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RESUMO *Screening* de atividade antibacteriana de extratos de plantas contra bactérias patogénicas

O uso prolongado e intensivo de antibióticos contribuiu para o desenvolvimento de bactérias resistentes, o que culminou na perda de opções terapêuticas disponíveis. A Organização Mundial de Saúde publicou uma lista de agentes patogénicos resistentes a antibióticos que inclui *Staphylococcus aureus* e *Pseudomonas aeruginosa*. É por isso imperativo que sejam desenvolvidas estratégias alternativas aos antibióticos eficazes contra os agentes patogénicos e há evidências que demonstram a atividade antibacteriana dos produtos naturais contra bactérias multirresistentes; contudo ainda há muito por explorar.

Assim, o objetivo deste estudo assenta na avaliação da atividade antibacteriana de plantas utilizadas no dia a dia, incluindo o alho, o gengibre e aa romã (casca, polpa e sumo) contra a *S. aureus* e a *P. aeruginosa*; e a novidade é a determinação do impacto das condições de extração (o solvente, a duração e temperatura de extração) na atividade antibacteriana de cada planta e o estabelecer de uma reação dessa atividade com a atividade antioxidante e o seu teor fenólico.

Os extratos foram obtidos a partir de diferentes solventes, etanol 96% e 70% e água destilada, em diferentes temperaturas (70 °C durante 1h e *overnight* à temperatura ambiente). A avaliação do conteúdo fenólico foi feita pelo método Folin-Ciocalteu, a atividade antioxidante pelos métodos *Ferric Reducing Antioxidant Power* e 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), e a atividade antibacteriana pela determinação da MIC e da MBC.

Os resultados demonstraram que os extratos etanólicos de plantas foram mais ativos contra as bactérias, particularmente a *S. aureus*. A sua atividade baseou-se principalmente na inativação do crescimento bacteriano, apesar de a erradicação também ter sido conseguida. Na maioria dos extratos, a atividade antibacteriana estava associada aos extratos com maior teor fenólico e capacidade antioxidante, extraídos com misturas etanol/água (EtOH (70%)). As condições de extração (70°C durante 1h e *overnight* à temperatura ambiente) afetam a análise do teor fenólico e da atividade antioxidante, que estão relacionadas com a atividade antibacteriana. De todos os extratos etanólicos 70%, o extrato da polpa de romã foi o mais promissor contra ambas as bactérias, pois foi o extrato que inibiu o seu crescimento a concentrações mais baixas e foi também o único capaz de erradicar a bactéria gram-negativa; pelo que deve ser explorado no futuro.

Palavras-chave: Extratos de plantas; Produtos naturais; Resistência a antibióticos; *Pseudomonas aeruginosa; Staphylococcus aureus*.

ABSTRACT Screening for antibacterial activity in plant extracts against pathogenic bacteria

The intensive and prolonged use of antibiotics contributed to the development of antibiotic resistance in bacteria, culminating in a loss of therapeutic options. World Health Organization list of antibiotic-resistant pathogens includes *Staphylococcus aureus* and *Pseudomonas aeruginosa*, reason that is imperative to evolve alternative non-antibiotic strategies that are effective against these infectious pathogens. A growing body of evidence have demonstrated the antimicrobial activity of natural products against multidrug-resistant bacteria and still much remains to be explored. Therefore, the aim of this work relies in the evaluation of the antibacterial activity of plants used in daily life, including as garlic, pomegranate (peel, pomace and juice) and ginger against *S. aureus and P. aeruginosa*. The novelty of this work is to determine the impact of extract conditions on the antibacterial activity of these plant extracts and to attempt to correlate with their antioxidant activity and total phenolic content.

The extracts were obtained using different solvents, including 96% and 70% ethanol and distilled water, at different temperatures (70 °C for 1h and overnight at room temperature). The evaluation of the total phenolic content was performed by the Folin-Ciocalteu method, the antioxidant activity by the Ferric Reducing Antioxidant Power and 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) method, and antibacterial activity by MIC and MBC determination.

Results demonstrated that ethanolic extracts of plants were the most active against bacteria, in particular *S. aureus*, than aqueous extracts. The activity of the ethanolic extracts was mainly based on inactivation of bacterial growth, but eradication was achieved in some cases. Frequently, this antibacterial activity was associated with the extracts with increased phenolic and antioxidant content, extracted with ethanol/water mixtures (EtOH (70%)). Extraction conditions (70 °C for 1h and overnight at room temperature) affect total phenolic content and antioxidant activity analysis, which has correlation with antibacterial activity. Among ethanolic 70% extracts, extracts of pomegranate peel were the most promising non-antibiotic drugs against both *S. aureus* and *P. aeruginosa* eradication, since it was the ethanolic 70% extract that inhibited their growth at the lowest concentration and was the only one able to eradicate the gram-negative bacteria.

In conclusion, pomegranate peel extracts exhibited promising potential as non-antibiotic drug to treat infections caused by *S. aureus* and should be further explored in near future.

Keywords: Antibiotic resistance; Natural products; Plant extract; *Pseudomonas aeruginosa; Staphylococcus aureus*.

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LIST OF ABBREVIATIONS

ABTS: 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)

AMR: Antimicrobial resistance

CF: Cystic Fibrosis

CFU: Colony Formation Unit

DMSO: Dimethyl sulfoxide

***E. coli*:** *Escherichia coli*

EOs: Essential Oils

EtOH: Ethanol

FDA: Food and Drug Administration

FRAP: Ferric Reducing Antioxidant Power

H_2O (d): Distilled water

K. pneumoniae: *Klebsiella pneumoniae*

MBC: Minimum Bactericidal Concentration

MDR: Multidrug resistant

MHA: Mueller-Hinton Agar Medium

MHB: Mueller-Hinton Broth Medium

MIC: Minimum Inhibitory Concentration

MRSA: Methicillin-resistant *Staphylococcus aureus*

MSSA: Methicillin-Susceptible *Staphylococcus aureus*

***P. aeruginosa*:** *Pseudomonas aeruginosa*

PJ: Pomegranate juice

PP: Pomegranate pomace

PPE: Pomegranate peel

***S. aureus*:** *Staphylococcus aureus*

TEAC: Trolox Equivalent Antioxidant Capacity

TPC: Total Phenolic Content

TSA: Tryptic Soy Agar

TSB: Tryptic Soy Broth

TTPZ: 2,4,6-tripyridyl-s-triazin

VISA: Vancomycin-Intermediate *Staphylococcus aureus*

VRE: Vancomycin resistance

WHO: World Health Organization

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the extract conditions, considering the same extract and the same solvent. The analysis starts with the letter “a” and “A” representing the highest value.

Figure 5 Trolox equivalent concentration, mmol Trolox Equivalent *per* g of extract (mmol/g), by FRAP analysis **(a)** and by ABTS analysis **(b)**, of garlic extracts.
Data presented represent the mean ± standard deviation of three assays in triplicate and each replicate included several garlics. The lowercase letter represents the significant comparison of the extraction solvents, considering the same extract and the same extraction conditions. The uppercase letter represents the significant comparison of the extract conditions, considering the same extract and the same solvent. The analysis starts with the letter “a” and “A” representing the highest value.

Figure 6 Trolox equivalent concentration, mmol Trolox Equivalent *per* g of extract (mmol/g), by FRAP analysis **(a)** and by ABTS analysis **(b)**, of ginger extracts.
Data presented represent the mean ± standard deviation of three assays in triplicate and each replicate included several ginger rhizomes. The lowercase letter represents the significant comparison of the extraction solvents, considering the same extract and the same extraction conditions. The uppercase letter represents the significant comparison of the extract conditions, considering the same extract and the same solvent. The analysis starts with the letter “a” and “A” representing the highest value.

Figure 7 Trolox equivalent concentration, mmol Trolox Equivalent *per* g of extract (mmol/g), by FRAP analysis **(a)** and by ABTS analysis **(b)**, of pomegranate peel extracts.
Data presented represent the mean ± standard deviation of three assays in triplicate and each replicate included several pomegranates. The lowercase letter represents the significant comparison of the extraction solvents, considering the same extract and the same extraction conditions. The uppercase letter represents the significant comparison of the extract conditions, considering the same extract and the same solvent. The analysis starts with the letter “a” and “A” representing the highest value.

Figure 8 Trolox equivalent concentration, mmol Trolox Equivalent *per* g of extract (mmol/g), by FRAP analysis **(a)** and by ABTS analysis **(b)**, of pomegranate pomace extracts.

Data presented represent the mean ± standard deviation of three assays in triplicate and each replicate included several pomegranates. The lowercase letter represents the significant comparison of the extraction solvents, considering the same extract and the same extraction conditions. The uppercase letter represents the significant comparison of the extract conditions, considering the same extract and the same solvent. The analysis starts with the letter "a" and "A" representing the highest value.

Figure 9 Trolox equivalent concentration, mmol Trolox Equivalent *per* g of extract (mmol/g), by FRAP and ABTS analysis, of pomegranate juice extracts. Data presented represent the mean ± standard deviation of three assays in triplicate and each replicate included several pomegranates.

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1. Introduction

1.1. Antibiotic Resistance

The administration of antibiotics was one of the most important medical interventions reductions of the human morbidity, mortality and increased life expectancy. Penicillin, discovered accidentally by Alexander Fleming in 1928, was the first natural antibiotic to be reported when the *Penicillium* fungus contaminated a culture plate in his laboratory, however, it was not developed for use until the late 1930s. Following the discovery of penicillin by Fleming, other scientists, including Rene Dubos and Selman Waksman, started a deliberate search for antibacterial agents among soil microorganisms, including bacteria and fungi.[1,2] The next biggest discovery came about in 1943, resulting in identification of streptomycin produced by *Streptomyces griseus*, that marked the beginning of the golden age of antibiotic discovery and development (1940–1990).[1,3] During the 1940s and early 1960s, antibiotic resistance to multiple antimicrobial agents was detected among enteric bacteria namely *Staphylococcus aureus* (*S. aureus*), *Salmonella*, *Shigella*, and *Escherichia coli* (*E. coli*)for the very first time.[2,4,5]

In 2015, it was estimated that in Europe 25,000 people die *per* year as a result of multidrug-resistant bacterial infections and it costs €1.5 billion annually to the European Union economy.[6,7] Patients with antibiotic-resistant bacterial infections need to stay in the hospital for at least 13 days, adding an additional cost annually.[2] Moreover, in the same year, MRSA killed more American people *per year* than HIV/AIDS, Parkinson's disease, emphysema, and homicide combined.[8]

Given this scenario, The World Health Organization (WHO) declared that medicine entered in the post-antibiotic era in which the current antibiotics become less effective overtime and medical advances are insufficient to face antibiotic resistance. Moreover, WHO classified antibiotic resistance as one of the three most important public health threats of the 21st century of worldwide dimension.[2,4,6,9]

The clinical relevance of antibiotics goes beyond simply preventing death and illness due to infection, in fact antibiotics also have successfully prevented or treated infections that can occur in patients who are receiving cancer treatment by chemotherapy or radiation therapy, or patients who have had surgeries such as organ transplants or cardiac surgery, for example.[10,11]

There are several reasons that can also lead for that antibiotic resistance. The overuse and misuse of antibiotics are one of key factors attributed to antibiotic resistance.[2,10] In 2015, 30% of the outpatient antibiotics prescribed were unnecessary, with acute respiratory infections holding the highest unnecessary use of antibiotics at 50%.[2] Another factor is the extensive use of antibiotics on

agriculture for growth promotion and prevention of disease, not to eradicate a bacterial infection. Therefore, antibiotic resistant bacteria may reach people indirectly through food chain by consumption of contaminated food or derived products.[11,12]

1.1.1. Mechanisms of antibiotic resistance

Antibiotics can be classified based on their structure and mode of action and at least seven major groups of antibiotics have been considered, including penicillin's, β-lactams, cephalosporins, aminoglycosides, fluoroquinolones, macrolides, tetracyclines, and glycopeptides.[1] The most common target for antibiotics are metabolically active cells, and so antibiotics act preferably on inhibition of the cell wall synthesis, depolarization of the cell membrane, inhibition of protein synthesis, inhibition of nucleic acid synthesis, and inhibition of metabolic pathways in bacteria.[13] However, bacteria are remarkably resourceful and, for that reason, they can easily adapt to a wide array of stressful conditions, including resist to antimicrobial agents. Various of these responsive mechanisms of resistance may have evolved in response to pressures applied by 'natural' antibiotics produced by other microorganisms with which these bacteria coexist. Therefore, most currently recognized antimicrobial resistance (AMR) mechanisms can be classified in three categories: altered target site for the antimicrobial agent; enzymatic inactivation of the antimicrobial agent; and decreased permeability of the bacterial envelope.[14]

Nevertheless, bacteria are not uniformly susceptible or resistant to antibiotic and thus they may exhibit different mechanisms for resistance: intrinsic resistance; acquired resistance and adaptive.[2,6] The intrinsic antibiotic resistance refers to the innate ability of bacteria to resist to the action of an antibiotic as a result of their genome encoding inherent structural or functional properties independent of previous antibiotic exposure. This kind of antibiotic resistance explains why some antibiotics are more active against gram-negative than gram-positive bacteria and vice-versa, due to their inherent distinct cell wall composition acting as barrier to the entrance of antibiotics into the cells.[1,6,15] Intrinsic mechanisms confer low level antibiotic resistance in the original host, however the normal commensal flora or environmental bacteria containing intrinsic mechanisms can become opportunistic pathogens in immunocompromised patients.[1]

