart rates above 90 beats/minute, respiratory rate higher than 20 breaths/minute or white cell count below 4×10^9/L or above 12×10^9/L, the infection is considered severe (Monteiro-Soares et al., 2019; Ramirez-Acuna et al., 2019).

After microbiological material collection from tissues or bones, empiric antibiotic therapy should be initiated. Though sometimes there needs to be an adjustment to a target antibiotic when the results of the microbiologic study and the antimicrobial agent's sensitivity are known (Lipsky et al., 2000; Mottola et al., 2016). Besides, the presence of sessile communities should also be considered. In fact, the occurrence of biofilms by *S. aureus* leads to the need of using very high concentrations of antibiotics when treating DFIs and frequently, as explained before, these treatments are not effective enough (Mottola et al., 2016). In this case, patients with prior antibiotic treatment, and recurring infection should be assumed to have MRSA infection and empiric treatment should be instituted (Bandyk, 2018).

As mentioned before, MRSA are frequently found in patients with DFIs, usually even in patients previously treated with antibiotic therapy or patients with a recent history of hospitalization (Clayton et al., 2009). In order to treat MRSA-associated infection, it is common to use a wide range of antibiotic therapy. Both Gram-positive bacteria, particularly *S. aureus*, and the most commonly isolated Gram-negative bacteria should be considered for antibiotic regimens. Furthermore, in severe infections, especially in the

presence of necrosis or recent antibiotic treatment, the antibiotic should also be addressed to anaerobes pathogens (Frykberg, 2002).

Taking into account that DFIs are usually polymicrobial, generally the treatment is a combination of different antibiotics and therapies. The most common used antibiotics and therapeutic regimens for each type of infection are presented in Table 1.

Table 1-Most common used antibiotics and/or therapeutic regimens for the treatment of DFIs according to its severity (adapted from Bader, 2008; Jneid et al., 2017)

Severity of the infection	Antibiotic/Therapeutic regimens
Mild infection	• Amoxicillin/Acid clavulanic • Ofloxacin • Ciprofloxacin • Povidone iodine 10 % • Chlorohexidine • Acetic acid 5 % • Silver Compounds

Moderate infection	• Piperacillin/Tazobactam
	• Beta-lactam allergy: metronidazole + levofloxacin + vancomycin or amoxicillin-clavulanate or ampicillin
	• Carbapenem IV:
	  ◦ Imipenem
	  ◦ Ertapenem
	  ◦ Meropenem
	• Dicloxacillin
	• Cephalosporin
	• Quinolones
	• Clindamycin

Severe	• Meropenem + vancomycin • Meropenem + linezolid • Piperacillin/tazobactam + clindamycin • Carbapenems • Ampicillin/Sulbactam • Vancomycin • Daptomycin • Cefepime • Ceftazidime • Metronidazole • Clindamycin • Quinolones • Tiracillin/Clavulanate

The most relevant agents used include antibiotics from fluoroquinolones and cephalosporins classes, and penicillin/β-lactamase inhibitors and cephalosporins. However, MRSA are usually resistant to most β-lactam antibiotics as well as to a wide range of other antimicrobials. This can result in persistent/recurrent infections, difficult to manage and expensive to treat (Clayton et al., 2009; Mottola et

al., 2016). There are only two fifth generation cephalosporins that showed effectiveness against MRSA, which are ceftaroline and ceftobripole. These antibiotics have a similar spectrum of activity and aim, which is to inhibit PBP2a (penicillin binding protein 2a), but their operating mode is different. PBP's are usually the bonding locals of the β-lactamic antibiotics, however, in the specific case of MRSA, this protein is different once it does not have the affinity to bond with the antibiotics (Santiago et al., 2015). While ceftaroline causes an allosteric change in PBP2a, ceftobiprole access to the active site of PBP2a by its R2 group (Mottola et al., 2016). This last one was approved by Food and Drug Administration (FDA) for the treatment of skin infections also caused by MRSA (Mottola et al., 2016).

Specific adjunctive therapies have been shown to aid in ulcer/wound healing, such as debridement and topical dressing. An advanced wound treatment may also include negative pressure wound therapy, and bioengineered skin (Bandyk, 2018).

Debridement consists of the removal of any obstacle that prevents new tissue formation, preparing the wound bed. This therapeutic technique involves physical or chemical debridement and transforms a stagnant wound into an acute healing process by removing pathogens from chronic infected wounds. There are a lot of different debridement techniques, even though the most effective method is still unknown (Edwards et al., 2010). The surgical debridement is considered a simple technique, requiring only the use of sterilized scissors or scalpels. It is a quick procedure with the disadvantage of possibility of open wound (Edwards et al., 2010). The wet-to-dry technique consists of soaking the wound in saline before the application of a moist gauze pad on the affected area. A few moments after the application, the devitalized tissue dries and gets attached to the gauze, that can be removed. This technique allows the removal of hardened necrosis, and it is relatively cheap. However, some granulating tissue may also be removed, and the procedure is usually painful for the patient (Kavitha, 2014). Another example of debridement technique is bio-surgery, using sterile maggots, placed right in the infected area. Being maggots feed only by the necrotic tissue, they discriminate the granulating tissue. However, there are still no clear evidence about the effectiveness of the treatment and the associated costs are high (Azad et al., 2016). Non-mechanical debridement is an easy application treatment and may be beneficial for healing of the wound (Edwards et al., 2010). The enzyme preparation has the purpose to digest the proteins fibrin, collagen, and elastin present on necrotic tissue of a wound. These formulations can be directly applied to the necrotic area (Kavitha, 2014). Polysaccharide beads or paste dextranomer polysaccharide are also common debridement treatments where the beads are removed by washing and the trapped necrotic material is removed due to the hydrophilic characteristic of the beads (Bradley et al., 1999).

Last but not least, the use of biologically inert hydrogels that contain high amount of water has emerged. Hydrogels consist of hydrophilic substances that stimulates autolytic debridement, maintaining the wound moist. On the other hand, hydrocolloids are hydrogel mixed with synthetic rubber and sticky materials. It stimulates autolytic debridement, maintaining the wound moist. While hydrogels are applicated in moderate-to-high exudative wounds, hydrocolloids are applied on severe exudative wounds (Kavitha, 2014; Moura et al., 2013).

Dressings are an essential part of the DFIs healing once they protect the wound from external exposure and at the same time reabsorb the exudate unpleasant odour. The applied dressing depends on the characteristics of the infection (Moura et al., 2013). They are usually inexpensive, well tolerated, and contribute to an atraumatic, moist wound environment (Clayton et al., 2009). However, topical medication and gels are more efficient when compared with saline wet-to-dry dressings (Frykberg, 2002).

Foams are composed by polyurethane or silicone. Semi permeability and antimicrobial activity are some of the characteristics of the foams. They are also used as secondary dressings with hydrogel or alginate dressings (Moura et al., 2013).

Alginate dressings are fibrous products derived from brown seaweed, which can form a gel after binding to wound exudate. They can be freely cut according to the shape of the wound, and they have excellent absorption properties. By optimizing the wound humidity, better outcomes are obtained with alginate dressings (Moura et al., 2013).

Film dressings are adhesive, porous, and thin transparent polyurethane, allowing oxygen, carbon dioxide, and water vapor pass through the dressing while liquids and bacteria are well-isolated (Ahmed et al., 2018).

Lastly, silver-impregnated dressings have anti-microbial activity, help controlling the pain and promote a faster wound contraction, by accelerating proliferation of fibroblasts and differentiation into myofibroblasts. Besides that, they are also used for odour absorption (Zhang et al., 2019).

In recent years, other more advanced therapeutic strategies have been developed, in which negative pressure wound therapy and bioengineered skin stand out. Negative pressure wound therapy (NPWT) consists of the use of a vacuum system to remove the excess fluid and to promote healing, both in acute and chronic wounds. Although the presence of osteomyelitis and necrotic tissue are contraindications to this therapy, it is proven that NPWT improves wound bed supply, ulcer size reduction and decrease of the bacteria load in DFIs (Zhang et al., 2014).

More recent studies show that bioengineered skin and human dermis enhance healing. It acts as a delivery system for growth factors and extracellular matrix components, through the activity of live human fibroblasts contained in their dermal elements (Frykberg, 2002). This therapy is usually applied in chronic non-infected DFUs.

2.4 Green and sustainable solution to efficiently face diabetic foot infection

Despite of the advances in DFIs care and management, there are still some cases in which the outcome is not the desired, especially when multi-drug resistant (MDR) bacteria are involved. Indeed, MDR bacteria are emerging around the world, mostly due to an abusive and inappropriate use of antibiotics (Oguntibeju, 2019). This problem got worse by the unavailability of the development of new antibacterial agents at the same pace as bacteria emerging. In view of that, there is an urgent demanding for finding

effective and fast solutions. Besides that, society has also increased their interest about natural products and on the use of extracts from plants as alternative medicine (Silva et al., 2021).

Accordingly, plants produce large number of compounds with known biological properties such as anti-inflammatory, antioxidant and antimicrobial effect (Silva et al., 2021; Xu et al., 2019). Several studies have been documented that extracts of medicinal plants can arrest bleeding from fresh wounds, inhibit microbial growth and promote wound healing (Oguntibeju, 2019; Shedoeva et al., 2019). One of the mechanisms reported was that plant extracts can stimulate fibroblasts, enhancing the wound healing process. Besides that, plant extracts have free radical scavenging action compounds, which can act singly or synergistically with antibiotics (Jimenez-Lopez et al., 2020; Oguntibeju, 2019). The wound healing process is related to the increase of strength of collagen fibres, preventing cell damage or promoting DNA synthesis. As mentioned before, the increase of ROS is a complication associated with diabetes that induces DNA, proteins, and lipids damage. The antioxidant properties of plant extracts and/or their components could be helpful as therapeutic agents against this (Oguntibeju, 2019).

Natural compounds extracted from plants may also have antimicrobial properties, especially when combined with commonly used antibiotics, enhancing their potential (Silva et al., 2021). Furthermore, they can also be considered as a sustainable solution once it is possible to reuse some less effective antibiotics existent in the market (Cheesman et al., 2017).

2.4.1 Biovalorisation of by-products from the wine industry – pomace

Wine production is one of the world's most important agricultural enterprises (Maicas et al., 2020). In Portugal, approximately 1.2 to 3.5 tons of waste are produced per year (Silva et al., 2021). For many years, wine waste was not valued as profitable, being mainly discarded, which leads to serious environmental issues such as water pollution, soil degradation, damage to vegetations, energy use and emission of gases and odours (Silva et al., 2021).

Grape seeds, skins and stalks are part of wine industry by-products, being GP the main one. GP concentrate BC of phenolic nature that may have antioxidant and antibacterial activities (Sanzuela et al., 2014). Phenolic compounds (PC) are a large group of secondary metabolites that can be found in vegetables and fruits, for example grapes, once it is one of the plants responses to adapt to their surroundings. PC such as flavonoids, tannins and lignins showed antioxidant and antimicrobial properties (Figure 2) (Altemimi et al., 2017; Dilas et al., 2009; Jimenez-Lopez et al., 2020). Besides that, PC compounds isolated from plants show low toxicity against human cells, which allows their use in therapy safely. This way extracts from plants may constitute an innovative solution for the future, not only by promoting new drug development but also to increase its sustainable production (Jimenez-Lopez et al., 2020).

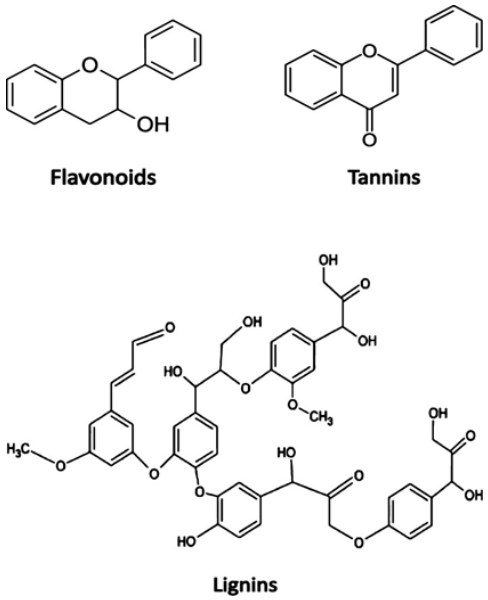

Figure 2-Chemical structures of the main phenolic classes.

The concept of CE is based on the ideal of closing loops and create complete cycles to a functional economy system. Moreover, CE is associated with 6Rs, reduce, recycle, redesign, remanufacture and recover. This theory can lead not only to develop a more sustainable production model of agriculture in general but can also promote economic growth and environmental conservation.

