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Antioxidant and Antimicrobial Activity of Plants from Portuguese Flora

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Resumo

Ao longo dos anos, as plantas têm desempenhado um importante papel na saúde, como recursos para a obtenção de fármacos. Porém, a biodiversidade existente faz com que as propriedades farmacológicas de muitas plantas sejam ainda desconhecidas. O trabalho tem como objetivo avaliar a atividade biológica *in vitro* de três plantas da flora portuguesa. Para tal, efetuou-se um estudo experimental com a obtenção de extratos aquosos (EA) e metanólicos (EM) de *Euphorbia paralias*, *Euphorbia hirsuta* e *Trifolium tomentosum.* Posteriormente, avaliou-se o conteúdo fenólico total (CFT), a capacidade de eliminação do radical DPPH e atividade quelante de iões de ferro, assim como a atividade antibacteriana contra *Escherichia coli* e *Staphylococcus aureus,* pelo método de difusão em disco e microdiluição em meio líquido. O género *Euphorbia* apresentou a maior capacidade em eliminar o radical DPPH (IC_{50} < 50 μg/ml). O EM de *E. paralias* exibiu o maior CFT (293.37 ± 20.25 mg GAE/g) e maior capacidade quelante. Este extrato, juntamente com o EM de *E. hirsuta* promoveram uma inibição moderada do crescimento de *S. aureus* (10 mg/ml) com valores de MIC de 1.0 e 2.0 mg/ml, respetivamente. Os resultados fornecem um conhecimento preliminar destas plantas, realçando as potencialidades inerentes ao género *Euphorbia*.

Palavras-chave: *Euphorbia*; *Trifolium*; antioxidantes; antibacterianos; fitoquímicos

Abstract

Through the years, we have seen the important role of plants in health, with valuable drugs being approved derived from phytochemicals. The diversity found in the plant kingdom makes that the properties of many medicinal plants remain unknown. Therefore, this research aims to evaluate the biological activity through antioxidant and antibacterial *in vitro* assays of three plants from Portuguese flora. It was prepared aqueous and methanolic extracts (AE and ME, respectively) from *Euphorbia paralias*, *Euphorbia hirsuta*, and *Trifolium tomentosum*. Their total phenolic content (TPC), capacity to scavenge DPPH radical, and ability to chelate iron ions were evaluated as well as their antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*, through disc diffusion and broth microdilution methods. *Euphorbia* species presented the higher radical scavenging capacity (IC_{50} < 50 μg/ml). The ME of *E. paralias* presented the highest TPC (293.37 ± 20.25 mg GAE/g) and also had the best performance in chelating iron ions. This same extract, along with ME of *E. hirsuta*, promoted moderate growth inhibition of *S. aureus* at 10 mg/ml with a MIC value of 1.0 and 2.0 mg/ml, respectively. Our findings provided preliminary knowledge about these plants and highlighted the potentialities of the *Euphorbia* genus.

Keywords: *Euphorbia*; *Trifolium*; antioxidants; antibacterial; phytochemicals

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Abbreviations, acronyms, and symbols

ABCB1	ATP-binding cassette sub-family B member 1
AE	Aqueous extract
BGI	Bacteria Growth Inhibition
CAT	Catalase
CFU	Colony-forming unit
CIP	Ciprofloxacin
DHPS	Dihydropteroate synthase
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
DW	Dry weight
EC_{50}	Half maximal effective concentration
EDTA	Ethylenediamine tetraacetic acid
EI	Ethnobotanicity index
ETC	Electron transport chain
g	Grams
GAE	Gallic acid equivalents
GEN	Gentamicin
GI_{50}	Half maximal inhibition of cell proliferation
GPx	Glutathione peroxidase
GSH/GSSG	Ratio of reduced glutathione to oxidized glutathione
H_2O_2	Hydrogen peroxide
HIV	Human immunodeficiency viruses
HO·	Hydroxyl radical
IC_{50}	Half maximal inhibitory concentration
ICA	Iron chelating activity
MDR	Multi-drug resistance
ME	Methanolic extract
mg	Miligrams
MHA	Müller-Hinton agar
MHB	Müller-Hinton broth
MIC	Minimum inhibitory concentration
min	Minutes
mL	Mililiters
mM	Milimolar
mm	Milimiter

NA	Nutrient Agar
Na_2CO_3	Sodium carbonate
NB	Nutrient broth
nm	Nanometers
NO·	Nitric oxide radical
O_2^-·	Superoxide anion radical
OECD	Organisation for Economic Co-Operation and Development
$ONOO^-$	Peroxynitrite
Q	Quercetin
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RSC	Radical Scavenging Capacity
SARS-COV-2	Severe acute respiratory syndrome coronavirus 2
SD	Standard deviation
SOD	Superoxide dismutase
spp	Several species
TPC	Total phenolic content
tRNA	Transfer ribonucleic acid
TRPV1	Transient receptor potential cation channel subfamily V member 1
UV/vis	Ultraviolet/visible
V/V	Volume/volume
WHO	World Health Organization
w/V	Weight/volume
µg	Micrograms
µM	Micromolar

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1. Importance of medicinal plants in health

Humans have long used naturally occurring substances as a source of food and for therapeutic purposes. Mainly, plants have played a leading medical role in most cultures (Beutler, 2009). The first written evidence of drugs' formulation using medicinal plants dates to the ancient Sumerian civilization (29th century BC). It comprised 12 recipes for drug preparation, referring to over 250 different plants. After that, the use of plants as health-promoting agents started to increase, following the natural evolution of societies, mainly due to the dissemination of knowledge between civilizations (Petrovska, 2012).

In the past, natural products were only applied based on empirical knowledge. Only at the beginning of the 19th century, plants began to be studied more closely to understand its medical use. Therefore, in 1804, Friedrich Sertürner discovered a drug, called morphine, from *Papaver somniferum* known as the opium poppy (Lockermann, 1951). After that, it began to emerge new researches to find bioactive compounds from natural sources.

It was in the early and mid-1990s that the panorama in pharmaceutical research changes with a slow decline in the investment of natural products. Therefore, if we analyse the natural-product pharmaceutical research in the years 1984–2003, results showed a period of increasing patent activity through the 1980s, a flattening or even slight decline from 1990 to 1999, and a pickup of activity between 2000–2003 (Koehn and Carter, 2005). This trend can be attributed to several factors: the establishment of high-throughput screening against defined molecular targets; the development of combinatorial chemistry allowing the discovery of new synthetic drugs, turning this procedure faster and economically valuable to the industry; lack of standardization procedures; and also the increase of competitiveness between pharmaceutical companies combined with strong regulations, once the results could be a compound not patented (Koehn and Carter, 2005; Schneider, 2018).

According to the World Health Organization (WHO), the global life expectancy reached 71.4 years in 2015, growing five years since 2000 (WHO, 2016) as a result of the progress in health systems worldwide. Life expectancy is now exceeding 80 years in most developed countries (Cho, 2017). Longer lives bring great opportunities that are very dependent on people maintaining good health into older age. However, health issues that confront more aged people are often associated with chronic conditions (DuGoff, Canudas-Romo, Buttorf, Leff, and Anderson, 2014). Consequently, aging has become a significant risk factor for several diseases, including cardiovascular disease, cancer, neurological disorders, diabetes, and obesity (Cho, 2017). So, it is crucial to the development of innovative drug discovery strategies, which includes the research of natural sources (Thomford et al., 2018). It is estimated that of the 300,000 plant species that exist in the world, only 15% have been evaluated to determine their pharmacological potential (De Luca, Salim, Atsumi, and Yu, 2012).

Cancer is a significant health problem in developing and developed countries (Kooti et al., 2017). Approximately 25% of all prescriptions contain one or more active ingredients from plants (Pan et al., 2013), and most of the anticancer drugs have been discovered from plant sources. Taxol is a clear example of a compound isolated from *Taxus brevifolia* in 1967, and, since then, it was subjected to various clinical trials showing great results as an antineoplastic agent in different types of cancers (Long, 1994). Currently, chemotherapy regimens include taxol, and it is still studied in combination with new formulations to improve cancer treatments (Suzuki et al., 2019). Similarly, salicylic acid was discovered from willow bark, which helps the development of acetylsalicylic acid, whose toxicity was lower. This compound maintains its antipyretic and analgesic effects and other prevention properties in cardiovascular disease and cancer (Desborough and Keeling, 2017; Drew et al., 2017).

Aging is a time-dependent decline in physiological organ function, and it is an essential factor in cancer development. People over the age of 65 years made almost two-thirds of all new cancer diagnoses (Hsu, 2016). In 2012, worldwide registered 14.1 million new cases and 8.2 million deaths (Ferlay et al., 2015), while in 2018, there were 18.1 million new cases and 9.6 million deaths (Ferlay et al., 2019). In the same year, breast, colorectal, lung, and prostate cancer represented half of the overall burden of cancer in Europe (Ferlay et al., 2018). Despite declining mortality rates for most cancer types globally, lung and liver cancer contradict these trends (Hashim et al., 2016), emphasizing the need to find alternative therapies. Therefore, natural compounds are a good source of molecules to be tested for its anticancer properties (Pereyra, Dantas, Ferreira, Gomes, and Silva-Jr, 2019).

Also, antioxidant molecules have raised much attention due to the many pharmacological properties (Ksouri et al., 2012). Reactive oxygen species (ROS) production is a natural process that occurs within cells, and during normal metabolism, ROS have physiological functions (Akanitapichat, Phraibung, Nuchklang, and Prompitakkul, 2010). When a disturbance in pro-oxidant/antioxidant balance emerges in favor of the pro-oxidant state, cell damages can occur (Valko, Rhodes, Moncol, Izakovic, and Mazur, 2006). If the cell is in good physiological condition, the antioxidant system is activated to neutralize ROS and to protect vital components (Sohn, Han, Lee, and Hwang, 2005). Attending to that, plants have long been a source of exogenous antioxidants because of their richness in bioactive compounds. It comprises mainly polyphenols (phenolic acids, flavonoids, anthocyanins, lignans, and stilbenes), carotenoids (xanthophylls and carotenes), and vitamins (vitamin E and C) (Baiano and Del Nobile, 2015; Manach, Scalbert, Morand, Rémésy, and Jiménez, 2004). For example, rosemary (*Rosmarinus officinalis*) is a clear example, with several studies revealing a good antioxidant activity and being a promisor anticancer agent (Bourhia et al., 2019; Moore, Yousef, and Tsiani, 2016). Species from *Trifolium* and *Euphorbia* genus are also good examples (Kolodziejczyk-Czepas, 2012; Salehi et al., 2019) with some compounds already identified and biologically tested (Kim et al., 2018; Zhang et al., 2017a). Since the discovery and subsequent isolation of ascorbic acid from plants (Szent-Györgyi, 1963), the antioxidant potential of plants has received

significant attention. Oxidative stress has been identified as a major factor in the development and progression of several diseases ranging from infection and inflammation to chronic cardiovascular, neurodegenerative, and metabolic disorders (Habtemariam, 2019).

Also important is the antibacterial drug resistance that currently threatens public health worldwide. It is noteworthy that in many WHO regions, the ratio of bacteria resistant to commonly used antibacterial drugs exceeded 50% for *E.coli, K. pneumoniae,* and *S. aureus* (WHO, 2014). This ineffectiveness is usually associated with its excessive use (Andersson and Hughes, 2010). Almost 30% of antibiotic prescriptions for outpatients were unnecessary in the United States (Fleming-Dutra et al., 2016). The Organisation for Economic Co-operation and Development (OECD) predicts that resistant microorganisms infections will be responsible for 2.4 million deaths in the next 30 years with an associated cost up to US$ 3.5 billion per year (OECD, 2019). Presently, multi-drug resistant (MDR) bacteria cause about 25,000 deaths in Europe each year (Cassini et al., 2019).

Hence, as the protection of antibiotic therapy is diminishing, new strategies are being studied to solve this public health problem (Ejim et al., 2011). Therefore, improvements can be made by providing new drugs to replace ineffective ones or prolonging the lifetime of current antibiotics (Annunziato, 2019). Naturally, plants have their defense mechanisms against pathogenic organisms. Following the assumption that humans pathogenic bacteria have a similar susceptibility to phytochemicals (Aruscavage, Lee, Miller and, LeJeune , 2006), plants can be of clinical value. Phenolic compounds and terpenoids produced by plants are showing promising results on the potentiation of antimicrobial activities (Zacchino et al., 2017). The potential antibacterial activity against several multidrug-resistant bacteria strains had already been observed in plants, including species of the *Euphorbia* genus (Hohmann et al., 2002; Voukeng, Beng, and Kuete , 2017), opening new perspectives to find more efficient and safe alternative therapies.

1.1. The role of antioxidants on the oxidative stress

When the antioxidant protective ability decay or the generation of reactive species increases, the result is a reduced capacity of the antioxidant system to fight against the oxidative attack, and cell damage can occur. At this stage, it is called oxidative stress (Valko et al., 2006).

Simultaneously to ROS generation, cells also produce reactive nitrogen species (RNS). Under aerobic conditions, most of the oxygen consumed is reduced directly to water by cytochrome oxidase in the electron transport chain (ETC), located in the inner membrane of mitochondria, without ROS release. Nevertheless, the primary source of ROS (over 90%) is from mitochondria in eukaryote cells. The electron escape from ETC may be the explanation whose interaction with molecular oxygen will form different types of ROS as superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^{\bullet}$) (Lushchak, 2014). In turn, the interaction of $O_2^{\bullet-}$ with free radical nitric oxide ($NO^{\bullet}$) causes the generation

of RNS such as peroxynitrite ($ONOO^-$), a powerful oxidant (Weidinger and Kozlov, 2015). Excessive production of reactive species could lead to the oxidation of essential biomolecules like proteins, lipids, and DNA with negative health consequences. For example, the lipid peroxidation of cell membranes is directly linked to mechanisms of neurodegeneration, cancer, cardiovascular or inflammatory diseases (Pisoschi and Pop, 2015).