In addition to intrinsic resistance, bacteria can acquire resistance to antibiotics. This kind of resistance is the major cause of the global crisis of antibiotic resistance.[6,15] It arises when bacteria becomes resistant through the acquisition and incorporation of new genetic material, such as plasmids, transposons, integrons or DNA from other microorganisms by horizontal gene transfer

or as a result of mutations of chromosomal genes. The acquisition may be temporary or permanent.[6,9,16]

Acquired resistance can be mediated by several mechanisms, which fall into three groups:

(i) Those that minimize the intracellular concentrations of the antibiotic as a result of poor penetration into the bacterium or of antibiotic efflux (membrane proteins that export antibiotics from the cell and maintain their low intracellular concentrations).[1,6,15,17]

(ii) those that modify the antibiotic target. Those changes that may consist of point mutations in the genes encoding the target site, enzymatic alterations of the binding site, and/or replacement or bypass of the original target. Regardless of the type of change, the final effect is identical: a decreased affinity of the antibiotic for the target site.[1,6,9,15,17]

(iii) and those that inactivate the antibiotic by hydrolysis or modification. The enzyme catalyzed modification of antibiotics is a major mechanism of antibiotic resistance that has been relevant since the first use of antibiotics.[1,6,15]

Moreover, bacteria can also produce an alternative target (usually an enzyme) that is resistant to inhibition of antibiotic and at the same time produce a native target too, which is sensitive to antibiotics, allowing bacteria to survive by adopting the role of a native protein.[17] Often, different mechanisms of resistance are combined, contributing to the expression of high levels of AMR.[15]

Furthermore, bacteria can develop other kind of resistance to antibiotics, which is called adaptive resistance. It can be define as a temporary increase in the ability of a bacterium to survive an antibiotic insult due to alterations in gene and/or protein expression as a result of exposure to an environmental trigger, such as pH, temperature, nutrient or oxygen limitation, ion densities and exposure to non-lethal doses of antibiotics.[15,18–20] Unlike intrinsic and acquired resistance, which are stable and can be transmitted vertically to subsequent generations, adaptive resistance is unstable, transient and highly dependent on the presence of antibiotics. It cannot be vertically transmitted and usually reverts at the liminal of the inducing status.[15,20] Because of its transient nature, adaptive resistance represents one of the biggest challenges in designing effective antimicrobial therapies, explaining the common differences found between *in vitro* and *in vivo* antibiotic susceptibilities exhibited by bacteria.[15] There are several mechanisms of adaptive resistance, including epigenetic inheritance, population heterogeneity, mutability, gene amplification, efflux pumps and biofilm formation.[15] Of all these mechanisms, biofilms represent one of the most effective antibiotic resistance strategies, as they have a 10 to 1000 times greater ability to resist the antibiotic than

planktonic cells. Moreover, they are responsible for approximately 80% of chronic and recurrent microbial infections in the human body.[21]

A biofilm can be defined as a community of cells attached to a substratum (biotic or abiotic), interface, or to each other that are embedded in a self-produced matrix of extracellular polymeric substance.[22] Biofilms may cause inflammation, because they are protected from antibiotics and the body's immune system.[23] Slow or arrested cell growth deep in the biofilm is known to decrease antibiotic susceptibility, and metabolic responses to nutrient limitation may control antibiotic tolerance in growth-arrested cells under these conditions.[24] Bacterial biofilms are resistant to antibiotics, disinfectant chemicals and to phagocytosis and other components of the innate and adaptive inflammatory defense system of the body. Combating this organization of cells usually requires high antibiotic doses for a prolonged time, and these approaches often fail, contributing to infection persistence.[25] The structure and composition of the biofilm matrix can contribute to antibiotic resistance. Exopolysaccharide and extracellular DNA in the biofilm matrix can act as a barrier to diffusion, preventing drugs from reaching living cells. The effectiveness of this barrier varies between antibiotics — large molecules, positively charged aminoglycosides, and antimicrobial peptides diffuse poorly in biofilms, but quinolones and β-lactams appear to move freely. For antibiotics that can penetrate the matrix, inactivation by resistance enzymes can produce collective resistance.[24]

1.1.2. Difficult-to-treat antibiotic resistant bacteria

In light of increasing antibiotic resistance, in February 2017, the WHO published a list of pathogens that includes the pathogens designated by the acronym ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter species*) to which were given the highest "priority status" since they represent the great threat to humans. These pathogens have evolved into multidrug-resistant (MDR) forms subsequent to antibiotic use and can cause severe and often fatal infectious diseases such as bloodstream infections and pneumonia.[3]

Among gram-positive pathogens, a global pandemic of resistant *S. aureus* currently poses the biggest threat.[11] In fact, this bacterium turned out to be one of the first causes of healthcare-associated infection, and in 1944, when *S. aureus* resistance was first identified, penicillin presented a solution. However, it offered only was only a short-term relief, because a few later (around 1950), Penicillin-Resistant *Staphylococcus aureus* appeared.[26,27] Methicillin was then

produced around 1960, and a year after their clinical use there were already records of Methicillin-resistant *Staphylococcus aureu*s (MRSA). Since then, MRSA infections have spread worldwide, appearing at a high incidence in several countries in Europe, America, and the Asia-Pacific region.[11,15,27,28] For many years vancomycin has been considered a last-resort antibiotic against severe MRSA and other resistant gram-positive infections. However, by the late 1980s vancomycin resistance first appeared in enterococci (VRE) and later, in 1997, Vancomycin-Intermediate *Staphylococcus aureus* (VISA). In recent years in Vancomycin-resistant *Staphylococcus aureus* (VRSA), which also emerged from MRSA. Nowadays, they are also recognized as high priority pathogens since, without containment and effective therapeutic solutions, they can cause serious infections that are impossible to control.[29]

Regarding gram-negative pathogens, they are particularly worrisome because they are becoming resistant to nearly all the antibiotic drug options available, creating situations reminiscent of the pre-antibiotic era. The most serious gram-negative infections occur in health care settings are caused by Enterobacteriaceae, mostly *Klebsiella pneumoniae* (*K. pneumoniae*), *Pseudomonas aeruginosa* (*P. aeruginosa*), and *Acinetobacter baumannii.*[11,15] Among these pathogens, *P. aeruginosa* infections are of particular importance due to the accumulation of resistance after exposure to nearly all antibiotics (including aminoglycosides, cephalosporins, fluoroquinolones, and carbapenems) and cross-resistance between agents, that may result in multidrug-resistant (MDR) *P. aeruginosa*.[11,30]

Understanding the resistance mechanisms of these bacteria is a key step towards the development of new antimicrobial strategies to tackle drug-resistant bacteria. Therefore, in the next sections it will be discussed the current state of antibiotic resistance in the most critical resistant gram-positive and gram-negative bacteria, *S. aureus* and *P. aeruginosa*, respectively, because WHO classification as critical threat to human health.

1.1.2.1. *Staphylococcus aureus*

S. aureus is a gram-positive ubiquitous bacterial species and a member of the Micrococcaceae family, that can be found in the environment and in normal human flora, skin and mucous membranes of most healthy individuals (approximately 20–25% of individuals have become persistently colonized and 75–80% intermittently or never colonized).[31–34] It is an opportunistic pathogen and the leading cause of a wide range of clinical infections, ranging from subclinical inflammation to severe infections causing pulmonary infections, pneumonia, endocarditis,

septicemia, skin and soft tissue infections, bacteremia, osteomyelitis, septic arthritis, gastroenteritis, meningitis, and urinary tract infections, bone, joints and infections associated with indwelling catheters or prosthetic devices.[25,31,34–36]

This pathogen is considered the most notorious superbug, which are microbes with higher morbidity and mortality rate increased due to several mutations being able to resist multiple classes of antibiotics, evading the majority of current therapies.[2,3,15] The intrinsic resistance mechanism mainly includes three aspects: outer membrane permeability, because when the cell membrane permeability is lowered, the energy metabolism of the bacteria is affected, and the drug absorption is reduced, which leads to drug resistance; active efflux systems, that have the ability to efflux drugs (exists in MRSA); and excessive production of β-lactamase (that also exists in MRSA), through two mechanisms.[26,37,38] One is the hydrolysis mechanism, where β-lactamase hydrolyses and inactivates β-lactam antibiotics and the other is the mechanism of pinching, where there is a large amount of β-lactamase binding to extracellular antibiotics, preventing the antibiotics from reaching the intracellular space, therefore the antibiotics are not able to reach the target site.[38]

Several mechanisms of acquired antibiotic resistance have been described, among which have been highlighted: resistance by mutations, there may be genetic mutations that alter the target DNA gyrase target or reduce outer membrane proteins, thereby reducing drug accumulation; acquisition of resistant genes, for example MRSA can obtain drug-resistant plasmids from Enterococcus; biofilm-mediated resistance, which allows bacteria to resist host immune responses and evade antibiotic killing; and persister cells, that can resist killing by reducing cell growth and metabolism, and even by becoming dormant and restart infection after antibiotic treatment.[26]

Treatment of *S. aureus* infections depends largely on the type of infection as well as the presence or absence of drug resistant strains. In general, penicillin remains the drug of choice if isolates are sensitive (MSSA, or methicillin sensitive *S. aureus* strains) and vancomycin in cause of MRSA infections.[26,39]

However, these are not the only drugs used. There are many others that are also described that can be used relatively effectively, despite their disadvantages such as norvancomycin, which is a glycopeptide antibiotic similar to vancomycin in its pharmacological effect; teicoplanin, clinically applicable when patients are allergic to β-lactam antibiotics; linezolid, mainly used to control systemic infection such as pneumonia; daptomycin, a cyclic peptide antibiotic with a fatty acid side chain that bind to the bacterial cytoplasmic membrane in the presence of calcium ions; tigecycline, which is specially applicable against gram-positive bacteria; quinupristin/dalfopristin, which has

comparable to or stronger than vancomycin; and ceftobiprole, used to treat skin and soft tissue infections and medical care related pneumonia.[26,29,40] In some cases, alternative therapy is necessary for addition to antimicrobial therapy, such as quorum sensing inhibition, that can inhibit the expression of bacterial virulence genes without affecting the growth and proliferation of bacteria, which makes the bacteria unable to develop resistance due to growth stress; lectin inhibition; iron chelation, which causes a lack of ions necessary for the growth and metabolic activity of pathogenic bacteria; nanoparticles; and phage therapy.[26,41] Most of bacteriophages utilize lysis systems through phage endolysins to hydrolyze the peptidoglycan of the infected bacteria and thereby destroy its cell wall.[41] Due to continuous increasing rate of MRSA infection, there is an urgent interest in agents that treat such infections.

1.1.2.2. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa (*P. aeruginosa*) is a gram-negative, rod-shaped bacteria that belongs to the family Pseudomonadaceae.[42] It is a non-lactose fermenting oxidase-positive opportunistic bacterium that causes a range of infections including acute and chronic infection that can persist for years[43,44], some of them in patients with compromised immune systems and/or disrupted epithelial barriers. It is consistently ranked among the most frequent pathogens found in nosocomial infections particularly in critically ill patients, such as pneumonia, urinary tract infections, and surgical site/soft tissue and blood infections.[42,45,46]

This pathogen is widespread in nature, inhabiting soil, water, plants and animals (including humans). It rarely causes disease in healthy people, but can multiply easily in immunocompromised patients.[47] It's actually the major cause of serious infection in many patients, particularly those who lack white cells as a result of hematologic malignancy or chemotherapy.[16]

Cystic fibrosis (CF) airway infections is an example of a *P. aeruginosa* chronic infection resistant to antibiotic treatments (mono and combinatorial therapy) resulting declined respiratory function and death of patients.[43,48,49] This opportunistic bacterial pathogen is the most prevalent pathogen and accounts for most of the morbidity and mortality in CF patients.[50] According to the survey conducted by the US National Healthcare Safety, *P. aeruginosa* was found to be the sixth most repeatedly occurring pathogen, the second most frequent cause of ventilator-associated pneumonia and the seventh commonest cause of catheter-related bloodstream infection.[45,42]

The treatment of *P. aeruginosa* infections has become a great challenge due to the ability of this bacterium to resist a variety of antibiotics, including aminoglycosides, quinolones and β-lactams.[51]

The mains intrinsic resistance mechanisms of this bacteria are over-expression of efflux pumps, that expel antibiotics out of the cell; decreasing outer membrane permeability; and the production of antibiotic-inactivating enzymes such as β-lactamases.[29,52–54]

Its acquired resistance can be achieved by horizontal transfer of resistance genes from other organisms via plasmids, transposons and bacteriophages or by mutational changes, that encode for proteins that control the passive diffusion of antibiotics across de outer membrane (in DNA gyrases and type IV topoisomerases, e.g.).[52,53,55,56]

The adaptive resistance of *P. aeruginosa* involves formation of biofilm, e.g. in the lungs of CF patients where the biofilm serves as a diffusion barrier to limit antibiotic access to the bacterial cells.[52,56] Moreover, persister cells are survive antibiotic attack, being responsible for prolonged and recurrent infections in CF patients.[52]

Current therapeutic options for *P. aeruginosa* treatment are the use of different antibiotic combinations and development of new antibiotics.[52] Polymyxins; carbapenems, such as doripenem which is a new carbapenem antibiotic with broad spectrum activity against bacteria, through inhibition of bacterial cell wall synthesis by binding to penicillin-binding proteins; antipseudomonal β-lactams; aminoglycosides, such as semisynthetic aminoglycoside antibiotic synthetically derived from the natural product sisomicin; and fosfomycin are currently available antimicrobials for the treatment of MDR *P. aeruginosa* infections.[52,55] Unfortunately, carbapenem-resistant *P. aeruginosa* and other resistant variants were detected and WHO has recently listed this resistant variant as critically human health threatening.[52]

Recent studies have reported several novel non-antibiotic therapeutic approaches that are highly effective in killing antibiotic-resistant *P. aeruginosa* strains. These approaches include inhibition of quorum sensing and bacterial lectins, use of iron chelation, phage therapy, vaccine strategy, nanoparticles, antimicrobial peptides and electrochemical scaffolds. These therapeutic approaches can be used as either an alternative to or in combination with conventional antibiotic treatments.[52]

Regardless the mechanisms involved, the prevalence of MDR *P. aeruginosa* is increasing worldwide over the last few decades. Furthermore, a significant proportion of MDR further restricts the treatment options available and, to date, few of these newer approaches cannot be used due to high cost, side effects and safety concerns.[52,55] So, there is an urgent need for the development of new strategies to treat these infections, with less side effects and costs and increased safety.

