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NATURAL EXTRACTS AS A PROMISING SOLUTION FOR GRAM-POSITIVE ANTIBIOTIC RESISTANCE: A COMPREHENSIVE REVIEW

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ABSTRACT

Background: Antibiotic resistance is currently one of the biggest problems in public health. Infectious diseases are the second human death cause, and the emergence of antimicrobial-resistant bacteria increases mortality and morbidity rates. There is a growing clinical need for the development of new antibiotics. In this line, WHO issued an alert about 12 bacteria with an urgent need to develop new antibiotics. **Aims:** This review aims to analyze the current knowledge of their antibacterial activity against the gram-positive pathogens listed by WHO and their extraction techniques. **Methods:** We systematically reviewed the literature in PubMed, searching publications describing the use of natural extracts as antibiotics over bacteria. The exclusion criteria consisted of limiting papers on natural extracts tested over the bacteria culture related to eleven selected bacteria, according to an alert issued by WHO in 2017, and seven plant extracts. **Results:** All the gram-positive bacteria present in the WHO alert have been treated, with different degrees of advance, with some of the plant extracts and plant-based compounds reviewed. Currently, they are in the preclinical stage. Edible herbs are more often used, as well as artemisia and wine byproducts. **Discussion:** Natural products based on plants have shown to be efficient in inhibiting bacterial growth, even in antibiotic-resistant strains. The classical extraction methods are still in use and have been improved with the available technology to improve efficiency and yield. **Conclusions:** Ongoing evidence shows that plant extracts and plant-based compounds are effective as antibacterial, with minimal effects on the host cell, a promising antibiotic source. Furthermore, they are sustainable, environmentally friendly, and renewable.

Keywords: *natural extract, bacteria, pathogen, Antibiotic-resistant, infectious diseases.*

1. INTRODUCTION

Infectious diseases are one of the most important public health problems. They are the second leading cause of death in the world. The first antibiotic was described in 1910, and opened the most fruitful period in this field. The 20th century, more precisely, the period from 1940 to 1970, is considered the golden age of antibiotics. Antibiotics are one of the most innovative medicines because of their ability to reduce morbidity and mortality caused by infections worldwide. Unfortunately, the misuse of antibiotics, especially the massive use and overuse of antibiotics, has led bacteria to develop mechanisms of resistance to antibiotics. This resistance has two main causes: through naturally occurring errors in DNA replication followed by the selection of drug-insensitive mutants that can later spread vertically in the bacterial population or

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through horizontal transfer of resistance genes between bacteria. This antibiotic resistance allows microorganisms in general, and bacteria in particular, to circumvent the action of the drug used against them. This decline in the effectiveness of antibiotics in treating bacterial infections has been the subject of multiple alerts (Diallo *et al.*, 2020; Laxminarayan *et al.*, 2013; McEwen & Collignon, 2018). Their role in emerging and reemerging bacterial diseases is affecting public health. Around the world, epidemiological surveillance programs are collaborating to diagnose antimicrobial resistance worldwide (Burnham, Leeds, Nordmann, O'Grady, & Patel, 2017; Christaki, Marcou, & Tofarides, 2020; Diallo *et al.*, 2020). The pathogens that need to be urgently addressed were mainly classified into two groups. One of them, shown in Figure 1, is the antimicrobial-resistant ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter* species) (De Oliveira *et al.*, 2020). These pathogens are included in a 2017 alert from WHO that calls researchers to find effective treatments against 12 bacteria (Table 1). In this fight against AR, several approaches are being taken to discover new antibiotics. In the past, plants have been used as medicines, and currently, they are back in the spotlight as an effective tool for developing new treatments for infectious diseases using plant extracts and natural compounds (Mulat, Pandita, and Khan, 2019). In the present review, we summarized the recent findings of antibacterial activity extracts and bioactive molecules and the most commonly used techniques to obtain these extracts. We focused our search on aromatic plants, wild plants, and plants used as infusions and byproducts of the wine industry.

2. DEVELOPMENT

A systematic review was performed as described previously (QUINTERO, Cristián Andrés; VALLEJO, Mariana Guadalupe; BONTTI, Sergio; PATIÑO, Sol; PEREZ-GIRABEL, Rocío, 2022). Briefly, the literature in PubMed was explored to search for publications describing the use of natural extracts as antibiotics over bacteria, collecting and analyzing data. In order to do so, we used the following words/terms in combination: bacteria: name) AND (natural extract) AND (plant name) AND (antibacterial activity or antibiotic). The exclusion criteria consisted of limiting papers on natural extracts tested over the gram-positive bacteria culture related to eleven selected bacteria, according to an alert issued by WHO in 2017, and seven plant extracts. The search was conducted on papers published until September 2021.

3. RESULTS AND DISCUSSION:

3.1.1 History of antibiotics

Although recent history dates the beginning of the age of antibiotics to 100 years ago, there is evidence of the use of antibiotic-producing microorganisms to treat infections 2000 years ago in China, Greece, Serbia, and Egypt. Even earlier, in 1550 BC, is the oldest record of antibiotics use, being the moldy bread and medicinal soils included in the Papyrus of Ebers and Smith as medicines (Ferber & Karnath, 1999). The modern era of antibiotics has two major highlights: The first was the use of salvarsan, a synthetic arsenic-based drug, to treat *Treponema pallidum*, the causative agent of the sexually transmitted disease syphilis, by Paul Ehrlich ("PROFESSOR EHRLICH'S NEW REMEDY FOR SYPHILIS.," 1910). The second was in 1929, when Sir Alexander Fleming published his findings on the inhibitory, bactericidal, and bacteriolytic effects of Penicillium cultures on various pathogenic bacteria (Fleming, 1929). A breakthrough came in the late 1930s when Selman Waksman systematized the discovery of new antibiotics based on microorganisms. He created an efficient tool for the search of compounds with antibiotic activity based on natural products, like microbes, being the filamentous Actinomycetales as their main source of them (WAKSMAN, 1947). Their definition of an antibiotic was "a compound made by a microbe to destroy other microbes". This was the beginning of the golden age of antibiotics, which lasted from 1940 to 1970.

3.1.2 The increasing need for new antibiotics

Antibiotics can be considered one of the greatest advances in modern medicine. They have helped and saved millions of people since they became common medicine. This massive use of antibiotics, as well as the overdose of antibiotics, not only in humans but also in animals and agriculture (Laxminarayan *et al.*, 2013), is considered to be the base of Antimicrobial Resistance (AR). Increasing AR is currently a major obstacle in infection management. Global reports have drawn attention to the situation, with numbers that must call all our attention: 25.000

deaths in Europe in 2009, according to the European Center for Disease Prevention and Control, 23.000 per year in 2013 in the U.S. alone, according to U.S. Centers for Disease Control and Prevention, and the number could reach 10 million deaths per year by 2050 (Diallo *et al.*, 2020). The ongoing antibiotics affect the life cycle of bacteria. They can either directly or indirectly affect the essential processes of the bacteria, as shown in Figure 2.