Nevertheless, the presence of antioxidants can delay or prevent ROS/RNS action. "Any substance that delays, prevents or removes oxidative damage to a target molecule" can be the definition of an antioxidant (Halliwell, 2007). The endogenous antioxidant system is naturally present in the cell. It includes enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and others non-enzymatic substances like proteins, glutathione, and scavengers with low molecular weight (e.g., lipoic acid, uric acid, and coenzyme Q) (Poljsak, Šuput, and Milisav, 2013). The protective mechanism is activated by SOD, which converts $O_2^{\bullet-}$ to H_2O_2 that later is catalyzed by CAT along with glutathione represented by the reduce and oxidize form (GSH/GSSG). GPx competes with CAT for H_2O_2 as a substrate and is the primary source of protection against low levels of oxidative stress. GPx can also neutralize $ONOO^-$ (Tong, Chuang, Wu, and Zuo, 2015). Antioxidants may exert their effect on biological systems by different mechanisms, including electron donation, metal ion chelation, co-antioxidants, or by gene expression regulation (Krinsky, 1992) (**Figure 1**).

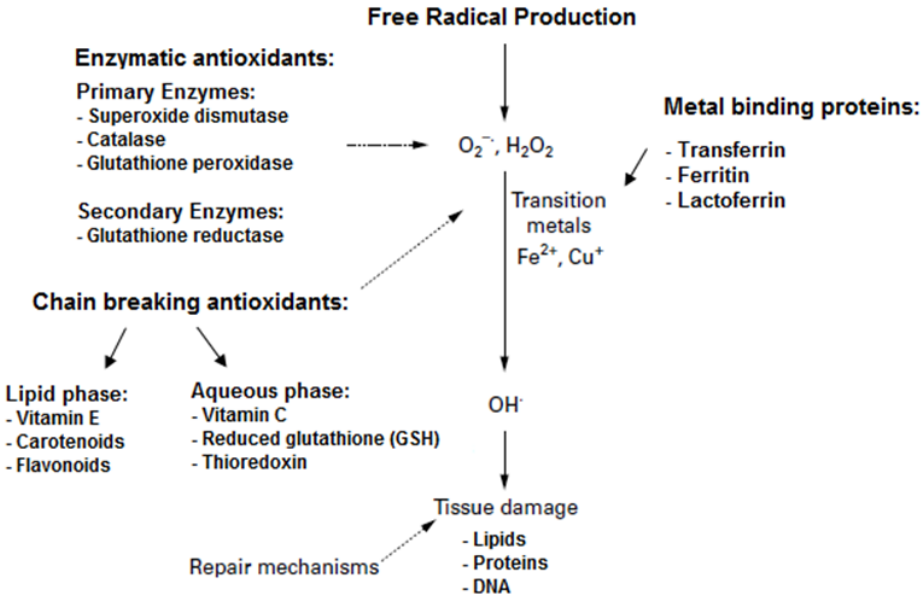

Figure 1. Antioxidant defenses against free radical attacks are divided into three main groups: antioxidant enzymes, chain-breaking antioxidants, and transition metal-binding proteins. Adapted from Young and Woodside, 2001.

Intake of exogenous antioxidants through dietary supplements proved to be valuable only when the oxidative stress is over normal or beyond the individual's stabilized level (Pisoschi and Pop, 2015). Its activity is often associated with the inhibition of radical chain reaction and oxidative enzyme, metal chelating, and antioxidant enzyme cofactors (Huang, Ou, and Prior, 2005).

Fruits and vegetables are a natural source of antioxidants and have a strong presence in human diet. In a cohort study where the relation between dietary factors and total mortality of more than 40 000 individuals was studied, it was found that reduced mortality was associated with high consumption of fresh fruit and vegetables (Agudo et al., 2007). Additionally, when male smokers combine antioxidants in their diet, lung cancer risk decreased (Wright et al., 2004). Medicinal plants proved to be also valuable sources of phytochemicals with antioxidant properties (Bourhia et al., 2019; Zhang et al., 2011).

However, some antioxidant compounds exhibit pro-oxidative actions under certain conditions like unbalanced intracellular condition, high antioxidant concentration and high O_2 levels (Ribeiro, Freitas, Silva, Carvalho, and Fernandes, 2018) . Quercetin is a flavonoid with great potential for medicinal applications. It has antioxidant properties by improving the endogenous glutathione production and quenching lipid peroxides caused by membrane degradation, but also pro-oxidant properties by inducing ROS generation followed by the sensitivity increase of the ovarian cancer cells to a specific treatment (Molina, Sanchez-Reus, Iglesias, and Benedi, 2003; Yi et al., 2014). These findings may create new opportunities for the development of new cancer therapies as well as in fighting the infection of multi-drug resistant bacteria once the increase of ROS generation leads to the attack of diverse targets of pathogens (Fang, 2011).

1.2. Drug-resistant bacteria

Antibiotic resistance occurs when a drug loses its ability to inhibit bacterial growth effectively. Therefore, bacteria become 'resistant' and continue to multiply in the presence of therapeutic levels of the antibiotics (Zaman et al., 2017). Antibiotic resistance is a growing health problem, with wide geographical variations between countries and regions of the same country. Antibiotic resistances are higher in southern and eastern Europe, where the consumption of these drugs is also higher compared to northern Europe. Portugal also observed this trend, and it is in primary health care that the most significant consumption of antibiotics occurs (Curto, Rosendo, and Santiago, 2019).

Worldwide, there are an estimated 700,000 deaths per year related to multidrug-resistant infections (Frieden, 2013; O'Neill, 2016). In Europe, the latest published data, relative to the year 2015, reported about 33,000 deaths, with the most significant impact on age groups under one and over 65 years old (Cassini et al., 2019). The same authors also reported that Portugal was the fourth country in the European Union and European Economic Space, with the most significant impact of these infections.

During the late 1950s and early 1960s, antibiotic resistance to multiple antimicrobial agents was detected, for the very first time, among enteric bacteria, namely *Salmonella*, *Shigella*, and *Escherichia coli*. Other antibiotic resistances were observed over the years, as seen in **Figure 2**.

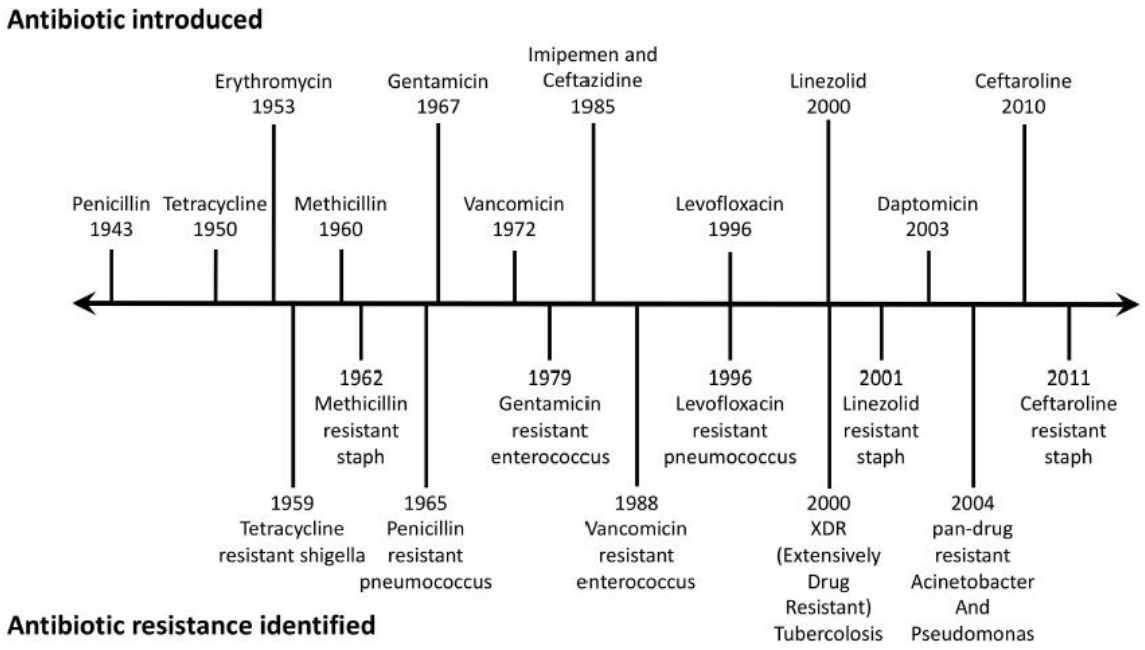

Figure 2. Antibiotic resistance pipeline (top: antibiotic introduced; bottom: antibiotic resistance identified) (Annunziato, 2019).

It is crucial to understand how antibiotics operate within bacteria cells before understanding what mechanisms are involving in the resistance process. Since the discovery of penicillin in 1928, other classes of antibiotics emerged with different targets giving to healthcare providers a broad spectrum of drugs to treat several types of infections. The main mechanisms of action of widely used antibiotics comprise (**Figure 3A**):

(1) Inhibition of cell wall synthesis mainly promoted by β-lactams antibiotics group, including penicillin and cephalosporins, which are responsible for inhibiting the transpeptidation cross-linking step of cell wall precursor, the peptidoglycan (Kong, Schneper, and Mathee, 2010);

(2) Inhibition of protein synthesis that is carried out by members of the family of macrolide antibiotics where erythromycin belongs and cause dissociation of tRNA from the ribosome 50s subunit (Tenson, Lovmar, and Ehrenberg, 2003);

(3) Inhibition of DNA/RNA synthesis with quinolone antibiotics being the most representative class once its target is topoisomerase IV and DNA gyrase, which are essential enzymes on the DNA replication process of bacteria (Fàbrega, Madurga, Giralt, and Vila, 2009);

(4) Inhibition of folate biosynthesis, whose action affects pathways of required precursors for cellular functions synthesis due to the link between dihydropteroate synthase (DPHS), an enzyme unique of prokaryotes once they cannot acquire folate from the environment, and sulfonamide antibiotics, leading to a downregulation process (Bourne, 2014).

However, bacteria can occasionally acquire resistance genes and transmit it to other bacterial communities living in the environment, through horizontal transfer genes, and whose phenomenon is strongly correlated with the anthropogenic activity (Boto, Pineda, and Pineda, 2019). There are multiple antibiotic resistance mechanisms in bacteria (**Figure 3B**) which include:

(1) Changes in the expression of genes for the efflux pump (Abdi et al., 2020). These proteins, which can extrude antibiotics out of the cell, are overexpressed by the bacteria to extrude the antibiotic. It is an essential mechanism of resistance in *P. aeruginosa* and *Acinetobacter* spp (Annunziato, 2019).

(2) Decreased uptake by changes in the outer membrane permeability mainly affecting porins, whose function is to allow molecules diffusion (Pagès, James, and Winterhalter, 2008).

(3) Enzymatic inactivation where bacteria produce enzymes that chemically modify or degrade antibiotics and disable the drugs. The most significant examples are beta-lactamase enzymes, which hydrolyze beta-lactams (penicillins, cephalosporins) (Annunziato, 2019).

(4) Modification of the drug target, which prevents the binding of the antibiotic and limits its potency (Annunziato, 2019).

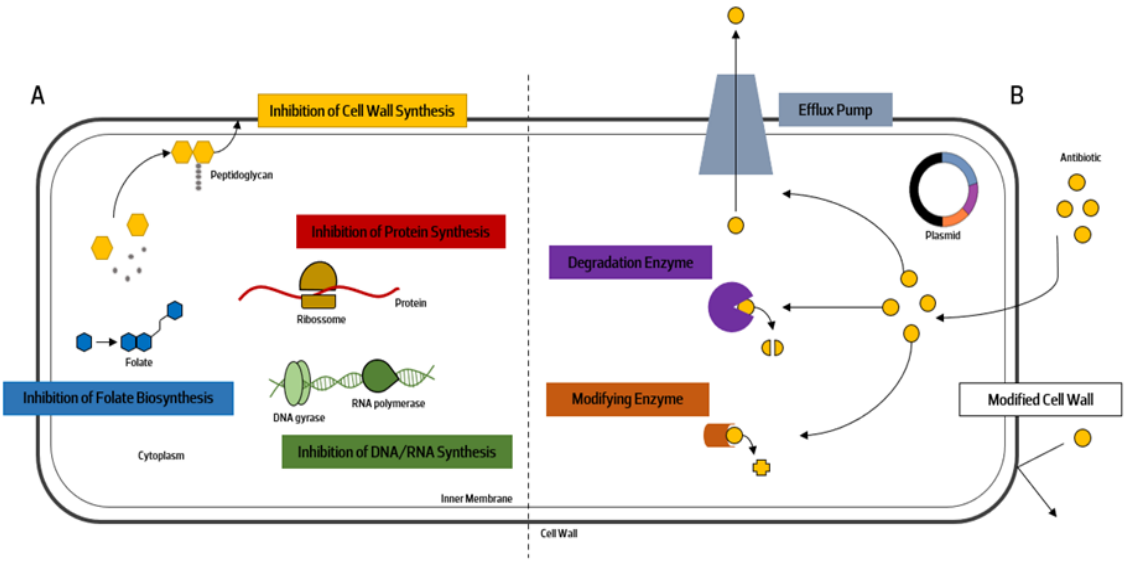

Figure 3. Mechanisms of action of widely used antibiotics (A) in bacteria as also as its multiple antibiotic resistance mechanisms (B).

