Extraction of phytochemicals from two invasive plant species and characterization of their bioactivity

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“The child is grown, the dream is gone.”

-Pink Floyd

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Abstract

Plants are recognized sources of compounds with high biotechnological impact. Among those compounds, phytochemicals have particular importance since are responsible for plants' protection against external factors like environmental stress and pathogens. Due to the sustainability inherent to naturally derived products, phytochemicals have been widely studied and used as food preservatives and pharmaceutical agents attending their antimicrobial and antioxidant capacities. This potential generates interest in these compounds since they may be part of the solution against antibiotic-resistant bacteria, potentiating antibiotics and other antimicrobials .

Invasive plant species are a group of plants that do not belong naturally to a habitat where they are present. Usually, they grow and develop faster than the autochthone species, harming the biodiversity of those places. The absence of legislation and measures to combat this issue has led to a global problem. There is a lack of studies about these species, especially from a metabolomics perspective – which mechanisms and bioactive compounds make these species stronger than native ones under specific conditions? This work aims at evaluating the phytochemical bioactivity of selected invasive plant species to validate the hypothesis that this underexplored biomass may have high biotechnological potential.

In that way, two invasive plant species, *Agave americana* (leaves) and *Tradescantia fluminensis* (stem and leaves, separately), were collected in the national territory and the antimicrobial activity of their extracts was evaluated. These were obtained by conventional solid-liquid extraction using water, hexane, ethanol, and methanol as solvents. The most promising solvents, ethanol and methanol, were also used to perform extraction by Soxhlet. The antimicrobial activity of the crude extracts was evaluated by the disc diffusion method against the bacteria *Escherichia coli* and *Staphylococcus epidermidis*, and the fungi *Saccharomyces cerevisiae*. Additionally, the total phenolic (TPC) and total flavonoid content (TFC) were accessed as also the antioxidant activity by the 2,2-azinobis (3-ethyl-benzothiazolina-6-sulfonic acid) and 2,2-diphenyl-1-picrylhydrazyl methods. The remaining biomass of *A. americana* from the extractions was then tested as a potential substrate for fermentation.

The Soxhlet extraction using methanol as solvent was the methodology and solvent that allowed to achieve the highest extraction efficiency of 43.8 ± 8.4 %. Tetracycline was the antibiotic whose antibacterial capacity was highly potentiated by the presence of the extracts, especially against *E. coli*, with the extract from *A. americana* producing the strongest positive effect (increase on the tetracycline inhibition halo by 2.5 times). Chloramphenicol was also potentiated against *E. coli*, and the extracts from the *T. fluminensis* stem produced relevant effects on the action of the antibiotics in both bacteria. *A. americana* crude extract was the only one that revealed antifungal activity against *S. cerevisiae* (11 ± 0.0 mm). Regarding the phytochemical characterization, the extract from *T. fluminensis* leave showed both the highest TPC and TFC of $2.75 \pm 4.23 \times 10^{-3}$ mg GAE/g extract and $3.48 \pm 3.22 \times 10^{-1}$ mg CE/g extract, respectively. *T. fluminenesis* also revealed higher antioxidant activity.

Resumo

As plantas são fontes reconhecidas de compostos com elevado impacto biotecnológico. Entre esses compostos, os fitoquímicos têm particular importância pois são responsáveis pela proteção das plantas contra fatores externos como stress ambiental e organismos patogénicos. Devido à sustentabilidade inerente aos produtos de origem natural, os fitoquímicos têm sido amplamente estudados e utilizados como conservantes de alimentos e agentes farmacêuticos, principalmente como agentes antimicrobianos e antioxidantes. Esse potencial gera interesse nesses compostos, uma vez que podem fazer parte da solução contra bactérias resistentes a antibióticos, potenciando a ação de antibióticos e outros antimicrobianos.

As plantas invasoras são um grupo de plantas que não pertencem naturalmente ao habitat onde estão presentes. Normalmente, crescem e desenvolvem-se mais rápido que as espécies autóctones, prejudicando a biodiversidade desses locais. A ausência de legislação e medidas para combater esta questão tem levado a um problema global. Há falta de estudos sobre essas espécies, principalmente do ponto de vista metabolómico – quais mecanismos e compostos bioativos que tornam essas espécies mais fortes do que as nativas em condições específicas. Assim, este trabalho visa avaliar o potencial fitoquímico de espécies vegetais invasoras selecionadas para validar a hipótese de que esta biomassa pouco explorada pode ter elevado potencial biotecnológico.