The small genome size and short time span of duplications have led to the ability of bacteria to acquire AR. This resistance can be intrinsic, acquired, or adaptive. The first two cases involve modifications in their DNA by transformation, transduction, or conjugation. The adaptive response is mainly through modulation of gene expression. (Christaki *et al.*, 2020). These modifications allow bacteria to survive in the presence of antibiotics through several mechanisms, including destruction or modification of the antibiotic by the bacteria, modification of the target (as target mutations, replacement, protection, or overproduction), as well as the reduced permeability or increased efflux of the molecule (Christaki *et al.*, 2020). The emergence of multidrug-resistant bacteria or superbugs is considered an important public health problem. Currently, several health agencies and organizations worldwide are implementing surveillance programs. In 2017, the WHO issued an alert highlighting 12 bacteria in urgent need of antibiotic development due to their increasing resistance (Table 1). The alert ranked urgency on a three-tier scale, with the presence of Gram-positive bacteria into priority 2 (*Staphylococcus aureus* and *Enterococcus)* and priority 3, *Streptococcus pneumoniae.*

3.1.3 Discovery of new antibacterial agents

Regardless of the obvious need for new antibiotics, no new classes of antibiotics have been discovered in the last 40 years, and the last new antibiotics were from the 1980s. One possible reason for this is the small profit that developing new molecules means for the big pharmaceutical companies. It takes them $ 2.5 billion and up to 15 years to discover and manufacture a new antibiotic (DiMasi, Grabowski, and Hansen, 2016).

There are several approaches to discovering new molecules with antibacterial activity (see (Cook & Wright, 2022; Durand, Raoult, & Dubourg, 2019; Wohlleben, Mast, Stegmann, & Ziemert, 2016)). The following main categories are distinguished.

I-Antibodies: specifically, monoclonal antibodies are growing exponentially in the pharmaceutical industry, with various uses, with more than 100 antibodies approved by the FDA for medical treatments (Mullard, 2021). The possibility of synthesizing them by recombinant methods (Moutel, Marjou, *et al.*, 2009; Moutel, Vielemeyer, *et al.*, 2009) indicates that antibodies are an excellent target for antibiotic development.

II- Antimicrobial Peptides (AMP), small peptides (i.e., less than 100 amino acids). They were described in 1957 and are derived from blood cells (SKARNES & WATSON, 1957). They are amphipathic molecules that have hydrophobic and cationic amino acids in their structure. They are expensive and prone to hydrolysis, which limits their application.

III- Antivirulence strategy. A novel strategy is to reduce the virulence of pathogenic bacteria by inhibiting the virulence factors rather than killing the pathogen. This has the advantage of avoiding the mechanisms of antimicrobial resistance. They can be used in combination with conventional antibiotics.

IV- Bacteriophages. These microorganisms are viruses that specifically infect bacteria. They are known as molecular biology tools and were traded as antibacterial agents after their discovery, but the appearance of penicillin has eclipsed them. In recent years, they have reemerged on the scene and have been successfully used against *Salmonella* spp. and *Shigella* spp., among others (Połaska & Sokołowska, 2019).

V- Combination strategies. The use of adjuvants or the combination of different antibiotics is useful. Adjuvants are designed to inhibit the intrinsic resistance mechanisms by which bacteria evade antibiotics. In addition, combinations of different antibiotics can have a synergistic effect, i.e., they are more active than independent applications (Ejim *et al.*, 2011).

VI- Natural Products (NP). The beginnings of the antibiotic era and most of the antibiotics currently in use are derived from natural products, mainly bacteria, fungi, and plants. Bacteria and fungi produce secondary metabolites to fight for their own niche, and they have been systematically studied, purified, and used in human and veterinary medicine and also in

agriculture (Katz & Baltz, 2016). On the other hand, plants have been used in various cultures as part of traditional medicine to treat diseases. In recent decades, they have come back into focus using plant extracts, essential oils, purified bioactive molecules, or even *in vitro* production of secondary metabolites (M, A, & F, 2019). The use of extracts and essential oils, as well as the purification of the molecule(s) responsible for antibacterial activity, has the advantage of using renewable raw materials, byproducts of industrial processes, making them environmentally friendly and sustainable. The identification and isolation of the main molecules exerting antibacterial activities allowed researchers to elucidate the mechanisms of action, with possible pathways being interaction with the cell wall, cell membrane, and bacterial proteins, altering processes like bacterial adhesion, metabolite, and ion equilibria, inhibiting biofilm formation or impairing DNA synthesis (QUINTERO, Cristián Andrés; VALLEJO, Mariana Guadalupe; BONTTI, Sergio; PATIÑO, Sol; PEREZ-GIRABEL, Rocio 2022; A. Silva *et al.*, 2021).

Extracts or essential oils (EO) from cultivated or wild plants and byproducts from industries that use plants as raw materials were selected for the present work. The selection included three aromatic plants: rosemary (*Salvia rosmarinus* Spenn., Lamiaceae), syn.: *Rosmarinus officinalis* L.], thyme (*Thymus vulgaris* L., Lamiaceae), and oregano (*Origanum vulgare* L., Lamiaceae); two wild plants: jarilla (*Larrea* spp., Zygophyllaceae) and mugwort or artemisia (*Artemisia vulgaris* L., Asteraceae) one cultivated plant with industrial interest: yerba mate (*Ilex paraguariensis* A.St.-Hil., Aquifoliaceae) and a byproduct of the industry, namely wine from *Vitis vinifera* L. (Vitaceae).

Plant extracts or EO can be used for the production of leaves, tails, roots, the whole plant, the aerial parts of the plant, and also from the fruits: skin, seeds, or the whole fruit. The experimental data of *I. paraguariensis* are based on the commercial presentation that can be used infusion, called “mate”. Finally, in the case of *V. vinifera*, we included byproducts of wine and wine as raw materials (Figure 3).

3.1.4 Bacteria

The Gram-positive bacteria have been treated with some of the above-mentioned natural extracts to test their antibacterial activity. The bacteria were selected according to the WHO alert (table 1). Gram-negative bacteria were reviewed previously (QUINTERO, Cristián Andrés; VALLEJO, Mariana Guadalupe; BONTTI, Sergio; PATIÑO, Sol; PEREZ-GIRABEL, 2022).