In 2017, WHO publishes a list of bacteria for which new antibiotics are urgently needed. This list is divided into three categories based on mortality, level of resistance, and treatability: critical, high, and medium priority (Annunziato, 2019).

The most critical group of all includes multidrug-resistant bacteria like *Acinetobacter*, *Pseudomonas,* and various Enterobacteriaceae (including *Klebsiella*, *E. coli*, *Serratia*, and *Proteus*), which have become resistant to a large number of antibiotics, including carbapenems and third-generation cephalosporins. Bacteria included in the high and medium priority categories can cause more common diseases such as gonorrhoea and food poisoning caused, for example, by salmon (WHO, 2017)

2. Biodiversity and Portuguese Flora

Portugal is located on the Iberian Peninsula in southwestern Europe. Most of its land falls within the Mediterranean macrobioclimate, except the north, which fits into the temperate macrobioclimate. This categorization is made following a bioclimatic classification system where climates, biomes, and biogeographical regions were considered, covering a wide territorial range. Within each macrobioclimate, there are different bioclimates represented by biological communities, ecoregions, and vegetation types, which are divided again by climatic conditions with specific plant formations and communities (Rivas-Martínez, Penas, Del Río, González, and Rivas-Sáenz 2017).

According to the bioclimatological map made by Monteiro-Henriques et al. (2015), Portugal has various types of climatic conditions that reflect the diversity of plants. In Portugal (excluding Madeira and Açores), there are 2844 species which represent 45.3% of the total Iberian flora, and 54% of the catalogued European plant species belong to the Iberian Peninsula (Aedo, Buira, Medina, and Férnandez-Albert, 2017). The vegetation is mainly Mediterranean in terms of both its structure and floristic composition (Pereira, Francisco, and Porto, 2016). In Portugal, the lack of information on vascular plant distribution in the territory led to the creation of an online platform by members of the Botanical Society of Portugal called Flora-On. Collaborators make the data input through merely observations or fieldwork as part of other externally funded projects, and the dataset is only provided upon request of the Botanical Society of Portugal, subjected to approbation. The identification of plant species is on the responsibility of the collaborator. However, most of them are experts in plant identification, and the taxon nomenclature is fully controlled through the use of an updated version of a reference checklist, avoiding spelling errors and outdated synonyms (Pereira et al., 2016).

Despite the presence of healthcare professionals, two-thirds of the population living in portuguese rural areas use folk medicine as their first resource in case of illness because of its cost-effectiveness, its perception that natural products are safe, and present fewer adverse reactions (Nunes and Esteves, 2006). The amount of medicinal plants that are beneficial for a population is given by the ethnobotanicity

index (EI), which is the ratio between the number of useful medicinal species reported and the total flora in the area, expressed as a percentage. In Trás-os-Montes, located in the north of Portugal, 16% of the plants are valuable in folk medicine (Neves, Matos, Moutinho, Queiroz, and Gomes, 2009). According to the same study, the mean of EI within the Iberia Peninsula is about 10%, which means that this value is high. However, Serra de São Mamede, situated in the inner part of the Alto Alentejo province, is the place where it was observed one of the highest EI values in the entire Iberia Peninsula (21.3%). It means that in every four plants of the flora founded, one is associated with medicinal properties (Camejo-Rodrigues, Ascensão, Bonet, and Vallès, 2003). This evidences also highlight the richness of the Portuguese flora and reinforce the need for more studies since many medicinal plants have not yet been extensively studied. Some of these plants belong to the genus *Euphorbia* (e.g., *Euphorbia paralias* and *Euphorbia hirsuta*) and *Trifolium* (e.g., *Trifolium tomentosum*).

2.1. *Euphorbia* genus

Euphorbia is the largest genus of the Euphorbiaceae family, including about 2000 species, and one of the most diverse genera of flowering plants. Its diversity makes the colonization of different environments, such as deserts, dunes, mountains, calcareous slopes, among others, possible (Salmaki, Zarre, Esser, and Heubl, 2011). More than 5% of species of *Euphorbia* are used in traditional medicine to treat digestive system disorders, skin ailments, infections, respiratory disorders, and inflammatory conditions (Ernst et al., 2015; Salehi et al., 2019). Depending on the desired effect, different parts of these plants can be used like roots, seeds, latex, wood, barks, and whole plant (Salehi et al., 2019).

Euphorbia plants are easily discernible by their toxic and highly skin irritant milky latex and particular inflorescences, designated as cyathia. The latex is the most valuable product obtained from *Euphorbia* species because, despite being toxic, it contains several biologically active compounds such as triterpenoids (Salehi et al., 2019).

In Portugal, the most recent update of *Euphorbia* species occurrences revealed at least 32 species belonging to this genus (Sociedade Portuguesa de Botânica, n.d.-a). In some of them, its latex has been used as medicinal agents by local populations, mainly for skin problems (warts and callus) (Camejo-Rodrigues et al., 2003; Gaspar et al., 2002; Vinagre, Vinagre, and Carrilho, 2019). Since the beginning of the seventies, the interest of the scientific community for this genus has increased over the years (**Figure 4**).

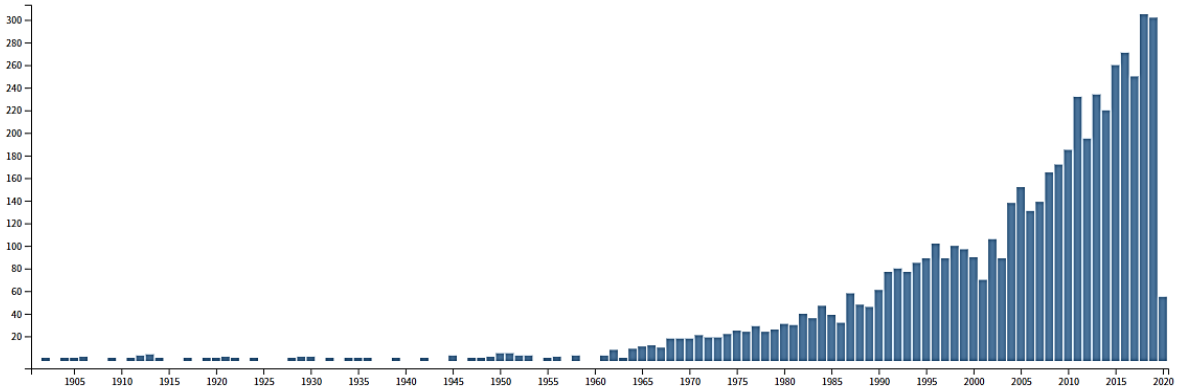

Figure 4. Number of publications per year on the online repository Web of Science (www.webofknowledge.com) with the search term "*Euphorbia*" with data from 1900 until the present.

2.1.1. Bioactive compounds and its biological activities

The secondary metabolism of *Euphorbia* species produces numerous phytochemicals with therapeutic potential, which include terpenoids and phenolic compounds (Salehi et al., 2019).

Terpenoids are a large class of secondary metabolites and consist of a modified type of terpenes (hydrocarbons) with different functional groups and whose classification depends on its carbon units. In *Euphorbia* plants, diterpenoids (compounds with 20 carbons units) are structurally variable due to the assortment of carbon skeletons (jatrophane, lathyrane, tiglianes, ingenane, myrsinols, among others), functional groups, and stereochemical complexity (Vasas, Rédei, Csupor, Molnár, and Hohmann, 2012). Triterpene alcohols found in *Euphorbia* species latex have been used as chemotaxonomic markers. Besides, sesquiterpenoids, phloracetophenones, cerebrosides, glycerols, flavonoids, and steroids were also obtained from the plant (Shi, Su, and Kiyota, 2008). The extracts and compounds isolated from the genus *Euphorbia* have shown different activities, including antiproliferation, mouldability of multi-drug resistance (MDR), cytotoxic, antimicrobial, and anti-inflammatory activity (Shi et al., 2008).

Over the years, attention has been given to the jatrophane and lathyrane diterpenes, biogenetic precursors of the polycyclic diterpenes, because of its essential biological activities (e.g., cytotoxic and MDR reversing action (Hohmann et al., 2002; Zheng, Cui, and Zhu, 1998).

Several lathyrane-type diterpenoids, including the jolkinols (compounds 1 and 2), 17-hydroxyjolkinols (compound 12), and lathyrols (compound 17), demonstrated great results as a cytotoxic agent against four cancer cell lines MCF-7 (human breast adenocarcinoma), C6 (rat brain glioma), HeLa (human cervix adenocarcinoma) and HepG2 (human hepatocellular carcinoma). Compounds 1, 7, 12, and 17 exhibited moderate cytotoxic activities against MCF-7 (IC_{50} 12.4–23.1 μM) and weak cytotoxicity against C6 (IC_{50} > 20 μM). Also, all tested compounds were inactive against the HeLa and HepG2 cell lines at 50 μM (Lu et

al., 2014). In another study, a jatrophane-type diterpene (esulatin M) isolated from *E. welwitschii* acted as an ABCB1 modulator and MDR-selective antiproliferative compound (Reis et al., 2016).

Phorbol esters were also isolated from *Euphorbia* species, and some compounds inhibited the growth of cancer cells. Xu et al. (2013) extracted 12-deoxyphorbol 13-palmitate from the roots of *E. fischeriana,* which inhibited the growth of BGC823 human gastric cells in a dose- and time-dependent manner. The growth inhibition ranged from 50 to 100%, with 40–80 μg/mL of the drug from 24–72 h (Xu et al., 2013). Also, five tigliane-type diterpenoids, including prostratin, fischeroside A, fischeroside B, fischeroside C, and 12-deoxyphorbol-13,20-diacetate isolated from *E. fischeriana*, were tested against T-cell line C8166. Prostratin exerted the most robust anti-HIV-1 activity, with an EC_{50} of 0.00006 μM (Pan et al., 2011).

Resiniferatoxin, a diterpene found in several members of the genus *Euphorbia* (e.g., from the latex of *E. resinifera* and *E. bicolor*) showed to act as an analgesic agent with different applications, for example, to treat several chronic pain disorders (Kissin and Szallasi, 2011) and it also has potential to improve the prognosis of infected patients with SARS-COV-2 once there are numerous TRPV1 receptors (resiniferatoxin target) in lungs cells which are responsible for pain transmission, immunomodulation and inflammation that, when antagonized, could lead to better outcomes and decrease elderly mortality (Nahama, Ramachandran, Cisternas, and Ji, 2020).

Phenolic compounds, which have one or more phenolic units, deprived of nitrogen-based functions and derived from shikimate and/or polyketide pathway, are also present in *Euphorbia* species. Various flavonoids were already identified in the *Euphorbia* genus like quercetin, kaempferol, rutin, and derivates (Noori, Chehreghani, and Kaveh, 2009). Both quercetin, known as a potent antioxidant, and kaempferol demonstrated to have excellent activity against microorganisms, which explain the facilitation of the wound-healing process observed in the presence of quercetin 3-*O*-glucoside and its derivates (Özbilgin et al., 2018; Singh and Kumar, 2013). Additionally, ellagitannin showed antifungal potential, and euphorbin exhibited antiallergic activity (Ascacio-Valdés et al., 2013; Kanoh, Hatano, Ito, Yoshida, and Akagi, 2000).

2.1.2. Toxicity

Considering all biological activities described for *Euphorbia* species, it is expected to cause acute toxicity in higher dosage. In Texas Poison Center Network, during 2000-2018, 60% of the reported adverse clinical effects of *E. tricucalli* exposure were ocular, following by dermal (14%) and gastrointestinal (12%) (Forrester, Layton, and Varney, 2020). Also, a patient reported extreme eye pain when exposed to *E. lathyris* latex (Ioannidis, Papageorgiou, and Andreou, 2009). However, other adverse effects were related to this genus. For example, there was an association with the consumption of *E. geniculata* by the cattle and its weakness and reduction in milk yield, as well as gastrointestinal irritation when they were fed with *E. hirta* (Bhatia, Manhas, Kumar, and Magotra, 2014). Kansui, the root of *E. kansui,* is considered a toxic

plant, causing severe skin, oral, and gastrointestinal irritation, hepatic injure, and tumor-promoting toxicity (Shen et al., 2016). Also, metabolomic analyses of rats' urines revealed significant changes of metabolites linked to liver and kidney damages and disparity of intestinal microbiome after being exposed for several days with *E. kansui* extract (Liu et al., 2013).

Ingestion of *E. paralias* can lead to renal toxicity. In 2013, it was reported a case of a 29 years-old man whose familial history of nephropathy was inexistent and suffered acute renal failure after ingesting the boiled plant as part of folk medicine to treat edema (Boubaker et al., 2013). The authors suggested that the acute renal failure may be associated with irritant substances presented in the latex of the plant, which is so characteristic of this genus.

Studies that relate diterpenes structure and irritant activity in *Euphorbia* species demonstrated that the presence of hydroxyl on C-20 is crucial for stimulation properties (Marston and Hecker, 1983; Seip and Hecker, 1982), and ingenane-type diterpenes with 3-unsaturated aliphatic chain possessed more vigorous cytotoxic activities which decreased when methyl and phenyl groups replaced the 3-unsaturated aliphatic chain (Zhang et al., 2012).

2.1.3. *Euphorbia paralias*

E. paralias has been poorly explored. Commonly known as "sea spurge", it is a hardy perennial plant that dwells sandy seashore (**Figure 5A**) and can be seen all over the Portuguese coastline, as supported by the distribution map on **Figure 5B**. Surveys of traditional applications of medicinal plants in Italy found that fishers commonly use the latex of this plant as an anesthetic against bites of weever fish as well as there are shreds of evidence of its use to treat edema and as purgative (Boubaker et al., 2013; Guarrera, 2005).