The concept of CE applied to wine production can be approached by two different points of view represented on Figure 3. The first one is related to the possibility of identification, quantification, extraction, and purification of BC like PC from pomace to produce added value products in food, cosmetic and even pharmaceutical industries (Ferri et al., 2020). It has been reported that skins contain anthocyanins, tannins, colorants and enzymes, and seeds have a lot of tannins with antioxidant and antimicrobial properties (Ferri et al., 2020; Fontana et al., 2017; Jimenez-lopez et al., 2020; Silva et al., 2018). The second one focuses on the use of the waste as raw materials as substrate or an additional nutritional supplement for cultivation of microorganisms. Both solutions presented can provide alternatives to reduce the environmental impact of winemaking activities and to add value to the commercialization of extracts rich in BC (Fontana et al., 2017; Maicas et al., 2020). In fact, wine industry has as one of the main goals the increase of sustainability of wine production, by transforming raw materials into high-value products. Not only is this strategy an implementation of the CE concept but also a business opportunity.

Driven by this quest *Sogrape Vinhos* S.A. (Portugal), one of the biggest Portuguese companies dedicated to cultivation, production, and exportation of wine, has also been working in ways to achieve a world in harmony with nature, celebrate diversity and inclusion, and sustainability. These directives have already led to its worldwide recognition as an example of sustainable viniculture/viticulture for the adoption

of excellent sustainability practices in the environmental, economic, and social areas. *Sogrape Vinhos* S.A. continue to be focused in finding ways to become better.

The work herein developed is part of the POMACE4Wounds project (IJUP2021-SOGRAPE-23), which was delineated to comply with *Sogrape Vinhos* S.A ambitions concerning a more sustainable wine production for the future.

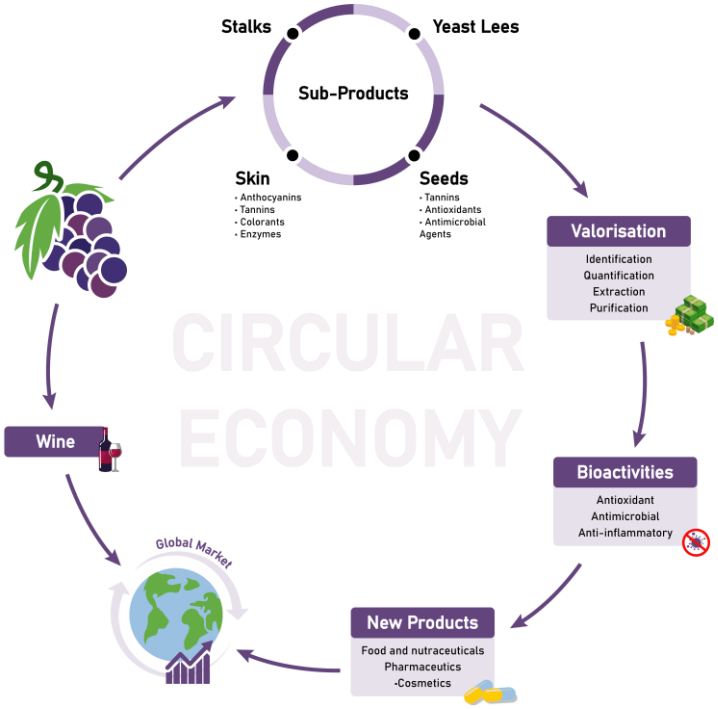

Figure 3- Schematic representation of winemaking production considering the circular economy perspective (adapted from Jiminez-Lopez et al., 2020)

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Chapter 3- Preparation of bioactive extracts from red grape pomace

3.1 Introduction

RGP, a by-product of red-wine production is constituted mainly by skins, seeds, and stalks (Sanzuela et al., 2014). RGP are rich in PC, such as flavonoids, tannins and lignins. The most predominant PC in RGP are anthocyanins, which belong to a class of red, purple and/or blue pigmented flavonoids that confer colour to the red wine and extracts (Allison et al., 2015; Ky et al., 2014).

Extract production is processed following four main steps. The first one is the solvent penetration into the solid matrix of the GP samples. After that, the solute dissolves in the solvents and causes the solute diffusion out of that solid matrix. Finally, the solutes can be collected (Zhang et al., 2018).

There are multiple extraction techniques named conventional extraction, which include solid liquid extraction (SLE), heating, griding and others. There are also more recent techniques that have been showing more efficiency and higher extraction yields, namely Micro-wave assisted extraction (MAE), ultrasonic assisted extraction (UAE), Soxhlet, Pulsed electrical field (PEF), sub- and supercritical fluid extraction (SFE), and pressurized liquid extraction (PLE), among others (Barba et al., 2016; Zhang et al., 2018). All of them have advantages and disadvantages and a consideration of the solute parameters is necessary when the selection is made.

SLE is a conventional technique involving the transference of solutes from a solid matrix to a solvent. It is a common unit operation used to recover many important components, such as phytochemicals from plants, or hydrocolloids from algae, among others. This technique, although it is one of the most common used to isolate PC, it also requires a large volume of solvents and a long extraction time (Aguilera, 2003; Bucić-Kojić et al., 2007). As a non-conventional extraction technique, MAE generates heat by the interaction of the polar compounds from the solvent with the organic components in plant matrixes in order to extract BC. This technique presents several advantages by increasing the extraction yield, reducing the losses of BC and the extraction time (Altemimi et al., 2017; Borges et al., 2020). It is also considered a green technology once it reduces the volume of organic solvent used. On the other hand, UAE uses ultrasound wave energy to disrupt the cell membrane, which facilitates the solvent penetration. It is known by being an easy technique, that requires only common laboratory equipment. UAE reduces both the extraction time, temperature and volume of solvent used. Although it is recommended for thermolabile and unstable compounds, ultrasonic probe has disadvantages related mainly with experimental repeatability and reproducibility (Altemimi et al., 2017; Zhang et al., 2018). Soxhlet is also a simple extraction technique, that allows the contact of the extract with fresh solvent in continue, and usually it is possible to obtain more mass than the MAE (Borges et al., 2020, Zhang et al., 2018). However, it takes a long time, and it has an

increase loss of the extractant. PEF treatment is a non-thermal method that significantly increases the extraction yield, decreases extraction time and minimizes the degradation of the thermolabile compounds. SFE uses a supercritical fluid, which can dissolve a lot of different natural compounds, and small pressure and temperature changes are applied. Lastly, PLE method decreases the extraction time by applying high pressure, which increases the solubility of compounds and penetration of solvent in the matrix (Zhang et al., 2018).

Added to the extraction technique, the extraction yield is affected by several properties, such as extraction solvent, the solvent-to-solid ratio, particle size of the materials, extraction temperature and time (Zhang et al., 2018). It has been reported that, for SLE, the selection of the extraction solvent is the most important parameter affecting the extraction yield (Borges et al., 2020). The choice of the used solvent must consider the polarity of the solute of interest. Similar polarities between the solvent and the solute promote a better dissolution, increasing the extraction yield. The most common used solvents are hexane, chloroform, ethyl acetate, acetone, methanol, ethanol, and water, being the first one mentioned the one with lower polarity and the last one the highest (Altemimi et al., 2017). In particular, aqueous mixtures of acetone, ethanol and methanol were reported to be the best solvents for the extraction of polyphenols from wine residues like pomace, once the water helps in the diffusion of extractable compounds through plant tissues (Cheng et al., 2012).

In this study, the SLE was the chosen extraction technique to obtain red grape pomace bioactive extracts (in terms of antimicrobial and antioxidant activity), since it is one of the most common used techniques for natural compounds extraction and requires simple laboratory equipment (Aguilera, 2013; Bucić-Kojić et al., 2007). Water and aqueous mixtures of acetone and ethanol, 50% and 70%, v/v, were used once they are considered green solvents. Besides that, they were reported to be the most reasonable choice to obtain grape pomace extracts rich in BC. Even though acetone is not considered a green solvent, it was also used on primary extractions once it is also one of the most common used solvents for GP extracts production and had better extraction yields than ethanol (Cheng et al., 2012).

3.2 Materials and Methods

3.2.1 Grape pomace sample collection and transport

Grapes arrived to the *Sogrape Vinhos* factory, and they were harvested by the removal of the stalks. (Figure 3). After that, the fermentation process and pressing of grapes was performed to produce wine. Right after the pressing, on the 20th of September of 2021, wine by-products (GP) were given by *Sogrape Vinhos* and transported to the FEUP laboratory. The pomace collected was mainly originated from the following red grape castes: *Touriga Nacional, Touriga Franca, Tinta Roriz* and *Tinta Barroca*.

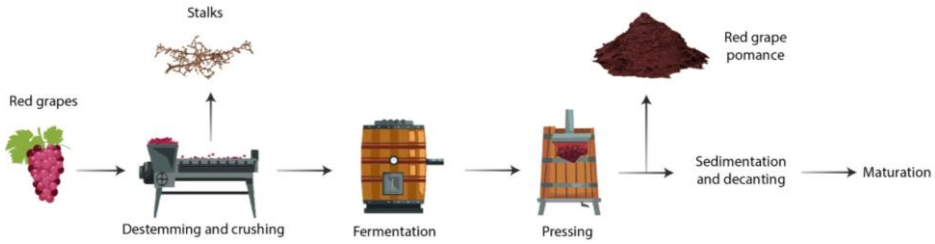


Figure 3-Grape pomace derived from the wine making process.

3.2.2 Grape pomace samples preparation: Drying and grinding

In the laboratory, firstly the GP was distributed in different traits and cups, weighed, and then put in the dryer at 60 ºC until complete dryness was obtained. This process happened as soon as the samples got to the laboratory in order to prevent deterioration and other processes that could interfere with the extract's activity.

3.2.3 Preparation of the grape pomace extracts

RPE were prepared using the SLE method according to Cheng et al. (2012) with minor alterations. For this, first the dried samples of the whole pomace were weighed and separated in three different fractions, seeds, skins, and stalks. After that, the samples were grinded and blended. Figure 4 illustrates this process.

Three different extractions were performed. On the first extraction, a proportion of solvent of 1:10 (w/v), i.e., for each g of solute, 10 mL of solvent were used. On the second and third extraction, a proportion of 1:3 (w/v) was used. On the first extraction, water, and aqueous solutions of acetone with concentrations of 50 and 70% and aqueous solutions of ethanol in concentrations of 50 and 70% were the chosen solvents. For the 2nd and 3rd extractions, only water and aqueous solutions of ethanol (50 and 70%) were used.

After this, the samples were agitated resorting to a magnet for 24 hours. The samples were then transferred to 50 mL cups and centrifugated at 3.772 g for 15 minutes (Eppendorf centrifuge 5810R; Eppendorf AG, Germany), collecting only the supernatant and discarding the rest. The samples were placed in a rotary evaporator (Büchi R-200, Flaiwil, Switzerland), operating at 60 º C until organic solvent evaporation (ethanol and acetone). Lastly, samples were lyophilized (SP Scientific, New York, USA), which allowed the evaporation of the aqueous solvent and consequently the formation of powder. The rest of the dried samples were stored in the refrigerator at 4 ºC.

Stock solutions with concentrations of 300 mg/mL and 200 mg/mL were prepared with DMSO on the 2nd and 3rd extraction, respectively.

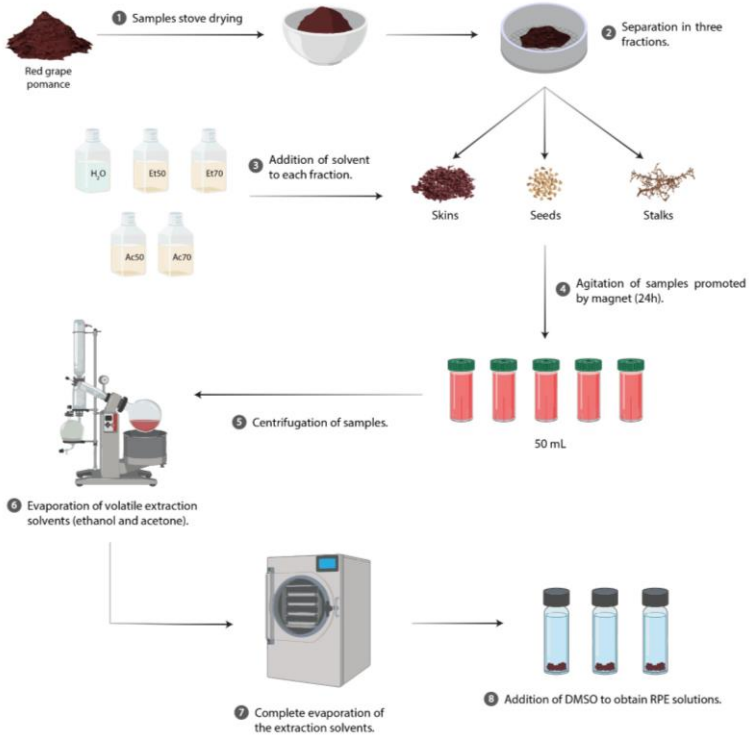


Figure 4-Extraction method used to obtain bioactive extracts from red grape pomace

3.3 Results and discussion

After separating the different components of the RGP, extractions were carried out to obtain the greatest amount of BC from the wine production by-products. First, five different solvents were used, namely, water, ethanol 50% and 70%, and acetone 50% and 70%. This extraction started with a proportion of 1:3 (w/v) of RGP separated (skins, seeds + residual skins, mixture (skins + seeds) and stalks), and each solvent. The total extraction masses, as well as the extraction yield are available in Table 2.