To discover the antibacterial activity of plant-based compounds, the most common first step is using inhibition disks. The protocol starts with by imbibing of the disk with the extract or the natural compound in solution, in serial dilutions, and applied over the bacterial biofilm. The compounds diffuse in the media, and produces a halo where the bacteria can not grow, called an “inhibition zone”. The diameter of the inhibition zone is a measure of the potency of the compound. Another option, alternative or posterior, is the use of the solutions directly added to the liquid or solid culture medium, and the measurement is made in function of the growth, which is monitored by counting the colonies. The quantification of the activity is based on critical parameters, such as Minimal Inhibitory Concentration (MIC), which is defined as the lowest concentration of the antimicrobial agent that inhibits the growth of the organism. Another important parameter is the Minimal Bactericidal Concentration (MBC), which provides information on the lowest concentration at or above the MIC required to kill a microorganism. Additionally, it is usual to inform specific parameters, like MIC_{90}, which indicates the minimum concentration at which 90% of the isolates were inhibited, or IC_{50}, the inhibitory or effective concentration for 50 % of all surveyed isolates of a strain (CLSI, 2020; John E. Bennett, 2020). Table 2 summarizes the extracts and essential oils tested over each bacteria.

3.1.4.1 Staphylococcus aureus

Staphylococcus aureus is an extracellular gram-positive bacterium with spherical morphology. It is a facultative anaerobe but also grows well under aerobic conditions. *S. aureus* is associated with human infections such as facial furuncles and carbuncles, inflammation of loose connective tissue, and postoperative wound infections, being an important complication in patients with underlying pathologies. The main complication of current infections by *S. aureus* is the wide dispersion of Methicillin-Resistant strains (MRSA), which makes using beta-lactam antibiotics practically impossible. In addition, it is more frequent the appearance of strains resistant to vancomycin(John E. Bennett, 2020).

3.1.4.1.1 Artemisia

Four different species of *Artemisia* were used to inhibit the growth of *S. aureus*. In the first work, the aerial parts of *A. rupestris* L. were collected and separated into two groups: stems and flowers plus leaves, for their extraction with different solvents using ultrasound or microwave methods. The inhibition was strong, regardless of the solvent, method, or part of the plant used, except for the aqueous extract of stems, which was the less effective. The methanolic extract of flowers and leaves was the strongest inhibitor (Nokerbek, Sakipova, Chalupová, Nejezchlebová, and Hošek, 2017). Working with flavonoids isolated from *A. rupestris*, artemetin, pachypodol, chrysosplenetin, penduletin, and chrysoeriol, Lan *et al.* did not find inhibition of *S. aureus* when used alone at concentrations of 128 and 256 μg/mL. However, they found a synergism between the last three and norfloxacin up to 16-fold, chrysoeriol reduced the MIC of ciprofloxacin128-fold, and oxacillin 8-fold (Lan *et al.*, 2021). Methanolic extracts of *A. vulgaris* were able to inhibit *S. aureus*, in a study that also included three other extracts of plants used in traditional medicine, being *A. vulgaris* the most effective against *S. aureus*, with a MIC of 25 mg/mL (Manandhar, Luitel, and Dahal, 2019). Methanolic extract of *A. absinthium* L. was assayed against *S. aureus* using a reference strain (*S. aureus* ATCC 6538) and nine clinical isolates. The reference strain was the most sensitive, with a MIC of 0.625 mg/mL, and the clinical samples showed a MIC >2.5 mg/mL (Boudjelal, Smeriglio, Ginestra, Denaro, and Trombetta, 2020). Interestingly, in the same study, the authors showed no dermal toxicity nor any sign of toxicity when the extract was applied or administrated orally in mice. In 2021, Mohamed *et al.* tested the extract of *A. herba-alba* Asso and seven different compounds isolated from it against bacteria, fungi, and yeast. Both gram-positive bacteria were sensitive to the extract and six of the isolated compounds, most effective in the whole extract into the generation of inhibition zone. The lower MIC was achieved with 11-epiartapshin and benzoic acid p-(β-D-glucopyranosyloxy)-methyl ester, with 25 μg/disk (Mohamed *et al.*, 2021).

3.1.4.1.2 Oregano

Several species of *Origanum* have been used to inhibit the growth of *S. aureus*, with different degrees of success. In 2019 was published a big screening where ethanolic extracts of 67 dietary spices were tested against antibiotic-resistant bacteria like *S. aureus* and *S. enteritidis*. Among the spices, *O. vulgare* showed inhibition zones of 14 and 12 mm for *S. aureus* resistant to antibiotics or normal. In contrast, *O. majorana* L. showed inhibition zones of 17 and 15 mm for the same strains. Only for *O. majorana* was determined MIC and MBC, 1.6 mg/mL (Zhang *et al.*, 2019). In the same year, a study was published in which hydroalcoholic extracts of *O. vulgare* were assayed alone and in addition to *Hypericum perforatum L*. (Hypericaceae) extracts. While the separated extracts showed inhibition zones of 16 and 13 mm, respectively, the used in combination was 21 mm (Bahmani *et al.*, 2019). Using supercritical fluid extraction, García-Pérez *et al.* obtained an extract able to inhibit the growth of *S. aureus*, with inhibition zones of 0.3-0.4 cm, with similar results for *E.coli* (García-Pérez *et al.*, 2019). Interestingly, the EO of *O. vulgare* seems to be more effective, as was shown by Lofa *et al.* They found inhibition zones from 15 to 22 mm, working with samples of *S. aureus* isolated from the pork supply chain, with MIC of 0.01-0.02 %(V/V), similar values showed by purified thymol and carvacrol (Lofa *et al.*, 2019).

3.1.4.1.3 Rosemary and Thyme

Extracts of these two aromatic herbs have been studied individually or in comparative studies. Del Campo *et al.* used a commercial oregano extract to test their activity, which showed a MIC of 0.5 %(V/V). Interestingly, with the addition of sodium chloride 10 % (W/V), they reached a MIC of 0.13 %(V/V). The pH was also analyzed, with an optimum of pH=4,5, with a MIC of 0.06 %(V/V) (Del Campo, Amiot, and Nguyen-The, 2000). When Zaïri *et al.* compared thyme [*T. algeriensis* Boiss. & Reut. and *T. capitatus* (L.) Hoffmanns. and Link] with rosemary (*S. rosmarinus*) over ten strains of S. aureus always found a better activity of thyme, especially *T. algeriensis.* They also compared activity of the different preparation methods, following a potency order decoction > infusion > methanolic extract. The lower MIC was obtained with the decoction of both strains of thyme, with 0,25 mg/mL (Zairi *et al.*, 2018). Remarkably, the same extracts were applied over *Staphylococcus epidermis*, with the same or lower MICs. As mentioned before, Zhang *et al.* performed a big screening, including *S. rosmarinus* and *T. vulgaris,* with better inhibition zones for rosemary. Also, the MIC was better, with 0,4 against 1,6 mg/mL for thyme (Zhang *et al.*, 2019). In another comparative study, Munekata *et al.* followed the growth rate of the bacteria when they were treated with extracts of rosemary and thyme obtained by conventional or ultrasound-

assisted extraction. Even when they did not find a significant diminution of the speed, the best results were obtained with rosemary extracted conventionally with ethanol (Munekata *et al.*, 2020).