Phytochemicals have been found in *E. paralias* with considerable biological activity. Abdelgaleil, Kassem, Doe, Baba, and Nakatani (2001) established the structure of several diterpenoids from the aerial parts of *E. paralias,* with some of them showing moderate insect anti-feeding and antiviral activity. Regarding antiviral activity against HIV-1 replication, only a diterpene paraliane showed a moderate activity (EC_{50} = 14 μg/ml) (Abdelgaleil et al., 2001). Also, acetone extract of *E. paralias* seeds demonstrated modest larvicidal activity (Hamad et al., 2019).

The apoptotic and antiproliferative effects of *E. terracina* and *E. paralias* extracts were investigated on human acute myeloid leukemia (THP1) and colon epithelial cancer (Caco2) cell lines, as well as on CD14+ normal monocytes and normal rat intestinal cell line IEC6. Results highlighted the presence of flavonoids and terpenoids in *Euphorbia* fractions, supporting some of the results observed in the study. THP1 was the most sensitive to the polar fraction of *E. paralias,* and no effect on normal monocytes viability was seen. Results also showed that polar fractions of both *Euphorbia* species have significant contents of saponins, which may explain, in part, the potent, selective cytotoxicity observed (Jannet et al., 2017).

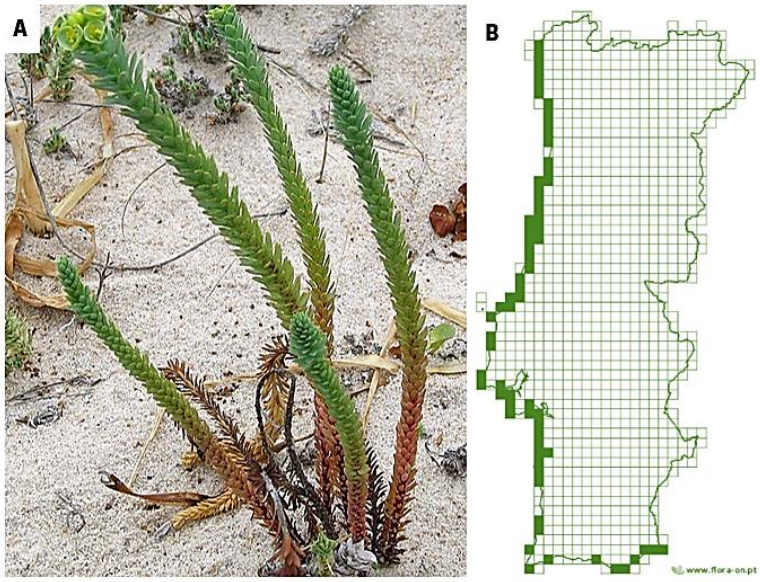

Figure 5. **(A)** Photograph of *Euphorbia paralias*. Reprinted from Almeida (n.d.). Copyright 2012–2020 by Creative Commons. **(B)** Distribution map of *Euphorbia paralias* in Portugal made with 217 observations by different Flora-On collaborators (Carapeto et al. 2020)

Many plants, including *E. paralias*, may offer a safe alternative for the discovery of potent antimicrobial agents and act as new anti-infective drugs. A clear example is a study performed by Hlila et al. (2017), where they observed more significant antimicrobial activity of *E. paralias* with chloroform extract than acetonic extract. Also, the methanolic fraction of the plant was the most active against *Mycobacterium* spp. when compared with other fractions, which was associated with the presence of the flavonoid quercetin-3-O-β-glucoside. According to the authors, this extract proved to be safe up to 10 g/Kg of body weight in albino mice (Safwat, Kashef, Aziz, Amer, and Ramadan, 2018).

2.1.4. *Euphorbia hirsuta*

Regarding *E. hirsuta*, which is the accepted name of the species in the genus *Euphorbia*, there is no information about it on scientific databases. Nevertheless, after seeking for "*Euphorbia pubescens* vahl", a synonym of the accepted name *E. hirsuta*, fewer results appeared (Govaerts, n.d.).

E. pubescens is a plant distributed in Portugal near banks of streams and rivers, whose chemical characterization has not been thoroughly investigated (Valente et al., 2003). It is a perennial, multi-tailed plant, more or less velvety (**Figure 6A**). **Figure 6B** represents its geographic distribution in Portugal. In Spain, its traditional use is associated with skin pathologies (González-Tejero et al., 2008).

About its phytochemicals, Valente et al. (2003) isolated three macrocyclic jatrophane diterpene polyesters, named pubescenes A, B, C; indole-3-aldehyde, and scopoletin. Other compounds were also isolated like pubescenes D (Valente et al., 2004a), pubescenol (jatrophane diterpene), helioscopinolide A

and B (two *ent*-abietanolide derivatives) (Valente et al., 2004b), euphopubescenol and euphopubescene (jatrophane diterpenes) (Valente et al., 2004c).

Pubescenes A, B, C showed moderate growth inhibitory effect on the human lung cancer NCI-H460 with GI_{50} values (concentrations that cause 50% inhibition of cell growth) of 31.7 ± 2.4 μM, 18.8 ± 2.5 μM, and 33.3 ± 5.9 μM, respectively. No capacity to inhibit cell growth of the human cancer cell lines MCF-7 and SF-268 (human glioblastoma) was observed with these compounds even at 50 μM (Valente et al., 2003).

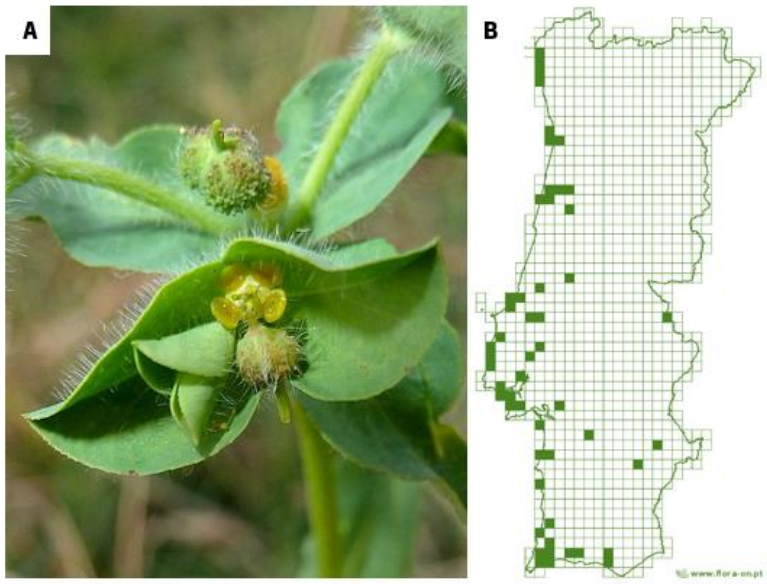

Figure 6. (A) Photograph of *Euphorbia hirsuta*. Reprinted from Farminhão (n.d.). Copyright 2012-2020 by Creative Commons. **(B)** Distribution map of *Euphorbia hirsuta* in Portugal made with 80 observations by different Flora-On collaborators (Clamote et al. 2020a),

Isolated polyester and lathyrane derivates diterpenes promoted moderate antiproliferative activity against specific cancer cell lines (MCF-7; NCI-H460; SF-268), supporting the idea of a tumor type-specific sensitivity of these compounds. In this study, euphopubescene exhibited the best result on the growth of human cancer cell lines with a GI_{50} value of 40.9 ± 0.8 μM for NCI-H460 (Valente et al., 2004c).

Moreover, it was found that some macrocyclic jatrophane diterpene polyester, named pubescenes, exhibited significant effects reversing multi-drug resistance on mouse lymphoma cells (Valente et al., 2004a).

Finally, pubescene D showed the best GI_{50} values in the inhibition of the growth of MCF-7 (37.50 ± 3.06 μM) and NCI-H460 (37.50 ± 0.65 μM). Helioscopinolide A and B also exhibited significant activity against *Staphylococcus aureus* (2.5 μg/spot) (Valente et al., 2004b).

2.2. *Trifolium* genus

Trifolium genus belongs to the sizeable Fabaceae family, which includes over 250 annual and perennial species and are known as clovers due to its trifoliate leaves with 5 to 9 leaflets. It is usually found in humid environments (Sabudak and Guler, 2009). Surveys of the traditional use of these species were already performed in distinct parts of the world. For example, in India, the seeds are eaten to treat dyspepsia, and the powdered of the whole plant is used in the form of tea as a therapy against dry cough, spasms, or dermatitis (Kumar, Sharma, Manhas, and Bhatia, 2015). *Trifolium repens* is the most important plant to treat circulatory diseases. In Iraq, *T. alexandrium* is used to cure colic, and in Italy, *T. phleiodes* is used as a diuretic, both prepared through decoction methods (Ahmed, 2016; Tuttolomondo et al., 2014).

As state by Sociedade Portuguesa de Botânica (n.d.-b), currently, there are 45 species of *Trifolium* in Portugal. Notably, there are records of the use of *T. angustifolium* to treat diarrhea in two different regions (Camejo-Rodrigues et al., 2003; Gaspar et al., 2002).

For a long time, clovers were out of scientific interest as a source of bioactive compounds despite ethnomedicinal evidence and agricultural value. Nevertheless, the trend changed due to the identification of several biological actions with potential applications as a nutraceutical, dietary supplements, and plant-based drugs (Kolodziejczyk-Czepas, 2016).

2.2.1. Bioactive compounds and their biological activities

The majority of the existing *Trifolium* species have not been phytochemically characterized. From the chemical analysis of the aerial parts of 57 *Trifolium* species, it was found a high content of total phenolics in several species, such as the phenolic acids and flavonoids (the most common compounds), followed by isoflavones and clomavide (Oleszek, Stochmal, and Janda, 2007). Quercetin and kaempferol derivates flavonoids were identified in *T. repens* (white clovers) (Kicel and Wolbis, 2012), while in numerous *Trifolium* seeds were found compounds like soyasponin I, II, and III (Oleszek and Stochmal, 2002).

Regarding the studies of the biological properties of clovers, the major ones are related to the phytoestrogenic action of the *T. pratense* (red clovers). Numerous studies demonstrated the benefits of this plant to treat menopause-related disorders. For instance, improvements in bone density, tissue integrity, and vaginal blood flow were observed in rabbits with surgically-induced menopause after 12 weeks of treatment with red clover isoflavones (Adaikan, Srilatha, and Wheat, 2009). In another study, the content of mineral bone, the strength of the tibia and femoral mass and density increased in a rat model of osteoporosis following 14 weeks of treatment with red clover isoflavones (Occhiuto et al., 2007). In a randomized clinical investigation with postmenopausal women, results showed a significant decrease in depressive and anxiety symptoms for those who were treated with red clover isoflavones (Lipovac et al.,

2010), revealing an alternative to the conventional treatment with synthetic estrogens. Formononetin is an isoflavone present in red clover extract and plays a role in cancer pathophysiology (Kim et al., 2018). Another isoflavone, biochanin A, exhibited anticancer, antioxidant, and anti-inflammatory activities, among others (Raheja, Girdhar, Lather, and Pandita, 2018).

Besides the most widely cultivated clovers *T. pratense* (red clover) and *T. repens* (white clover), other *Trifolium* plants such as *T. hybridum* (alsike clover), *T. fragiferum* (strawberry clover), *T. resupinatum* (Persian clover), *T. incarnatum* (crimson clover), *T. alexandrinum*, and *T. subterraneum* (subterranean clover) are known (Kolodziejczyk-Czepas, Krzyzanowska-Kowalczyk, Sieradzka, Nowak, and Stochmal, 2017). Moreover the *Trifolium* species synthesize important compounds like flavonoids, saponins, clovamides (caffeic acid esters), and other phenolic compounds (Oleszek et al., 2007; Oleszek and Stochmal, 2002).

In its work, Kolodziejczyk-Czepas et al. (2013) studied the effects of extracts from *T. pallidum* (phenolic fraction and clovamides fraction) and *T. scabrum* (phenolic fraction) on the functions of human blood platelets, *in vitro*. Extracts from both plants revealed antiplatelet properties. However, phenolic fractions from *T. pallidum* and *T. scabrum* had more substantial antiplatelet properties (measured by the platelet adhesion and aggregation) than the clovamide fraction from *T. pallidum* (Kolodziejczyk-Czepas et al., 2013).

Sabudak, Ozturk, Goren, Kolak, and Topcu (2009) showed the potential antioxidant activity of hexane extracts of five *Trifolium* species (*T. balansae*, *T. stellatum*, *T. nigrescens* subsp. *petrisavi*, *T. constantinopolitanum*, and *T. resupinatum* var. *resupinatum*). In DPPH assay, relatively better inhibitions were seen for *T. stellatum* and *T. constantinopolitanum* oils, with % inhibition values of 21.05 ± 0.25 and 27.22 ± 0.86 for the concentration of 100 μg, respectively (Sabudak et al., 2009).

Also, the hepatoprotective effects of *T. alexandrinum* extract were noticed in rats' acetaminophen-induced hepatotoxicity (Sakeran, Zidan, Rehman, Aziz, and Saggu, 2014). Also, non-polar and polar extracts of *T. alexandrinum* inhibited the growth of 18 bacteria strains with the most potent antibacterial activity promoted by polar extracts (Khan, Ahmed, Shukla, and Khan, 2012). Likewise, saponin-rich extracts of three *Trifolium species* seemed to inhibit the formation of germ tubes and the invasive capacity of the fungus *Candida albicans* (Budzyńska et al., 2014).