Table 2-Extraction results for the first extraction

	Skins		Seads + residual skins		Mix (skins + seeds)		Stalks	
Extraction solvent	**Total extraction mass (g)**	**Extraction yield (%)**	**Total extraction mass (g)**	**Extraction yield (%)**	**Total extraction mass (g)**	**Extraction yield (%)**	**Total extraction mass (g)**	**Extraction yield (%)**
Water	0.32	10.7	0.32	10.7	0.40	13.3	0.73	24.3
Ethanol 50 %	0.29	9.7	0.39	13.0	0.29	9.7	0.91	30.3
Ethanol 70 %	0.25	8.3	0.24	8.0	0.24	8.0	0.73	24.3
Acetone 50 %	0.32	10.7	0.47	15.7	0.35	11.7	0.61	20.3
Acetone 70 %	0.44	14.7	0.46	15.3	0.36	12.0	0.86	28.7

It is possible to verify that the fraction of RGP had an impact on the extraction yield. The highest extraction yields were obtained for stalks, resulting in extraction yields in a range of 20.3 to 30.3%. Similar extraction yields were obtained for seed+residual skins and skins+seeds, being around 8.0 to 14.7%.

Overall, the most profitable extractions were performed with acetone. Besides that, it is also possible to notice differences on the extraction yield of ethanolic solvents when comparing to water. In general, the use of aqueous 50% ethanolic solvent showed better extraction yields. These data are according to Brazinha et al. (2014), in which it was reported that aqueous ethanolic solvents performed better than only using water.

Although studies indicate a higher BC in the seeds, they also indicate that grapes are rich in polyphenols, such as anthocyanins, located mainly in the skins, and catechins, present in the skins, seeds, and stalks. Thus, it was decided to proceed with the samples obtained from the extraction of the mixture of skins and seeds, since it was supposed to have a higher BC and, consequently, greater antimicrobial and antioxidant activities (Silva et al., 2018; Ky et al., 2014).

After the extraction process, it was expected to obtain a powder. However, only samples extracted with acetone showed this characteristic. The samples extracted with water and ethanol had a gelatinous and had a viscous consistency, which made dissolution with DMSO quite complicated.

Despite having concluded in the results of the first extraction, that acetone was the solvent with the highest extraction yield, only water and ethanol are considered green solvents (Torres-Valenzuela et al., 2020). Besides that, it was reported that methanol and water were the solvents with higher extraction efficiency, once water helps in the diffusion of the extractable compounds, which is not according to the results of this study (Borges et al., 2020). Thus, and considering that the project also has the intention of applying more sustainable solutions for the use of wine by-products, on the second and third extractions, only water and aqueous ethanolic solutions (50 and 70%) were used as solvents. Results obtained are shown in Table 3.

Table 3-Extraction results for the second and third extractions performed for the mix of skins and seeds of RGP

	Extraction solvent	**Total extraction mass (g)**	**Extraction yield (%)**
2nd Extraction	**Water**	2.19	5.5
	Ethanol 50 %	2.00	5.0
	Ethanol 70 %	1.84	4.6
3rd Extraction	**Ethanol 50 %**	3.83	6.4
	Ethanol 70 %	2.20	3.7

First, it is possible to verify the differences between the extraction yields for the 2nd and 3rd extractions compared to the first. This decrease of yield may be due to the proportion of used solvent. In

fact, according to Zhang et al. (2018), solvent ratio is one of the parameters that has higher influence on the obtained extraction yields. Thus, other ratios should be applied in order to obtain the best extraction yield.

Besides that, it is possible to verify that the yields for these extractions are in the range of 3.7 to 5.5%. Ruberto et al. (2007), obtained similar extraction yields (1.03-5.75%) for RPE. However, in this study, methanol and n-hexane were the solvents used. These low yields are mostly caused by losses throughout the process, due to the gelatinous consistency of the samples, which makes their collection quite difficult, once the samples were attached to the cup walls.

For the 3rd extraction, it is possible to verify the increase of extraction yield when using aqueous ethanolic 50% as a solvent when compared to aqueous ethanolic 70%. Brazinha et al. (2014) reported that the total BC was higher for extractions using an aqueous ethanolic solvent at 67%, v/v. However, Cheng et al. (2012) reported that there should not be significant differences between aqueous 50% and 70% ethanol solutions (v/v).

3.4 Conclusions

In this study, extraction of BC from wine by-products production was performed. The yields obtained were in the range of 4 to 30%, and the most profitable solvent in terms of extraction yield was acetone. It was possible to verify that the extraction yields not only are dependent on the fraction of RGP, but also on the solvent used and the solute/solvent ratio. Lower extraction yields were obtained in this study which can be due to the chosen extraction technique and also because of the losses throughout all process.

The main goal of this study was to extract the highest BC possible from RGP in order to find a more sustainable treatment of wine by-products production, so it was imperative that the solvents used from a certain point, namely water and aqueous solutions of ethanol, were green solvents. Besides that, this choice also considered the fact that these RPE may have a pharmaceutical application, namely as antioxidant and antimicrobial agents against *S. aureus*, which are the most common microorganisms present in DFI.

To conclude, other extraction techniques should be applied and different solvents and solute/solvent ratios to optimize the process, obtaining higher extraction yields, and consequently, higher amount of BC.

Chapter 4- Bioactivity of Red Grape Pomace Extracts

4.1 Introduction

The effectiveness of antibiotics has been decreasing due to the emergence of bacteria resistant to these drugs, increasing morbidity, mortality and health costs (Kebede et al., 2021). Thereby, the search for innovative antibiotic produced from natural products is ultimately important.

GP is a rich source of polyphenols, which leads the RPE to exhibit a wide range of biological effects, such as antibacterial, antifungal, antiviral, anti-inflammatory, anticarcinogenic and vasodilatory effect (Aires et al., 2009; Ky et al., 2014; Silva et al., 2021). Resveratrol, procyanidins, tannins, flavanols, among others, are part of the high phenolic content in grapes, providing them interesting antimicrobial properties. Antimicrobial effects have been mainly attributed to its ability to breakdown the structure of the bacterial cytoplasmic membrane causing loss of integrity and eventual cell death (Silva et al., 2018; Silva et al., 2021).

It is also believed that extracts of medicinal plants with antioxidant activity could be helpful therapeutic agent in promoting wound healing. There are a lot of diversity when it comes to natural antioxidant species known. Its antioxidant power may be due to its content in PC (Melo et al., 2015). It has been reported that wine by-products can be considered antioxidant sources once they are rich in PC. Typically, plants produce PC as a natural way to protect themselves against tissue lesions, infections, and UV radiation (Moreno-Arribas et al., 2009).

PC basic structure consists in an aromatic ring to which several or only one hydroxyl group is attached, and their antioxidant efficiency is related to the functional group present, its position in the molecule and chain size. PC mode of action is based on the capacity to protect the biological systems against the action of free radicals. Free radicals are chemical species with a unpair electron, which gives them great electron affinity and, consequently, a huge reactivity (Soares, 2002). It has been proven that several components with antioxidant properties have the capacity to neutralize those free radicals, preventing or reducing the lesions caused by them (Magalhães et al., 2008; Re et al., 1998).

In this study, the antimicrobial proprieties of by-products from wine industry, more specifically RPE, against the most common pathogen isolated from DFI, *S. aureus*, were evaluated. Firstly, MIC and MBC of the RPE against two different strains of *S. aureus* (CECT976 and RN4220) were tested. After that, a modified disc diffusion method allowed the study of the effect of the RPE and the evaluation of possible synergies with several antibiotics. Lastly, the antioxidant capacity of the RPE was evaluated by ABTS (2,2-azinobis (3-ethyl-benzothiazolina-6-sulfonic acid)) method.

4.2 Materials and methods

4.2.1 Antimicrobial activity

4.2.1.1 Bacterial strains and culture conditions

The microorganisms used in this study were the Gram-positive bacterium *S. aureus* CECT976, obtained from the Spanish Type Culture Collection and *S. aureus* RN4220. The last strain mentioned is resistant to 14- and 15-membered macrolides including erythromycin and contains multicopies of plasmid pU5054 that carries the gene encoding the MsrA macrolide efflux protein (Oliveira et al., 2019; Tintino et al., 2016). The bacteria were preserved at -80 ºC in Mueller–Hinton broth (MHB, Merck) containing 30% (v/v) glycerol. For the inoculum preparation, *S. aureus* was grown overnight in MHB at 37 ºC under 160 rpm of agitation before all the experiments.

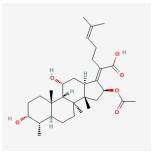
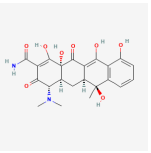
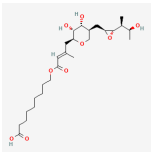
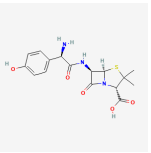
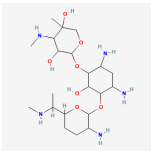
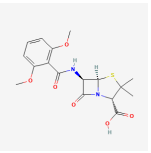
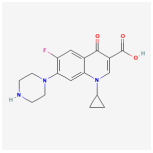
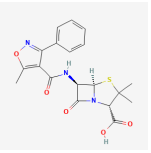
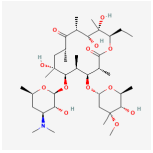
4.2.1.2 Antibiotics

For this work were selected nine antibiotics of topical application that belong to different biosynthetic classes and include: fusidic acid (FA), amoxicillin (AM), oxacillin (OX), erythromycin (ER), ciprofloxacin (CI), and tetracycline (TE) from Sigma-Aldrich (St. Louis, USA), mupirocin (MU) and gentamicin (GE) from AppliChem GmbH (Darmstadt, Germany), and methicillin (ME) from Thermo Fisher Scientific (Massachusetts, USA). Excepting MU, CI and TE that were prepared by adding DMSO, all the remaining antibiotics were prepared in water at specific concentrations (Table 4) according to the guidelines of Clinical & Laboratory Standards Institute (CLSI). (NCCLS/CLSI, 2015) 2 mL of water or DMSO were added, according to the antibiotic, as mentioned before, and stock solutions were prepared in 15 mL cups with the concentrations, represented on Table 4, and 2 times that concentration in order to perform the tests with the pretended concentration.

4.2.1.3 Red Grape Pomace Extracts

For these assays, previous stock solutions of crude ethanolic extracts (50 and 70%) were used. The stock solutions prepared had a final concentration of 300 mg/mL and 200 mg/mL for the 2nd and 3rd extraction, respectively. After MIC and MBC determination, only stock crude ethanolic extracts (50 and 70%) in a concentration of 200 mg/mL were used.

Table 4-Antibiotics, concentrations tested in the disc diffusion method, class, and their chemical structure (PubChem Compounds) (NCCLS/CLSI, 2015)

Antibiotic	µg/mL	Class	Chemical structure	Antibiotic	µg/mL	Class	Chemical structure
Fusidic acid (FA)	10	Fusidanes		**Tetracycline (TE)**	30	Tetracyclines	
Mupirocin (MU)	200	Monocarboxylic acids		**Amoxicillin (AM)**	20	Penicillins	
Gentamicin (GE)	10	Aminoglycosides		**Methicillin (ME)**	20	Penicillins	
Ciprofloxacin (CI)	5	Fluoroquinolones		**Oxacillin (OX)**	4	Penicillins	
Erythromycin (ER)	15	Macrolides					

4.2.1.4 Minimum inhibitory and minimum bactericidal concentration (MIC and MBC)

The determination of MIC and MBC was carried out in two different steps using the broth microdilution method as represented on Figure 5.

On the first trial, overnight cultures were adjusted to an optical density (OD_{600nm}) of 0.132 ± 0.02 in MHB. Subsequently, 180 µl of the adjusted bacterial suspension were added to sterile 96-well polystyrene microtiter plates with 20 µl of each crude ethanolic extract (50 and 70%) at different concentrations (6.25 to 1000 µg/mL). The crude ethanolic extracts (50 and 70%) did not exceed 10% (v/v) of the well volume. Bacterial suspensions without RPE and bacterial suspensions with DMSO (10%, v/v) were used as controls. Then, the plate was incubated at 37°C for 24 h and 150 rpm. The experiments were performed in triplicate. After that, 10 µL of each well was dropped on plate count agar (PCA, Merck) plates. Finally, after 24 h of incubation at 37 ºC, the plates were analysed.

On the second trial, overnight cultures were adjusted to an OD_{600nm} of 0.264 ± 0.02 in MHB. Subsequently, 100 µL of MHB were added to 96-well polystyrene microtiter plates; then 100 µL of each RPE was added on the first well with a concentration of 200 mg/mL and successive two-fold dilutions were carried out for the next wells up to a concentration of 25 mg/mL (4 wells). After that, 100 µL of the bacterial suspension was added to each well, causing the concentration in the wells to be halved, i.e., the concentrations tested were in the range of 50 to 12.5 mg/mL. Bacterial suspensions without RPE and bacterial suspensions with DMSO (25%, v/v) were used as controls. Then, the plates were incubated at 37 °C for 24 h and 150 rpm. After that, 10 µL of each well was dropped on plate count agar (PCA, Merck) plates. Finally, after 24 h of incubation at 37 ºC, the plates were analysed. The experiments were performed in triplicate and three independent repeats.