1.2. Strategies to combat antibiotic resistance

The rapid increase in resistance, along with the emergence of microbial pathogens resistant to broad-spectrum antibiotics, which include antibacterial agents such as ampicillin, amoxicillin, streptomycin, chloramphenicol, and tetracycline, as well as the slow discovery of new antibiotics, threatens to undermine future options for antibiotic therapy.[57,58]

Moreover, development and dissemination of resistant strains against carbapenems has a devastating impact on the healthcare system across the globe, because these antibiotics are employed in last resource to treat multidrug-resistant bacterial infections.[58] Therefore innovative strategies are urgently required to treat the development and dissemination of multidrug-resistant pathogens.

Drug combination therapies have become a powerful approach to fight against complex diseases in recent years.[59] The administration of multiple licensed therapeutic agents has been employed as an alternative strategy to treat microbial diseases that do not respond to conventional drugs.[58] Clinical trials show higher synergy outcomes for proper combinations, such as higher efficacy and less toxicity, and many approaches neglect the toxicity and efficacy of drug combinations.[59,60] Moreover, this method is advantageous, as different drugs are directed against different therapeutic targets simultaneously. A single drug typically targets a single protein or pathway, so traditional therapies need to go beyond the 'one disease, one drug, one target' paradigm, thus, combination therapy is more efficient and, for that reason, is becoming more regular.[58,59]

This therapy is a strategy for preventing infections caused by MDR gram-negative pathogens. New combinations are increasingly proposed as a therapeutic option. The combinations include antibiotics plus drugs without antibiotic activity, or antibiotics plus other antibiotics.[61]

With the recent advances, Food and Drug Administration (FDA) approved new drug therapies. For example, combination of dolutegravir and lamivudine blocks the HIV-1 multiplication, treating HIV-1 infection and neutralizing emerging drug-resistant HIV strains.[58,59] In addition, this therapy has been utilized in the treatment of fungal diseases, e.g., by combining fluconazole and dexamethasone, replication of drug-resistant *Candida albicans* has been inhibited; and drug resistant tubercolosis, with the activity of moxifloxacin and linezolid, because the anti-efflux pump molecules timcodar and verapamil destabilize the *Mycobacterium tuberculosis* cell wall.[58] It has also been evaluated as a therapeutic method to treat and regulate the spread of malaria, by using tafenoquine and chloroquine along with six artemisinin drugs.[58] Moreover, plazomicin can be used in combination with tazobactam/piperacillin or ceftazidime against multidrug-resistant (to β-lactam

and aminoglycoside antibiotics) *Enterobacteriaceae* species, such as *E. coli* and various *Klebsiella* and *Enterobacter* species.[58]

Not only pairwise combinations but also triple and quadruple combinations are emerging recently. As an example, the combination of oravirine, lamivudine and tenofovir was approved to deal with HIV-1 infection.[59] Additionally, the triple combination of elexacaftor, tezacaftor and ivacaftor (ETI) has been demonstrated to improve lung function, weight and quality of life in CF patients.[62] Moreover, the impact of administering plazomicin, dalbavancin, and ceftobiprole was assessed against methicillin-resistant *S. aureus* strains.[58] Although it is a very promising therapy, the rationale underlying combinatorial therapy is not often well established due to lack of understandings of the specific pathways responding to the drugs, and their temporal dynamics following each treatment.[63] Moreover, current knowledge of drug combination therapies is limited because of adverse drug effects, toxicity and cell line heterogeneity, apart from the fact that identification of combinatorial drugs is expensive and time consuming.[59,64]

Cycling, or mixing therapy, have also been investigated quite extensively for fight antibiotic resitance.[57] For more than 30 years, the cycling strategy has been in doubt as to its ability to alleviate the problem of antibiotic resistance, and yet this remains an open problem.[65] Antibiotic cycling is the crop rotation idea applied to antibiotics.[65,66] Different antibiotics are prioritized against specific infections for a period of time, only for that period of drug prioritization to be replaced by one of restriction at a pre-determined later time, which could be many months.[65] Unfortunately, the cumulative evidence to date suggests that antibiotic cycling has limited efficacy for preventing antibiotic resistance.[67]

Like cycling, mixing has been tested in at least three prior clinical studies. It did contribute to a reduction in resistant gram-negative infections in a hospital-wide study but fared less well when implemented in an intensive care unit.[57,65,68] It was partially successful in one study where it may have contributed to a reduction in MRSA infection, but without impacting on gram-negative infections. For example, suppose the drug order of cefepime, ciprofloxacin, piperacillin-tazobactam, and imipenemcilastatin in the quarterly cycles described to tackle drug resistant *P. aeruginosa*.[65]

Drug repositioning is another strategy to combat antibiotic resistance, that aims identifying new uses for drugs that are outside the scope of the original medical indication.[69,70] It has attracted considerable attention due to its efficiency in saving time and cost over the traditional *de novo* drug development approaches.[71] This procedure takes into account data previously acquired, in particular on the drug's safety and toxicity which makes the risk of failure is lower and also make

the initial phases of development for a repositioned drug considerably faster, and therefore cheaper.[69,70] The concept of drug repositioning thus excludes any structural modification of the drug. Instead, repositioning makes use of a new indication of the biological properties for which the drug has already been approved through the elucidation of the human genome, since some diseases share common biological targets. For example, Parkinson's disease and Alzheimer's disease share 48 genes and four signaling pathways, which suggests that a given drug might have efficacy against both conditions. In addition, the pleiotropic effect of the drugs used can also confer properties that lead to drug repositioning.[69,71]

The first example of successful drug repositioning mainly came about through serendipity like acetylsalicylic acid (aspirin). Initially marketed as an analgesic and still widely used today to prevent cardiovascular events and also indicated for colorectal cancer.[69,70] Rituximab was original indicated for various cancers and in 2006 had a new indication for rheumatoid arthritis; topiramate was initially indicated for epilepsy and in 2012 for obesity treatment; raloxifene was first indicated for osteoporosis and in 2007 was approved for breast cancer; and many other drugs can be added to this list.[69–72] The main challenges faced in this therapy lie in the relatively weak intellectual property protection afforded to such medicinal products, which can reduce their return on investment and discourage companies from developing them, and some regulatory issues.[69]

It is then imperative to evolve alternative non-antibiotic strategies that are safer to humans and effective against infectious pathogens. Some of the approaches used nowadays for treating those antibiotic-resistant infections include the use of bacteriophage, quorum sensing inhibition, lectin inhibition, iron chelation, nanoparticles, antimicrobial peptides or bacteriocins, antimicrobial adjuvants, fecal microbiota transplant, and competitive exclusion of pathogens through genetically modified probiotics and postbiotics.[26,73,74]

It is important to note that many prescribed antibiotics have disadvantages, mainly adverse reactions, such as local pain injection, allergic reactions, fever, liver and kidney disfunction, digestive tract reactions, nausea, vomiting, muscle weakness, diarrhea, among others.[26] Moreover, the alternative therapies mentioned above also have some limitations in terms of efficacy, because they represent narrow-spectrum molecules; toxicity, which is usually high; and safety, since there is some uncertainty about their viability for human diseases treatment.[26] A major concern is the development of antiphage antibodies during the application of the therapy, and it is also possible that eventually the bacteria will become resistant to phage lysis in the same way that antibiotic resistance has emerged. Other problems of this therapy include the observation that some MRSA

seem to be inherently less susceptible to bacteriophages than antibiotic-susceptible *S. aureus*; and the possibility of lysogenic conversion, whereby bacteriophage could acquire various toxin genes and introduce these into susceptible bacteria.[75]

It is also important to mention that 59% of all drugs approved to treat bacterial infections were naturally derived or inspired and that fraction increases to 74% when vaccines are exclude looking at only small molecule drugs, indicating that natural products are by far the most significant source of antibiotics available. In addition, natural products have many advantages, such as being better tolerated in the human body with fewer side effects and being moderately priced, which gives them an advantage over other methods of combating antibiotic resistance.[26,73,76] Therefore, only strategies for combating antibiotic resistance based on natural products, especially natural products obtained from plants, will be mentioned from now on, though these may be used in combination with other strategies mentioned above.

1.3. The role of natural products in the combat of antibiotic resistance

Some small pharmaceutical and biotechnology companies are developing antibiotics, but most depend on venture capital rather than sales income, and with the present regulations, they face huge barriers to enter the market. While this is happening, resistance continues to increase. However, there are some bright possibilities associated to natural products.[77]

Since ancient civilization that natural products have been used with medicinal purposes which has allowed to gather a vast knowledge about their diverse bioactive potential.[78,79] In the mid-20th century, in full golden era of discovery of novel antibiotics, natural products served as powerful scaffolds against pathogenic bacteria. Several antimicrobial compounds were discovered, such as marine-derived actinomycetes, oil derived natural products, plants derived natural products, among others.[80,81] Natural products with industrial applications can be produced from primary or secondary metabolism of living organisms (plants, animals or microorganisms). The number of natural compounds discovered exceeds 1 million and among them, 50–60% are produced by plants (alkaloids, flavonoids, terpenoids, steroids, carbohydrates, etc.) and 5% have a microbial origin.[77]

One of the most promising strategies is the use of uncultured microorganisms. Of the 22,500 biologically active compounds that have been obtained from microbes so far, 45% are produced by actinomycetes, 38% by fungi, and 17% by single-celled bacteria.[77] They remain a group of interest because in addition to producing many primary metabolites, such as amino acids, vitamins, and

nucleotides, they can also produce secondary metabolites, which constitute half of the pharmaceuticals currently on the market.[82,83]

Considering that 99% of bacteria and 95% of fungi have yet to be cultivated in the laboratory, efforts to find ways to cultivate such microorganisms are being well spent, as they may allow access to a vast untapped repertoire of genetic and metabolic diversity that could lead to the discovery of natural products with interesting activities.[77,82] Moreover, researchers seek to extract bacterial DNA from marine habitats, express them in a host bacterium and screening the library for new antibiotics, because almost 70% of Earth's surface is covered by ocean, representing a huge reserve of natural biological and chemical diversity on our planet.[77,83]

Natural products extracts or their semisynthetic analogues have been widely used as chemical drugs against human diseases and thus natural products continue to be important raw materials for the development of new drugs.[78,84,85] In fact, some analgesics with pain relieving properties, have derivate from salicylic acid (hydrolyzed salicin) and acetylsalicylic acid (more known as aspirin). Furthermore, the first antibiotic penicillin is a natural product obtained from the mold of *Penicillium Notatum*.[79] Many other drugs were discovered from natural products including tetracycline, artemisinin and doxorubicin.[85]

The medicinal potential of plants such as fruits, herbs, roots, seeds used for centuries for the treatment and management of various ailments including infections throughout human history should be noted. They are well known for the production of biologically active compounds and actually, they represent one of the most promising sources of antibiotic compounds because of their structural diversity, safety, and nontoxic quality.[78,86] In fact, bacteria are less likely to develop resistance to plant-derived antibacterial agents because these products typically contain bioactive moieties with diverse chemical designs and modes of action unlike antibiotics that mostly involve a single target.[80,87,88]

Essential oils (EOs) are also an available natural strategy against antibiotic resistant and are among the most economically relevant plant-derived products, being frequently responsible for several health-promoting properties. These products are potential reservoirs of many bioactive compounds with several beneficial properties, and they are aligned with the current consumer preference for natural products.[73] Moreover, they are complex mixtures, so resistance is less likely to develop following their use, as is the case with single synthetic compounds.[89] Several molecules present in EOs are endowed with antibacterial properties, especially phenols, alcohols and aldehydes.[73]

Despite all presented advantages for microorganisms and natural products from plants, more properly, EOs, there are also some disadvantages. The work with microorganisms in biodiscovery may present other obstacles such difficulties in the isolation or cultivation of the organisms and in identification or distinction of compounds that are likely to be new or likely to be known.[90] And EOs, although they are natural, have been reported to cause toxic effects, in high enough doses.[73] Moreover, EOs contain complex compounds that are very photosensitive and susceptible to degradation, which requires better storage conditions. Another challenge for the rational exploitation of EOs by relevant industries is their quality control, as well as the legislation texts regarding their application.[73]

For that reason, it is imperative to find other alternatives and lately the scientific community has shown interest in natural products from plant extracts, other than EOs, because they are relatively safe, environment friendly, increase the shelf life of foods, are widely accepted by consumers, and have the potential to be exploited for multiple uses. They are also economical, easily available and chemically diverse.[89,91]