2.2.2. Toxicity

In the literature, there is a lack of toxicological reports of *Trifolium* species in humans. As was mentioned before, red clover is the most studied species of the genus, and controlled trials were already performed to evaluate its safety and efficacy. A phase I trial of standardized red clover extract with three isoflavone doses (40 mg, 80 mg, and 120 mg) showed no evidence of acute toxicity or adverse effects and confirmed

the short-term safety of the formulation (Piersen et al., 2004). On the other hand, another clinical trial with a standardized ethanolic extract of red clover (120 mg isoflavones) was found to be safe for 12 months (Geller et al., 2009).

A case report of a 52-years-old woman revealed an interaction between methotrexate injection to treat severe psoriasis and red clover supplementation, which caused severe vomiting and epigastric pain (Orr and Parker, 2013). Another case of a 28-year-old woman with a bleeding disorder was associated with the consumption of 5-6 daily cups during two weeks of tea of red clover and alfalfa (Karimpour-Reihan, Firuzei, Khosravi, and Abbaszade, 2018).

2.2.3. *Trifolium tomentosum*

Scientific information about *T. tomentosum* is scarce. The plant is recognized as woolly clover and is an annual herb that can grow up to 8-22 cm high (**Figure 7A**). Inhabits, especially cold weather environments, and it is distributed across South Europe, Cyprus, Egypt, western Syria, North Iran, India, West Australia, and America (Singh and Srivastava, 2017). In Portugal, it has a wide distribution through the territory (**Figure 7B**). It is traditionally used as a diuretic as well to treat fever, vomiting, and cough (Arnold, Baydoun, Chalak, and Raus, 2015)

In *T. tomentosum* seeds, the only flavonoid detectable was quercetin (1.73 mg/g dry matter). There was also seen the presence of saponins, more precisely soyasaponin I (1.86 mg/g dry matter) (Oleszek and Stochmal, 2002). Soyasaponin I demonstrated the cytoprotective effects on human cells of the digestive system against a mycotoxin presented in some foods (Vila-Donat, Fernández-Blanco, Sagratini, Font, Ruiz, 2015). Other compounds like linamarin and lotaustralin were also identified in the aerial part of *T. tomentosum* (Muzashvili, Moniuszko-Szajwaj, Pecio, Oleszek, and Stochmal, 2014). Both compounds are cyanogenic glucosides that promote the chemical defense response against herbivores and pathogens (Møller, 2010). It was not reported any case of toxicity or adverse events associated with the use of this plant.

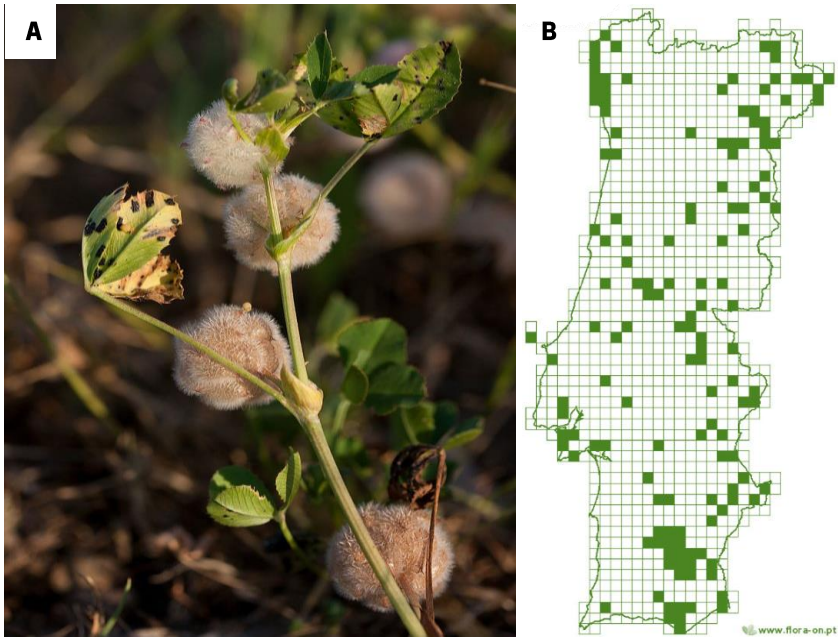

Figure 7. (A) Photograph of *Trifolium tomentosum*. Reprinted from Porto (n.d.). Copyright 2012–2020 by Creative Commons. **(B)** Distribution map of *Trifolium tomentosum* in Portugal made with 226 observations by different Flora-On collaborators (Clamote et al. 2020b).

3. Objectives

Regarding the existing biodiversity of Portuguese plants and after analyzing the biological properties and potentialities of some plants from *Trifollium* and *Euphorbia* genus, this research aims to:

- Perform a phytochemical screening assay for the presence of phenolic compounds, polyphenols, flavonoids, tannins, terpenoids, diterpenes, and alkaloids in aqueous and methanolic extracts of *E. paralias*, *E. hirsuta*, and *T. tomentosum*;
- Determine total phenolic content and antioxidant activity of aqueous and methanolic extracts of *E. paralias*, *E. hirsuta* and *T. tomentosum* through DPPH radical scavenging assay and metal chelation;
- Evaluate the antibacterial activity of aqueous and methanolic extracts of *E. paralias*, *E. hirsuta* and *T. tomentosum* through disc diffusion and broth microdilution tests against *Escherichia coli* and *Staphylococcus aureus*.

4. Material and Methods

4.1. Chemical and reagents

Dimethyl sulphoxide (DMSO), iron (III) chloride anhydrous, Dragendorff's reagent, sodium carbonate anhydrous, EDTA disodium salt dihydrate, Folin-Ciocalteu's reagent, iron (II) sulfate heptahydrate, and Mueller-Hinton broth (MHB) were obtained from VWR Chemicals (Radnor, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-*p,p'*-disulfonic acid monosodium salt hydrate (Ferrozine), quercetin, and gallic acid were obtained from Sigma-Aldrich (St. Louis, USA). Methanol, ethanol, chloroform, and sulphuric acid were obtained from Fisher Chemical (Waltham, USA). Nutrient broth (NB) and lead (II) acetate trihydrate were obtained from Merck KGaA (Darmstadt, Germany). Agar was obtained from Labchem (Zelienople, USA), and Mueller-Hinton Agar (MHA) was obtained from HIMEDIA (Mumbai, India).

4.2. Plant material and preparation of the extracts

In this study, the plants used were collected from Barrinha de Esmoriz (40°57'45.9"N | 8°38'07.7"W) in July 2019. With the cooperation of specialists from the Faculty of Science of the University of Porto, plants were identified using literature and comparisons with herbarium exsiccates. The different botanical taxa studied in this work are shown in **Table 1**. Freshly collected plant material was air-dried and ground in a laboratory mill to a moderately fine powder (particle size ≤ 0.5 mm). Powdered material of the aerial parts was submitted to extraction with two solvents, methanol-water (80:20, V/V) and water in a proportion of 1:10 dried plant/solvent, during 48h and 24h respectively, at room temperature and in the dark. After that, the mixture was filtered through Whatman number 1 paper, concentrated to dryness under vacuum at 40 °C by using a rotary evaporator (VWR, Ika RV8) (methanolic extract) and freeze-dried (FreeZone 4.5 liter benchtop freeze dry system, LabConco©). The resulting powders were stored at -20 °C for further use.

Table 1. Scientific and common names, parts, and traditional uses of plants used in this study.

Scientific name (family)	Common name	Plant part used	Traditional uses	References
Euphorbia paralias (Euphorbiaceae)	Morganheira-das-praias	Aerial parts	Local anesthetic and purgative. It is also used to treat edema.	(Boubaker et al., 2013)
Euphorbia hirsuta (Euphorbiaceae)	Ésula-lanosa; Titímalo-lanoso	Aerial parts	Skin pathologies	(González-Tejero et al., 2008)
Trifollium tomentosum (Fabaceae)	Trevo-tomentoso	Aerial parts	Diuretic and to treat fever, cough, and vomiting.	(Arnold et al., 2015)

4.3. Phytochemical screening of secondary metabolites

For all plant extracts the presence of phenolic compounds, polyphenols, flavonoids, tannins, terpenoids, diterpenes, and alkaloids was determined according to standard methods (Georgé, Brat, Alter, and Amiot, 2005; Tamokou, Mbaveng, and Kuete, 2017). All experiments were performed in triplicate, and results were expressed as absence (–), presence (+), or strong presence (++) of the secondary metabolites.

4.3.1. Detection of phenolic compounds

Extracts were treated with 5–10 drops of 5% of ferric chloride. Changing color to bluish dark indicates the presence of phenols.

4.3.2. Detection of polyphenols

It was added to 100 µL of extract, 500 µL of Folin–Ciocalteu reagent (1:10 V/V), and 400 µL of 7.5% Na_2CO_3. Then, the sample was incubated for 2 hours at room temperature and in the dark. Changing color to dark blue indicates the presence of polyphenols.

4.3.3. Detection of flavonoids

A few drops of 5% of lead acetate was added to 2.5 mL of extract. A white, yellow precipitate indicates the presence of flavonoids.

4.3.4. Detection of tannins

Extracts were treated with 5–10 drops of 1% of ferric chloride. A bluish dark color indicates the presence of tannins.

4.3.5. Detection of terpenoids (Salkowski's test)

For this test, 3.0 mL of extract was added with 1.0 mL of chloroform and 1.5 mL of concentrated sulphuric acid. Brownish–red ring development reveals the presence of terpenoids.

4.3.6. Detection of diterpenes

To 1 mL of the extract, 4–10 drops of copper acetate at 5% were added. Changing color to emerald green indicates the presence of diterpenes.

4.3.7. Detection of alkaloids (Dragendorff's test)

To the extract (2.5 mL), 2 drops of Dragendroff's reagent were added. The appearance of an orange precipitate indicates the presence of alkaloids.

4.4. Parameters related to antioxidant activity

4.4.1. DPPH radical scavenging activity

The DPPH radical scavenging activity was evaluated according to the procedure previously described by Lima et al. (2007). Briefly, selected concentrations (ranging 1–2500 μg/mL) of each extract were added to DPPH (90 μM) in a 96-well microplate, and the percentage of remaining DPPH· was determined at 5 in 5 minutes from the absorbance at 515 nm measured by a plate reader spectrophotometer (Dynex Technologies MRX II Microplate Reader). As the reaction reached steady state after 1 hour, the radical scavenging capacity of each sample was obtained following the formula in RSC (%) = $[(Ab_c-(Ab_s-Ab_b)/Ab_c)$ x 100], where Ab_c is the absorbance of the control, Ab_s is the absorbance of the sample, and Ab_b is the absorbance of the blank. The concentration of plant extracts needed to reduce 50% of free radical DPPH (IC_{50}) was determined by plotting the percentage of inhibition against the sample concentrations. Quercetin was used as a standard.

4.4.2. Iron (II) chelating activity

The iron(II) chelating activity of plant extracts was measured according to Russo et al. (2005) with some modifications. Briefly, in each well of the microplate was added 50 μL of the plant extract at different concentrations as well as 0.12 mM of iron (II) sulfate ($FeSO_4$) (50 μl) and the same volume of 0.6 mM of ferrozine. After a vigorously shake, the plate stood at room temperature for 10 min and protected from light. The absorbance was measured at 562 nm in a spectrophotometer (Dynex Technologies MRX II Microplate Reader). Iron chelating activity (ICA) in percentage (%) was calculated following the next formula: $ICA(\%)=100x[(Ab_c-Ab_s)/Ab_c]$, where Ab_c is the absorbance of the control and Ab_s is the absorbance of the sample. The concentration of the extract, which chelates 50% of the ferrous ion (IC_{50}), was calculated plotting the ICA(%) against extract concentrations. EDTA was used as a positive control.

4.4.3. Determination of Total Phenolic Content (TPC)

The total phenolic content (TPC) was determined following the Folin–Ciocalteu procedure described by Singleton and Rossi (1965) with minor modifications (Alves et al., 2010).

The extracts were prepared at 1 mg/ml with respective solvents (metanol or water) and further dilutions were made to fit the calibration curve.

Briefly, it was mixed 500 µL of the extract with 2.5 mL Folin-Ciocalteu reagent (1:10, V/V) and 2 mL of 7.5 % (w/v) of sodium carbonate (Na_2CO_3) solution. Then, the mixture was firstly incubated at 45 °C, for 15 min, at room temperature for 30 min. The absorbance was read at 765 nm against a blank (solvent + Folin-Ciocalteu reagent + Na_2CO_3), in a UV/Vis spectrophotometer (Jenway 6300). A standard solution of gallic acid was used to make a calibration curve to obtain a correlation between the sample and standard concentration (linearity range = 5 – 100 mg/ml; r > 0.999). The results obtained of TPC were expressed as milligrams of gallic acid equivalents (GAE) per grams of dried extract.

4.5. Antibacterial susceptibility test

4.5.1. Bacterial strains and growth conditions

Gram-negative bacteria, *Escherichia coli* (DSM 1576), and gram-positive bacteria *Staphylococcus aureus* (DSM 346) were obtained from the DSMZ-German collection of microorganisms and cell cultures GmbH. Both bacteria strains were maintained in glycerol stock at -80 °C.