Dessa forma, duas espécies vegetais invasoras, *Agave americana* (folhas) e *Tradescantia fluminensis* (caule e folhas, separadamente), foram colhidas em território nacional e a atividade antimicrobiana dos seus extratos foi avaliada. Estes foram obtidos por extração sólido-líquido convencional usando água, hexano, etanol e metanol como solventes. Os solventes mais promissores, etanol e metanol, também foram usados para realizar a extração pelo método Soxhlet. A atividade antimicrobiana dos extratos brutos foi avaliada pelo método de difusão em disco contra as bactérias *Escherichia coli* e *Staphylococcus epidermidis*, e o fungo *Saccharomyces cerevisiae*. Adicionalmente, o teor de fenólicos totais (TPC) e flavonoides totais (TFC) foram avaliados, bem como a atividade antioxidante pelos métodos 2,2-azinobis (3-etil-benzotiazolina-6-acido sulfónico) e 2,2-difenil-1-picrilhidrazila. A biomassa remanescente das extrações de *A. americana* foi ainda testada como um potencial substrato para fermentação.

A extração Soxhlet usando metanol como solvente foi a metodologia que alcançou a maior eficiência de extração 43,8 ± 8,4 %. A tetraciclina foi o antibiótico cuja capacidade antibacteriana foi mais potenciada pela presença dos extratos, principalmente contra *E. coli*, com o extrato de *A. americana* a produzir o efeito positivo (aumento do halo de inibição da tetraciclina em 2,5 vezes). O cloranfenicol também foi potenciado contra *E. coli* e os extratos do caule de *T. fluminensis* produziram efeitos relevantes na ação dos antibióticos em ambas as bactérias. O extrato bruto de *A. americana* foi o único que revelou atividade antifúngica contra *S. cerevisiae* (11 ± 0,0 mm). Em relação à caracterização fitoquímica, o extrato da folha de *T. fluminensis* apresentou tanto o maior TPC quanto o TFC de 2,75 ± 4,23 × 10^{-3} mg GAE/g extract e 3,48 ± 3,22 × 10^{-1} mg CE/g extract, respectivamente. *T. fluminenesis* também revelou maior atividade antioxidante.

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1. Introduction

Multidrug-resistant bacteria are a group of microorganisms that developed mechanisms to protect themselves from a diversity of antibiotics. This is one of the greatest challenges in the health system nowadays, representing a serious problem for public health [1]. Microorganisms have shown the ability to protect and adapt since the appearance of the first antibiotics. *Staphylococcus aureus*, for example, dealt with the introduction of penicillin by producing β-lactamases, resulting in a 75% increase in its resistance [2], [3]. However, globalization, and excessive and inappropriate use – not only in humans but also in animal husbandry and aquaculture – have caused the emergence of multidrug-resistant pathogens and the spread of this problem outside of the hospital environment [1], [4]. The World Health Organization (WHO) has established a list of bacteria for which new antibiotics are urgently needed, specifically the known ESKAPE bacteria which includes *Enterococcus faecium, S. aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* species classified as critical (level 1 of priority) [5]. These microorganisms usually lead to consequences like chronic illness, complicated treatment, and an increase in morbidity, mortality, and medical expenses [4], [6]. Bacteria have different ways to combat the effect of the antibiotic like decreasing its ability to penetrate the cell, expelling it from the cell through the efflux pump mechanism, inactivating it via modification or degradation, and modification in its target within the microorganism (Figure 1) [7], [8]. Among different strategies like immuno-antibiotics, and biochemical approaches, phytochemicals emerge as one of the most promising alternatives for complementing antibiotics [6], [8]. These compounds are usually extracted from plants using different solvents like methanol, ethanol, hexane, or water, and are part of different groups such as phenols, flavonoids, and terpenes [6], [9].