In both trials, the OD (λ = 600 nm) was measured using a microplate reader (FLUOstar Omega; BMG LABTECH; Germany), and the lowest concentration of components at which no growth was detected ($ODt = 24\ h \leq ODt = 0\ h$) was defined as the MIC. The PCA plates were also analysed, and the MBC of each RPE corresponded to the minimum concentration causing no bacterial growth on the PCA plates (Borges et al., 2013).

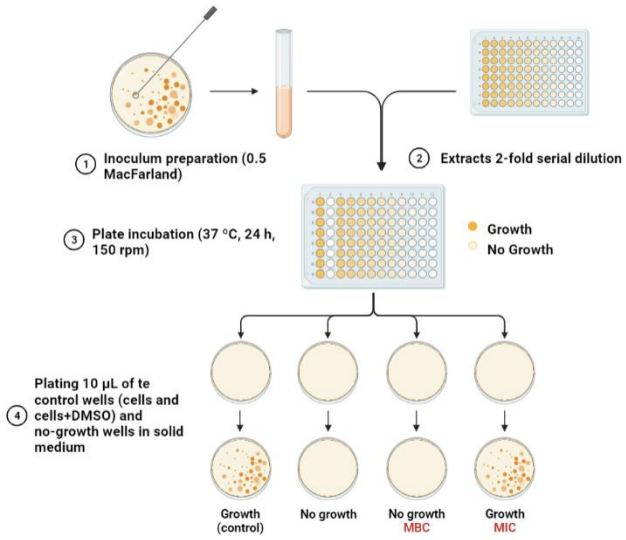


Figure 5-Schematic illustration on the MIC and MBC determination method.

4.2.1.5 Modified Disc Diffusion Method

First, Mueller-Hinton agar (MHA) medium was prepared, autoclaved, and then cooled until approximately 30 ºC. The medium was then poured into 90 mm Petri dishes to give a uniform depth of approximately 4 mm (~ 20 mL).

Bacterial cells were grown overnight in solid agar medium (MHA) at 37±2°C. After the overnight growth, the cultures were adjusted to OD of 0.132 ± 0.02 at λ = 600 nm (corresponding to 0.5 McFarland standard). For this, some isolated colonies were resuspended in falcons containing saline solution (NaCl, 0.85%). Bacterial suspension was seeded in MHA plates using a sterilized cotton swab.

Disks with 6 mm of diameter were removed from the MHA plates to form wells in the agar. A volume of 10 µL of each antibiotic prepared according to the CLSI guidelines (AM- 20 µg/mL, ME- 5 µg/mL, OX- 1 µg/mL, ER- 15 µg/mL, TE- 30 µg/mL, CI- 5 µg/mL, FA- 10µg/mL, MU- 200 µg/mL and GE- 10 µg/mL) was added to the wells. After that, 10 µL of the RPE at concentrations of 50 mg/mL, 25 mg/mL and 12.5 mg/mL were also added to the wells (NCCLS/CLSI, 2015).

Wells containing only each antibiotic and wells containing only RPE were used as positive controls. The plates were incubated at 37 °C for 24 h. After incubation, each inhibition zone diameter (IZD) was recorded. Figure 6 illustrates the modified disc diffusion method performed. Regarding antibiotics singly, depending on the IZD obtained, they were classified according to CLSI guidelines in one of the following categories: susceptible (S), intermediate (I), or resistant (R) (NCCLS/CLSI, 2015).

A classification of the RPE was expressed according to Aires et al., in which a comparison between the IZD of each RPE (IZDe) and the IZD of each antibiotic (IZDa) used was established. This way, the RPE were classified accordingly to: non-effective (-) if: IZDe = 0; moderate efficacy (+) if: 0 < IZDe < IZDa; good efficacy (++) if: IZDa < IZDe < 2xIZDa; strong efficacy (+++) if IZDe > 2xIZDa (Aires et al., 2009),

In order to classify the combinatorial effect between the RPE and antibiotics, the IZDa of each MH Broth plate (IZDantibiotic) and each well with RPE (IZDantib+ RPE) were determined. The interaction was classified accordingly to: negative (N) if: (IZDa – (IZDa + e)) ≥ 6 mm (-); indifferent (I) if: ((IZDa + e) – IZDa) < 4 mm (+); additive (A) if: 4 mm ≤ ((IZDa + e) – IZDa) < 6 mm (++); potentiation (P): if ((IZDa + e) – IZDa) ≥ 6 (+++) mm, being a = antibiotic and e = RPE (Borges et al., 2013; Ali et al., 2019).

All tests were performed in duplicate with two replicates. The antibacterial activity was expressed as the mean of IZD (in mm).

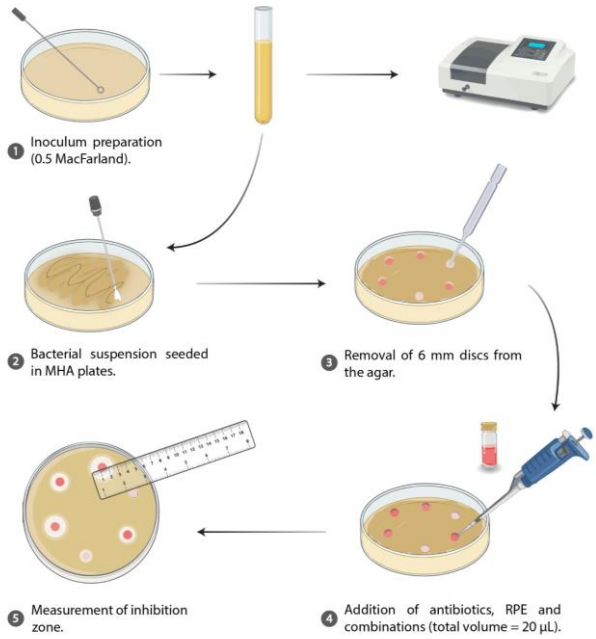


Figure 6-Schematic illustration on the modified disc diffusion method.

4.2.2 Antioxidant activity

The antioxidant capacity of RPE in concentrations of 20 mg/mL was estimated by the evaluation of ABTS (2,2-azinobis (3-ethyl-benzothiazolina-6-sulfonic acid) radical scavenging activities, according

to Xiao et al. (2020), with minor alterations. Briefly, the ABTS+ solution was prepared by the addition of 7 mM ABTS to 2.45 mM of potassium persulphate. This solution was mixed and allowed to stand in the dark for 12–16 h at room temperature to produce ABTS radical cation. The working solution of ABTS·+ was obtained by the dilution of the previously made ABTS solution with ethanol and adjusted to an absorvance of 0.70 at 734 nm. Each sample and Trolox standards were added to ABTS·+ solution and the absorvance was read 10 min after mixing. The percentage of inhibition was calculated by the following formula: % inhibition = 100 × (A0-A)/A0 where A0 was the initial absorbance obtained by measuring the solvent absorvance, and A was the final absorbance of each tested RPE sample at 734 nm. The calibration curve between % inhibition and Trolox (1.25-25 μM) solutions was then established. The radical-scavenging activity of each RPE was expressed in Trolox equivalent antioxidant capacity (TEAC mg Trolox/g RPE).

4.2.3 Statistical analysis

Both mean and standard deviation (SD) within samples were calculated for all cases. At least two independent experiments were performed for each condition tested. One-way ANOVA with Bonferroni test was performed. Statistical analysis was based on a confidence level ≥95% ($p<0.05$ was considered statistically significant).

4.3 Results and discussion

4.3.1 Inhibitory and bactericidal activities

The evaluation of MIC and MBC *in vitro* allows microbiologists to access the antibiotic effect *in vivo* and their influence in planktonic microorganisms in the exponential phase of growth. MIC is interpreted as the minimum concentration of an antibiotic or RPE that clearly shows an inhibition on microbial growth. On the other hand, the evaluation of MBC gives the information on at which concentration there is completely killing of bacteria (Borges et al., 2013; Ceri et al., 1999; Fux et al., 2005).

The antibacterial activity of the RPE was evaluated on planktonic *S. aureus* cells (both RN4220 and CECT976 strains) in two different steps by determining MIC and MBC.

On the first trial, the concentrations tested were in the range of 62.5 to 1000 μg/mL. These values were chosen, once phytochemicals are routinely classified as antimicrobials based on susceptibility tests that produce inhibitory concentrations in the range of 100 and 1000 μg/mL (Borges et al., 2012). In this study, it was not possible to obtain reliable conclusions about the MIC, once it was not verified a reduction of the absorbance values at the concentrations tested. Actually, an increase on the absorbance was noticeable, which allowed to conclude that the RPE at those concentrations did not have any antimicrobial effect against both bacterial strains. Regarding the MBC, no values were also obtained for any concentration of the RPE in both strains, since the bacteria grew similarly for all the concentrations tested.

Since it was not possible to obtain any MIC or MBC values for the concentrations tested on the first trial, it was decided to proceed with higher concentrations. As mentioned before, on the second trial the concentrations tested were in the range of 12.5 to 100 mg/mL. The MIC and MBC obtained in this study are summarized in the Table 5.

Table 5-MIC and MBC values obtained for both *S. aureus* strains (CECT976 and RN4220) with the crude ethanolic extract (50% and 70%)

	RPE (%)	**MIC (mg/mL)**	**MBC (mg/mL)**
***S. aureus* CECT976**	Et 50	25	25
	Et 70	25	25
***S. aureus* RN4220**	Et 50	50	-
	Et 70	25	-

The crude ethanolic extracts (50% and 70%) showed both inhibitory and bactericidal proprieties against *S. aureus* CECT976 strain at concentrations of 25 mg/mL. For *S. aureus* RN4220 strain the MIC value of crude ethanolic extract (50%) was 50 mg/mL, however, no MBC was found at the range of the tested concentrations. On the other hand, crude ethanolic extract (70%) showed inhibitory effect at 25 mg/mL but no MBC was also found at the tested concentrations. These results indicate that *S. aureus* RN4220 strain displayed a more resistant profile than *S. aureus* CECT976. Actually, this is expected as it is known that *S. aureus* RN4220 strain expresses efflux pumps which make them in fact more resistant (Oliveira et al., 2019; Tintino et al., 2016).

Previous studies also corroborate this behaviour, verifying that in general *S. aureus* RN4220 stain was more resistant to antibiotics and phytochemicals than *S. aureus* CECT976 strain (Oliveira et al., 2019; Tintino et al., 2016). This way, the results obtained in this study are in accordance with others works.

Oliveira et al. (2013) reported MIC values of 0.63-0.75 mg/mL of *Merlot* Pomace extracts against *S. aureus*. However, Jayaprakasha et al. (2013) obtained much higher MIC results, with inhibition only at 998.9 mg/mL. Besides that, values of 0.5 mg/mL were also obtained in Sanhueza et al. (2014) for GPE against *S. aureus*. However, in a more intensive study on Red Grape Extracts (RGE), values of MIC and MBC obtained for *S. aureus* were in a range of 1-900 mg/mL (Silva et al., 2021). This fact may be due to the parameters that affect the metabolism of flavonoid in grapes, and consequently the PC in RPE, which include temperature, UV radiation exposure, and nutrient supply. RPE and its content can also be affected by the extraction method as well as maceration (Allison et al., 2015; Melo et al., 2015).

Duarte et al. (2017) and Wang et al. (2008) classified the RPE as “strong inhibitors” for MIC values below 0.5 mg/mL, “moderate inhibitors” for MIC values between 0.6-1.5 mg/mL and “weak inhibitors” for MIC values above 1.6 mg/mL. Thus, all of the RPE of this study can be classified as weak inhibitors (Oliveira et al., 2013).

4.3.2 Combinatorial activity of antibiotics and crude grape pomace extracts

A variety of laboratory procedures may be used to evaluate the *in vitro* antimicrobial activity and synergies between different compounds. Disk diffusion method is one of the most basic and simple methods to assess these characteristics of either antibiotics or plant extracts and their combination (Balouiri et al., 2016). In previous studies, RPE inhibited β-lactamases in *S. aureus*, affected the cytoplasmic membrane stability and inhibited enzymes involved in the synthesis of folic acid, reducing MIC of some known drugs by 30 to 75 times. These data proved that different combinations of antibiotics and natural products may improve the interaction of the antibiotic with its target (Aires et al., 2009; Sanhueza et al., 2017).

To classify the effect of the extracts against *S. aureus*, and the combinatorial effect between the RPE and antibiotics, the IZD of antibiotics singly, RPE singly and antibiotics in the presence of RPE were determined and the effect classified. In the following Tables (Table 6, 7 and 8), it is possible to see the obtained results.