3.1.4.1.4 Wine byproducts

In the wine industry, the obtention of byproducts has great importance and diversity. One byproduct of wine production is the lees. A work published in 2013 showed the lees could inhibit the growth of *S. aureus*, requiring the previous activation: they must be irradiated with light at 400 nm. Even more, it depended on the time of irradiation: LED-light irradiation for 10 min effectively killed the bacteria with an approximate 3-log reduction, while LED-light irradiation for 20 min achieved a 5-log reduction (Tsukada, Sheng, Kamachi, and Niwano, 2016). The grape pomace was also described as a synergist of several antibiotics, as was the case for Cabernet Sauvignon grape pomace extracts and their activity in couple with antibiotics over clinical isolates of *S. aureus*, and MR *S. aureus* (Peixoto et al., 2018). The addition of grape pomace extracts augmented 30 to 75-fold the activity of antibiotics like ciprofloxacin, norfloxacin, or levofloxacin (Sanhueza *et al.*, 2017). The authors also showed synergism between grape pomace extracts and purified phenolic compounds (found in grape extracts), with an improvement of the activity from 8 to 64-fold, with a maximum for gallic acid and vanillic acid.

3.1.4.1.5. Yerba mate

Commercial brands produce yerba mate with small differences in their harvesting, drying, and grinding criteria, besides the differences in their region of origin. In this regard, Burris *et al*. performed a screening using different commercial brands of yerba mate, three from Argentina and one from Uruguay, for the extract preparation. All of them inhibited the growth of *S. aureus* ATCC 27708 and *S. aureus* SA113. The MIC was 25 μg/mL for all brands when tested over *S. aureus* ATCC 27708, >50 μg/mL for *S. aureus* SA113 for three of them, and 25 μg/mL for one brand (Burris, Davidson, Stewart, and Harte, 2011). Intriguingly, the same study showed that the MIC for *E. coli* was between 4 and 8-fold higher.

3.1.4.2 Enterococcus faecium

Enterococci are gram-positive bacteria of coccoid morphology, facultative anaerobes, and extracellular. They are an important cause of hospital-acquired infections such as septicemia and intra-abdominal sepsis, Central Nervous System infections, skin and soft tissue infections, endocarditis, and pneumonia. Of the members of the *Enterococci* family*, Enterococcus faecium* shows a greater risk for the patient since it presents a high degree of resistance to antimicrobials, including vancomycin, a glycopeptide with a good effect on the cell wall of *Enterococci* (John E. Bennett, 2020).

3.1.4.2.1 Artemisia

In two different works, *Artemisia* spp. was used to inhibit *E. faecium*, in both cases using *A. absinthium*. The first of them confronted nine plant extracts against six multidrug-resistant bacteria. The extracts were prepared with ethanol or water; the only active ones were the ethanolic extracts. The *A. absinthium* extract was just able to inhibit the growth of *E. faecium* partially, with an IC_{50} of 256 μg/mL, without reaching an inhibition greater than 90% in the maximum concentration assayed (256 μg/mL) (Khan *et al.*, 2018). However, in a later work, the water extracts did not inhibit the growth of *E. faecium* or *E. faecalis* when they were assayed in inhibition disks. Interestingly, the EO of *A. absinthium* inhibited the growth in liquid broth cultures of *E. faecium* but did not inhibit *E. faecalis* growth. Also, in both cases, none of the aqueous extracts assayed inhibited bacterial growth (Bartkiene *et al.*, 2020).

3.1.4.2.2 Oregano.

In 2009 a screening of *Enterococci* isolated from piglets was published. Over 55 enterococcal strains were isolated. They found 37 *E. faecium* and 4 *E. faecalis*. Ten selected strains were treated with *O. vulgare* extracts, which inhibited the growth of all strains (Strompfová and Lauková, 2009). In a later work, Silva and col used eight EO from herbs used in gastronomy against ten foodborne and spoilage bacteria. Among them, *E. faecium* and *E faecalis* were sensitive to oregano (*O. vulgare*) EO, reaching inhibition zones of 25 and 19 mm, respectively. Interestingly, the MIC determined was higher for *E. faecium*, 15% (V/V), while 5% (V/V) was the MIC for *E faecalis* (N. Silva, Alves, Gonçalves, Amaral, & Poeta, 2013). Another species of *Origanum*, *O. hirtum*, was used to prepare EO and hydrolates for testing their capacity to inhibit the *E. faecium* and *E. faecalis* growth, among other bacteria. The EO of *O. hirtum* showed high activity against all the tested bacteria, with a MIC and MLC of 0,125 % (V/V) for both

strains. The hydrolates, on the other hand, showed a MIC and MBC major to 50% (V/V), showing the EOs always had better antimicrobial activity than the corresponding hydrolates (Di Vito *et al.*, 2021).

3.1.4.2.3 Thyme and Rosemary

As mentioned before, Silva *et al. (2013)* tested eight EO, including thyme and rosemary. The MIC determined for thyme was 5 and 15 % (V/V) for *E. faecium* and *E. faecalis*, while for rosemary, it was above 50 % (V/V) in both cases (N. Silva et al., 2013). Thyme (*T. vulgaris*) EO showed one of the strongest effects against E. faecium and *E. faecalis*, with inhibition zones of 37 and 30 mm, respectively. On the other hand, rosemary (*S. rosmarinus)* EO was considered not inhibitory against both bacteria.

3.1.4.2.4 Wine byproducts

An interesting study was published in 2010 by Corrales and col. (Corrales *et al.*, 2010), where grape skin extracts were assayed. Riesling grapes were collected in two vineyards, one with traditional agriculture and the other with organic agriculture practices. Despite the differences in their composition, levels of quercetin and kaempferol were significantly higher in organic samples, while the content of flavonoids, catechin, epicatechin, and procyanidin B1 was higher in the conventional grape skin extracts; the antibacterial activity was similar in both extracts. Even residual pesticides in conventional farming grapes did not influence their ability to inhibit *E. faecium* or *E. faecalis* (Corrales *et al.*, 2010).