The cultivation medium for *E. coli* and *S. aureus* was Nutrient Broth or Agar (NB, NA). For bacterial growth, the bacteria cultures were prepared by dipping the swab into the glycerol stock and then suspended in 5 mL of NB. The culture was grown aerobically for 20 h at 37 °C. Then, the bacteria were streaking to isolate pure cultures and kept at 37 °C for another 20 h. For antibacterial assays, isolated colonies were picked up and diluted in MHB medium and adjusted to 0.5 McFarland turbidity, which corresponds to 10^8 colony-forming unit (CFU)/mL.

4.5.2. Screening for antibacterial activity

The selected plant extracts were subjected to a general screening for antibacterial activity through the disc diffusion method described by EUCAST (2020) with minor modifications. Isolate colonies, previously adjusted to 0.5 McFarland in MHB medium, were inoculated into MHA plates (depth level 4.0 ± 0.5 mm) with a sterile cotton swab. In the case of Gram-negative bacteria, the swab was pressed against the inside of the tube to avoid over-inoculation, whereas Gram-positive bacteria do not. It was guaranteed no gaps between streaks. For the application of antibacterial discs, 20 µl of a fixed concentration (10 mg/ml) of each plant extract dissolved in 2.5% DMSO was aseptically dropped (single dose) into blank discs and firmly employed to the surface of the inoculated agar plate. As a reference antibacterial agent, it was used

ciprofloxacin discs (Liofilchem, Italy) at 5 μg, and DMSO control was also made to ensure that this solvent is not implicated in the antibacterial activity. The plates were incubated at 37 °C for 20 h. The diameters of inhibition zones were measured (in mm) after incubation.

4.5.3. Broth microdilution method

To determine the minimal inhibitory concentration (MIC) of the extracts, it was performed the broth microdilution test described by Wiegand, Hilpert, and Hancock (2008) with some adaptations.

Initially, serial two-fold dilutions were made directly in a sterile 96-well microplate of the plant extract in the MHB medium covering a broad spectrum of concentrations tested (4-0.008 mg/mL). After that, each bacterial suspension in MHB medium previously adjusted to 10^8 CFU/mL was diluted (1:100), and 100 μL was added to the wells. The final volume of each well was 200 μL with the desired inoculum of 5×10^5 CFU/mL. Parallel to this, it was made sterility control (only medium), growth control (bacterial suspension and medium), and the respective extract blank of each dilution (extract and medium). A small volume (10 μL) was taken of the growth control, immediately after inoculation, and successively diluted in MHB (1:1000) to be plated onto NA plates. Both 96-well microplates and plates were incubated at 37 °C for 16-20h. Gentamicin was used as a positive control for both strains.

After the incubation period, it was necessary to validate the test so that the number of cells in the plates was counted. It is expected that after the dilution (1:1000) of the final inoculum, there is about 20-80 colonies when the correct inoculum density (5×10^5 CFU/mL) is used. The MIC value is defined as the lowest concentration of the plant extract, where there was no visible growth of the tested isolate as observed with naked-eye.

To complement MIC visual analysis, due to the extracts coloration, the absorbance was read at 630 nm, and a dose-response curve was constructed to complement the visual analysis (Patton, Barret, Brennan, and Moran, 2006) to determine the bacteria growth inhibition following the formula BGI (%) = $[(Ab_c-(Ab_s-Ab_b)/Ab_c) \times 100]$, where Ab_c is the absorbance of the control, Ab_s is the absorbance of the sample, and Ab_b is the absorbance of the blank. In this case, MIC was determined as the lowest concentration of the plant extract, which results in 100% of inhibition growth.

4.6. Statistical analysis

Data were expressed as mean values ± SD of at least three independent experiments. The IC_{50} was calculated from the dose-response curve obtained by plotting the percentage of inhibition versus the concentrations. Data were analyzed and compared by one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons and by unpaired two-samples t-test. All the statistical analyses were

performed using GraphPad Prism 8.0 (GraphPad Software, Inc., San Diego, USA), and p values ≤ 0.05 were considered statistically significant.

5. Results

5.1. Phytochemical constituents assessment

5.1.1. Qualitative analysis

The qualitative phytochemical assessment of the selected plant species with different extraction solvents is represented in **Table 2** for various chemical constituents.

Table 2. Qualitative phytochemical screening of aqueous and methanolic extracts of each studied plant for the occurrence of phenolic compounds, polyphenols, flavonoids, tannins, terpenoids, diterpenes, and alkaloids.

Phytochemicals	*E. paralias*		*E. hirsuta*		*T. tomentosum*	
	AE	ME	AE	ME	AE	ME
Phenolic compounds	++	++	++	++	+	+
Polyphenols	++	++	++	+	+	-
Flavonoids	+	++	++	++	+	+
Tannins	++	++	++	++	+	+
Terpenoids	-	+	-	-	-	-
Diterpenes	-	-	-	+	+	++
Alkaloids	++	++	++	++	++	++

AE: aqueous extract; ME: methanolic extract

The results are expressed as strongly present (++), present (+), or absence (-) from three independent assays (n=3).

As noted, both species of the *Euphorbia* genus showed a strong presence of phenolic compounds even for the different extractive solvents, and the same occurred for the other classes of phenolic compounds such as polyphenols, flavonoids, and tannins. However, the presence of polyphenols in ME of *E. hirsuta* was less evident, as was for flavonoids in the AE of *E. paralias*. Terpenoids occurred only in ME of *E. paralias* and diterpenes in ME of *E. hirsuta*. All selected plants strongly showed the presence of alkaloids as being part of its phytochemical constituents.

Even though *T. tomentosum* had positive results for phenolic compounds, polyphenols (except ME), flavonoids and tannins, it can be seen that the presence of phenolic compounds is much lighter when compared with the other two *Euphorbia* plants. By contrast, diterpenes were detected in both AE and ME of *T. tomentosum*.

5.2. *In vitro* antioxidant activity

The potential antioxidant activity of both aqueous and methanolic extracts was evaluated through common methods such as DPPH· scavenging and iron (II) chelating activity assays. All plant extracts presented antioxidant activities, although with different values (**Table 3**).

Table 3. *In vitro* antioxidant activities of aqueous and methanolic extract of each studied plant along with respective standards for each assay, quercetin, and EDTA.

Plant species	DPPH· scavenging (IC_{50}, μg/ml)			Iron (II) chelating (IC_{50}, μg/ml)		
	AE	ME	Q	AE	ME	EDTA
E. paralias	9.7 ± 0.5 [a]	43.0 ± 4.2 [b]		580.8 ± 47.2 [a]	49.6 ± 2.9 [c]	
E. hirsuta	17.5 ± 1.4 [a]	14.9 ± 1.0 [b]	1.8 ± 0.2 [c]	199.6 ± 7.9 [a]	198.2 ± 4.6 [a]	1.2 ± 0.0 [c]
T. tomentosum	163.0 ± 0.8 [a]	129.9 ± 8.1 [b]		1325.8 ± 23.7 [a]	243.3 ± 5.6 [b]	

AE: aqueous extract; ME: methanolic extract; Q: quercetin; EDTA: ethylenediaminetetraacetic acid

The IC_{50} values (μg/ml) are expressed as mean ± standard deviation from three independent assays (n = 3). Multiple comparisons were performed between AE and ME for each plant and the positive control. Significant differences ($p \leq 0.05$) are represented with different letters.

For both assays and all plant extracts, antioxidant activity was observed in concentration-dependent patterns (Appendice, Figure 1 and Figure 2) to calculate the IC_{50}.

Regarding the capacity of the DPPH· scavenging, the aqueous extract of *E. paralias* was the most effective with an IC_{50} value of 9.7 ± 0.5 μg/mL followed by both extracts of *E. hirsuta*. However, the ME of *E. hirsuta* was significantly more potent in neutralizing DPPH· than the AE (14.9 ± 1.0 and 17.5 ± 1.4 μg/mL, respectively). It was the *T. tomentosum* extracts that obtained the lowest radical scavenging capacity, although ME had a better performance than the AE for this specie. The IC_{50} values for all extracts were higher than the positive control, quercetin (1.8 ± 0.2 μg/ml).

On the other hand, the ME of *E. paralias* was shown to be the most efficient in chelating iron ions (IC_{50} = 49.6 ± 2.9 μg/mL). Both extracts of *E. hirsuta* presented similar activity, and no significant differences were seen between them (199.6 ± 7.9 and 198.2 ± 4.6 μg/mL for AE and ME, respectively). Concerning *T. tomentosum*, the AE was substantially weaker than the ME with an IC_{50} value significantly higher (1325.8 ± 23.7 μg/ml) ($p \leq 0.05$) comparing to 243.3 ± 5.6 μg/mL.

5.2.1. Total phenolic content

The results of total phenolic content quantification are represented in Table 4. From the analyzed extracts, those that had the highest TPC (**Table 4**) were ME of *E. paralias* (293.37 ± 20.25 mg GAE/g) followed by the AE of *E. paralias* (165.80 ± 14.24 mg GAE/g).

Table 4. Total phenolic content of aqueous and methanolic extract of the plants studied.

Plant species	Total phenolic content (mg GAE/g)	
	AE	ME
E. paralias	165.80 ± 14.24 [a]	293.37 ± 20.25 [b]
E. hirsuta	12.01 ± 0.30 [a]	21.24 ± 1.01 [b]
T. tomentosum	4.53 ± 0.32 [a]	17.51 ± 0.32 [b]

AE: aqueous extract; ME: methanolic extract; GAE: gallic acid equivalents;

The results are expressed in mg gallic acid equivalents (GAE) per gram of dried extract, and the values are the mean ± standard deviation from four independent assays (n=4). Comparisons between AE and ME of each plant were performed, and significant differences ($p \leq 0.05$) are represented with different letters.

In general, extracts made only with water were significantly lower in phenolic content when compared with methanol–water extracts ($p \leq 0.05$).

5.3. Antibacterial activity

Screening of the tested plant extracts for their antibacterial activity was performed against *S. aureus* and *E. coli*. In agreement with the results in **Table 5**, *E. coli* was not susceptible to any of the extracts but to the antibiotic (41.0 ± 1.0 mm). However, *S. aureus*, when subjected to 10 mg/ml of the methanolic extracts of *E. paralias* and *E. hirsuta*, exhibited a slight inhibition with a diameter of 8.3 ± 0.6 mm and 9.7 ± 0.6 mm, respectively when compared to the CIP (32.0 ± 1.0 mm).

Then, the extracts which promoted growth inhibition bacteria at 10 mg/ml were tested for MIC determination. The results are shown in **Table 6,** and the dose–response curve of the growth inhibition of bacteria promoted by the plant extracts are represented in Appendice, Figure 3.

In particular, the ME of *E. paralias* and *E. hirsuta* exhibited good activity against *S. aureus*. The MIC values of these extracts were 1.0 and 2.0 mg/mL, respectively, against the tested bacteria strain. The most potent of all was the antibacterial activity of *E. paralias*. However, gentamicin was more efficient in inhibiting bacteria growth of *S. aureus* with a MIC value of 0.0052 mg/mL than the plant extracts.

Table 5. Inhibition zone measured around the discs containing 10 mg/mL of the extracts of the studied plants and ciprofloxacin at 5 µg/disc against *Staphylococcus aureus* and *Escherichia coli*.

Plant species	*S. aureus* (Inhibition zone, mm ± SD)			*E. coli* (Inhibition zone, mm ± SD)		
	AE	ME	CIP 5 µg/disc	AE	ME	CIP 5 µg/disc
E. paralias	–	8.3 ± 0.6		–	–	
E. hirsuta	–	9.7 ± 0.6	32.0 ± 1.0	–	–	41.0 ± 1.0
T. tomentosum	–	–		–	–	

AE: aqueous extract; ME: methanolic extract; CIP: ciprofloxacin; –: no inhibition zone is observed

The results are expressed in mm ± SD from three independent assays (n=3).

Table 6. MIC determination of the methanolic extracts of the *Euphorbia* plants and gentamicin against *Staphylococcus aureus*.

Plant species	*S. aureus* MIC values (mg/ml)	
	ME	GEN
E. paralias	1.0	0.0052
E. hirsuta	2.0	

ME: methanolic extract; GEN: gentamicin; MIC: minimum inhibition concentration

The results are expressed in mg/ml from three independent assays (n = 3)

6. Discussion

In the present study, the antioxidant activity of *E. paralias*, *E. hirsuta,* and *T. tomentosum* extracts was investigated through DPPH and iron-chelating assays as well as its antibacterial activity against *E. coli* and *S. aureus.* In order to identify the putative chemical class of compounds engaged with antioxidant and antibacterial activities, plant extracts were screened for secondary metabolite classes like phenolic compounds, polyphenols, flavonoids, tannins, terpenoids, diterpenes, and alkaloids.

Both extracts of the *Euphorbia* genus presented the same group of compounds based on the phytochemical screening, except for the methanolic extracts where it was positive for terpenoids, in *E. paralias*, and positive for diterpenes, in *E. hirsuta*. A previous study, where the authors performed a phytochemical characterization of *E. paralias,* also revealed the presence of terpenoids in the aqueous-methanol fraction (Jannet et al., 2017). However, they examined the plant in its early stages of development, which can possibly explain the inexistence of alkaloids in their extract contrary to what was observed in our study where fully grown plants were used. Other *Euphorbia* species presented alkaloids in its composition like *E. hirta* and *E. golondrina* (Ndam et al., 2016; Tuhin et al., 2017).

Also, chemical constituents of *E. paralias* have been investigated previously and the presence of irritant and cytotoxic ingenanes (Sayed et al., 1980), paralinones (Öksüz et al., 1997), segetanes, jatrophanes, paralianes and another tetracyclic diterpenoids (Jakupovic et al., 1998a; Jakupovic, Morgenstern, Marcot, and Berendsohn, 1998b), as well as triterpenoids and flavonoids (Rizk et al., 1974, 1976), have been reported.