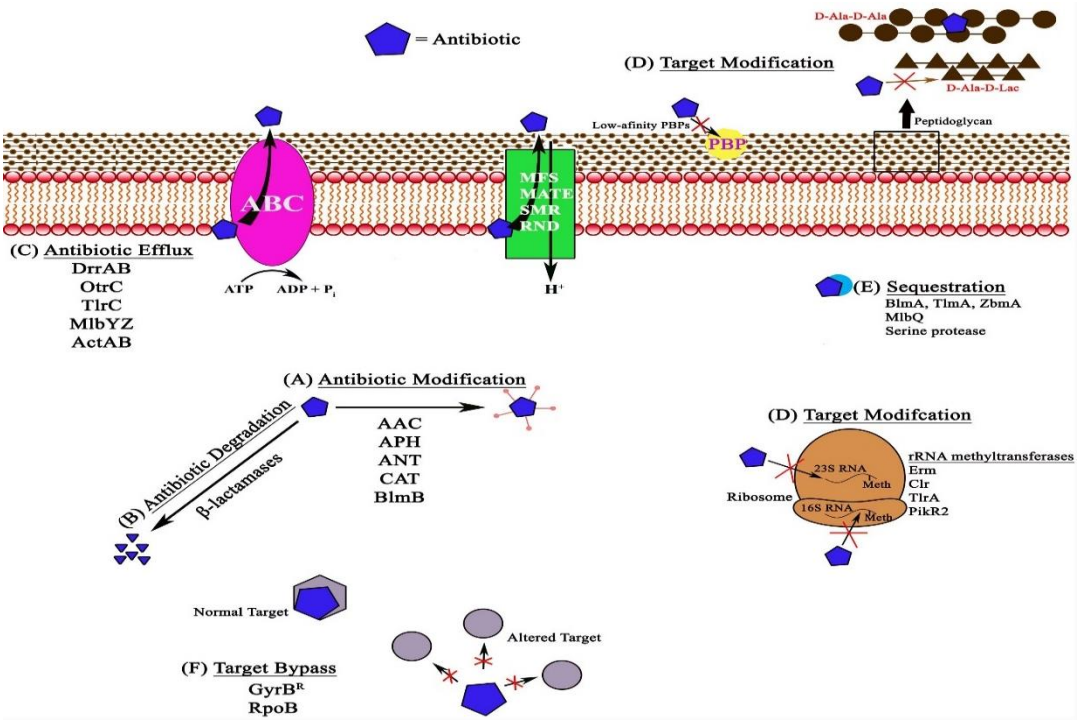


Figure 1 - Different antibiotic resistance mechanisms (From [7]).

Approximately 30% of the planet Earth is covered by land, and 30% of the land surface is covered by plants in all different forms – forests, shrubs, grass, crops, and urban gardens [10]. Plants has diverse indispensable roles on the planet and in the specific ecosystems that they are part of, such as: being the habitat for several living beings, maintaining the air quality by converting carbon dioxide (CO_2) into oxygen (O_2) during photosynthesis, sustaining the soil and maintaining soils nutrients balance, and constituting a food source for several species. More related to humans, plants are also seen as a source of medicine, and contribute to well-being in several ways – green city parks, and house gardens.

Since ancient times, plant extracts have been used for several purposes due to the bioactive compounds produced in plants metabolism, some of those are still included in the actual activities [11]. Among those, phytochemicals have particular importance since they are responsible for plants' protection against external factors like environmental stress and pathogens [12]. Nowadays, plant extracts and phytochemical molecules are used as food preservatives and pharmaceutical agents, due to their antimicrobial and antioxidant activities [9]. Hundreds of plant species are or have been used, for example, bearberry (*Arctostaphylos uva-ursi*) to treat urinary tract infections [13], or rosemary (*Rosmarinus officinalis*) to preserve and delay food oxidation [14], [15]. Beyond the sustainability inherent to naturally derived products, the observed activities generate interest in them since they may be part of the solution against antibiotic-resistant bacteria, potentiating antibiotics and other antimicrobials [16].