3.1.4.3 Streptococcus pneumoniae

Streptococcus pneumoniae has a spherical or slightly elliptical morphology, mostly in pairs, with a gram-positive structure. They are extracellular bacteria. *S. pneumoniae* is a facultative anaerobe with moderate growth requirements. *S. pneumoniae* primarily colonizes the upper respiratory tract of healthy individuals and can be isolated from clinical specimens of tonsillitis, pneumonia, meningitis, and otitis media in community patients. It presents an important public health problem worldwide due to its high morbidity and mortality rates, especially in children and the elderly. Over the years, *S. pneumoniae* has developed resistance mechanisms that gave rise to strains resistant to penicillin and macrolides (John E. Bennett, 2020).

3.1.4.3.1 Oregano and thyme

S. pneumoniae has been treated only with EO from *O. vulgare*, *T. daenensis* Čelak. and *T. vulgare L*. Working with *O. vulgare* and *T. daenensis,* Sharifi *et al.* found that the formation of biofilms of *S. pneumoniae* was reduced by the EOs, proportionally to their concentration. The same assay was used to successfully prove the decrease in the number of adherent bacteria and the size of aggregates. Subsequently, the expression of genes related to biofilm synthesis was analyzed by quantitative real-time RT-PCR (qPCR) and showed a significant reduction when treated with EOs. Finally, the EOs were also shown to inhibit the growth of *S. pneumoniae* at MICs between 0.625 and 10 µL/mL, with *T. daenensis* being the most effective (Sharifi, Ahmadi, and Mohammadzadeh, 2018). They also found that the EOs have a total or partial synergistic effect with ciprofloxacin and ethidium bromide. In addition, they proved by RT-PCR that the efflux pump activity was inhibited using sub-MIC concentration of the EOs (Ghafari, Sharifi, Ahmadi, and Nayeri Fasaei, 2018). Ács and col worked with seven different EO against six respiratory tract pathogens in the same year. *T. vulgare L* EO showed moderate activity against *S. pneumoniae.* Two methods tested the antibacterial activity of commercial EO: broth macrodilution test to determine MIC and MBC and vapor phase test to measure MIC produced by the volatile compounds only, showing a MIC and MBC of 0.11 and 0.22 mg/mL, respectively, for the EO, while volatile compounds MIC was 90 µL of EO/L of airspace volume (Ács *et al.*, 2018).

3.1.5 Extracts Preparation

The extracts can be obtained from the leaves, stems, bark, seeds, flowers, fruits, roots, or the whole plant, or even byproducts of the industry, which are used as raw materials for any plant. Fresh or dry vegetal material (drug) can be used, depending mainly on the stability of the components to be isolated. Usually, desiccation is required to stop enzymatic reactions that can modify the quality of the material, avoiding fungal infections as well (rust). However, for products as EOs, a fresh material is sometimes preferred due to the volatile nature of the components and their possible chemical alterations.

The next step is grinding to decrease the particle size, augment surface exposure and promote solvent penetration through cell walls that have been partially lysed in previous stages by

cutting, chopping, or pulverizing the raw material (Guglielmi, Pontecorvi, and Rotondi, 2020). Finally, the process continues with the extraction with solvent or solvent systems, including water, ethanol, methanol, and acetone, among others, in different proportions. Nowadays, Natural Deep Eutectic Solvents (NADES) are used as an innovative strategy to minimize the environmental impact of organic solvents. As extra values, NADES obtained mixtures seem to be 'ready-to-use' extracts (removal of solvent would not be necessary) and provide better biopharmaceutical properties (D. T. da Silva *et al.*, 2021).

In order to improve the extraction, temperature, time, stirring, herbal material/solvent ratio, and stability of the components are important variables to be considered. The solubility of the active ingredients, for instance, is modulated by the chosen solvent and other components in the plant matrix.

We describe the most used procedures, from traditional techniques to advanced technologies. A summary is shown in Figure 4.

3.1.5.1 Conventional techniques

3.1.5.1.1 Maceration

One of the simplest methods used for hundreds of years, maceration involves contacting the ground drug in a closed container at room temperature, with occasional stirring. It is suitable for thermolabile bioactive components. The usual time required (3-14 days) can be shortened with constant stirring (dynamic maceration). If total extraction of the components is desired, the solvent must be renewed (R. M. L. da Silva, Couto, & Bresolin, 2012).

3.1.5.1.2 Percolation

In this technique, the solvent flows through to the vegetal material contained in a cylindrical or cone-shaped vessel (percolator). After macerating at room temperature for a certain period (2-4 h) in this closed container, the liquid is slowly dropped by a tap at the bottom of the percolator (Handa SS, Khanuja, SPS, Longo G, 2018). This way, the extraction process done by initial maceration is complemented, and an enriched liquid is obtained. It is often used to prepare tinctures or fluid extracts with high concentrations of active ingredients.

3.1.5.1.3 Digestion

To increase the efficiency of the extractive process, moderate heating is applied (40-60 °C) to the vegetal material/solvent system in a short period (4-6 h), avoiding thermal decomposition of the active ingredients. Water, hydro-alcoholic solution, ethanol, or other organic solvents are used. Frequently, poorly soluble components are extracted by digestion (Hussain MK, Saquib M, 2019).

3.1.5.1.4 Infusion

Boiling water is used as an extraction solvent, added to the herbal drug, and allowed to stand for a short period (5-20 min), and then the extract is filtered and collected. As a result, highly water-soluble (polar) components are obtained and used for aromatic plants with volatile metabolites (R. M. L. da Silva *et al.*, 2012).

3.1.5.1.5 Decoction

Unlike infusion, in decoction, the herbal drug and water (at room temperature) are placed together and then boiled for a defined time (5-20 min), proceeding to filtration and collection. A higher concentration of active compounds is achieved, but due to the increased temperature (approximately 100 °C), it is applied for extracting thermo-resistant compounds, and usually, hard vegetal materials are used (e.g., roots or bark) (Azwanida, 2015; R. M. L. da Silva *et al.*, 2012).

3.1.5.1.6 Soxhlet

All previously mentioned techniques are non-continuous processes: unless an amount of fresh solvent is added and extraction is repeated, it will not be exhaustive. Soxhlet extraction is a continuous and exhausting technique. In a Soxhlet apparatus, the drug is packed into the chamber of the equipment (Soxhlet extractor), usually in a cartridge. At the same time, the organic solvent (e.g., ethanol, acetone, ethyl acetate) is contained in a glass flask and submitted to heating. A condenser is added to prevent evaporation. A side tube leads the solvent vapor from the flask to the condenser, filling the cartridge and extracting the material. A second tube, called a siphon, connects the Soxhlet extractor to the flask and evacuates the liquid extract once its maximum level is reached, returning to the flask. After extraction, waste material and enriched extract are obtained. A lower amount of solvent is required to determine the weight of the drug. Only thermo-resistant

compounds that stay unaltered in the hot solvent for long can be extracted.