Table 6-IZD ± SD of the antibiotics for both *S. aureus* CECT 976 and RN 4220 strains. Classification of the antibiotics according to CLSI guidelines

Bacterial Strain	**CECT 976**		**RN4220**	
Antibiotic	**IZD (mm)***	**Classification****	**IZD (mm)***	**Classification****
AM	14.5±0.0	R	0.0±0.0	R
ME	0.0±0.0	R	0.0±0.0	R
OX	0.0±0.0	R	0.0±0.0	R
ER	8.8±0.0	R	0.0±0.0	R
TE	3.3±0.4	R	0.0±0.0	R
CI	11.5±0.7	R	0.0±0.0	R
FA	8.3±0.4	-	0.0±0.0	-
MU	19.3±0.4	-	0.0±0.0	-
GE	0.0±0.0	R	0.0±0.0	R

** The IZD values do not consider the well diameter (6mm); ** susceptible (S), intermediate (I), or resistant (R); (-) – No data was found.*

Based on the obtained IZD of antibiotic AM, ER, TE, CI, FA and MU it was possible to verify some efficacy against the *S. aureus* CECT976 strain. However, ME, OX and GE did not have any inhibitory effect against this strain. The most effective antibiotic was MU (Table 3).

On the other hand, for *S. aureus* RN4220 strain there was not a formation of an IZD for all tested antibiotics, which allowed to conclude that the expression of efflux pumps make them in fact more resistant to the various antibiotic classes tested (Oliveira et al., 2019; Tintino et al., 2016).

RPE were also tested singly at different concentrations to classify their effect against both strains when compared to the antibiotic effect. On Table 7 it is possible to consult the IZD measured in mm and the standard deviation as well as the classification.

Table 7-IZD ± SD (mm) values of the crude ethanolic (50 and 70%) extracts at different concentrations (50, 25 and 12.5 mg/mL) for both *S. aureus* CECT976 and RN4220 strains. Classification of the RPE relatively to antibiotics (MA, ME, OX, ER, TE, CI, FA, MU and GE)

				Classification*								
Bacterial Strain	**Ethanolic Extracts**	**Concentration (mg/mL)**	**IZD (mm)****	**AM**	**ME**	**OX**	**ER**	**TE**	**CI**	**FA**	**MU**	**GE**
***S. aureus* CECT976**	Et 50%	50	8.5±0.7	+	+++	+++	++	+++	+	++	+	+++
		25	7.0±0.0	+	+++	+++	+	+++	+	+	+	+++
		12.5	7.5±0.7	+	+++	+++	+	+++	+	+	+	+++
	Et 70%	50	9.0±0.0	+	+++	+++	++	+++	+	++	+	+++
		25	7.0±0.0	+	+++	+++	+	+++	+	+	+	+++
		12.5	5.5±0.7	+	+++	+++	+	++	+	+	+	+++
***S. aureus* RN4220**	Et 50%	50	6.0±0.7	+++	+++	+++	+++	+++	+++	+++	+++	+++
		25	3.8±0.4	+++	+++	+++	+++	+++	+++	+++	+++	+++
		12.5	3.0±0.0	+++	+++	+++	+++	+++	+++	+++	+++	+++
	Et 70%	50	9.5±0.7	+++	+++	+++	+++	+++	+++	+++	+++	+++
		25	4.3±0.4	+++	+++	+++	+++	+++	+++	+++	+++	+++
		12.5	3.8±1.1	+++	+++	+++	+++	+++	+++	+++	+++	+++

**Non-effective (-); Moderate efficacy (+); Good efficacy (++); Strong efficacy (+++)*

*** The IZD values do not consider the well diameter (6mm)*

The results obtained in these trials showed promising conclusions about the effect of the RPE against both *S. aureus* strains, since the non-effective classification was not applied to any of the tested compounds. *S. aureus* CECT976 was the strain that experienced the highest exhibitory effects with a IZD of 9.0 mm, when it was in contact with the crude ethanolic extracts (70%) at a concentration of 50 mg/mL. This results a in a moderate efficacy when compared to AM, CI and MU, good efficacy compared to ER and FA and strong efficacy when compared to ME, TE and GE. The second most effective RPE was the crude ethanolic extract (50%) at 50 mg/mL, showing an IZD of 8.5 mm. This RPE was classified by having moderate efficacy when compared to AM, CI and MU, good efficacy relatively to ER and FA and strong efficacy relatively to ME, TE and GE.

Both crude ethanolic extracts (50 and 70%) at the concentration of 25 mg/mL showed a IZD of 7.0 mm against *S. aureus* CECT976, showing a moderate efficacy relatively to MA, ER, CI, FA and MU and a strong efficacy relatively to ME, OX, TE and GE. Besides that, it is possible to see that the observed effect is concentration dependent (ranging from 5.5 to 9.0 mm).

According to the statistical analysis conducted, all RPE showed significant inhibition differences when compared with AM, ME, OX, ER, CI, FA and MU ($p<0.05$). However, it was not found statistically significant differences between the crude ethanolic extracts (50 and 70%) with TE and GE. ($p>0.05$). Besides that, there was not a significant inhibitory difference between all RPE ($p>0.05$) against *S. aureus* CECT976, which allowed to conclude that independently of the extraction solvents used the results displayed were similar.

For *S. aureus* RN4220, the IZD measured were lower than for the CECT976 strain, except for the crude ethanolic extract (70%). The range of IZD obtained is from 3.0 to 9.5 mm and it is possible to see an increase of the IZD as the concentration of the RPE increases, which may be due to the higher phenolic content

of the RPE. Since the IZD of antibiotics was 0.0 mm for this strain, and the RPE showed a clear inhibition halo, all the tested solutions were classified as strong efficacy.

According to the statistical analysis, all RPE showed significant increased inhibitory results when compared with AM, ME, OX, ER, TE, CI, and FA ($p<0.05$). However, both crude ethanolic extracts (50 and 70%) in a concentration of 25 and 12.5 mg/mL did not show significant inhibitory increase when compared to MU. Crude ethanolic extract (70%) in a concentration of 50 mg/mL ($p>0.05$) also did not show significant increase of the inhibitory effect when compared to GE ($p>0.05$). Besides that, there was not a significant inhibitory difference between all RPE ($p>0.05$) against *S. aureus* RN4220, which allows to conclude that there are not significant differences between the concentration of the RPE neither between the percentage of ethanol used for extraction. Oliveira et al. (2013) reported IZD values from 10-12 mm against *S. aureus* ATCC25923 strain. Same range of values were reported by Xu et al. (2019), obtaining IZD of 8.2-13.5 mm against a *S. aureus* MRSA, which are according to the values obtained in this study for RPE.

The synergies obtained from this screening show promising results. As it is possible to see on Table 8, 108 combinations between the crude ethanolic extracts and antibiotics were tested. From those, 74.1% show a potentiator effect. Besides that, 18.5% of the combinations have an addictive effect and only 7.4% showed indifferent effect.

Even though results seam promising against *S. aureus* CECT976, statistical analysis only showed a significant inhibitory difference ($p<0.05$) for crude ethanolic extracts (50%) in concentrations of 50 and 25 mg/mL and crude ethanolic extracts (70%) in concentrations of 50 and 12.5 mg/mL when compared to ME; and crude ethanolic extract (50%) in concentration of 25 mg/mL when compared to OX. The rest of results obtained for the inhibitory effect did not show a significant difference when compared with the control samples ($p>0.05$). Moreover, statistical analysis was also performed to study the influence of concentration and the aqueous volume of ethanol on the inhibitory effect against *S. aureus* CECT976, showing that none of the variables have a significant difference ($p>0.05$).

When comparing the results obtained for *S. aureus* RN4220 strain, the statistical analysis indicates that combinations of all crude ethanolic extracts, and GE have the highest significant difference comparing with the control ($p<0.05$). Even though combinations of the crude ethanolic extracts with CI are classified as having potentiation effect, statistical analysis shows the lowest significant difference with the CI control measurements ($p>0.05$).

Table 8-IZD ± SD (mm) values of the combinations of antibiotics (AM, ME, OX, ER, TE, CI, FA, MU and GE) with crude ethanolic extracts (50 and 70%) at different concentrations (50, 25 and 12.5 mg/mL) for both *S. aureus* CECT 976 and RN4220 strains. Synergies of the combinations according to Aires et al., 2019.

		RPE											
		Et 50%						Et 70%					
	Concentration (mg/mL)	50		25		12.5		50		25		12.5	
Bacterial Strain	Antibiotic	IZD (mm)**	C*	IZD (mm)**	C*	IZD (mm)**	C*	IZD (mm)**	C*	IZD (mm)**	C*	IZD (mm)**	C*
CECT 976	AM	19.5±0.0	++	20.5±1.4	+++	18.8±1.8	+++	18.5±0.0	++	19.3±0.4	++	20.0±0.7	++
	ME	6.3±0.4	+++	6.0±0.0	+++	1.3±3.2	+++	6.5±0.7	+++	1.0±0.0	+++	4.3±0.4	+++
	OX	6.5±0.0	+++	7.0±0.0	+++	0.0±0.4	+++	6.3±0.4	+++	3.5±3.5	+++	4.8±0.4	+++
	ER	12.5±0.7	++	14.5±0.7	+++	13.5±0.0	+++	11±0.0	+	12.0±0.7	++	14.0±0.0	+++
	TE	12.8±0.4	+++	13.0±0.0	+++	11.5±0.0	+++	12.3±1.1	+++	12.3±0.4	+++	12.0±0.0	+++
	CI	15.8±3.2	++	15.3±0.4	+	14.8±0.4	+++	13.0±0.0	+	15.8±1.8	++	16.0±0.0	++
	FA	16.0±0.0	+++	16.3±1.8	+++	16.3±1.1	+++	15.5±0.0	+++	15.3±0.4	+++	14.8±0.4	+++
	MU	26.8±0.4	+++	30.3±1.1	+++	27.3±0.4	+++	27.0±0.0	+++	26.0±0.7	+++	28.3±1.1	+++
	GE	6.5±0.7	+++	8.5±0.0	+++	5.3±0.4	++	7.3±0.4	+++	3.0±3.0	++	5.5±0.0	+++
RN4220	AM	5.8±0.4	+++	0.0±0.0	+++	0.0±2.8	+	4.8±0.4	+++	4.0±0.7	+++	4.8±0.4	+++
	ME	2.8±3.9	+++	3.5±0.7	+++	0.0±0.0	++	5.0±0.0	+++	5.5±0.0	+++	3.5±0.0	+++
	OX	5.8±0.4	+++	4.0±0.0	+++	0.0±0.0	++	6.0±0.7	+++	5.0±0.0	+++	4.0±0.0	+++
	ER	5.8±0.4	+++	0.0±0.0	++	0.0±0.0	++	5.3±0.4	+++	4.5±0.7	+++	3.8±0.4	+++
	TE	7.0±0.7	+++	0.0±0.0	++	0.0±0.0	++	5.8±1.1	+++	5.0±0.0	+++	4.0±0.0	+++
	CI	6.5±0.7	+++	5.3±0.4	+++	2.0±0.0	+++	5.8±0.4	+++	6.8±1.1	+++	4.0±0.0	+++
	FA	5.8±0.4	+++	3.8±0.4	+++	0.0±0.0	+	5.3±0.4	+++	5.0±0.0	+++	1.5±3.5	+++
	MU	6.5±0.7	+++	4.5±0.0	+++	0.0±0.0	+	6.0±0.0	+++	6.0±0.7	+++	5.3±0.4	+++
	GE	2.5±4.2	+	4.3±0.4	++	0.0±0.0	+	5.0±0.0	++	4.8±0.4	++	1.8±3.2	++

**Classification: Negative (-); Indifferent (+); Additive (++); Potentiation (+++)*

*** The IZD values do not consider the well diameter (6mm)*

Additionally, statistical analysis was also performed to evaluate the influence of concentration and the aqueous volume of ethanol on the inhibitory effect against *S. aureus* RN4220. The obtained results showed significant inhibitory differences between concentrations of 50 and 12.5 mg/mL of the crude ethanolic (50%) combined with AM and TE ($p<0.05$). Besides that, there was also a significant inhibitory difference between concentrations of 50 and 12.5 mg/mL of crude ethanolic extracts (50%) and between concentrations of 25 and 12.5 mg/mL of crude ethanolic extracts (50%) for the combinations with FA, MU and GE ($p<0.05$). Furthermore, when combined with MU and GE, RPE at concentration of 12.5 mg/mL showed a significant difference between the 50 and 70% of ethanol v/v ($p<0.05$). The remaining results did not show a significant difference in these parameters on the inhibitory effect ($p>0.05$).

Despite of the high number of studies about the use of RPE as antimicrobial agents, there is still a lack of literature information involving similar combinations, especially when it comes to combinations of these extracts with antibiotics.

4.3.4 Antioxidant properties of RPE

The antioxidant activity of RPE was investigated with a view to their exploitation as a potential source of natural antioxidants. Recently, it has been highly recommended the use of more than one type of antioxidant activity measurement due to the differences between the various free radical scavenging assay systems (Ky et al., 2014; Ruberto et al., 2007). In this sense, several methods can be applied to assess the antioxidant capacity. To name a few, ABTS (2,2-azinobis (3-ethyl-benzothiazolina-6-sulfonic acid), FRAP (Ferric Antioxidant Power), DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) and DMPD (N, N-dimethyl-ρ-phenylenediamine) can assess the antioxidant capacity by evaluating the action of an antioxidant to inhibit the free radicals by donating a hydrogen atom (Magalhães et al., 2008; Re et al., 1998).