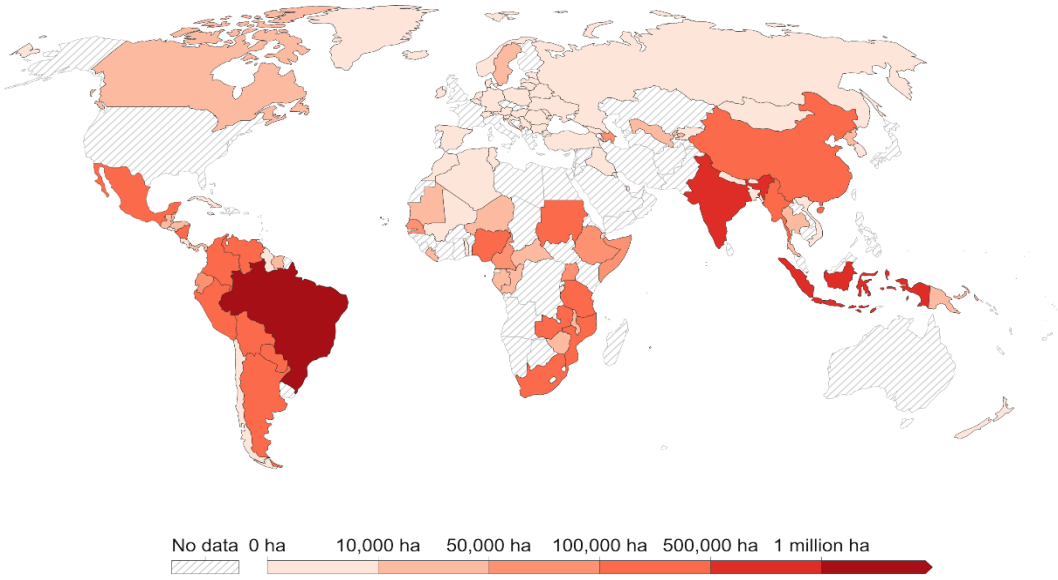


Figure 2 - Annual deforestation 2015-2020 [17].

However, we are facing a reduction of the green area, special the forest area, which plays a major influence on the above-presented roles (Figure 2) [17]. Deforestation is the destruction of the forest by natural occurrences or by the action of men for the use of the land for different purposes. The main causes of this phenomenon are industrial agriculture and livestock, urbanization, wood resources overexploitation, climate changes, and natural disasters [18]. When there is land space available, new plants can grow in the places once occupied by the native ones [19]. Invasive plant species are a group of exotic plants – not naturally belonging to a habitat where they are present – that grow faster than autochthonous

species, harming the biodiversity of those places. For example, several eucalypt species are known for their capacity to massively regenerate through seeds in recently burned areas, becoming an ecological problem in regions where the species is not native [20].

To be considered an invasive species, an exotic plant has to: reproduce autonomously and numerously; spread away from its natural place, in time and space, independently from the environment disruption degree and without men's intervention; reach high densities; promote environmental changes and/or socioeconomic prejudices [21]–[23]. They are seen as one of the major threats to the ecosystems because of the following negative impacts:

- Economic impacts: especially when invading crops' fields, forests, or aquacultures, they represent a loss in production and costs in measures to control and recover the places
- Public health impacts: when the species are toxic, cutting or a vector to plagues
- Groundwater impacts: some species have high water demand, increasing the problems' dimension with the fast growth
- Ecosystem impacts: for example, changing the biogeochemical cycles, fires susceptibility, food chains, and competition with native species – even replacing them totally in some cases

Invasive plant species have some characteristics that potentiate their fast and wide spread, such as fast growth, competing more efficiently for the available resources than the native ones, pyrophyte species – plants that are adapted and are favored by fire –, do not have natural predators in the places where they are invading, and vegetative reproduction [21]–[23].

The European Commission has already established the EU Biodiversity Strategy for 2030, which contains the commitment to manage established invasive alien species. The Invasive Alien Species Regulation (Regulation (EU) 1143/2014) includes a set of measures to be taken across the EU concerning invasive alien species aiming to prevent, minimize and mitigate the adverse impacts posed by those plants with steps of action: prevention, early detection and rapid eradication, and management [24], [25]. In Portugal, the European regulation is applied via the *Decreto-Lei 92/2019*, which confers the responsibility of elaborating the control plans, contention, or eradication to the *Instituto da Conservação da Natureza e das Florestas* (ICNF), that should maintain this information available and updated in their respective website [26]. However, after consulting the website, it was not found any objective strategy despite the obligation of eradication if you own any invasive plant species [27].