3.1.5.1.7 Liquid-liquid extraction

As a derivative of the funnel separation partition, the liquid-liquid extraction is a continuous technique in which the active ingredients are partitioned between an aqueous and an organic phase according to their log P using glass equipment. Usually, this is the next step for purifying a previously obtained extract (e.g., infusion), and a succession of solvents is used as their polarities increase: hexane, dichloromethane, ethyl acetate, and butanol. As a result, a group of metabolites, from non-polar to polar compounds, are selectively obtained in each fraction, which is useful for performing bio-guided assays.

3.1.5.1.8 Hydrodistillation

In EO extraction, special techniques are performed according to the volatile nature of the components. One of the most used in industry is hydrodistillation, which comprises three types of techniques: water distillation, water, and steam distillation, and direct steam distillation. The latter is the most used for producing EO on a large scale. The steam is generated in a boiler, separated from the plant material, that stands in a perforated grid. As the steam enters the inlet to the grid, extraction is exerted at a temperature not exceeding 100°C, so no thermal alteration of the components should occur. As advantages, the amount of steam is easily regulated, and a highly purified EO is obtained (Handa SS, Khanuja, SPS, Longo G, 2018)

3.1.5.2 Advanced techniques

3.1.5.2.1 Microwave-Assisted Extraction (MAE)

Microwave radiation is applied to the solvent-soaked drug, turning the electromagnetic energy into heat. Solvent penetration into the drug is facilitated by heating, and active metabolites are extracted. Microwave radiation generates a dipole rotation in molecules of polar solvents. Heating is provided when polar molecules try to align to a magnetic field direction (at 2450 MHz), and it is generated near the surface of the material, being transferred by conduction to the rest. As a disadvantage, only dielectric absorption is generated in non-polar solvents, and small heating occurs (Hussain MK, Saquib M, 2019).

3.1.5.2.2 Ultrasonication

In ultrasound-assisted extraction, high-frequency sound energy is applied (> 20 KHz) to the vegetal material/solvent macerate to promote the disruption of the plant cell wall. This accelerates the process by reducing the extraction time. An increase in permeability produces cavitation and facilitates the release of bioactive metabolites. Nevertheless, free radicals can be generated at the high frequency used and eventually degrade the components of the sample (Hussain MK, Saquib M, 2019).

3.1.5.2.2 Ohmic heating

An alternating electric flow is applied and is forced to pass through the sample, which must have electrical conduction. Otherwise, an electrolyte is added, like NaCl, or the use of an organic solvent is advisable. The movement of ions of the sample towards the electrodes of opposite charges produces an electrical resistant heating (Joule effect), and this thermal energy produces the extraction. As an advantage, heating is more homogeneous than other techniques (e.g., MAE), and undesired effects on the sample characteristics are minimal, especially in food products (Alkanan, Altemimi, Al-Hilphy, Watson, and Pratap-Singh, 2021).

3.1.5.2.3 Supercritical Fluid Extraction (SFE)

Some substances, when subjected to high pressure and temperature beyond their critical point, behave as supercritical fluids, having properties of both gas (vaporization) and liquid (solvating characteristics). SFE offers several benefits as the replacement of organic solvents (with no solvent residues) and an increment in extraction efficiency. CO_2 is often used to produce EO due to its low cost, safety, inertness, and availability, among other characteristics. In addition, the absence of oxygen minimizes oxidative reactions during conventional extractions. Nowadays, other areas using SFE are food and nutraceuticals production. The main limiting factor to adopting this technology is the important initial capital investment (Handa SS, Khanuja, SPS, Longo G, 2018; Hussain MK, Saquib M, 2019).

3.1.5.2.4 Counter-Current Extraction (CCE)

Soaked plant material is disintegrated to produce a fine slurry. This material moves in one direction through a cylinder forced by a pump.

Solvent flow is opposite to that of the slurry; active components are partitioned between two phases, and a concentrated extract is obtained at one end of the extractor while vegetal waste material is recovered on the other side. This technique has several goals, such as using a lower amount of solvents compared to traditional ones, the extraction is executed at room temperature, which is suitable for thermolabile compounds, and heat produced during pulverization is absorbed by water in the wet preparation. Additionally, it has been reported as more efficient and effective than continuous hot extraction techniques (Handa SS, Khanuja, SPS, Longo G, 2018).

Once the extract is obtained, the following step is solvent elimination, usually employing evaporation under reduced pressure, and a completely dry sample can be obtained by lyophilization.

3.2 DISCUSSION

Microorganisms, in general, like fungi, parasites, and bacteria, can affect human health, as well as animal and plant health. They can cause several diseases with a wide range of symptoms and consequences that can even lead to death. Infectious diseases currently affect an enormous proportion of the population, which translates into very high economic costs for public health care and, even more important, being the second leading cause of death in the world.

The appearance of antibiotics in the early 20th century represented a breakthrough in modern medicine. They significantly reduced morbidity and mortality caused by infections worldwide. However, the increasing antibiotic resistance of various pathogenic bacteria made the development of new antibiotics inevitable and urgent.

Even though various strategies exist to discover and develop new molecules with antimicrobial properties, no new molecules have been approved in the last 40 years. New technologies and the restoration and improvement of old technologies lead to different strategies for the discovery of new molecules effective against bacteria. The use of natural extracts, especially plant extracts, is a promising approach to finding bioactive compounds or even an extract or EO as a therapeutic agent.

In this review, we have highlighted recent advances in the field related to gram-positive bacteria, which are included in a 2017 alert from WHO. There is a wide range of results, from wild plants to herbs used in gastronomy, to byproducts of the wine industry, including wine itself.

We have reviewed conventional and advanced techniques used for producing plant extracts, where several methods have been improved. Much remains to be done in order to improve those methods with new technologies such as nanotechnology, biotechnology, and cellular and molecular biology to improve their ability to penetrate the cell wall, their activity, their targeting, and their delivery.

Replacing classical solvents with natural deep eutectic solvents would help to reduce environmental impact. The natural extracts are active, sustainable, environmentally friendly, and renewable, in line with the latest trends in industrial development. It is necessary to continue research, including the mechanism of action, activity at the molecular level, toxicity to the host cell, and clinical phases in humans.

4. CONCLUSIONS:

Humanity is facing an alarming situation due to antimicrobial resistance and the lack of fully effective treatments for bacterial infections. This applies to human health as well as veterinary and agricultural treatments. It is a global public health problem that currently affects all countries. There is no single solution, but multidisciplinary approaches are the way to go. Equally important is rapid and accurate diagnosis to find the right treatment. Most clinics have upgraded their equipment to molecular biology techniques, according to COVID, which allows for improved diagnosis.