The ABTS method allows the evaluation of the antioxidant activity of a sample through its ability to reduce ABTS•+ cation radicals. When adding potassium persulfate to ABTS in the reaction mixture, ABTS is oxidized to the ABTS•+ cation, which has a dark blue color with maximum absorbance at wavelengths of 415, 645, 734 and 815 nm. Adding antioxidant species to this solution promotes the reduction of the ABTS•+ cation to ABTS, which leads to a reduction of the blue color. The DPPH method is one of the most common used techniques for the evaluation of antioxidant capacity of extract once it is a simple, efficient, and cheap. It is based on the measurement of the antiradical ability of a compound against the 2,2-diphenyl-1-picrylhydrazyl (DPPH•) radical. By the action of an antioxidant compound, the DPPH• radical is reduced to its stable form DPPH-H (diphenyl-1-picrylhydrazine). This reaction is monitored by color change from violet (DPPH•) to yellow (DPPH-H), followed by measurement of absorbance at 517 nm. The greater the free radical scavenging capacity of an antioxidant compound, the greater the reduction in DPPH and the less persistent purple color in the sample (Boots et al., 2008; Rodrigo et al., 2009).

However, due to the limitation of time and RPE quantity, the determination of the antioxidant activity of the RPE was carried out only using the ABTS method, in which stronger antioxidant properties induce higher ABTS inhibitions (Brazinha et al., 2014). Total antioxidant activity of RPE was expressed in % of scavenging and in mg of TEAC (Trolox equivalent antioxidant capacity)/ g of extract.

Regarding Table 9, it is possible to see that crude ethanolic extract (50%) had greater % of scavenging activity (68%), and consequently greater antioxidant activity (0.40±0.06 TEAC/ g of extract). Antioxidant activity was not registered for the crude ethanolic extract (70%).

The data obtained showed that the crude ethanolic extract (50%) is a free radical inhibitor, and this effect may be due to the presence of polyphenols, which may act with free radicals to convert them to more stable product and terminate free radical chain reaction (Jayaprakasha et al., 2003). Besides that, antioxidants can act as sacrificial subtracts to prevent or inhibit the generation of reactive oxygen species (ROS), which are already at higher levels in DFI (Apelqvist, 2012; Tang et al., 2020; Zhu et al., 2019).

Table 9-Antioxidant properties of the crude ethanolic 50 and 70% extracts expressed in terms of % of scavenging and Trolox equivalent/g of extract (average ± %SD)

% Scavenging		**TEAC***	
Et50	**Et70**	**Et50**	**Et70**
68.0±1.4	-56.9±6.9	0.40±0.06	-0,12±0,02

**TEAC – Trolox equivalent (mg TEAC/ g RPE)*

On a previous study, Ky et al. (2003), evaluated the antioxidant activity of grapes and GP from six different French grape, obtaining a mean value of 23.7 mg TEAC/g using ABTS method. Besides that, Melo et al. (2015) also evaluated the antioxidant capacity of Chenin Blanc, Petit Verdot, and Syrah grape by-products by ABTS method, obtaining a mean value of 201.5 mg TEAC/g.

Despite the values discrepancy, in both studies the extracts were obtained using methanol as solvent, so it is possible that the RPE contain different PC composition and consequently, different antioxidant activities. However, Ruberto et al. (2007) reported values in the range of 1.58-2.24 mg TEAC/g for RPE, which are closer to the values of the crude ethanolic extract (50%) in the present study. Relatively to the crude ethanolic extracts (70%), the lack of antioxidant activity may be due to the high ethanol content used in the extraction. Melo et al. (2015) reported that the highest antioxidant capacities were obtained for the extracts in which 40-55% of aqueous solutions of ethanol were used in the extraction and that the increase of ethanol concentration used in the extraction did not correspond to a proportional increase in the antioxidant capacity. Besides that, these RPE may be photosensitive due to their composition in PC, therefore, the crude ethanolic extract (70%) may have had some contact with light during laboratory procedures, affecting the antioxidant capacity.

4.4 Conclusions

The overall study had the intent of evaluating the bioactivity of the RPE as a green sustainable source of new broad-spectrum antimicrobial and antioxidant products. Antimicrobial activity of RPE against *S. aureus* was confirmed, since MIC values of 25 mg/mL were obtained for *S. aureus* CECT976 strain and 25 and 50 mg/mL were obtained for *S. aureus* RN4220 for the crude ethanolic 70 and 50% extracts, respectively. However, MBC values of 25 mg/mL were obtained only for *S. aureus* CECT976 strain. The study on the inhibitory effect and synergies between the RPE and antibiotics suggested a promising potentiation effect. However, further analysis should be performed to have a better understanding on the activity of RPE and antibiotic combination. Besides that, antioxidant activity was detected for the crude ethanolic extract (50%) and non-detectable for the crude ethanolic extract (70%). Since the evaluation on this study was only regarding ABTS method, other antioxidant assays should be performed to assess this activity. A high number of studies on antimicrobial and antioxidant activities of RPE have been performed. However, there is still a lack of literature information and standardization on the possibilities of combinations of raw material type, extraction methods and solvents, as well as microbiological assays applied.

Chapter 5- Insight on the antimicrobial and antipathogenic mode of action of Red Grape Pomace Extracts against *S. aureus*

5.1 Introduction

The specific processes underlying the inhibitory impact of GP extracts/constituents on microorganisms are yet unknown, partly due to their chemical complexity and their composition as mentioned before. Besides that, because concentrations vary greatly in grape skin, grape seeds, and combinations, another impacting factor is the distribution of active antimicrobial chemicals among pomace fractions. There are a lot of suggested mechanisms for GP extracts with antimicrobial proprieties acting synergistically, increasing the antibacterial effects (Hassan et al., 2019). To name a few, GP extracts may induce shorted oligopeptides that disrupt cellular membranes, inactivate bacterial exoenzymes, minimizing the chances of bacteria to develop resistance trough functional mutations, deprive bacteria of essential nutrients and metals such as iron, which are essential for bacteria to grow, and interfere with porin functionalities, permeabilization, causing rupture, and disintegration of cytoplasmic membranes. They can also reduce the pH gradient across the cell membrane and inhibit proton exchangers and inhibit multicomponent efflux transporters/multidrug resistance pumps. More recent studies noticed a promotion of the oxidation of polyphenols coupled with the reduction of dissolved oxygen that leads to the generation of hydrogen peroxide (H_2O_2), which later inhibits bacterial growth. Furthermore, GP extracts also inactivate bacterial cytoplasmic proteins and enzymes directly through binding or precipitation (Fiallos et al., 2020; Hassan et al., 2019).

Bacterial viability assays are widely used to evaluate antimicrobial properties and its mode of action, being most of the methods focused on nucleic acid stains, cytoplasmic membrane potential, and reporter gene systems as well as pathogenic determinants such as the production of exoproteins (Nakatsuji et al., 2016; Stiefel et al., 2015).

It is known that *S. aureus* expresses more than 50 extracellular virulence factors, including exotoxins, surface proteins and secreted enzymes, such as proteases, nucleases, and lipases. Proteases may interact with the epidermal barrier in DFI, converting local host tissue into nutrients required for bacterial growth. Thus, proteases promote tissue colonization and damage (Nakatsuji et al., 2016). Proteases are exoproteins secreted by *S. aureus* that can be divided in three catalytic classes, namely papin-, serin-, and metallo-like cysteine proteases, being gelatinase included on the last class (Bien et al., 2011; Gottschall et al., 1995; Kantyka et al., 2011). Gelatinase has been identified as one of the virulence factors of *S. aureus*,

and it has been proven that its production inhibition can selectively treat *S. aureus* DFIs with a beneficial wound healing effect (Qiu et al., 2021).

Besides that, siderophore production is also an essential virulence factor of *S. aureus*. This compound has a high iron affinity, and it is produced by bacteria to compensate iron starvation (Courcol et al., 1997; Dale et al., 2004).

The lack of clear information on the antibacterial mode of action of RPE and particularly its involvement on pathogens "disarm" motivated further investigation on the mechanism underlying its activities. In this study, it was applied a combination of techniques to investigate the possible mechanism by which RPE might inhibit/kill *S. aureus*, as well as preclude its ability to interfere with *S. aureus* virulence/pathogenicity and thus skin colonization, infection and wound non-healing. Firstly, viability staining with SYTO9, and PI was performed to evaluate the effect of RPE on cytoplasmic membrane damage. After that, potassium leakage was measured to corroborate the membrane damage results obtained before. Lastly, the effect of RPE on the virulence factors production by *S. aureus*, namely proteases, gelatinase and siderophores was studied.

5.2 Materials and methods

5.2.1 Assessment of membrane integrity due to propidium iodide uptake

For the inoculum preparation, both *S. aureus* CECT976 and RN4220 strains grown overnight in MHB at 37 ºC under 160 rpm of agitation before all the experiments. Overnight cultures were adjusted to a OD_{600nm} = 0.149 ± 0.02 in MHB which corresponds to a cell density of 1×10^6 cells/mL after the dilution necessary for the methods described below.

The Live/Dead BacLightTM kit (Invitrogen) allows the assessment of membrane integrity by selective stain exclusion. This method was proceeded according to Simões et al. (2005) with minor alterations. This method was applied to estimate not only viable cells but also the total counts of bacteria as it is possible to consult on Figure 7. BacLight is composed of two nucleic acid binding stains: SYTO 9TM and propidium iodide (PI). SYTO 9TM penetrates bacterial membranes, staining the cells green. However, PI only penetrates cells with damaged membranes, binding to single- and double-stranded nucleic acids. The combination of these two stains generates red fluorescing cells. After being exposed for 60 minutes to the RPE at 20 mg/mL of concentration, the bacterial suspensions were transferred to saline solution and diluted 1:10. Three hundred microliters of each diluted suspension were filtered through a Nucleopore® (Whatman) black polycarbonate membrane (pore size 0.22 μm) and stained with 250 μL of diluted SYTO 9 TM and 50 μL of diluted component PI. The dyes were left to react for 7 min in the dark. The membrane was then mounted on BacLight mounting oil, as described in the instructions provided by the manufacturer. The microscope used for observation of the stained bacteria was a LEICA DMLB2 with a mercury lamp HBO/100W/3 incorporating a CCD camera to acquire images using IM50 software (LEICA) and a 100× oil immersion fluorescence objective. The optical filter combination for optimal viewing of stained mounts

consisted of a 480 to 500 nm excitation filter in combination with a 485 nm emission filter (Chroma 61000-V2 DAPI/FITC/TRITC). A program path (Scan Pro 5) involving object measurement and data output was used to obtain the total number of cells (both stains) and the number of PI-stained cells. Both the total number of cells and the number of PI-stained cells on each membrane was estimated from counts of a minimum of 15 fields of view. The experimental was carried out for both *S. aureus* CECT926 and RN4220 strains and two independent experiments were performed for each condition tested as it is illustrated on Figure 7.

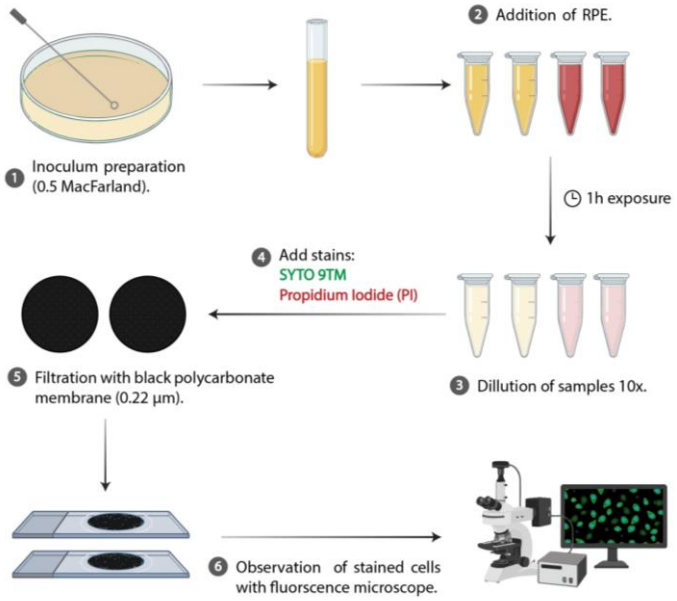


Figure 7-Schematic representation of the assessment of membrane integrity due to propidium iodide uptake.

5.2.2 Potassium (K^+) leakage

Potassium leakage is another indicator method of cell damage membrane. The principle of this method is the detection of the leak of ions when membrane damage occurs, which can be easily detected by atomic absorption spectrophotometry (Cooling et al., 2005).

The flame emission and atomic absorption spectroscopy was used for the K^+ titration in solutions of *S. aureus* CECT926 and RN4220 with RPE (crude ethanolic extracts (50 and 70%)).

For the inoculum preparation, both *S. aureus* CECT976 and RN4220 strains were grown overnight in MHB at 37 ºC under 160 rpm of agitation before all the experiments. Then, the bacterial suspensions

were transferred to a sterilized 50 mL tubes centrifuged for 10 minutes at 4000 rpm and the supernatant was discarded. The bacterial cells were washed with saline solution and the same process was repeated.