1.4. Antimicrobial plant-derived products

Plants are sedentary, which 'forced' them to find strategies to overcome threats from the environment.[87] Plant synthesizes a variety of secondary metabolites (phytochemicals) involved in plant defense mechanism, making them a major source of molecules with most beneficial effects on health as, for instance, antioxidants and antimicrobial.[88]

Plant-derived antimicrobial compounds exert their antimicrobial activity in several different ways, including:

(i) disruption the bacterial membrane. For example, flavonoids extracted from *Aspilia mossambicensis* (wild sunflower), *Ocimum gratissimum* (African basil), and *Toddalia asiatica* (orange climber) show activity against MRSA and *P. aeruginosa*, by interacting with membrane proteins that resulted in increased cell membrane permeability and consequently disruption of the cell wall.[87] Moreover, it is also described that catechins alter membrane fluidity, by targeting bacterial membrane protein, fatty acid synthase, β-lactamase, and other bacterial enzymes.[88] Epigallocatechin gallate, a polyphenol obtained from green tea, black tea and cocoa, for example, shows intensive activity, perturbs membranes of bacteria and causes leakage of membranes isolated from *E. coli*.[88] Carvacrol, thymol and eugenol are also described phytochemicals responsible for microbial membranes disruption.[86]

(ii) inhibition of cell wall and protein synthesis. For example, quinones (2,6-dimethoxy-1,4-benzoquinone (DMBQ) extracted from wheat germ), were described as antimicrobial against *S. aureus* and *Bacillus cereus*, by providing free radicals to irreversibly bind to the nucleophilic amino acids in microbial protein, causing protein function loss.[87] Protein synthesis was also significantly inhibited by genistein, an isoflavone.[88]

(iii) damage and inhibition of the synthesis and function of DNA and RNA. For example, alkaloids present in Berberine extracted from roots and stem-bark of Berberis species showed antibacterial, by inserting DNA to RNA polymerase, gyrase and topoisomerase IV, and nucleic acid.[87] Additionally, flavonoids (such as kaempferol and myrecetin) usually found in citrus peel possess broad spectrum antimicrobial activity against *E. coli* and *S. aureus*. Kaempferol also show strongest antibacterial activity against *E. coli* DNA gyrase, by inhibiting the activity of gyrase enzyme that holds the key role in DNA supercoiling and bacterial growth.[88] Quercetin, one of the ubiquitous flavonoids, impedes the DNA supercoiling, and causes its cleavage. Can be obtained from yellow onion skin and showed effect on antibiotic-resistant bacteria *Helicobacter pylori*.[88]

(iv) interference with intermediary metabolism. In bacterial cell the energy is required for the transport of solutes, uptake of metabolites, and biosynthesis of macromolecules. This energy comes from the respiratory chains like electron transport chain. Some antioxidants, such as reterochalcones from *Glycyrrhiza Inflata* (chinese licorice), inhibit the respiratory chains at any step and thus depriving the cell of the energy necessary for growth and was proved being effective against *Micrococcus luteus*, *S. aureus* and *P. aeruginosa*.[88] In addiction, flavonoids weaken mechanism of energy formation and metabolism. For example, cinnamaldehyde from cinnamon was tested against some bacteria and showed promising result was against *B. cereus*.[88,92]

(v) interruption of normal cell communication by alkaloids, flavonoids, quinones, tannins, coumarins, terpenes, lectins and saponins.[88]

(vi) control of biofilm formation by trans-cinnamaldehyde, carvacrol, thymol or geraniol, specifically.[86]

(vii) inhibition of bacterial capsule production by salicylic acid and its derivates.[86]

(viii) attenuation of bacterial virulence by controlling quorum-sensing (anti-virulence agents).[86]

(ix) reduction of microbial toxin production by dihydroisosteviol.[86]

(x) induction of coagulation of cytoplasmic constituents.[87]

Besides the mentioned examples, other examples of plants with compounds that present antimicrobial activity can be reported, such as piperine isolated from *Piper nigrum* that has shown to enhance antimicrobial activity of mupirocin against *S. aureus* strains including MASA through the inhibition of efflux of ethidium bromide[76]; ethanol extract of *Momordica charantia L.* (bitter-melon) which displayed the antibiotic activity against MRSA strain[76]; the ethanol extract of *Hypericum perforatum L.* (St. John's wort) that exerts strong antimicrobial activity against *Streptococcus mutans*, *Streptococcus sobrinus*, *Lactobacillus plantarum* and *Enterococcus faecalis*, as well as its water extracts that display strong antibacterial activity against *Streptococcus sobrinus* and *Lactobacillus plantarum*[76]; coumarins, which can be extracted from *Melilotus albus* (honey-clover) and whose extracts with ethanol, acetone, and ethyl acetate have been shown to be active against *Bacillus subtilis* and *S. aureus*[93]; flavonoid-rich water-ethanol (70%) extract of *Equisetum arvense L.* (common horsetail) had antibacterial activity against *S. aureus*[93]; quercetin and hydroxycinnamic derivatives from 70% ethanol extract of *Urtica dioica* (stinging nettle) showed activity against MSSA and MRSA[93]. Many more examples could be included here.

More specifically with regard to phenolics compounds and plant extracts rich in these substances, it is important to mention that these can be excellent inhibitors of bacteria. Some examples are mentioned below. For example, bergamot peel has been found to be effective against gram-negative foodborne pathogens *E. coli* and *Salmonella enterica* and *Bacillus subtilis*; quince peel against *E. coli*, *P. aeruginosa* and *S. aureus*; mango kernel against *E. coli*. Other fruits such as jackfruit, papaya, plum, guava, and tamarind and their seed and many more examples that could me mentioned, have also shown antimicrobial activity against both gram-positive and gram-negative bacteria.[94]

In addition to all that has already been mentioned, it is also important to note that plants also have high antioxidant activity, which, although it is not yet fully understood, may be connected to antimicrobial activity.[88] It perhaps can be attributed to their capacity to chelate iron, vital for the survival of almost all bacteria and due to their capacity to eliminate free radicals.[88,95]

Antioxidants, such as phenolic compounds existing in plants mentioned before, are responsible for inhibiting oxidation at several points, depriving cells of energy, which may result in interrupting nucleic acid synthesis.[88] Moreover, their hydroxyl (–OH) groups are thought to cause inhibitory action by interacting with the cell membrane of bacteria to disrupt disrupting microbial membranes or impairing cellular metabolism.[88,94] Gram-negative and gram-positive bacterial cell walls play a very

important role in osmotic protection of cell and many researchers have demonstrated that the interaction of phenols and polyphenols with bacterial cell wall is different for gram-negative and gram-positive bacteria, because their cell wall composition differs significantly.[88,95]

Nowadays, plant phenols and polyphenols enjoy an ever-increasing recognition not only by the scientific community but also, and most remarkably, by the general public because of their presence and abundance in fruits, seeds, vegetables, and derived foodstuffs, whose regular consumption has been claimed to be beneficial for human health.[96] So the focus of this study relies on plants with antioxidant properties used daily, as is the case of garlic, pomegranate and ginger.

1.4.1. *Allium sativum* (Garlic)

Allium sativum, more known as garlic, is among the oldest cultivated plants and one of the most important bulb vegetables. It has been used as a spice and flavoring agent, and in folklore medicine for over 4000 years, and consequently is a widely researched medicinal plant.[97,98] It has been used and investigated for diverse medicinal properties, such as anticancer, anti-inflammatory, antifungal, antiviral and antioxidant properties, and in 1858, Louis Pasteur reported its antibacterial properties.[97–102] More recently, garlic has been proven to be effective against gram-positive and gram-negative bacteria, including *P. aeruginosa*, *S. aureus*, *E. coli*, *Salmonella enterica*, *Klebsiella aerogenes* and *Mycobacterium*.[98,103]

Most of the health benefits of garlic are attributed to a myriad of cysteine-derived sulfur-containing organic compounds present in garlic, mainly alliin and its crushing converts it into allicin.[103–106] Allicin is a highly reactive, very unstable with low bioavailability compound, that degrades and rearranges itself into different sulfides or ajoene.[102,105] The extraction procedure results in concentrating a particular compound rather than providing a pure compound and the extraction of garlic with water or ethanol followed by the concentrating of the extract will provide an allicin-rich product and it was noticed that yield with ethanol is better compared to water.[98]

An *in vitro* study with allicin vapors showed that they were able to exhibit bactericidal activity against MDR lung pathogenic bacteria such as *P. aeruginosa* and *Streptococcus pyogenes*.[105] Nevertheless, it was also described that aqueous extract showed antibacterial activity against *S. aureus*, *K. pneumoniae* and *Bacillus subtilis*[98], which means that garlic has effectively a large potential as an antibacterial agent. Moreover, ethanolic extract of garlic revealed that it contains various thoisulfinates, being the major one also allicin, and exhibited some degree of antibacterial activity against test enteropathogenic bacterial strains.[105]

1.4.2. *Zingiber officinale* (Ginger)

Ginger, the rhizome of *Zingiber officinale*, is a member of the Zingiberaceae family that has been used as a spice globally for over 2000 years because of its characteristic spicy aroma and taste.[107–110] It is a perennial herb originated South-East Asia (today's northeast India) and now cultivated in many different countries.[107,111,112] Chemical analysis of ginger shows that it contains over 400 different compounds, being carbohydrates (50–70%), lipids (3–8%), terpenes, and phenolic compounds e its pharmacological activity is mainly attributed to its active phytocompounds 6-gingerol and 6-shogaol, beside other phenolics and flavonoids.[108,109,113,114] The rhizomes have been used in many oxidative stress related medical conditions, but in recent years, it has also been described as a potential antibacterial agent.[107,109,112,115–118]

Ginger antimicrobial activity is due to its phenolic compounds insoluble in water and, thus, its aqueous extracts exhibit lower antimicrobial activity than organic extracts, because ginger hydrophobic compounds interact with the lipophilic part of the membrane and isolated mitochondria, promoting its integrity and function disrupt.[108,113] Actually, it was showed that ethanolic ginger extract has antimicrobial activity against *E. coli, Salmonella typhi*, *Bacillus subtilis, Candida albicans*[113,119,120] and also can inhibit the growth of a multidrug-resistant strain of *P. aeruginosa*, by affecting membrane integrity and inhibit biofilm formation.[117]

1.4.3. *Punica granatum* (Pomegranate)

Pomegranate (*Punica granatum L.*) is a plant of Punicaceae family cultivated and naturalized over the whole Mediterranean region since ancient times that has prominent medical history and possesses remarkable medicinal properties.[121–123]

The antioxidant activity of the pomegranate peel extract (PPE) is attributed to the bioactive phenolic compounds ranging from simple phenolic acids, such as hydroxybenzoate to complex polyphenols, such as tannins and water-soluble polyphenolic compounds, such as ellagitannins). [124,125] Tannins may be toxic to the microorganisms, since their hydrophilic parts may interact with the polar region of membrane whereas the hydrophobic part is immersed in the non-polar inner region of the bacterial membrane, causing instability of the membrane.[126] Nevertheless, it is important to note that the content of the tannins can have large variations between different pomegranate cultivars. For instance, it has been described that in pomegranate fruits of Egyptian origin, the punicalagin (an ellagitannin, a type of phenolic compound) concentration in aqueous methanol extracts of peels

was reported to be 98.02 mg/g, while extracts from pomegranates from Israel presented a considerably higher content, at about 612.8 mg/g.[127]Moreover, chlorogenic acid, one of the major compounds found in the PPE can also interact with the bacterial outer membrane, and rupture the cell membrane, deplete intracellular content and release macromolecules from the cytoplasm, leading to bacterial death.[128] In general, phenolic compounds can inhibit the activity of essential proteins by interacting with the sulfhydryl groups.[128] The literature has widely discussed the efficacy of PPE at inhibiting or reducing the growth of a wide range of microorganisms, such as *S. aureus*, *Staphylococcus epidermidis, Lactobacillus acidophilus, Streptococcus mutans* and *Streptococcus salivarius*.[128–130]

The Pomegranate pomace (PP) is rich in carbohydrates and fibers and has high water-absorption capacity. Its active phenolic compounds belong to three groups: ellagitannins, ellagic acid derivatives, and gallic acid derivates and they represent the reason why PP has such a high antioxidant activity and has the capacity to inhibit the growth of pathogenic bacteria such as *P. aeruginosa* and *K. pneumonia*.[131,132]

The pomegranate juice (PJ) contains considerable amounts of total soluble solids, total sugars, reducing sugars, anthocyanins, phenolics, ascorbic acid and proteins and has also been reported to be a rich source of antioxidants.[133] Its antioxidant activity is mainly attributed to their flavonoid content, such as anthocyanins ,catechins, and tannins, that together account for 92% of their antioxidant activities.[123,126,134–136] PJ was tested against 60 clinical strains of *S. epidermidis* isolated from ocular infections and resistant to ampicillin and it completely inhibited the growth of all 60 strains. In this study, ampicillin was used as a control and various bacteria shown to be resistant to the antibiotic and sensitive to PJ.[126]

In general, pomegranate's extracts exhibited bactericidal activity against various pathogenic bacteria including *S. aureus, P. aeruginosa*, *E. coli*, *K. pneumoniae, Streptococcus pneumoniae* and *Candida albicans*.[124,130,136,137] Although it has been described that *P. aeruginosa* was sensitive to the pomegranate extracts, in general, various studies reported that gram-positive bacteria were more sensitive than gram-negative.[130,137]

1.5. Objectives

Antibiotic resistance is a global public health threat and it is urgent to solve it or at least minimize it. One of the strategies is to replace or complement antibiotic treatment with natural products that typically exhibited antioxidant properties that perhaps can be correlated with their antibacterial

properties so the aim of this study is to investigate the impact of the extract conditions, specifically the solvent, the duration and temperature on the antibacterial activity of garlic, ginger and pomegranate (peel, pomace and juice) against *P. aeruginosa* and *S. aureus*. Moreover, it is aimed to correlate the enhanced antibacterial activity of plant extracts with antioxidant activity and total phenolic content in order to understand the underlying mechanisms of action and possibly to accelerate the screening of bioactive potential of other plants for antibacterial purposes.