Current literature shows that plant extracts and natural compounds have antibacterial activity and minimal effects on the host cell. They are also sustainable, environmentally friendly, and renewable in line with global trends. The regional economy can benefit from the added value of industrial processes such as wine and infusions production and juice production using byproducts of the manufacturing process itself.

The natural extracts and their bioactive components can be used alone or in combination with traditional treatments. In this sense, they must

all be validated *in vitro* and *in vivo* and successfully pass all 5 phases to be used in humans. Most plant-based antibiotics are still in the pre-clinical stage, either in the initial phase or *in vitro*, and some have already been tested in eukaryotic cells. In some cases, they have received the patent to be used(Guglielmi *et al.*, 2020).

Of the three bacteria gram-positive, *Staphylococcus aureus* is the most studied. While the extracts used have varied in success, artemisia and wine byproducts show the best results, even when used alone or in synergy with currently used antibiotics. *S. pneumoniae* has been tested the least with natural extracts. With fewer reports, *Enterococcus faecium* has been treated with extracts, and certain compounds present in wine have been identified as responsible for antibacterial activity. Although *Streptococcus pneumoniae* has not been extensively studied, a study shows the molecular and genetic mechanism involved in the activity of the use of thyme and origano EO.

Although in ancient times, medicinal plants were used applying rudimentary extraction methods, like teas and tisanes (infusions) or poultices, the revival of therapies based on natural products in the last century encouraged the development of different extraction technologies. A study of the factors that influence the extraction processes and the physicochemical properties of the bioactive components allows the selection of optimal methods for medicinal, aromatic, or edible species. Components like polyphenols or EO, which are often mentioned in the present work, represent a challenge for extraction, not only because of their low solubility but also because of their potential decomposition over time. Even when conventional methods offer products of high quality and reduced time of operation, innovative methods are sought after by pharmaceutical and food industries since similar or higher yields of active ingredients are obtained, harmless solvents are used, and they are in line with the profile of current consumers, who prefer greener options. However, due to the variables that affect the extraction process and, therefore, the product obtained, it is necessary to standardize both the processes and the content of active components in these mixtures in order to guarantee reproducibility in biological activity tests, with potential application in antimicrobial therapy (Jha & Sit, 2022).

Taken together, it is clear that a multiple approach is needed to identify and produce new molecules with antimicrobial activity. Collaboration between academia, small biotech startups, large pharmaceutical companies, and the national governments is the best way to find effective treatments for numerous infections that currently cannot be treated.

5. DECLARATIONS

5.1. Study Limitations

The study is limited to the selected bibliography.

5.2. Acknowledgements

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5.4. Competing Interests

The authors declare that they have no competing interests.

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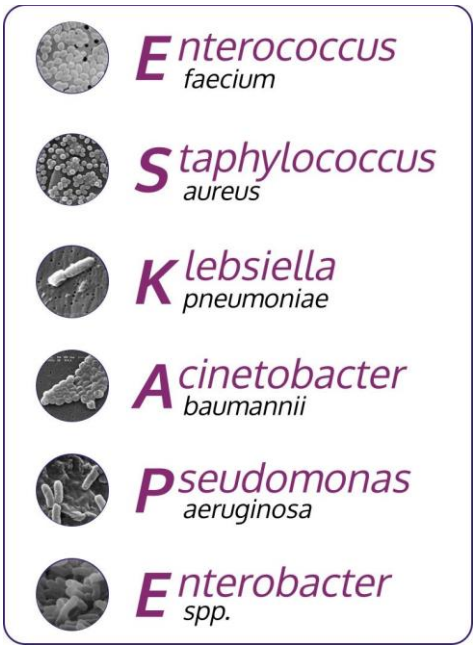

Figure 1- Antimicrobial-resistant ESKAPE pathogens. The group of pathogens responsible for several hospital infections with antimicrobial resistance in 2008.

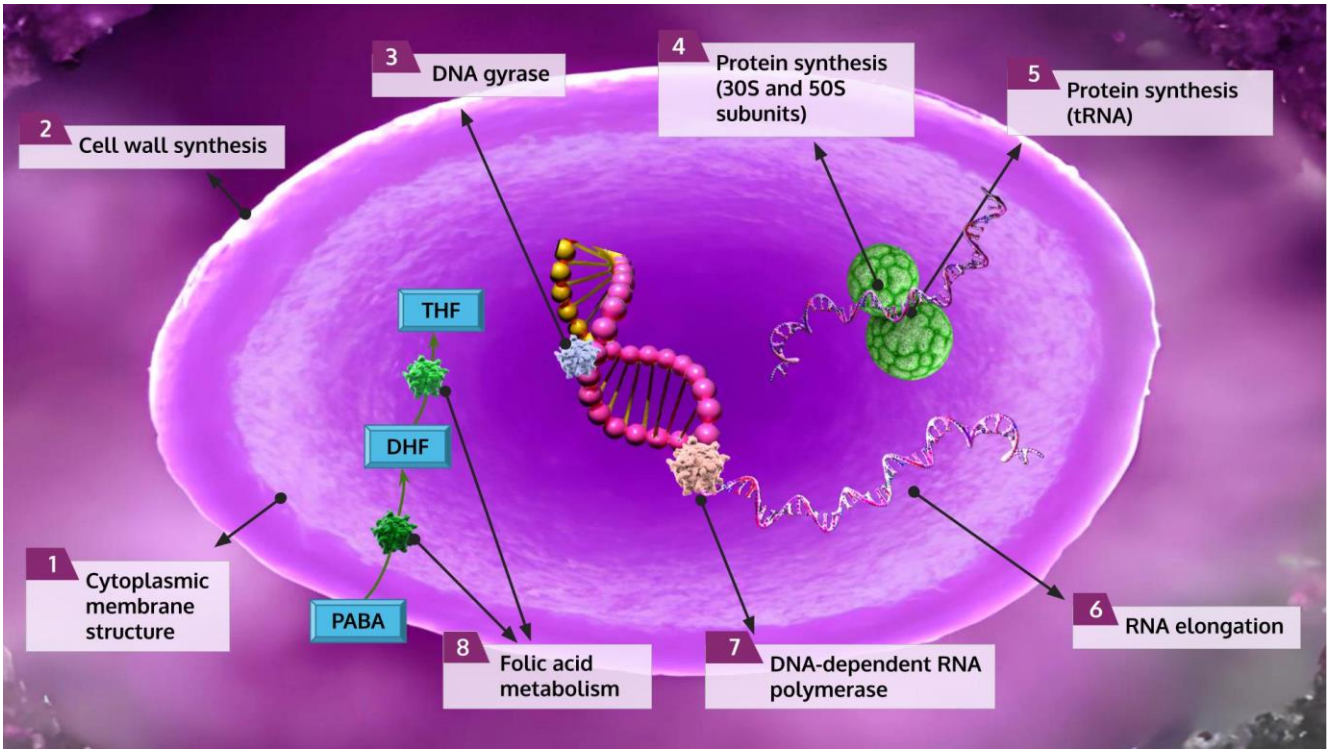

Figure 2. Action mechanisms of antibiotics

1: Changes in permeability or damage to the cytoplasmic membrane. 2: Inhibition of cell wall synthesis. 3: Inhibition of DNA gyrase. 4: Reversible inhibition of protein synthesis by subunit binding (30S or 50S). 5: Inhibition of protein synthesis by prevention of t-RNA binding to the A site. 6: Formation of a stable complex with DNA and RNA elongation prevention. 7: Inhibition of DNA-dependent RNA polymerase. 8: Inhibition of dihydropteroate synthase and dihydropteroate reductase