After that, bacterial cells were adjusted to a OD_{600nm} = 0.149 ± 0.02 in saline solution (0.85% NaCl). In 15 mL tubes, bacterial suspensions were put in contact with the crude ethanolic extracts (50 and 70%) in concentrations of 20 mg/mL for 1 hour. After that, the samples were filtrated and then 0,25 g of NaCl was added to each sample and stored in the refrigerator (4 ºC). The samples were analysed in a Flame Atomic Absorption Spectrometer (plus device GBC AAS 932) and the data acquisition with GBC Avante 1.33 software. The samples were measured at two independent experiments.

5.2.3 Virulence assays

The virulence assays were performed according to Borges et al., with minor alterations. Wells of 6 mm diameter were made in the agar plates, prepared according to the procedure explained below. Then, 15 µL of the bacterial suspensions exposed or not to RPE (prepared as described in the Section 5.2.2) were placed on the wells of each plate.

Proteases production

Production of proteases was determined on Plate Count Agar (PCA; Oxoid) containing 1% skim milk powder (Merck) and supplemented with crude 50 and 70% ethanolic extracts at the desired concentration (20 mg/mL). Briefly, 15 µl of the solutions prepared previously (section 5.2.2) were placed out on the plates. Plates without extract were used as negative controls. The plates were incubated for 72 h at 30 °C. After the incubation time, the plates were swamped with 1 M HCl. Protease production was observed by a transparent halo formed around the cells (Dogan & Boor 2003). All tests were performed in duplicate with two repeats.

Gelatinases production

In order to evaluate the effect of the extract on gelatinases production, gelatine agar (5 g l−1 peptone (Merck), 3 g l−1 yeast extract (Oxoid, Basingstoke, UK), 30 g l−1 gelatine (Oxoid), 15 g l−1 agar (Scharlau, Barcelona, Spain), pH 7) supplemented with crude 50 and 70% ethanolic extracts at the desired concentration (20 mg/mL) were used. Solutions without extract were used as negative controls. After incubation at 37 °C for 48 h, the plates were swamped with a saturated solution of ammonium sulphate (VWR, Leuven, Belgium). Gelatinase production was observed by a transparent halo formed around the wells (Su et al. 1991). All tests were performed in duplicate with two repeats.

Siderophores production

The detection of siderophores production was carried out in chrome azurol S (CAS; Fluka, Steinheim am Albuch, Germany) agar plates. For the preparation of the plates, 1 L of CAS agar, 60.5 mg of CAS were dissolved in 50 ml water and mixed with 10 ml iron (III) solution (1 mM FeCl3.6H20 (Panreac, Maryland Heights, MO, USA), 10 mM HCl). This solution was gradually added to 72.9 mg hexadecyltrimethylammonium bromide (HDTMA; Merck) dissolved in 40 ml of water under stirring. The

resultant dark blue liquid as well as basal agar medium (830 ml of H2O; 30 g of 3-(N-morpholino) propane sulfonic acid (MOPS; Fisher Scientific); 0.5 g of NaCl; 0.3 g of potassium phosphate (K2PO4; Chem-Lab); 0.1 g of ammonium chloride (NH4Cl; Riedel-de Haen, Seelze, Germany); 5 g of L-asparagine (AppliChem, Darmstadt, Germany); 15 g of agar, pH 6.8) were autoclaved. After cooling to 50 °C, 20 mL of glucose (50 %) (Sigma-Aldrich) as well as 100 mL of CAS indicator solution were finally added along the glass wall, with moderated agitation to prevent generation of foam. Each plate received 20 ml of CAS agar, and 15µL of the previous prepared solutions (section 5.2.2) were placed out on the wells of the plates. Solutions without RPE were used as negative controls. The plates were incubated at 37 °C, for 48 h. Siderophore production was observed by an orange halo formed around the cells (Schwyn & Neilands 1987). All tests were performed in duplicate with two repeats.

The methods applied for K^+ assay and virulence assays are schematically represented on Figure 8.

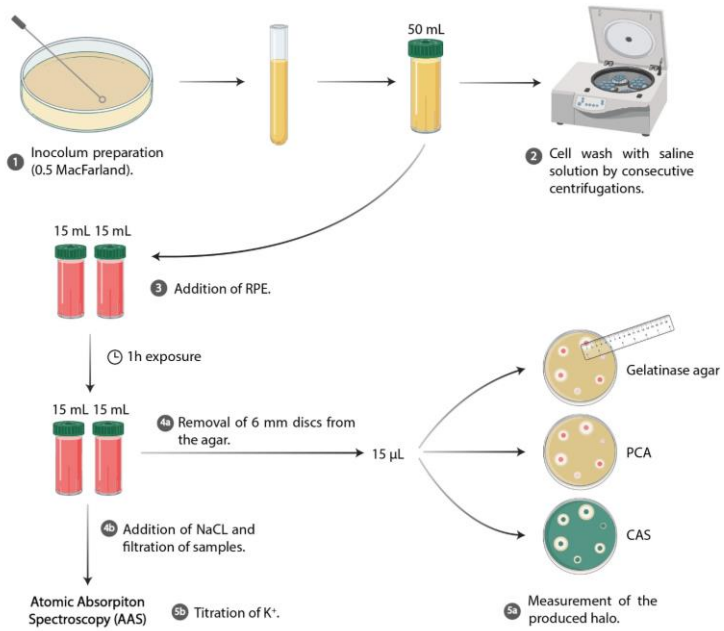


Figure 8-Schematic representation of Potassium leakage assay and virulence assays

5.2.4 Statistical analysis

Both mean and standard deviation (SD) within samples were calculated for all cases. At least two independent experiments were performed for each condition tested. One-way ANOVA and Turkey test was performed. Statistical analysis was based on a confidence level ≥95%, ($p < 0.05$ was considered statistically significant).

5.3 Results and Discussion

5.3.1 Effect of RPE on *S. aureus* cytoplasmic membrane integrity

One of the criterions mostly used to assess the cytoplasmic cellular membrane integrity, which helps to define if a bacteria cell is viable or dead, is PI uptake. In fact, viability staining with SYTO9 and PI is a frequently used method once it is a quick and simple procedure, in which the assumptions that viable cells have an intact membrane and non-viable cells have damaged membrane are made. SYTO9 enters live and dead cells bounding to nucleic acids, inducing a fluorescent signal. The identification of dead cells is acquired by the PI staining, which only penetrates cells with cytoplasmic membrane damage. This last stain has a higher affinity to nucleic acids, consequently, in the presence of both stains, SYTO9 is displaced by PI. This method, however, is not considered accurate and can result in an under- or overestimation of the viable cells, Thus, several other methods may be applied to corroborate the results (Stiefel et al., 2015).

In order to evaluate the effect of RPE on *S. aureus* cytoplasmic membrane integrity, it was determined the PI uptake after 1 hour of exposure to RPE at 20 mg/mL (Figures 9, 10 and 11). PI is a nucleic acid stain to which cell membrane is usually impermeable. This characteristic is the cause for being used as an indicator of the integrity of cytoplasmic membrane (Simões et al. 2007; Borges et al. 2013).

Results suggest that RPE did not compromised the integrity of the cytoplasmic membrane in *S. aureus* CECT976 strain, as there were no statistically significant differences between the samples with RPE and the control solutions ($p>0.05$). However, for *S. aureus* RN4220, the percentage of damaged cells increased after the exposure to RPE for both crude ethanolic extracts (50 and 70%) ($p<0.05$).

There are a few limitations of this method to assess membrane integrity, namely bleaching effects of SYTO9, different bidding affinities of the dyes to cells and background fluorescence that can lead to an under or overestimation of the number of non-viable cells (Stiefel et al. 2015). As it is possible to see on Figures 10 and 11, a red spot was formed, possibly due to the fact that the RPE used is red colour. Thus, other tests should be performed to confirm the results obtained.

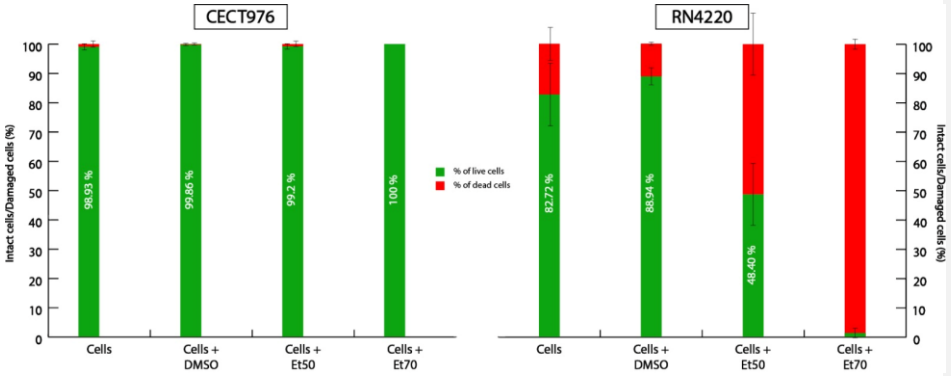


Figure 9-Percentage of intact cells/damaged cells for *S. aureus* CECT976 and RN4220 after 1 hour exposure to RPE.

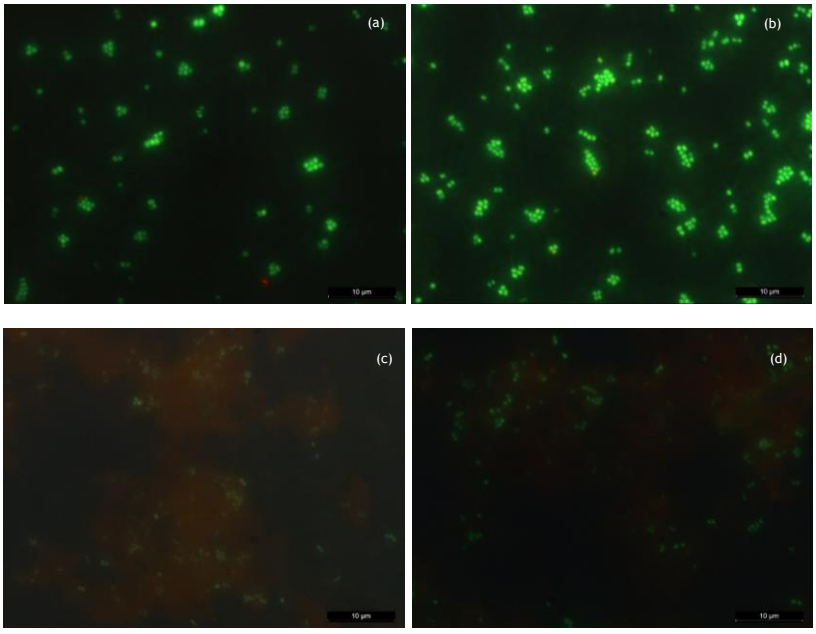


Figure 10-Representative images of PI uptake (magnification 1000×). (a) *S. aureus* CECT976 without treatment; (b) *S. aureus* CECT976 exposed to DMSO (control) for 1 h; (c) *S. aureus* CECT976 exposed to crude ethanolic extract 50% for 1 h; (d) *S. aureus* CECT976 exposed to crude ethanolic extract 70% for 1 h.

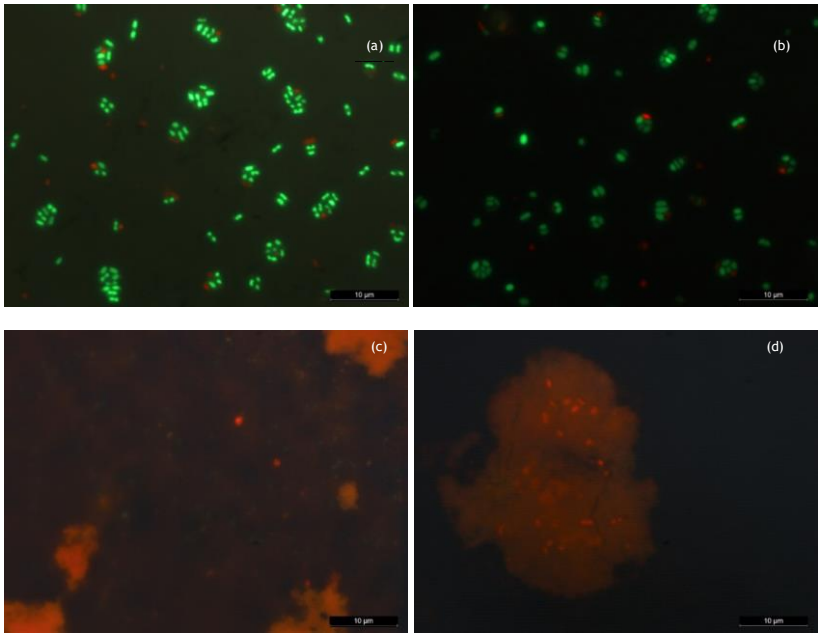


Figure 11-Representative images of PI uptake (magnification 1000×). (a) *S. aureus* RN4220 without treatment; (b) *S. aureus* RN4220 exposed to DMSO (control) for 1 h; (c) *S. aureus* RN4220 exposed to crude ethanolic extract 50% for 1 h; (d) *S. aureus* RN4220 exposed to crude ethanolic extract 70% for 1 h.