2. Materials and methods

2.1. Plant material

All edible biomasses used in this work were provided by a local supermarket: *Allium sativum* (garlic); *Zingiber officinale* (ginger) and *Punica granatum* (pomegranate).

Garlic, ginger and pomegranate were peeled, cut into small pieces and ground with a coffee mill. Garlic was then centrifuged for 10 minutes, 200 rpm (RS LAB, HIGUGE-GJ6). In the case of ginger, as a very wet sample was obtained, it was dried in an oven at 60 °C, until a powder was obtained. The pomegranates were divided into three fractions: the peel, the pomace and the juice, which were then frozen in sample tubes and lyophilized. All samples were then stored at -18 °C, until further use.

2.2. Preparation of plant extracts

The extractions were performed at two different temperatures (overnight at room temperature and at 70 °C for 1 hour) with different water/ethanol mixtures as solvents: EtOH (96%), EtOH (70%) and $H_2O_{(d)}$. 1 mg of each biomass was mixed with 20 mL of solvent, in duplicate, both for the overnight at room temperature extraction and for the extraction at 70 °C for 1 hour. Then, for the first one, the samples were placed on a tray covered with aluminum foil (no shaking in this process); and for the second, they were placed in a water bath with shaker at 70 °C. Subsequently, the extracts were filtered and stored at -18 °C, until further use.

To assess the extraction yield, the solid content of each extract was determined as follows: 1 mL of each extract was placed in a pre-weighted aluminum crucible, which were taken to the oven at 105 °C overnight, until constant weight. The analysis was performed in triplicate, and the yield value was obtained using the following formula:

$$\text{Extraction yield} = (\text{dried sample concentration} \times \text{volume of extraction} / \text{mass of matrix}) \times 100 \quad \text{(eq. 1)}$$

2.3. Determination of Total Phenolic Content (TPC)

Phenolic content was determined using the Folin–Ciocalteu assay. The Folin-Ciocalteu method is an electron transfer-based assay, and assesses the reducing capacity which is expressed as phenolic content (that is highly dependent of extraction yield and solvent).[138] The calibration curve was done with different concentrations of gallic acid (0.200, 0.150, 0.100, 0.075, 0.050, 0.025, 0.010 and 0.005 mg/mL) and distilled water was used as a blank. The solvent used in each extraction (EtOH (96%), EtOH (70%) and H_2O $_{(d)}$) was taken as the blank for each sample. Sample solutions were added to microplate with 100 µL of Folin-Ciocalteu reagent (1:10 in H_2O). 80 µL of Na_2CO_3 were also added to each well and the reaction was incubated at 42 °C, protected from light, for 30 minutes. The absorbance was measured with a microplate reader at 750 nm (Thermo Fisher Scientific, Lisboa, Portugal). The total phenolic content was calculated as gallic acid equivalent (mg/mL) by using gallic acid calibration curve.

2.4. Determination of antioxidant activity

Antioxidant potential of extracts was assed using two different methods: Ferric Reducing Antioxidant Power (FRAP) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) method.

2.4.1. Ferric Reducing Antioxidant Power

It has been 27 years since the Ferric Reducing Antioxidant Power (FRAP) assay method was first described, by Benzie & Strain. This method monitors the reaction of Fe^{2+} with 2,4,6- Tripyridyl-s-Triazine (TPTZ) to form a violet-blue color with an absorbance maximum at 593 nm.[139,140]

Sample or standard Trolox solutions (20 µL) were added directly to the 96-well microplate followed by 280 µL of FRAP working solution. The mixtures were shaken, incubated at 37 °C protected from light, for 30 minutes. The absorbance was read at 593 nm using a microplate reader (Thermo Fisher Scientific, Lisboa, Portugal).

The extracts from garlic, ginger and pomegranate (peel, pomace and juice) were used. Some dilutions were done in order to obtain absorbances within the linear range of the calibration curve, allowing the equivalent Trolox concentration (mg/mL) calculus. The Trolox Equivalent Antioxidant Capacity (TEAC) was calculated using the pre-determined calibration curve, using Trolox as

standard in concentrations (1.250, 0.9, 0.625, 0.313, 0.156, 0.078 and 0.039 mM) and Methanol/Water (70:30, v/v) as the blank control:

$$abs = 2{,}47 \times [TEAC] - 8{,}01 \times 10^{3} \quad \text{(eq. 2)}$$

The solvent used in each extraction (EtOH (96%), EtOH (70%) and $H_2O_{(d)}$) was taken as the blank for each sample.

2.4.2. 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid

The ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid, was first described by Miller et al. (1997). It is based on the ABTS radical (ABTS•+) that absorbs at 743 nm, formed by the loss of an electron by the nitrogen atom of ABTS. The ABTS•+ radical is strongly colored (blue-green color), but ABTS is colorless. When Trolox is present, the nitrogen atom quenches the hydrogen atom, the ABTS•+ declines and the solution decolorize, which causes the absorbance at 743 nm to decrease and allows the evaluation of compounds antioxidant capacity.[140,141]

As with the FRAP assay, the extracts from garlic, pomegranate (peel, pomace and juice) and ginger were used with some dilutions in order to obtain absorbances within the linear range of the calibration curve, which was obtained using Trolox as standard in concentrations (0.200, 0.140, 0.098, 0.069, 0.048 and 0.034 mM) and Methanol/Water (70:30, v/v) as the blank control. The solvent used in each extraction (EtOH (96%), EtOH (70%) and $H_2O_{(d)}$) was taken as the control for each sample.

In a 96-well plate, 180 µl ABTS working solution and 20 µl sample or control solution were added, shook well, and protected from light for 30 min. The absorbance was measured in a microplate reader at 734 nm (Thermo Fisher Scientific, Lisboa, Portugal). The Trolox equivalent concentration was calculated using the pre-determined calibration curve and the percentage of inhibition indicating the ABTS radical scavenging capacity was calculated as follows:

$$\%_{\text{Inhibition SAMPLE}} = 100 \times (Abs_{734}\text{ABTS}_{\text{BLANK}} - Abs_{734\text{ SAMPLE}})/Abs_{734}\text{ABTS}_{\text{BLANK}} \quad \text{(eq. 3)}$$

Where Abs_{734}sample is the absorbance of each sample;

The TEAC (Trolox Equivalent Antioxidant Capacity) of samples is calculated as follows:

$$TEAC_{SAMPLE}\ (mg/mL) = (\%\ inhibition - 1.33)/102 \quad \text{(eq. 4)}$$

2.5. Determination of antibacterial activity

2.5.1. Bacterial species and growth conditions

In this study *Pseudomonas aeruginosa clinical isolate* U147016-1 and *Staphylococcus aureus* ATCC 25923 were used. Bacteria were routinely cultured on Tryptic Soy Broth (TSB, Liofilchem) or Tryptic Soy Agar (TSA, Liofilchem) at 37 °C. All strains were preserved in cryovials (Nalgene) with TSB supplemented with 20% glycerol at -80 ± 2 °C to minimize putative adaptation to the laboratory environment. Prior to each experiment, bacterial cells were grown on TSA plates overnight at 37 °C.

2.5.2. Determination of Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

The antimicrobial activity of the extracts was established by determining the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) using the microdilution method following the recommendations of the Clinical and Laboratory Standards Institute.[142] Before the experiment, ethanolic extracts were prepared to antibacterial activity analysis with 5% aqueous DMSO (aqueous extracts suffer no alteration).

MIC and MBC were assayed using a 96-well plate with different concentrations of plant extract described in **Table 1**. All extracts were prepared in Mueller Hinton Broth (MHB). Bacteria were added to the wells to obtain a final concentration of $5x10^5$ CFU/mL (Colony Formation Unit) and incubated at 37 °C, 120 rpm for 18 to 21 hours. Afterward, cultures were plated onto Mueller Hinton Agar. MIC was defined as the lowest concentration of an extract that inhibited the 99% bacterial growth determined by optical density reading at 620 nm using a microplate reader (Biochrom EZ Read 800 Plus, Cambridge, UK). The lowest concentration of crude extracts with the absence of growth on solid medium after overnight incubation at 37 °C was considered as MBC. All tests were performed at least in duplicate.

Table 1 Range of tested extract concentration obtained from different plants under study (mg/mL)

Plant	Solvent	Extraction Conditions	Range of tested [extract] (mg/mL)
Garlic	EtOH (96%)	70 °C ≈ 1H	0.002 - 1.1
		Overnight	0.001 - 0.4
	EtOH (70%)	70 °C ≈ 1H	0.013 - 6.8
		Overnight	0.010 - 5.1
	H_2O (d)	70 °C ≈ 1H	0.015 - 7.9
		Overnight	0.014 - 7.3
Ginger	EtOH (96%)	70 °C ≈ 1H	0.004 - 1.8
		Overnight	0.002 - 1.2
	EtOH (70%)	70 °C ≈ 1H	0.009 - 4.7
		Overnight	0.005 - 2.5
	H_2O (d)	70 °C ≈ 1H	0.012 - 6.3
		Overnight	0.016 - 8.0
Pomegranate Peel (PPE)	EtOH (96%)	70 °C ≈ 1H	0.010 - 5.1
		Overnight	0.009 - 4.7
	EtOH (70%)	70 °C ≈ 1H	0.011 - 6.0
		Overnight	0.010 - 5.3
	H_2O (d)	70 °C ≈ 1H	0.011 - 5.6
		Overnight	0.010 - 5.3
Pomegranate Pomace (PP)	EtOH (96%)	70 °C ≈ 1H	0.008 - 4.0
		Overnight	0.007 - 3.5
	EtOH (70%)	70 °C ≈ 1H	0.008 - 4.2
		Overnight	0.007 - 3.8
	H_2O (d)	70 °C ≈ 1H	0.007 - 3.7
		Overnight	0.008 - 4.1
Pomegranate Juice (PJ)	-	-	0.117 - 90.4

2.6. Statistical analysis

All data were analyzed using GraphPad Prism 6 software. Data were compared by two-way analysis of variance (ANOVA) followed by Turkey multiple comparisons test.

3. Results and Discussion

3.1. Plant extraction yield

The extraction of active compounds can be performed using various solvents with distinct polarities resulting in the solubilization of distinct bioactive compounds and with the extraction efficiencies.[143]

Water, methanol, ethanol, and acetone are amongst the most used solvents for the extraction of bioactive compounds from plants[143], and in this study ethanol (96% and 70%) and water were selected to evaluate their impact on the antibacterial activity and their correlation with antioxidant activity and phenolic content

In garlic extracts, the extraction yield was higher on aqueous extracts ($0.32 \pm 2.12 \times 10^{-4}$ and $0.29 \pm 2.12 \times 10^{-3}$ at 70 °C and overnight extraction, respectively) followed by the ethanolic extracts. It was noted that as ethanol concentration increased, the lower the extraction yield was as expected (**Figure 1(a)**).[143–145] One of the factors that may account for the high yield of the aqueous extracts may be the high percentage of carbohydrates (approximately 30%) in its composition.[146] Nevertheless, this does not implies that these will be the most active extracts, as the bioactivity in garlic is not commonly associated with its composition in carbohydrates.

Regarding the extraction yield of ginger extracts, the same trend was verified, as the higher the percentage of ethanol in the solvent, the lower the yield ($0.25 \pm 1.84 \times 10^{-3}$ mg/mg and $0.32 \pm 6.36 \times 10^{-4}$ mg/mg in 70 °C and overnight extractions, respectively) (**Figure 1(b)**). This is justified by the fact that carbohydrates represent the vast majority of the constitution of ginger (50-70%), as mentioned above, since carbohydrates are highly soluble in water, given the presence of -OH groups.[147] However, this trend was not verified in several studies.

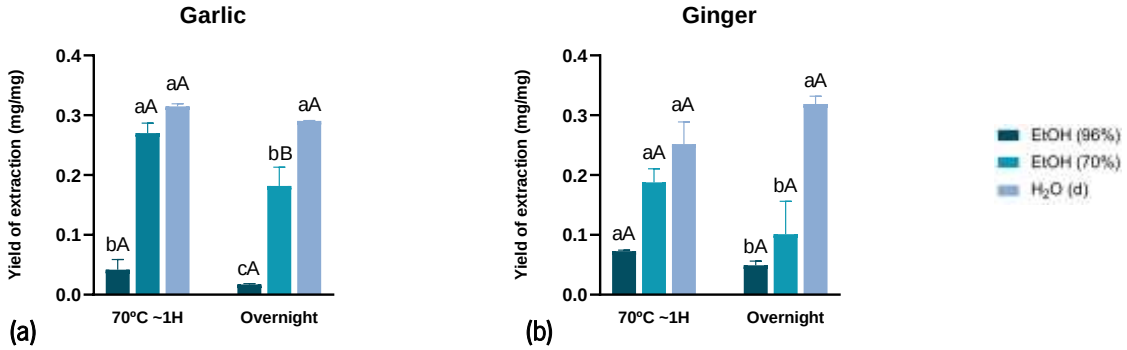

Figure 1 Extraction yield of extracts obtained from plants under study. The result is presented in mg of extract *per* mg of matrix of garlic **(a)** and ginger **(b)** extracts.