Figure 3. Raw material for extracts and essential oil preparation. The starting material for the extracts and EO is, in general, stems, leaves (fresh or dried), flowers and inflorescences, and for Vitis vinifera, seeds, fruit skin, whole fruit, and also byproducts of the wine industry as well as the wine itself.

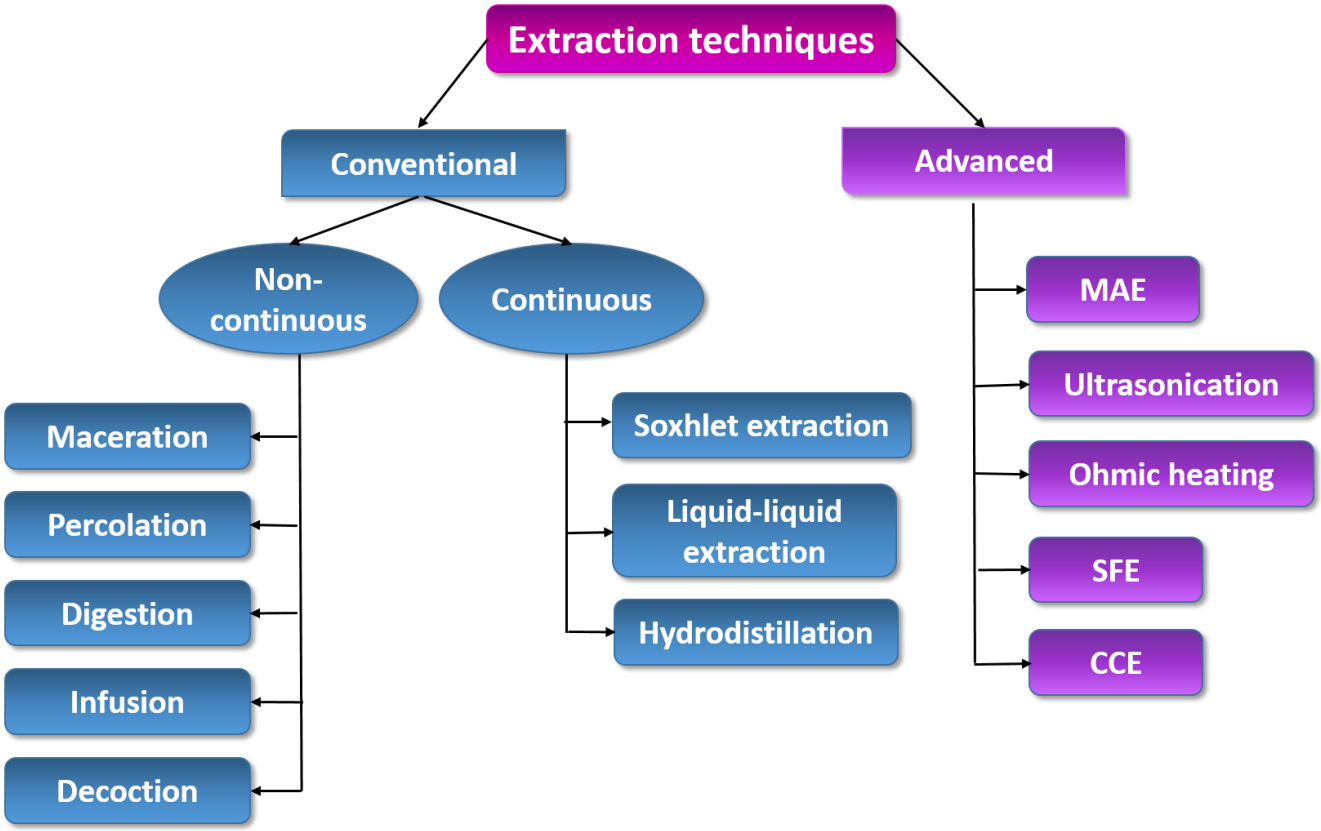

Figure 4. Schematic representation of extraction techniques. Traditional and advanced extraction techniques for isolation of bioactive metabolites from medicinal plants.

Table 1- WHO priority list of pathogens for antibiotics research

Priority	*Bacteria*	Resistance	Gram
Priority 1: CRITICAL	*Acinetobacter baumannii*	carbapenem-resistant	-
	Pseudomonas aeruginosa,	carbapenem-resistant	-
	*Enterobacteriaceae**	carbapenem-resistant, 3rd generation cephalosporin-resistant	-
Priority 2: HIGH	*Enterococcus faecium*	vancomycin-resistant	+
	Staphylococcus aureus	methicillin-resistant, vancomycin intermediate and resistant	+
	Helicobacter pylori	clarithromycin-resistant	-
	Campylobacter	fluoroquinolone-resistant	-
	Salmonella spp.	fluoroquinolone-resistant	-
	Neisseria gonorrhoeae	3rd generation cephalosporin-resistant, fluoroquinolone-resistant	-
Priority 3: MEDIUM	*Streptococcus pneumoniae*	penicillin-non-susceptible	+
	Haemophilus influenzae	ampicillin-resistant	-
	Shigella spp.	fluoroquinolone-resistant	-

*Enterobacteriaceae include: *Klebsiella pneumonia*, *Escherichia coli*, *Enterobacter* spp., *Serratia* spp., *Proteus* spp., and *Providencia* spp, *Morganella* spp

Table 2. Extracts and EOs used for the bacterial treatment. References: 1: artemisa, 2: jarilla, 3: oregano, 4: rosemary, 5: thyme, 6: wine, 7: yerba mate. +: used, -: not used

Bacterial strain	Extract-Essential Oil						
	1	2	3	4	5	6	7
Staphylococcus aureus	+	-	+	+	+	+	+
Enterococcus faecium	+	-	+	+	+	+	-
Streptococcus pneumoniae	-	-	+	-	+	-	-