There is a lack of studies about these species, especially from a metabolomics perspective – which mechanisms and bioactive compounds make these species stronger than native ones in specific conditions? This work aims at evaluating the phytochemical potential of selected invasive plant species to validate the hypothesis that this underexplored biomass may have high biotechnological potential, giving an end to this undervalued class of plants. Additionally, the fermentative potential of the remaining biomass was studied, contributing to the sustainability of the global process, and promoting a zero-waste approach. The potential use

of extraction by-products as a new source/raw material for bioenergy production also contributes to a positive impact on other concerning issue like global warming.

2. State of the art

2.1. Antimicrobial resistance impacts

Antibiotics are chemical compounds generated from microorganisms used as medicine to treat bacterial infections, inhibiting or destroying pathogens [28], [29]. Antibiotic resistance, on the other hand, happens when a bacterium changes as a consequence of antibiotic use [8]. In 1929, Sir Alexandre Fleming discovered penicillin, the first antibiotic with special action in *Staphylococcus* and *Streptococcus* species [30]. After it gained extensive usage in 1940, the antibiotic resistance challenge arises, causing a big search for new alternatives. In 1944 streptomycin appeared, followed by methicillin, but once again resistant strains were developed almost immediately [31]–[33]. Nowadays, it is estimated that 70% of all pathogenic species are resistant to at least one commercially available antibiotic [34]. The excessive use of antibiotics is considered a determinant factor leading to the emergence of bacterial resistance [32]. Due to the selective pressure imposed by antibiotics – the more often antibiotics are used, the greater the pressure – and to the short life cycle of bacteria, this capacity rapidly spreads and becomes present in a major part of the bacterial population. Besides mutations, the direct DNA transfer via plasmids between bacteria plays an important part in the circulation of antibiotic resistance, a process that can be induced by antibiotics exposure [35], [36]. Some situations can turn into resistance transmission vectors when procedures like antibiotic use and disinfection are not correctly applied. Some examples are sanitation settings, infection control standards, water quality, the standard of drugs, diagnostics and treatments, and quarantine migrations [6]. It is also believed that the co-existence of the antibiotic-producer bacteria and the antibiotic-target bacteria contributes to the spread of antibiotic resistance mechanisms. Naturally, organisms that produce antimicrobial compounds have a self-defense mechanism against them, which may be transferred to the pathogens. Resistance determinants found in these two groups of bacteria support this hypothesis [37], [38]. Some of the reported processes used by these multi-drug resistant (MDR) pathogens are the upregulation of efflux pumps, structural modification of porins, enzyme synthesis, and cell-to-cell communication [39]. As antibiotics become less effective, some diseases like pneumonia, tuberculosis, blood poisoning, gonorrhea, and food-borne become harder to treat, if not impossible in some cases [40]. About 64% of people infected with MDR bacteria species have higher chances to die than people infected with other species. Actually, it is estimated that MDR infections take around 10 million lives per year, costing the world economy about $100 trillion [41].

2.2. Phytochemicals for microbial control

With the failure of screening synthetic combinatory compounds libraries, it has consequently demonstrated the potential of phytochemicals and the importance to study them further [42]. Phytochemicals are chemical compounds synthesized by plants to protect themselves from predators and pathogens [6], [12]. These natural products have the advantages of interacting with biological systems, low-associated toxicity, low-associated cost,

multiple targets and pathways, and inherent sustainability [43], [44]. Many phytochemicals have shown the ability to combat imminent development and risk of antibiotic resistance. These phytochemicals are typically obtained from crude extracts, using different techniques and solvents for the extraction process, providing different efficiencies and chemical selectivity [45].

These compounds are present in a variety of plants, from small flowers to big trees, and in the different parts of them such as roots, leaves, fruit, and stalk. They can be divided into four big chemical groups: alkaloids, organosulfur compounds, phenolics and their derivatives (like flavonoids), and terpenoids [6]. Despite terpenes being the larger class of compounds, phenolics have the larger spectrum of different bioactivities, as will be explored further in this work [39]. Table 1 presents several plant species used to extract phytochemicals and the respective total phenolic content (TPC), a common way to quantify phytochemicals in an extract.