Data presented represent the mean ± standard deviation of three assays in triplicate. The lowercase letter represents the significant comparison of the extraction solvents, considering the same extract and the same extraction conditions. The uppercase letter represents the significant comparison of the extract conditions, considering the same extract and the same solvent. The analysis starts with the letter "a" and "A" representing the highest value.

Regarding the PPE extracts, a different trend was observed. Extraction with ethanol produced increased yield compared to water. In a study done on PPE extracts with various solvents of different ethanol concentrations, the pure solvent (ethanol 99%) showed higher extraction yield than ethanol (70%), which in turn also showed higher extraction yield than water.[148] In this work, the results were in line with what was expected for the extracts obtained at 70 °C, in which the highest value was obtained by the ethanolic (96%) extract (0.29 ± 7.07E-04 mg/mg), which was very similar to the extract described in the literature with ethanol (99%), followed by the ethanolic (70%) extract and then the water, maybe due to high content in phenolic compounds (**Figure 2(a)**).[149] Nevertheless, it is also important to emphasize that the high yield obtained in the aqueous extracts perhaps may be attributed to the amount and type of tannins present in the PPE, which are both be water-soluble and insoluble compounds, and to the presence of soluble carbohydrates (such as pectins).[150] However, in the overnight extracts, this was not the case, which reinforces that soluble carbohydrates may be responsible for the higher yields achieved in the aqueous extracts. Further this could perhaps indicate that temperature or agitation had an influence on the extraction, but further studies would be needed to validate this assumption. It is also important to mention that the extracts obtained at 70 °C showed higher yields than the extracts obtained overnight, which is validated by the fact that it has been shown that temperature is an important parameter for the efficiency of bioactive compounds extraction. Previous study indicates that an increase of temperature means an increase of efficiency of bioactive compounds extraction, which translates into an increase in extraction yield.[151] However, temperature can cause degradation of the most sensitive bioactive compounds thus possibly decreasing the final intended functionality.

The extraction yields of the PP (**Figure 2 (b)**) were slightly lower than those of the PPE. Here there was not a very large difference either between solvents or extraction conditions, which may be related to the fact that pomegranate has some highly water-soluble and other insoluble compounds, as mentioned above.

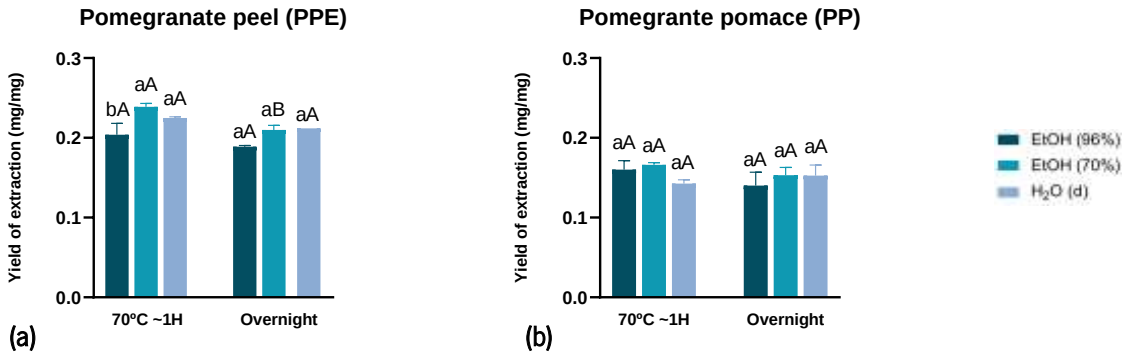

Figure 2 Extraction yield of extracts obtained from plants under study. The result is presented in mg of extract *per* mg of matrix of pomegranate peel (PPE) **(a)** and pomegranate pomace (PP) **(b)** extracts.

Data presented represent the mean ± standard deviation of three assays in triplicate. The lowercase letter represents the significant comparison of the extraction solvents, considering the same extract and the same extraction conditions. The uppercase letter represents the significant comparison of the extract conditions, considering the same extract and the same solvent. The analysis starts with the letter "a" and "A" representing the highest value.

3.2. Total Phenolic Content analysis

Total Phenolic Content (TPC) assay allows assessing the amount of phenolic content in the samples, though it can also indirectly assess the antioxidant potential of the extracts as both are usually well correlated. Folin–Ciocalteu reagent contain complex polymeric ions formed from phosphomolybdic and phosphotungstic heteropoly acids. Its reduction results in the formation of a blue color, which can be measured spectrophotometrically at a wide range of wavelengths, with 750 nm or 760 nm. Color formation by a sample is compared to color formation of a standard compound, gallic acid, and then are reported as gallic acid equivalents.[140,152] Phenolic compounds existing in plants have redox properties, that allows them to act as antioxidants.[152]

In the extractions at 70 °C, there were no significant differences in the content of phenolic compounds of garlic extracts obtained with different solvents (**Figure 3 (a)**). However, in the overnight extraction, this difference exists. The ethanolic extracts exhibited much more phenolic content than the aqueous extracts (40.61 ± 3.55 and 55.28 ± 4.58 in EtOH (96%) and EtOH (70%) extracts, respectively; and 03.35 ± 0.60 in aqueous extracts), as it was expected according to literature. M. Bar (2022) described a greater ability of polar solvents to solubilize biotic compounds, particularly polyphenols, compared to water.[143] Moreover, it is also important to note that overnight ethanolic extracts had higher phenolic content than the ones extracted at 70 °C, probably due to

degradation of some bioactive compounds at high temperatures.[153] The phenolic content of garlic is mainly due to quercetin, however, it is important to note that garlic is a plant with low phenolic content, according to what is described in the literature[143,153], which justifies why this is one of the extracts with the lowest total phenolic content.

Although the extraction yield was quite low in the ginger ethanolic (96%) extracts, they have high phenolic content, which means that different compounds were being extracted (109.76 ± 7.08 mg/mg and 96.08 ± 13.09 mg/mg, in 70 °C and overnight extractions, respectively) (**Figure 3 (b)**). Both 6-gingerol and 6-shogaol, mentioned above, are highly insoluble in water[154,155], which is why the total phenolic content was much higher in the ethanolic extracts and in particular in the ethanolic (96%) extracts.

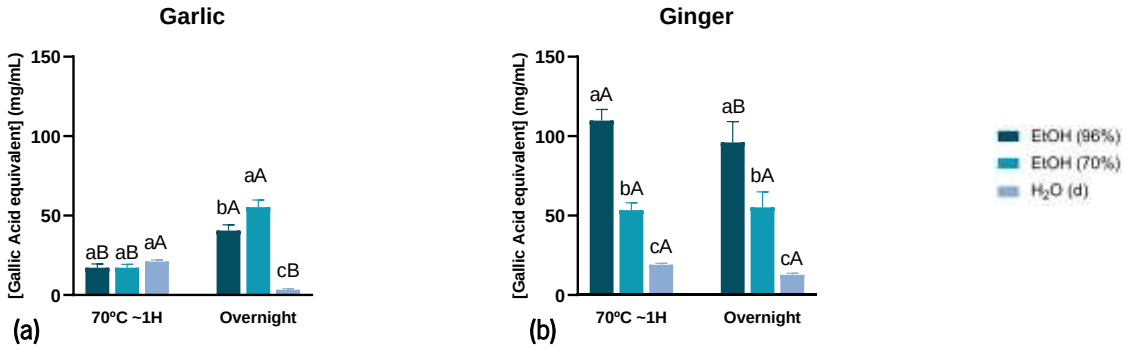

Figure 3 Total Phenolic Content (TPC) analysis, in gallic acid equivalent concentration (mg/mL) of garlic **(a)** and ginger **(b)** extracts.

Data presented represent the mean ± standard deviation of three assays in triplicate. The lowercase letter represents the significant comparison of the extraction solvents, considering the same extract and the same extraction conditions. The uppercase letter represents the significant comparison of the extract conditions, considering the same extract and the same solvent. The analysis starts with the letter "a" and "A" representing the highest value.

It is described that PPE ethanolic extracts have higher phenolic content than aqueous extracts[127], which is actually in agreement with the result obtained in this study (**Figure 4 (a)**). The fact that the PPE is rich in tannins, makes a mixture of solvents (EtOH (70%)) show better results. Furthermore, it is also important to mention that this compound is more easily solubilized at higher temperatures, which may account for the fact that the values were higher in the extraction at 70 °C.[149] Finally, it is noteworthy that the PPE was, of all plants tested, the one with the highest phenolics content.

As described above for PPE, the same is true for pomegranate pomace, since the compounds that composes PP are phenolic compounds both soluble and insoluble in water, so it was indeed expected that a mixture of solvents would perform better, as seen in this study (21.84 ± 1.87 mg/mg and 19.40 ± 1.14 mg/mg, in extraction at 70 °C and overnight, respectively) (**Figure 4 (b)**). Moreover, it is important to note that the phenolic content of the PP was considerably lower than that of the PPE.

PJ has even lower phenolic content than pomace, despite its high potential as an antioxidant (12.15 ± 2.13 mg/mg (**Figure (c)**). However, these should not be compared, as no extraction procedure was performed and the PJ has probably a much higher water content.

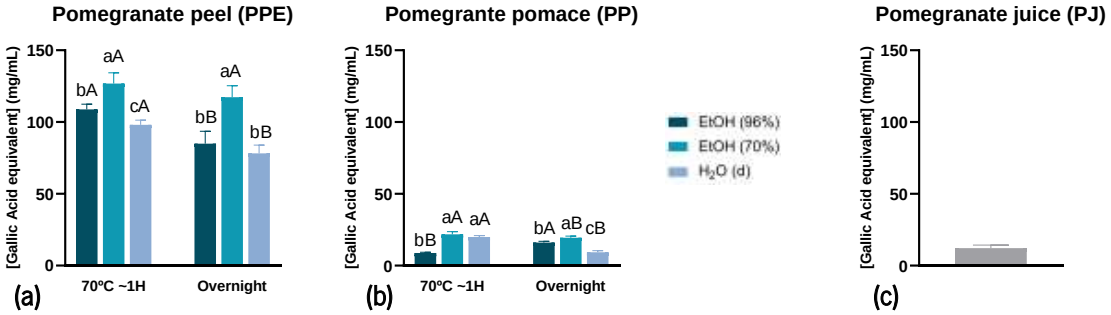

Figure 4 Total Phenolic Content (TPC) analysis, in gallic acid equivalent concentration (mg/mL) of pomegranate peel (PPE) **(a)**, pomegranate pomace (PP) **(b)** and pomegranate juice **(PJ)** extracts. Data presented represent the mean ± standard deviation of three assays in triplicate. The lowercase letter represents the significant comparison of the extraction solvents, considering the same extract and the same extraction conditions. The uppercase letter represents the significant comparison of the extract conditions, considering the same extract and the same solvent. The analysis starts with the letter "a" and "A" representing the highest value.

3.3. Determination of antioxidant activity

The FRAP assay is the only assay that directly measures antioxidant activity, with ABTS measuring the scavenging of a free radical. Nevertheless, it is important to note that not all reductants capable of reducing Fe^{3+} are antioxidants, any substances that are electron donors, even without antioxidant properties, can contribute to the FRAP value and overestimate the results.[156] The values it expresses represent the corresponding concentration of electron-donating antioxidants with the reduction in the ferric iron (Fe^{3+}) to the ferrous ion (Fe^{2+}).

The ABTS assay utilizes the stable nitrogen-based ABTS•+ radical. ABTS•+ methods first generate high ABTS•+ concentrations, followed by the antioxidant addition and monitoring ABTS•+ decline over a time period.[140] The result of the assay performed in this study will be presented as TEAC (Trolox Equivalent Antioxidant Capacity), which translates the ability of the sample to eliminate the radical, meaning that the higher the TEAC, the higher the antioxidant capacity of the extract.

In **Figure 5 (a)**, it is possible to verify that in garlic extracts, the Fe^{3+}-TPTZ complex was reduced most strongly to the ferrous (Fe^{2+}) by ethanolic (96%) extracts and, particularly by the one extracted overnight (0.077 ± 0.02 mmol/g), with a significative difference for ethanolic (96%) extract at 70 °C (0.036 ± 0.01 mmol/g) ($p<0.0001$). Since the main responsible for the antioxidant activity of garlic is allicin, and despite it is a low polarity compound, it is easily dissolved in ethanol[106], it was expected that the ethanolic (96%) extracts exhibited much higher antioxidant activity, as it was found here. Moreover, although the difference between the ethanolic (70%) extracts and the aqueous extracts is small in both scenarios, the ethanolic extracts mostly show slightly more antioxidant activity, which is also in line with what has been found in the literature. There was even a study that compared the antioxidant activity of ethanolic (50%) extracts with aqueous extracts, in which it was described that the ethanolic has more antioxidant activity.[144] In **Figure 5 (b)**, the antioxidant activity analysis was done based in the ABTS radical, in which the behavior pattern was the same as in the FRAP analysis. The highest antioxidant activity was also produced by ethanolic (96%) at overnight extraction (0.121 ± 0.01 mmol/g), followed by ethanolic (96%) extracted at 70 °C (0.070 ± 0.01 mmol/g), with a significative difference ($p<0.0001$).