Despite ethnomedicinal uses and agricultural value of clovers *(Trifolium* genus), the chemical composition and pharmacological properties of clover-derived preparations were, for a long time, out of scientific interest. However, the recent years have provided a noticeable increase of interest in clovers as a valuable source of bioactive compounds (Kolodziejczyk-Czepas, 2016). The phytochemical profile of *T. tomentosum* comprises diterpenes in both extracts (AE and ME), and it is in agreement with the study carried by Vlaisavljevic et al. (2014) where they found several diterpenes in the essential oils of red clover. The medium-polar nature of diterpenes made them susceptible to detection in polar solvents, too (Mukherjee, 2019). Several studies have shown the presence of different phytochemicals in species of *Trifolium*, like cyanogenic glucosides (Muzashvili et al., 2014), triterpene glycosides, identified as oleanane derivatives (Pawelec et al., 2013), and flavonoids (Sabudak, Demirkiran, Ozturk, and Topcu, 2013).

Phenolics are secondary plant metabolites and are very important for antioxidant activity. This is believed to be mainly due to their redox properties, which play an essential role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Meghashri, Kumar, and Gopal, 2010). In the present work, it is clear the richness of phenolic compounds in the methanolic extract of *E. paralias* (293.37 mg GAE/g). Similar results were observed in the study of

Kefayati, Motamed, Shojaii, Noori, and Ghods (2017), but with *E. splendida*. The authors evaluated the antioxidant activity of total methanolic extract and subfractions of *E. splendida* Mobayen. The methanolic extract showed the highest TPC values (270.74 ± 0.005 mg/g). The extract and subfractions of *E. splendida* are found to have different levels of antioxidant activity and phenolic contents. Plant polyphenols, like quercetin and rutin, act as reducing agents and antioxidants by the hydrogen-donating property of their hydroxyl groups, so these compounds may be responsible for the observed antioxidant activity (Kefayati et al., 2017). Kawashty, Abdalla, El-Hadidi, and Saleh (1990) showed the abundance of flavonoids in *E. paralias*, a well known class of bioactive compounds with strong antioxidant activity, which may explain the marked antioxidant capacity of this plant, in study.

Regarding the TPC found for *E. hirsuta*, values ranged from 12.01 ± 0.30 mg GAE/g (in AE) to 21.24 ± 1.01 mg GAE/g (in ME). There are no studies, to the best of our knowledge, in the lliterature, regardind TPC values for *E. hirsuta*. However, higher values of TPC were obtained from ethanolic and aqueous extracts of *E. hirta*, with 293.74±2.48 mg GAE/g and 277.64±2.46 mg GAE/g, respectively (Venkatachalam, Thavamani, Muddukrishniah, and Vijayan, 2018). Basma, Zakaria, Latha, and Sasidharan (2011) also found higher values in the same species. Leaves extract had the highest total phenolic content (206.17±1.95 mg GAE/g), followed by flowers, roots and stems extracts (117.08±3.10 mg GAE/g, 83.15±1.19 mg GAE/g, and 65.70±1.72 mg GAE/g, respectively).

The discrepancy of values observed in this work, in comparison to other authors might be due to the different species of *Euphorbia*; the geographical origin of the plants; or to the plant's development stage (Bajalan, Mohammadi, Alaei, and Pirbalouti, 2016; Pirbalouti, Siahpoosh, Setayesh, and Craker, 2014). The choice of solvents used and studied organs may also affect the secondary metabolite pool extracted, therefore the antioxidant level (Trabelsi et al., 2010).

On the other hand, *T. tomentosum* showed lower values in phenolic compounds when compared with the other studied plants. However, TPC of the ME (17.51 mg GAE/g), was higher comparing to the value obtained by (Oleszek et al., 2007) from the of the aerial parts of *T. tomentosum* (13.54 mg/g).

A clear behavioural trend can be observed when we compare the values of TPC for AE and ME in all the studied plants; namely that the use of a polar organic solvent, such as methanol, possesses a higher partition coefficient with respect to the capture of phenolic bioactives (Munro et al., 2015).

In recent years, there is an increasing interest in finding antioxidant phytochemicals, because they can inhibit the propagation of free radical reactions, and thereby protect the human body from diseases. Antioxidant activity is a complex process usually occurring through several mechanisms, which include direct quenching free radicals to terminate the radical chain reaction, chelating transition metals, acting as reducing agents, or stimulating the antioxidative enzyme activities (Lima et al., 2006). Moreover, the complex composition of phytochemicals, with different functional groups, polarity, and chemical behavior,

could lead to mixed results, depending on the assays employed. Therefore, an approach with multiple assays in order to evaluate the total antioxidant activity is highly advisable.

The DPPH radical is widely used to evaluate the antioxidant capacity of extracts from different plant materials. It appraises the antiradical ability of the extract by measuring the reduction in the absorbance of DPPH radical. When antioxidants are in the presence of DPPH·, they donate hydrogen to form a stable DPPH molecule with a decrease in the absorbance (Matthäus, 2002). Visually, the color changes from violet to yellow as the radical molecule is scavenged.

Naturally, iron is found within living organisms in its reduced and oxidized form what makes it an intrinsic producer of ROS through Fenton reaction (Liu, Zhou, Ziegler, Dimitrion, and Zuo, 2017). Compounds with metal-binding properties can form a complex with iron ion breaking the chain reaction of ROS production (Valko et al., 2006). The assay to evaluate the iron-chelating activity is based on the reaction between ferrozine and iron ions, which produces a purple complex. A color reduction means that the extract has chelating agents capable of breakdown the complex formation (Adjimani and Asare, 2015).

In the present work, it was studied the antioxidant activity of methanolic and aqueous extracts of selected plants assessing the DPPH scavenging capacity and iron chelating activity. Samples will be considered to have a high or significant antioxidant capacity with $IC_{50} < 50$ μg/ml, moderate antioxidant capacity with $50 < IC_{50} < 100$ μg/ml, and low antioxidant capacity with $IC_{50} > 100$ μg/ml (Kuete and Efferth, 2010). Omisore et al. (2005) also considered the cut-off point for antioxidant activity as 50 μg/ml. Samples with $IC_{50} > 50$ μg/ml were classified as being moderately active, while samples with $IC_{50} < 50$ μg/ml were judged as having high antioxidant capacity (Omisore et al., 2005) .

Euphorbia species showed strong antiradical activity for both extracts with significant antioxidant capacity ($p < 0.05$). For *E. hirsuta*, and as of our knowledge, this is the first antioxidant activity report. On the other hand, *E. paralias* was already described with a modest antioxidant activity in aqueous and methanolic extracts (Abou-Enein, El-Ela, Shalaby, and El-Shemy, 2012), which make our results better regarding the DPPH scavenging potency. However, a more recent study supports our data with an IC_{50} value of 5.25 μg/ml in the polar fraction of *E. paralias* (Jannet et al., 2017). Some factors, like geographical region of the plant, extraction procedure, solvents used in the extraction, or the development stage, might be the explanation for such variations (Kumar, Yadav, Yadav, and Yadav, 2017; Mokrani and Madani, 2016). The abundance of flavonoids in *E. paralias* was shown by Kawashty et al. (1990), and compounds with vigorous antioxidant activities, like quercetin, was found to be representative of the phenolic class in *Euphorbia hirta* (Tona et al., 2004).

Known actions of quercetin include antioxidant, anti-inflammatory, anti-bacterial, anti-aggregatory, and anti-carcinogenic (Loh, Er, and Chen, 2009). The same authors concluded that quercetin is present in its glycosidic forms in the ME. The absence of quercetin and rutin in the AE could be explained by the less

polar nature of these flavonoids (water is a polar solvent) and thus would have been extracted in the less polar solvents like chloroform and methanol during the sequential extraction.

Zhang et al. (2017b) indicated that the extract of *E. lathyris* has significant antioxidant activities ($p < 0.05$), perhaps due to its high contents of phenolics and flavonoids. Several reports have suggested that the antioxidant properties in Euphorbiaceae members are mainly due to the presence of high contents of secondary metabolites, such as different types of flavonoids (Kadri, Gharsallah, Damak, and Gdoura, 2011; Subhan et al., 2008).

The aqueous extract of *E. paralias* was the best in neutralize DPPH radical with 4.3-fold lower total phenolic content than the methanolic extract. Results reported in the literature demonstrate that other species of the *Euphorbia* genus showed a positive correlation of quenching DPPH free radical and total phenolic content (Basma et al., 2011; Zhang et al., 2017b). Nevertheless, some medicinal plants, like *Eucommia ulmoides* and *Sarcandra glabre* presented high antioxidant activity despite having low levels of phenolic content, which could be explained by the implication of other phytochemicals in their antioxidant activities (Zhang et al., 2011). For example, alkaloids already demonstrated its efficiency in scavenging the stable free radical DPPH (Zhao et al., 2006).

In terms of metal chelating ability, methanolic extract of *E. paralias* was the only with high iron-chelating activity ($IC_{50} < 50$ μg/ml). This finding suggests that the chemical structure of phenolic compounds in the extract possessed more than one hydroxyl group. According to Zhou, Yin, and Yu (2006), phenolic compounds with only one hydroxyl group exhibiting weak transition metal chelating ability . This specific structure affects the negative charge density at the chelation site in a positive way, and that is the reason why quercetin has higher chelating capacity than other flavonoids like luteolin, rutin and kaempferol (Mira et al., 2002).

Concerning the low DPPH scavenging capacity of *T. tomentosum* extract ($IC_{50} = 163.0 \pm 0.8$ μg/ml in AE; $IC_{50} = 129.9 \pm 8.1$ μg/ml in ME), it can be said that it is in agreement with the weaker antioxidant activity found in *T. repens* ($IC_{50} = 276 \pm 14$ μg/ml) (Liu, Li, Sun and Tian, 2014). Notably, the *Trifolium* genus is well-known for their content of isoflavones and clovamides (Oleszek et al., 2007). However, none of these compounds were found in *T. tomentosum*, as reported by the same authors. That absence can support our results once isoflavone already demonstrated substantial antiradical activity, *in vitro* (Kładna, Berczyński, Kruk, Piechowska, and Aboul-Enein, 2016) as well as clovamide for scavenging DPPH free radical (EC_{50}: 9.24 μM) (Arlorio et al., 2008). Furthermore, clovamide-rich extracts of three *Trifolium* species displayed significant antioxidant activity (Kolodziejczyk-Czepas et al., 2017). On the other hand, regarding the metal-chelating activity of *T. tomentosum* extract, it is observed the same pattern which reinforces the low antioxidant capacity of these plant extracts. Besides that, it is considered that the AE of this plant did not have any iron-binding activity ($IC_{50} > 1000$ μg/ml), having also the lower TPC of all extracts studied. The chemical structure of phenolic compounds has some functional sites where transitions metals bind (Guo

et al., 2007). This fact can explain the positive correlations between TPC and metal-chelating action observed in several studies (Gonçalves, Gomes, Costa, and Romano, 2013; Granato, Grevink, Zielinski, Nunes, and van Ruth, 2014) as also support the significant iron-chelating activity of *E. paralias*, with methanolic extract comprising the highest TPC.

Regarding the screening for antibacterial activity performed with all plants studied at a fixed concentration of 10 mg/ml, none of the extracts promoted the inhibition growth in the *E. coli*. However, only the ME of *E. paralias* and *E. hirsuta* showed modest antibacterial activity against *S. aureus* (8.3 and 9.7 mm, respectively). None of the aqueous extracts had an effective antibacterial activity. In traditional medicine there are some reports of using *Euphorbia* against some bacterial infections thus, these results may be due to the different extraction solvents and type of constituents. For example, aromatic or saturated organic compounds are characterized by their high reactivity against microorganisms, and they are easily extracted by using ethanol or methanol solvents (Cowan, 1999). A low concentration of antibacterial compounds or its absence may explain the water extract inactivity.

The methanolic extract of different parts of *E. hirta* has previously been reported to have good antibacterial activity, with the highest inhibition zone endorsed by leaves and flowers (28 mm) in *S. aureus* and only by the leaves (18 mm) in *E. coli* (Rajeh, Zuraini, Sasidharan, Latha, and Amutha, 2010). It is essential to highlight the fact that our concentration test was 10-fold lower than described by the authors. So, the absence of antibacterial activity against *E. coli* does not mean that the plants studied are inactive; rather, the crude extract may have an insufficient active compound(s) at the tested concentration. Besides, non-polar compounds tend to diffuse more slowly in a culture medium, which could mask their action (Moreno, Scheyer, Romano, and Vojnov, 2006). Moreover, the natural morphology of Gram-positive bacteria, where *S. aureus* belongs, made them more permeable to the environment factors due to the lack of a thick phospholipid and lipopolysaccharide outer membrane layer (Rohde, 2019). An example is an investigation provided by Ogbulie, Ogueke, Okoli, and Anyanwu (2007). Their results revealed the inhibition zones of crude ethanolic extract of *E. hirta* against numerous bacteria increasing in a concentration-dependent manner. According to the same study, the plant extract did not inhibit the growth of *E. coli* at 50 mg/ml, whereas the extract, at its highest concentration tested, exhibited a larger inhibition zone (11.9 mm). Another *Euphorbia* specie, *E. helioscopia*, had a similar behavior (Lone et al., 2013).