The cytoplasmic membrane acts as a barrier between the cytoplasm and the extracellular medium, allowing different ion compositions between the internal and external environment. Usually, cytoplasmic medium is rich in K^+, thus, potassium leakage is a good indicator of the cytoplasmic membrane damage (Codling et al., 2003; Lambert et al., 1973). It has been reported in other studies that RPE showed a possible membrane activity against *S. aureus*, as potassium leakage occurred after treatment (Cooling et al., 2005; Lambert et al., 1973). The antimicrobial activity of RPE has been previously attributed to the presence of PC, which specifically induce cell membrane damage (Fiallos et al., 2020).

The results obtained for K^+ release from *S. aureus* after 1 hour exposure to RPE are presented on Table 10.

The results indicated alteration in the cytoplasmic membrane permeability for both strains tested, once it is clear that concentrations of K^+ increased more than 100 times comparing to the control samples ($p<0.05$). Besides that, it was not found statistically significant difference between the crude ethanolic extracts (50 and 70%) for both tested strains. ($p>0.05$). It was also possible to compare K^+ release between the strains exposed to crude ethanolic extracts (70%), indicating a significant difference and higher

membrane damage for *S. aureus* RN4220 strain. ($p<0.05$). However, it was not found statistically significant differences between the two strains exposed to crude ethanolic extracts (50%) ($p>0.05$).

The evaluation of bacterial cytoplasmic membrane integrity by quantification of K^+ efflux can be correlated with PI uptake, since PI only penetrates cells that have lost membrane integrity. Despite the impossibility of conclusion about *S. aureus* CECT976 strain, once the results seamed promising on K^+ release, but they are not according to the results obtained for PI assay. The results obtained for *S. aureus* RN4220 strain may indicate that RPE interfere with the membrane permeability.

Table 10-Concentration of K^+ (μg/mL) in solution after contact of *S. aureus* CECT976 and RN4220 strains with crude ethanolic extracts for 1h. Mean values ± SD for at least two independent experiments are illustrated.

		Concentration of K+ in solution (μg/mL)
***S. aureus* CECT976**	**Cell**	0.93±0.17
	Cell+DMSO	0.14±0.01
	Cell+Et50	187.45±3.35
	Cell+Et70	74.03±2.77
***S. aureus* RN4220**	**Cell**	0.74±0.07
	Cell+DMSO	0.12±0.01
	Cell+Et50	199.93±9.83
	Cell+Et70	146.13±3.43

5.3.2 Effect of RPE on virulence factors production

The most common methods for detection of these last-mentioned virulence factors are based on medium inoculation with targeted compounds. Siderophore detection, for example, is based on a competition for iron between the ferric complex of the indicator dye chrome azurol S (CAS) and microorganism-produced siderophores. Siderophore, which appears to have a higher affinity for iron, removes the iron from CAS (III). The positive reaction causes the CAS reagent to change colour, usually from blue to orange (Milagres et al., 1999).

Relatively to gelatinase and protease expression, it was not possible to take any conclusions. The results obtained were not reliable, once it has been reported that gelatinase and protease are characteristic features of *S. aureus* virulence, and plates of control did not show any halo as it was expected (Bein at al., 2011; Qiu et al., 2021). By inhibiting the activity of these virulence factors, it is possible to indirectly decrease the bacteria capacity of colonization and infection development, i.e., antivirulence drugs targeting bacterial virulence factor, instead of killing or inhibit bacterial growth, have the capacity to disarm pathogens, constituting thus an alternative to antibiotics (Totsika, 2017).

The effect of the RPE on siderophore production by *S. aureus* was evaluated by an indicator dye chrome azurol S (CAS). According to Milagres et al. (1999), the positive reaction, i.e., the removal of iron

from CAS, results in a colour change, usually from blue to orange. As it is possible to visualize on Figure 12, the halo and the yellow colour of the medium increased for both of *S. aureus* strains after 1 hour exposure with RPE.

Two hypotheses may be suggested by this increase in halo iron consumption: either *S. aureus* siderophore production was potentiated by RPE or the increase was due to release of intracellular siderophores during cell lysis. The second hypothesis seams more reliable, once it was proven on the other trials (PI uptake and K^+ release), that RPE effectively can lead to membrane damage. In fact, siderophore production allows a higher affinity for iron present in the medium, which is an essential component for bacteria to growth (Courcol et al., 1997; Dale et al., 2004). This way, further studies should be done to clarify this effect.

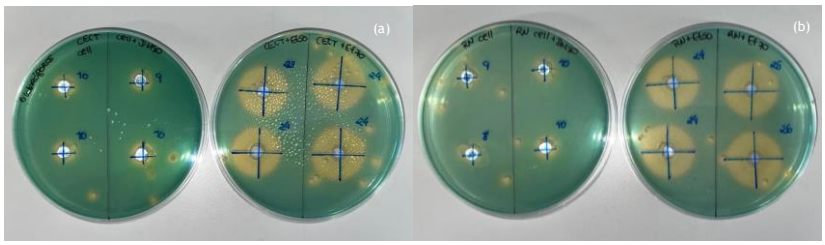


Figure 12- Halos produced in the siderophores assay (a) by *S. aureus* CECT976; (b) and by *S. aureus* RN4220, controls and after one hour exposure with RPE; control plates are on the left side of the image and samples on the right side for both (a) and (b).

5.4 Conclusions

The assessment of bacterial cytoplasmic membranal integrity by PI uptake after 1 hour exposure to RPE revealed permeation to PI for *S. aureus* RN4220 strain. However, this effect was not detected for *S. aureus* CECT976. Besides that, leakage of intracellular K^+ indicated that the antibacterial action of RPE was due to the cytoplasmic membrane damage, showing that crude ethanolic extracts (50%) had a higher antibacterial effect.

Furthermore, virulence factors of *S. aureus* were evaluated. Although, no reliable results were obtained for gelatinase and protease production, siderophores assay showed an increase of the produced halo, indicating in a primary evaluation that RPE increase the siderophores production.

Due to these results, further methods and assays should be applied to reach more clear conclusions about the effect of RPE in the mode of action of *S. aureus* present in DFI.

Chapter 6-Conclusions and Future Remarks

6.1 Conclusions

In order to overcome the issues of DFI and its association with MDR bacteria (for example, the most common isolated pathogen - *S. aureus*), there is an urgent need to discover new compounds with antimicrobial properties. Alongside with this problem, it is also imperative the increase of urge to achieve a more sustainable wine production. In this work, the extraction of BC from wine by-products and the RPE antimicrobial, antioxidant properties against *S. aureus*, as well as their effect on virulence factors production was evaluated.

The RPE extraction yields obtained were in the range of 4 to 30%, and the most profitable solvent in terms of extraction yield was acetone. However, for the rest of the study, only crude ethanolic extracts (50 and 70%) were applied. Through this study, it was possible to verify that the extraction yields not only are dependent on the fraction of RGP, but also on the solvent used and the solute/solvent ratio.

The present work confirmed that RPE have antimicrobial activity against *S. aureus*, since MIC values of 25 mg/mL were obtained for *S. aureus* CECT976 strain and 25 and 50 mg/mL were obtained for S. *aureus* RN4220 for the crude ethanolic extracts (70 and 50%), respectively. However, MBC values of 25 mg/mL were obtained only for *S. aureus* CECT976 strain. Besides that, the study on the inhibitory effect and synergies between the RPE and antibiotics suggested a promising potentiation effects on several combinations. The antioxidant activity was detected by ABTS assay for the crude ethanolic extract (50%) and non-detectable for the crude ethanolic extract (70%).

The exposure of *S. aureus* to RPE elicited cell permeation to PI, and release of intracellular K^+ for *S. aureus* RN4220 strain, supporting the statement that the antibacterial action of RPE may be through cytoplasmic membrane destabilization. However, none of the effects mentioned before were observed for *S. aureus* CECT976. After contact with RPE, *S. aureus* cell suffered damage. Relatively to the effect of RPE on virulence factors production, no conclusions were obtained for proteases and gelatinases production. Furthermore, for both strains of *S. aureus*, it was possible to observe an increase of the produced halo, indicating in a primary evaluation that RPE increase the siderophore production.

Therefore, taking all together, RPE may constitute a good antimicrobial agent against *S. aureus* present on DFI, however, further studies and methods should be applied in order to take more assertive conclusions. Some suggestions for the future work are explained on the next section.

6.2 Future Remarks

This work set out be a step forward in exploring wine industry by-products for targeting *S. aureus* in DFI. In it is the hope of creating a more sustainable way of wine production by adding value to its by-

products. MDR also constitute an emerging problem on the society; therefore, natural sources of antimicrobial agents can present a future solution. However, as it is possible to understand by the ongoing discussion throughout this thesis, some questions remain unanswered or unclear and would benefit from further research. This way, suggestions for future work are presented herein.

In terms of extraction, this process could be optimized by the application of other extraction techniques. For example, Soxhlet extraction, as well as different solvents, solute-solvent ration, and different concentrations of the aqueous solutions. This way, it may be possible to extract bioactive compounds more effectively, increasing the extraction yield and possibly the bioactivity of the RPE.

Although different methods were applied to study the antimicrobial properties of the extracts, it was not possible in this study to assess the percentage of bioactive compounds present on the RPE, neither its real composition in PC. In this sense, the content in PC of the RPE could be determined by the Folin-Ciocalteu method. Besides that, LCMS (Liquid chromatography–mass spectrometry) analysis could also be performed for identification of the discriminated monomeric PC present in RPE. Furthermore, a HPLC (High-performance liquid chromatography) analysis would allow the determination of the content in discriminated PC such as gallic acid, catechin, epicatechin, among others.

After the studies mentioned before, antimicrobial, antioxidant activities and effect on the virulence could be assessed with the RPE that presented better results, i.e., higher extraction yields and higher content in PC. For that, MIC, and MBC determination as well as synergies between RPE and antibiotics should be repeated. Besides that, it has been highly recommended the use of more than one type of antioxidant activity measurement due to the differences between the various free radical scavenging assay systems. In this sense, another method such as DPPH should be performed to corroborate the results obtained in this study. It would also be interesting to assess the RPE capacity to inhibit *S. aureus* efflux pumps. For that, Ethidium Bromide-Agar Cartwheel Method could be performed. Ethidium bromide is a broad range efflux pump substrate and has been explored to assess efflux activity in *S. aureus*.

For the insight on the antimicrobial and antipathogenic mode of action of RPE against *S. aureus*, assays performed herein should be repeated, i.e., it was possible to conclude that RPE affect membrane integrity, but no clear conclusions were obtained for one of the tested strains. Another proposal could be an evaluation of the RPE effect on the physicochemical properties of cell surface of *S. aureus*, namely, hydrophobicity and ion charge. For that, the determination of contact angles and zeta potential could be performed. Besides that, *S. aureus* has more than 50 virulence effects, and RPE also have a wide variety of compounds. This way, different extracts with different phenolic composition can interact differently with different virulence factors, which is translated on a vast possibility of combinations to evaluate. One of the virulence factors produced by *S. aureus* in chronic wounds is staphylokinase (Sak). The RPE effect on this protein production could be evaluated by adding a plasmin-specific chromogenic substrate to the samples and monitoring colour development.

After all the suggested methods, there could be also an assessment of the RPE with *in vitro* models from wounds and perform toxicity tests. Another suggestion could be the capacity of RPE to penetrate skin barriers, which could be assessed by a Transwell System. Future work can also be developed to create a cream formulation using RPE or its incorporation in wound patches.

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Annex I – Modified Disc Diffusion Method

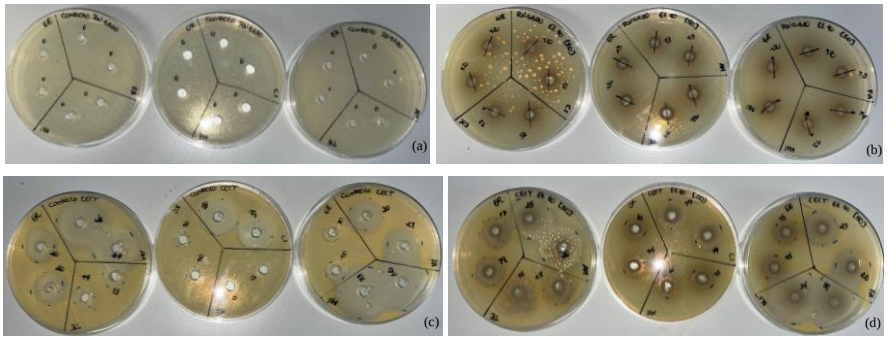

Figure A 1- Examples of the IZD values obtained for antibiotics and RPE singly and combined for *S. aureus* CECT976 and *S. aureus* RN4220 strains. (a) IZD of antibiotics for *S. aureus* RN4220; (b) IZD of RPE combined with antibiotics for *S. aureus* RN4220; (c) IZD of antibiotics for *S. aureus* CECT976; (d) IZD of RPE combined with antibiotics for *S. aureus* CECT976.