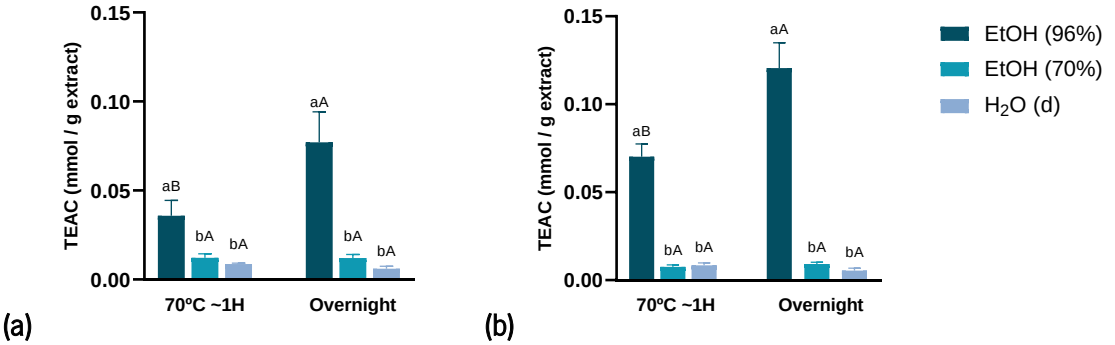

Figure 5 Trolox equivalent concentration, mmol Trolox Equivalent *per* g of extract (mmol/g), by FRAP analysis **(a)** and by ABTS analysis **(b)**, of garlic extracts.

Data presented represent the mean ± standard deviation of three assays in triplicate and each replicate included several garlics. The lowercase letter represents the significant comparison of the extraction solvents, considering the same extract and the same extraction conditions. The uppercase letter represents the significant comparison of the extract conditions, considering the same extract and the same solvent. The analysis starts with the letter "a" and "A" representing the highest value.

In ginger extracts, the Fe^{3+}-TPTZ complex was also reduced most strongly to the ferrous (Fe^{2+}) by EtOH (96%) extracts (1.50 ± 0.16 mmol/g and 1.50 ± 0.18 mmol/g in 70 °C and overnight extractions, respectively) (**Figure 6(a)**). It can be seen the lower the percentage of ethanol in the solvent, the lower the ginger antioxidant activity, which can be explained by the fact that the major bioactive compounds of garlic, 6-gingerol and 6-shagol are poorly water soluble, as it was mentioned above. This is in agreement with the literature, in which it was described that ethanolic ginger extracts exhibited higher antioxidant activity than aqueous extracts.[157,158] The same behavior can be seen on ABTS analysis (**Figure 6(b)**), in which the extracts with the highest antioxidant activity were also the ethanolic (96%) extracts (1.31 ± 0.13 mmol/g and 1.46 ± 0.09 mmol/g in 70 °C and overnight extractions, respectively).

Considering all the extracts, it can be observed that the extracts with the highest antioxidant potential were the pomegranate peel and ginger extracts, particularly those that were obtained with ethanol. Thus, it is expected that these extracts have great antibacterial potential, given the connection that is believed to exist between the two bioactivities.

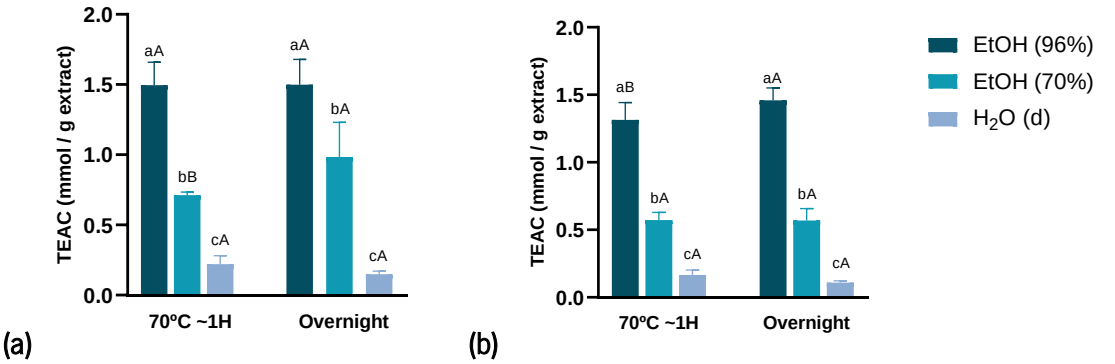

Figure 6 Trolox equivalent concentration, mmol Trolox Equivalent *per* g of extract (mmol/g), by FRAP analysis **(a)** and by ABTS analysis **(b)**, of ginger extracts.

Data presented represent the mean ± standard deviation of three assays in triplicate and each replicate included several ginger rhizomes. The lowercase letter represents the significant comparison of the extraction solvents, considering the same extract and the same extraction conditions. The uppercase letter represents the significant comparison of the extract conditions, considering the same extract and the same solvent. The analysis starts with the letter "a" and "A" representing the highest value.

In PPE extracts (**Figure 7(a)**), the Fe^{3+}-TPTZ complex was most strongly reduced to Fe^{2+} by ethanolic (70%) overnight extract (1.66 ± 0.12 mmol/g) and aqueous extract, 70 °C for 1 hour (1.615 ± 0.15 mmol/g), with no significative difference. On the other hand, the lowest value was obtained by aqueous overnight extract (1.09 ± 0.6 mmol/g).

In **Figure 7(b)**, the results were in line with what was expected, ethanolic (70%) extracts showed higher antioxidant capacity in both 70 °C and overnight extraction according to the ABTS method, (1.41 ± 0.08 mmol/g and 1.316 ± 0.09 mmol/g, respectively). As was the case in the evaluation of the phenolic compounds (**Figure 4(a)**), generically the highest antioxidant activity was also obtained when the extraction solvent used was EtOH (70%), which may mean that the phenolic compounds are responsible for the antioxidant activity. This becomes even more evident, knowing that this extract contains a wide range of phenolic compounds, some soluble in water and others mostly insoluble, that make a mixture of solvents more effective, as described in literature.[159]

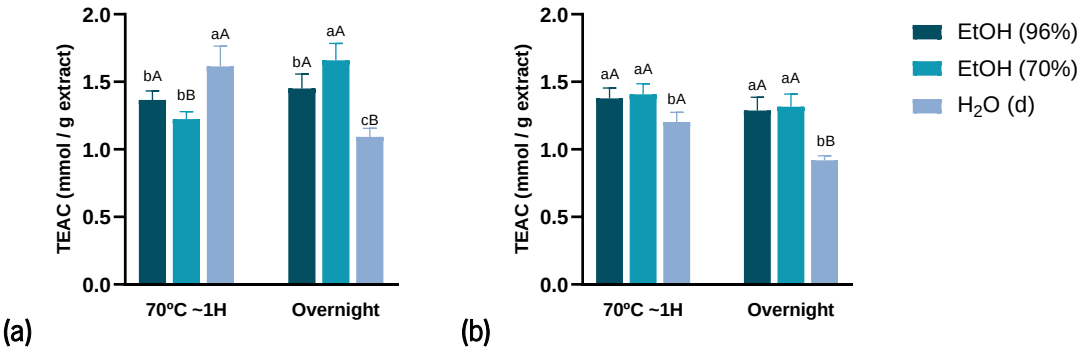

Figure 7 Trolox equivalent concentration, mmol Trolox Equivalent *per* g of extract (mmol/g), by FRAP analysis **(a)** and by ABTS analysis **(b)**, of pomegranate peel extracts.

Data presented represent the mean ± standard deviation of three assays in triplicate and each replicate included several pomegranates. The lowercase letter represents the significant comparison of the extraction solvents, considering the same extract and the same extraction

conditions. The uppercase letter represents the significant comparison of the extract conditions, considering the same extract and the same solvent. The analysis starts with the letter "a" and "A" representing the highest value.

As described above for PPE, the same is true for PP, since the compounds that compose that shows its antioxidant activity are mostly ellagitannins, ellagic acid derivatives and gallic acid, where ellagic acid and gallic acid are more soluble in ethanol than in water[160,161] and ellagitannins are soluble in water[162], so it was indeed expected that a mixture of solvents would perform better antioxidant activity. In **Figure 8** is possible to verify that both FRAP **(a)** and ABTS **(b)** analysis, the highest antioxidant activity was performed by ethanolic (70%) extract, but in FRAP analysis it happened in overnight extraction (0.34 ± 0.07 mmol/g), with no significative difference for the one extracted at 70 °C; and in ABTS in 70 °C extraction (0.29 ± 0.01 mmol/g), also with no significative difference with the overnight extract.

It is also important to note that at 70 °C, the aqueous extracts present an antioxidant activity close to the ethanolic (70%) extracts, while overnight, this similarity is obtained between the ethanolic extracts (96%) and (70%). However, the same was verified in the analysis of phenolic compounds (**Figure 4(b)**), which may be another indicator that the activity of PP is due to its phenolic content.

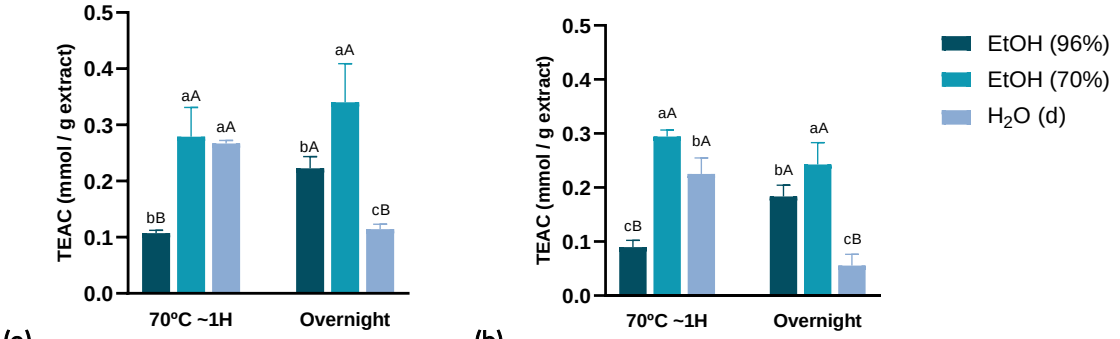

Figure 8 Trolox equivalent concentration, mmol Trolox Equivalent *per* g of extract (mmol/g), by FRAP analysis **(a)** and by ABTS analysis **(b)**, of pomegranate pomace extracts.

Data presented represent the mean ± standard deviation of three assays in triplicate and each replicate included several pomegranates. The lowercase letter represents the significant comparison of the extraction solvents, considering the same extract and the same extraction conditions. The uppercase letter represents the significant comparison of the extract conditions,

considering the same extract and the same solvent. The analysis starts with the letter "a" and "A" representing the highest value.

Although the antioxidant activity of PJ is mainly attributed to compounds that also exist in PPE and PP, such as anthocyanins, catechins, and tannins, this extract has a lower antioxidant activity *per* g extract than PPE and PP, both in FRAP (0.10 ± 0.01 mmol/g) and ABTS analysis (0.14 ± 0.01 mmol/g) (**Figure 9**).

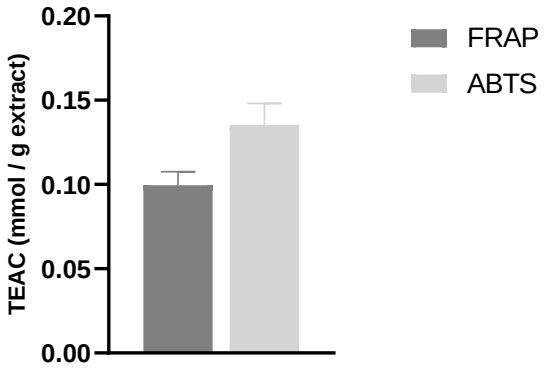

Figure 9 Trolox equivalent concentration, mmol Trolox Equivalent *per* g of extract (mmol/g), by FRAP and ABTS analysis, of pomegranate juice extracts. Data presented represent the mean ± standard deviation of three assays in triplicate and each replicate included several pomegranates.

3.4. Determination of antibacterial activity

Natural biomasses as plants are composed by panoply of compounds with different bioactive activities. The use of various solvents affects the solubility of different phytochemicals and, consequently, the antimicrobial properties of each extract can be distinct.[143] In this study, different extraction conditions were used in order to evaluate their impact on antibacterial activity of the selected plant biomasses, garlic, ginger and pomegranate peel, pomace and juice, against *P. aeruginosa* and *S. aureus*. Antibacterial activity was stipulated according to the MIC and MBC values.

Analyzing the results (**Table 2**), only the ethanolic extract 96% overnight inhibited *S. aureus* growth but it was not able to eradicate bacteria and o activity against *P. aeruginosa* was recorded. Its activity against gram-positive bacteria rather than gram-negative was reported previously in literature.[163] This difference resulted from the distinct cell wall structure between these major classes of bacteria, since gram-negative bacteria are surrounded by a thin peptidoglycan cell wall,

which itself is surrounded by an outer membrane containing lipopolysaccharide. Gram-positive bacteria lack an outer membrane but are surrounded by layers of peptidoglycan many times thicker than is found in the gram-negatives.[164,165]

Considering the previous results, the antibacterial activity of the ethanolic (96%) extracts of garlic against *S. aureus* might be related with the antioxidant activity and the total phenolic content (TPC). The ethanolic (96%) extract was the one with highest antioxidant activity (**Figure 5**) and with significant TPC (**Figure 3(a)**). Being allicin described as one of the compounds responsible for antioxidant activity of garlic[102], as mentioned above, and being this a compound more easily solubilized in ethanol, this bioactivity may be due to its presence. This is also in agreement with a study done with garlic extract, in which it was found that the higher the allicin content in the extract, the higher its antibacterial activity, and when the formation of allicin was inhibited during extraction, the extract lost its activity.[166] It is also described that phenolics can play an important role in antibacterial activity, although, as mentioned earlier, the main contributors are organosulfur compounds (such as allicin).[167] Moreover, it is important to emphasize that being the same compound the primarily responsible for both antioxidant and antibacterial properties of garlic, there may be effectively a relationship between the two bioactivities.