Once screened the antibacterial activity of the studied plants, the broth microdilution method was performed for the methanolic extract of *E. paralias* and *E. hirsuta* in order to determine MIC values against *S. aureus*. In an investigation, *S. aureus* showed good sensitivity to the ethanolic extract of the *E. characias* leaves with a MIC value of 1.25 mg/ml, which support our evidence with MIC values of 1.0 mg/ml and 2.0 mg/ml for ME of *E. paralias* and *E. hirsuta*, respectively (Pisano et al., 2016). Curiously, it was found in this species several quercetin-derivated substances such as quercetin 3-*O*-glucoside (1.94 g/L), the same

compound isolated from the *E. paralias* (Safwat et al., 2018). According to the authors, methanolic extract of *E. paralias* was the best displaying growth inhibition in three different *Mycobacterium* species with MIC ranges of 3.12–5.0 mg/ml. However, when the authors tested the quercetin 3–*O*–glucoside isolated from the plant, more promising results were obtained even against *M. tuberculosis* since it is believed to inhibit the glutamine synthase enzyme, the most crucial virulence factor for the tuberculosis disease. Also, quercetin was found to interact with DNA through inhibition of the supercoiling activity of bacterial gyrase leading to DNA cleavage (Plaper et al., 2003).

Besides flavonoids, other phytochemicals presented in the extract may be exerting their antibacterial action against *S. aureus*. For example, changes in alkaloid contents of plants from Turkey and northern Cyprus influenced their antimicrobial activities (Çoban, Toplan, Özbek, Gürer, and Sarıyar, 2017). Also, compounds belonging to terpenoids showed great antibacterial activity and, some of them revealed synergetic effects in combination with antibiotics used in the standard clinical practice (Barbieri et al., 2017).

The broth microdilution method is mainly described for testing isolate compounds for their antimicrobial activities. Notwithstanding, the manipulation of isolated substances and plant extracts is very distinct, which can bring some difficulties when applying the method. Precipitation of compounds present in the plant extracts, along with its coloration, was the significant problems at the time to visually determine the MIC value. The same difficulties were reported by numerous authors (Eloff, 1998; Klančnik, Piskernik, Jeršek, and Možina, 2010; Zgoda and Porter, 2001). So, the dose–response curve, which relates to the concentration of the plant extracts with the inhibition of bacteria growth, helped us to obtain a MIC value. However, it is crucial to perform other methods for the determination of microbial sensitivity to ensure that disparity between techniques is avoided (Patton et al., 2006).

7. Conclusion

Plants have been used as medicines for thousands of years and current research in drug discovery from medicinal plants involves a multifaceted approach including botanical, phytochemical, biological, and molecular techniques. This research aimed to identify the biological activity of three Portuguese plants. Based on the evaluation of the antioxidant and antibacterial activities, it can be concluded that the extractive solvent is an essential factor in the plant's biological potency. The results indicate that *E. paralias* is the most active plant with effective antioxidant and antibacterial properties, followed by *E. hirsuta*. By analyzing findings of *T. tomensotum* and its biological performance in each assay, this work showed that polar solvents are not efficient in extract presumed bioactive compounds from this plant that assign one of the tested biological properties. However, all the plants studied exhibited antioxidant activity but with different levels of intensity.

The *in vitro* assays performed in this study provides a reinforce insight into the importance of Portuguese medicinal plants and their potential contribution in clinical practice. Even so, the limitations of the indirect methods could lead to a considerable overestimation of the antioxidant activity. To better understand the implications of these results, future studies could address cell-based assays to observe interactions of the plant extracts with the biological machinery of a living cell, and also test the extracts against a broader spectrum of bacteria strains and fungi. Another future perspective is the identification of main compounds in all studied extracts using, for example high-performance liquid chromatography.

Medicinal plants are a breeding ground for bioactive compounds that have served, for many years, as the first line of treatment for various diseases. However, to fulfill the needs of the market, pharmaceutical industries exchange the exploration of natural products to synthetic chemistry of new drugs, setting aside this whole world of potentialities. Unfortunately, most of the medicinal plants have not yet extensively explored, and many of its properties remain unknown. Thus, in all the work reported here, a contribution was made to the elucidation of some biological effects of *E. paralias*, *E. hirsuta* and *T. tomentosum*. Our findings highlight the great potential of *E. paralias,* which should be subject to further investigation, like future bio-guided fractionation and isolation of potential bioactive compounds, in order to understand its clinical relevance.

8. References

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9. Appendices

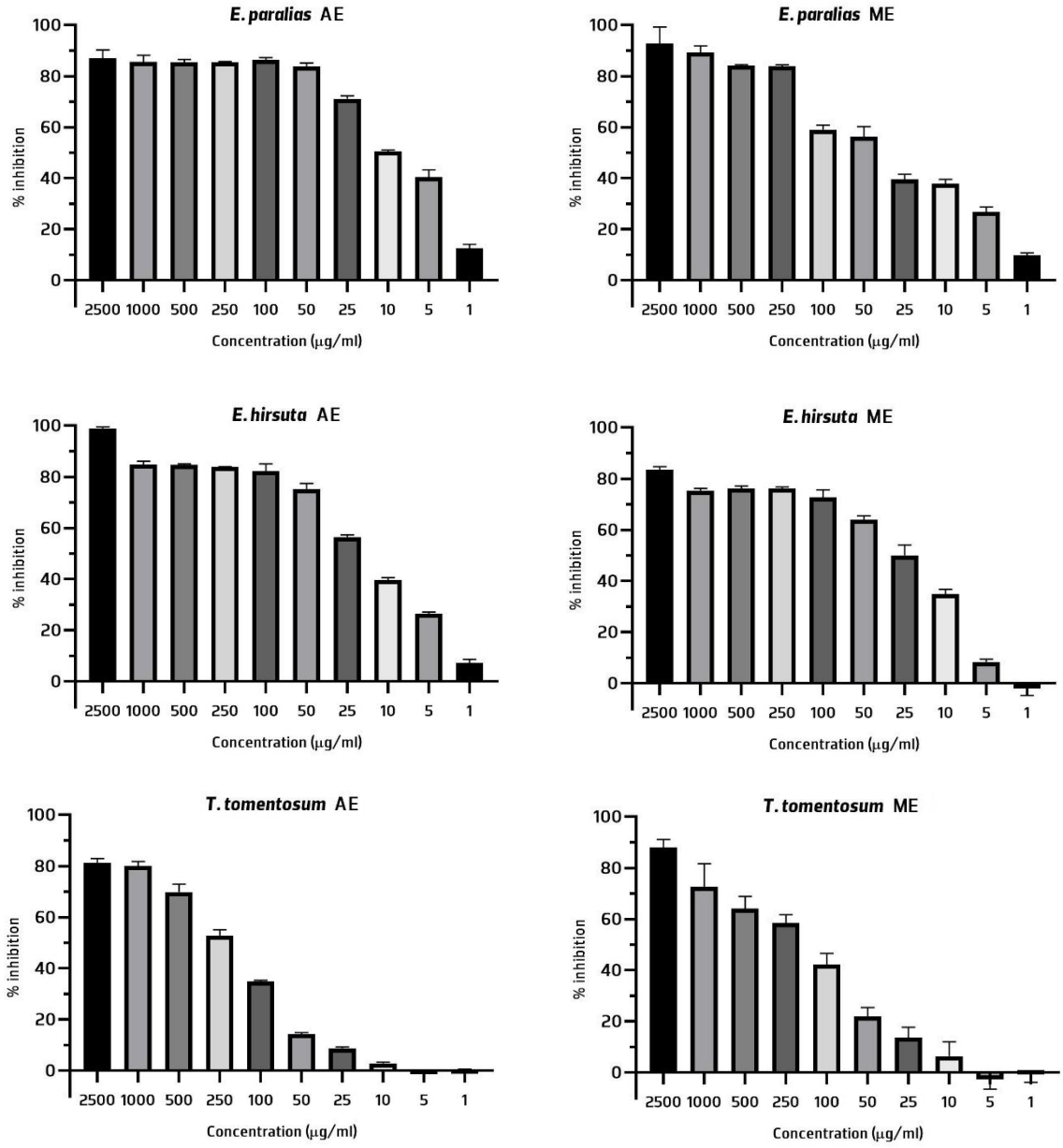

Figure 1. DPPH scavenging capacity of the plant extracts at different concentrations (2500–1 µg/ml)

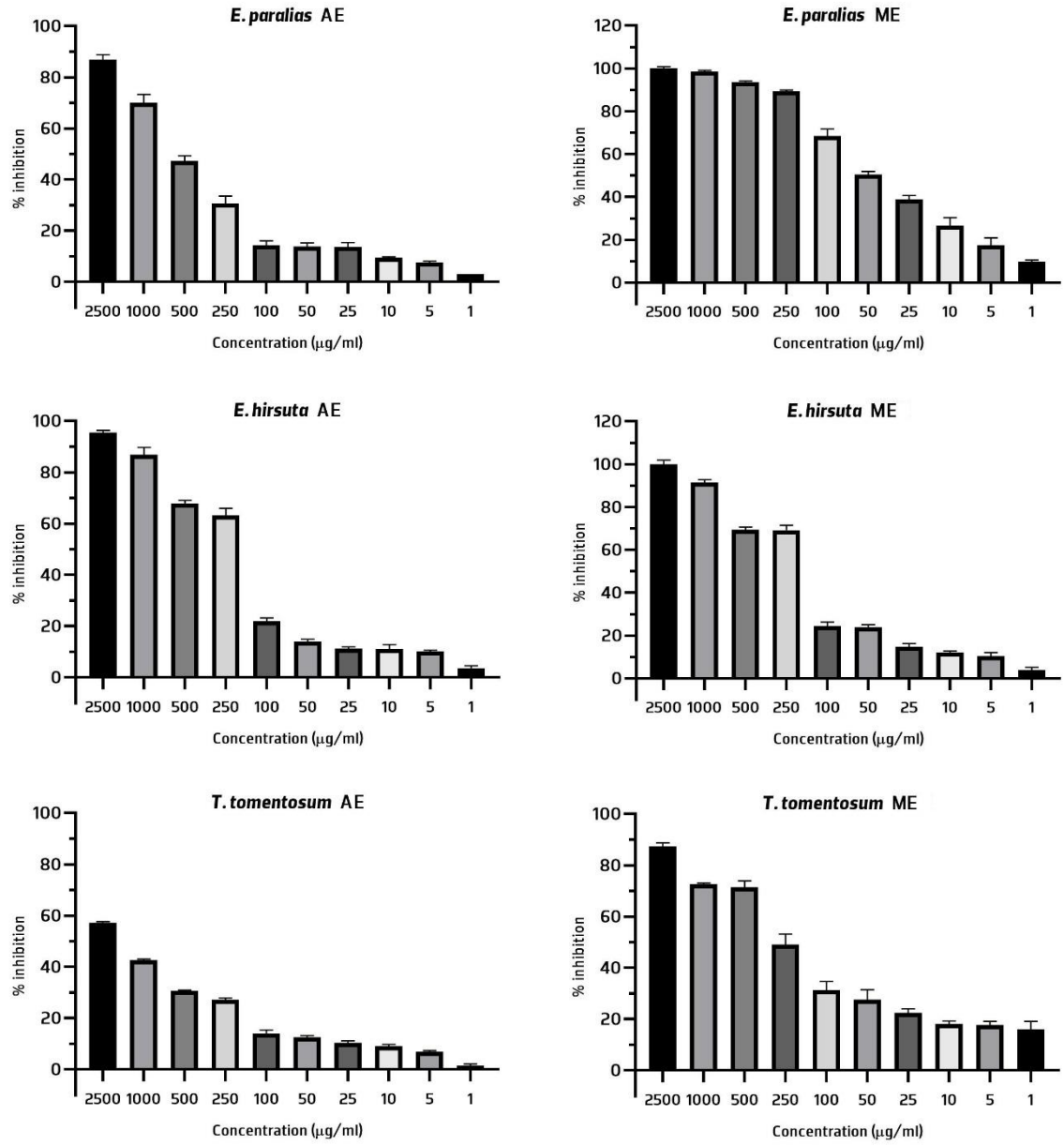

Figure 2. Iron chelating activity of the plant extracts at different concentrations (2500–1 µg/ml).

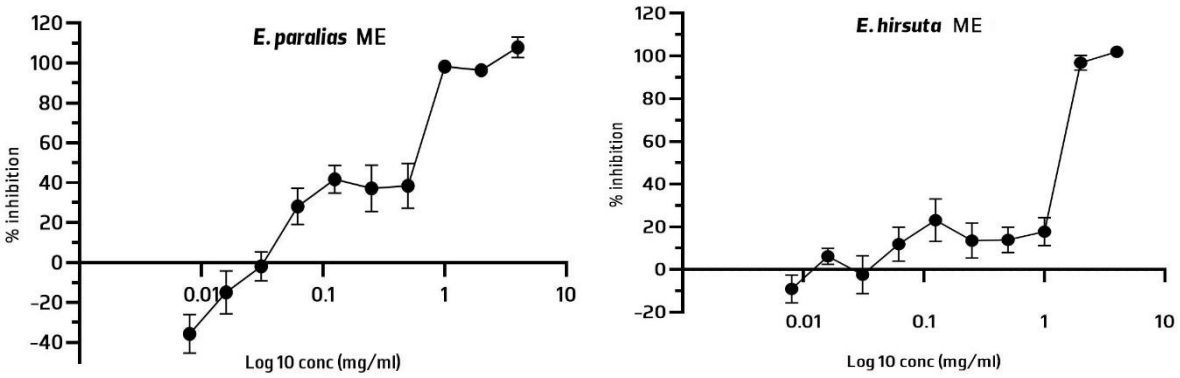

Figure 3. Dose-response curve between different concentrations (4-0.008 mg/ml) of plant extracts under two-fold serial dilutions, and the inhibition growth of *S. aureus*.