Few studies reported that aqueous extracts of garlic can be antibacterial[98] but in this work no inhibition or eradication was observed for both species. Mozaffari Nejad et al described the minimum concentration of aqueous garlic extract that prevents the growth of gram-positive microorganisms is between 15.6 and 48.3 mg/mL and gram-negative microorganisms is between 14.9 and 37.2 mg/mL[168], which are much higher extract concentrations than those that were tested in the present work.

Furthermore, it is important to emphasize that the only extract capable of inhibit the growth of *S. aureus* was the extract obtained overnight, which was in line with the literature, since it is described that temperature may degrade important garlic bioactive compounds.[153]

Table 2 Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of garlic extract obtained from different extraction conditions tested on *S. aureus* and *P. aeruginosa*

Bacteria	Solvent	Extraction conditions	MIC_{99} (mg/mL)	MBC (mg/mL)	Maximum concentration tested (mg/mL)
S. aureus	EtOH 96%	70 °C ~1H	> 1.1	> 1.1	1.1
	EtOH 70%		> 6.8	> 6.8	6.8
	H_2O (d)		> 7.9	> 7.9	7.9
	EtOH 96%	Overnight	0.4	> 0.4	0.4
	EtOH 70%		> 5.1	> 5.1	5.1
	H_2O (d)		> 1.8	> 7.3	7.3
P. aeruginosa	EtOH 96%	70 °C ~1H	> 1.1	> 1.1	1.1
	EtOH 70%		> 6.8	> 6.8	6.8
	H_2O (d)		> 7.9	> 7.9	7.9
	EtOH 96%	Overnight	> 0.4	> 0.4	0.4
	EtOH 70%		> 5.1	> 5.1	5.1
	H_2O (d)		> 7.3	> 7.3	7.3

Among the ginger extracts tested, only ethanolic extract (96%, at 70 °C, 1h) was able to inhibit the growth of *S. aureus* (**Table 3**). Although in both antioxidant analyses it showed slightly less activity than ethanolic (96%) extract overnight (**Figure 6**), with regard to the phenolic content analysis, the ethanolic extracts were also the ginger extracts that showed the highest phenolic content, especially the extract obtained at 70 °C (**Figure 3(b)**), which could perhaps justify the fact that this extract showed higher antibacterial activity, which is usually associated with compounds such as 6-gingerol and 6-shogaol, as already mentioned.

According to the literature, ginger extracts were expected to also inhibit the growth of *P. aeruginosa*[169], although this did not occur at the extract concentrations tested in this study.

It is also important to note that no extracts obtained from ginger demonstrated bactericidal activity.

Table 3 Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of ginger extracts obtained from different extraction conditions tested on *S. aureus* and *P. aeruginosa*

Bacteria	Solvent	Extraction conditions	MIC_{99} (mg/mL)	MBC (mg/mL)	Maximum concentration tested (mg/mL)
S. aureus	EtOH 96%	70 °C ~1H	0.9	0.9	1.8
	EtOH 70%		>4.7	>4.7	4.7
	H_2O (d)		>6.3	>6.3	6.3
	EtOH 96%	Overnight	>1.2	>1.2	1.2
	EtOH 70%		>2.5	>2.5	2.5
	H_2O (d)		>8.0	>8.0	8.0
P. aeruginosa	EtOH 96%	70 °C ~1H	>1.8	>1.8	1.8
	EtOH 70%		>4.7	>4.7	4.7
	H_2O (d)		>6.3	>6.3	6.3
	EtOH 96%	Overnight	>1.2	>1.2	1.2
	EtOH 70%		>2.5	>2.5	2.5
	H_2O (d)		>8.0	>8.0	8.0

PPE extracts revealed to be the most active against *S. aureus* and *P. aeruginosa* (**Table 4**). The extracts with the highest ability to cause growth inhibition at lower concentrations were the extracts obtained with EtOH (70%), that corresponded to PPE extracts with highest total phenolic content (**Figure 4(a)**) and antioxidant activity (**Figure 7**). This can be justified by the fact the active bioactive compounds in PPE are more soluble in solvent mixtures, as already mentioned. However, this mixture between ethanol and water seems to be more efficient in the 70:30 (v/v), since it was found that in ethanol (80%) extracts tested, the MIC was between 15.62 and 19.5 mg/mL[170] and that in ethanolic (50%) extracts the MIC was around 10 mg/mL.[171] This may indicate that the percentage of ethanol in the solvent has high influence in the extracted compounds. However, studies with a wider range of ethanol concentrations used as an extraction solvent would be needed to validate this information.

Aqueous overnight extracts were effective against *S. aureus* using the microdilution method, but these results cannot be compared, because no studies were found about aqueous extracts obtained with the same extraction method at similar conditions or against the same microorganisms.

Both overnight and 70 °C and overnight ethanolic (70%) extracts were effective against both bacteria. However, it is possible to verify that overnight extracts were effective at lower

concentrations than extracts obtained at 70 °C, indicating that the temperature may have affected the extraction of some compounds responsible for antibacterial activity.

It is also important to mention that *S. aureus* is more susceptible than *P. aeruginosa*, since there was no bactericidal activity against the gram-negative bacteria, which is also described in literature.[128]

Table 4 Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of pomegranate peel extracts obtained from different extraction conditions tested on *S. aureus* and *P. aeruginosa*

Bacteria	Solvent	Extraction conditions	MIC_{99} (mg/mL)	MBC (mg/mL)	Maximum concentration tested (mg/mL)
S. aureus	EtOH 96%	70 °C ~1H	>5.1	>5.1	5.1
	EtOH 70%		1.5 - 2.9	2.9	6.0
	H_2O (d)		≥5.6	≥5.6	5.6
	EtOH 96%	Overnight	≥4.7	≥4.7	4.7
	EtOH 70%		0.7-2.6	2.6	5.3
	H_2O (d)		2.7-5.3	5.3	5.3
P. aeruginosa	EtOH 96%	70 °C ~1H	≥5.1	≥5.1	5.1
	EtOH 70%		1.5-2.9	≥5.8	6.0
	H_2O (d)		≥5.6	≥5.6	5.6
	EtOH 96%	Overnight	>4.7	>4.7	4.7
	EtOH 70%		0.7-2.6	>5.3	5.3
	H_2O (d)		≥5.3	≥5.3	5.3

Analyzing **Table 5**, it is possible to verify that the extracts of PP with increased antibacterial activity were the ethanolic (70%) extracts, corresponding to the extract with highest total phenolic content (**Table 2(c)**) and the antioxidant activity (**Figure 3**). Furthermore, it is important to note that the extract obtained overnight was able to inhibit the growth of *S. aureus* at a lower concentration than the extract at 70 °C, which is in agreement with the result obtained in the FRAP analysis (**Figure 3(a)**), although in the ABTS analysis the opposite was expected (**Figure 3(b)**).

It is important to note that the aqueous extract obtained at 70 °C showed inhibition of the growth of *P. aeruginosa*, which is noteworthy because this extract showed high antioxidant activity and a high content of phenolic compounds. This also helps validate the fact that the antibacterial activity of PP can result from its phenolic compounds. This study is the first one reporting the antibacterial activity of PP for *S. aureus* and *P. aeruginosa.*

None of the extracts showed bactericidal activity at the concentrations tested against both species.

Table 5 Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of pomegranate pomace extracts obtained from different extraction conditions tested on *S. aureus* and *P. aeruginosa*

Bacteria	Solvent	Extraction conditions	MIC_{99} (mg/mL)	MBC (mg/mL)	Maximum concentration tested (mg/mL)
S. aureus	EtOH 96%	70 °C ~1H	>4.0	>4.0	4.0
	EtOH 70%		≥4.2	>4.2	4.2
	H_2O (d)		>3.7	>3.7	3.7
	EtOH 96%	Overnight	>3.5	>3.5	3-5
	EtOH 70%		≥3.8	>3.8	3.8
	H_2O (d)		>4.1	>4.1	4.1
P. aeruginosa	EtOH 96%	70 °C ~1H	>4.0	>4.0	4.0
	EtOH 70%		>4.2	>4.2	4.2
	H_2O (d)		≥3.7	≥3.7	3.7
	EtOH 96%	Overnight	>3.5	>3.5	3-5
	EtOH 70%		≥3.8	≥3.8	3.8
	H_2O (d)		>4.1	>4.1	4.1

PJ is one of the extracts with lower content of total phenolics (**Figure 4(c)**) and, when compared to PPE and PP extracts, it is possible to verify that it also presents lower antioxidant activity (**Table 6**). However, it is possible to register the growth inhibition of both bacteria, although maybe this inhibition has to do with the higher concentration of the extract used and not exactly with the extract itself. The values obtained for *S. aureus* are not very different from those found in the literature, although the extracts in the study in question refer to juice extracted with EtOH (50%), which in this case were MIC = 25 μg/μL, equivalent to 25 mg/mL and MBC = 40 μg/μL, equivalent to 40 mg/mL.[171]

Being PJ rich in sugars[133], as mentioned before, and being sugars growth enhancers for bacteria[172], it would be interesting to perform an ethanolic extraction of PJ in order to extract more phenolic compounds, which are mainly attributed to antibacterial activity, so as to possibly obtain an extract with even more activity. Nevertheless, it is important to note that the tested extracts demonstrated bactericidal activity against both bacteria.

Table 6 Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of pomegranate juice tested on *S. aureus* and *P. aeruginosa*

Bacteria	MIC_{99} (mg/mL)	MBC (mg/mL)	Maximum concentration tested (mg/mL)
S. aureus	22.6	45.2	**90.4**
P. aeruginosa	90.4	90.4	**90.4**

It is important to note that PPE was also able to inhibit the growth of both bacteria (although only with some solvents, as mentioned earlier), but it was able to do so at considerably lower extract concentrations than those tested with the juice, suggesting that PPE not only has more potential as an antioxidant, but also has more potential as an antibacterial.

4. Conclusion and future perspectives

The aim of this study was to identify non-antibiotic alternatives with potential to address, or at least minimize, the problem of antibiotic resistance, one of the biggest crises in the world. It has been described that natural products, and in particular plants, have a high potential as antibacterial drugs, often associated with their high antioxidant activity. In this study, total phenolic content, antioxidant and antibacterial properties of extracts from five different biomasses, garlic, pomegranate (peel, pomace and juice) and ginger, were evaluated.

Regarding the extraction yields, no optimal solvent was found for the three biomasses used. Deionized water demonstrated to produce higher yield of extracts of garlic and ginger, while ethanol (70%) was the best solvent for the pomegranate extraction (both peel and pomace). In contrast, in most the extractions 70 °C for 1 hour results in increased yield than overnight extraction at room temperature, because mass transfer rates and target's compounds solubility are generally higher at higher temperatures. Further, temperature may also have a positive effect in damaging the plant structure, thus facilitating extraction.

In the analysis of total phenolic content, most of the ethanolic extracts showed more phenolic content than the aqueous extracts and, ethanol (70%) showed to solubilize more content of phenolic compounds than ethanol (96%). In aqueous extracts, the extraction performed at 70 °C always promoted a higher extraction of phenolic content, although in less quantity than the ethanolic ones, than the overnight extraction. The extracts that showed higher phenolic content were the PPE extracts obtained with ethanol (70%), at 70 °C and overnight.

Analyzing the antioxidant capacity of the different extracts, it was concluded that the ethanolic solvents produced extracts with higher antioxidant activity than the aqueous extracts. The concentration of ethanol used (70 and 96%) did not seem to influence the antioxidant activity of the extracts. Furthermore, it was possible to conclude that in the aqueous extracts the antioxidant activity was higher when the extraction was done at 70 °C than when it was performed overnight. On the other hand, in most of the ethanolic extracts, higher antioxidant activity was obtained in the overnight extracts than in the extractions done at 70 °C, which allows the conclusion that for this specific type of evaluation, the extraction at 70 °C for is not the best option. Eventually, the more active fractions are also more thermolabile and their functionality was impaired at higher temperatures. Therefore, temperature is an important operational parameter to control and optimize when dealing with the extraction of functional fractions from natural biomasses, to achieve a reasonable balance between process feasibility (including extraction yield) and expressed bioactivity.

Finally, regarding the antibacterial activity, in most extracts, it is associated with the extracts with higher phenolic and higher antioxidant content. Among ethanolic extracts, PPE extracts were the most promising non-antibiotic drugs against *S. aureus* and *P. aeruginosa* eradication and must be deeply explored in near future.

As future work, it would be interesting to complement this work with a phytochemical analysis of plant extracts in order to validate some assumptions made throughout this work. Moreover, investigation of the activity of plant extracts on antibiotic resistant isolates, multi-resistant and extensively resistant isolates to evaluate its true potential and eventually against biofilms could be included. Moreover, it could be investigated the synergistic activity of the plant extracts with antibiotics on antibiotic resistant isolates and multi-resistant and extensively resistant isolates.

In a long-term perspective, it could be appealing to evaluate the potential of other extracts through further analysis of total phenolic content and antioxidant activity determination with the extracts at the same concentration and to test these and other extracts on other bacteria from the ESKAPE group, given their relevance.

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