Table 1 - Total phenolic content (TPC) of plant species and respective selected solvent and extraction technique.
**not the TPC, but the extraction yield in g of extract/g of dried plant (%); GAE: Galic acid equivalent; DW: Dry weight; FW: Fresh weight*

Technique	Solvent	Plant specie	TPC	Reference
Solid-liquid	Water	*Olea europaea*	5.7*	[9]
		Acacia dealbata	8.1*	[9]
		Syzygium cumini	24.2 mg GAE/g DW	[46]
		Punica granatum	14 mg GAE/g plant	[47]
		Juglans regia	7 mg GAE/g plant	[47]
		Moringa oleifera	8 mg GAE/g plant	[47]
		Cassia fistula	5 mg GAE/g plant	[47]
		Olea europaea	7.9*	[48]
		Tradescantia fluminensis (leaves)	4.69 mg GAE/g plant	[49]
		Tradescantia fluminensis (stem)	2.96 mg GAE/g plant	[49]
	Methanol	*Olea europaea*	7.2*	[9]
		Acacia dealbata	7.9*	[9]
		Salacia chinensis	46 mg GAE/g DW	[50]
		Olea europaea	131.7 mg GAE/g DW	[51]
		Salvia officinalis	22.6 mg GAE/g plant extract	[52]
		Salvia sclarea	24.0 mg GAE/g plant extract	[52]
		Salvia glutinosa	17.1 mg GAE/g plant extract	[52]
		Salvia pratensis	9.7 mg GAE/g plant extract	[52]
		Lavandula angustifolia	5.4 mg GAE/g plant extract	[52]
		Calendula officinalis	6.6 mg GAE/g plant extract	[52]
		Matricaria recutita	7.5 mg GAE/g plant extract	[52]
		Echinacea purpurea	4.1 mg GAE/g plant extract	[52]
		Rhaponticum carthamoides	13.3 mg GAE/g plant extract	[52]
		Juglans regia	11.5 mg GAE/g plant extract	[52]
		Melilotus officinalis	4.3 mg GAE/g plant extract	[52]
		Geranium macrorrhizum	25.9 mg GAE/g plant extract	[52]
		Potentilla fruticosa	37.9 mg GAE/g plant extract	[52]

		Olea europaea	30*	[48]
		Agave americana	15.1*	[53]
	Ethanol	*Olea europaea*	4.1*	[9]
		Acacia dealbata	6*	[9]
		Rosmarinus officinalis	46.5 mg GAE/g plant	[54]
		Salvia officinalis	41.6 mg GAE/g plant	[54]
		Bidens pilosa	21.45 mg/g	[55]
		Abutilon theophrasti	3.34 mg GAE/g FW	[56]
		Amaranthus retroflexus	1.87 mg GAE/g FW	[56]
		Ambrosia artemisiifolia	3.42 mg GAE/g FW	[56]
		Datura stramonium	2.86 mg GAE/g FW	[56]
		Erigeron annuus	13.66 mg GAE/g FW	[56]
		Galinsoga ciliata	1.43 mg GAE/g FW	[56]
		Reynoutria japonica	9.38 mg GAE/g FW	[56]
		Solidago gigantea	9.06 mg GAE/g FW	[56]
		Sorghum halepense	5.07 mg GAE/g FW	[56]
		Tradescantia fluminensis (leaves)	3.01 mg GAE/g plant	[49]
		Tradescantia fluminensis (stem)	1.92 mg GAE/g plant	[49]
	Acetone	*Olea europaea*	6.8*	[9]
		Acacia dealbata	5.1*	[9]
	Hexane	*Olea europaea*	0.9*	[9]
		Acacia dealbata	7.8*	[9]
		Tradescantia fluminensis (leaves)	2.04 mg GAE/g plant	[49]
		Tradescantia fluminensis (stem)	8.45 mg GAE/g plant	[49]
Ultra-sound	Water	*Olea europaea*	6.2*	[9]
		Acacia dealbata	6.4*	[9]
		Punica granatum	16 mg GAE/g plant	[47]
		Juglans regia	8 mg GAE/g plant	[47]
		Moringa oleifera	12 mg GAE/g plant	[47]
		Cassia fistula	8 mg GAE/g plant	[47]
	Methanol	*Olea europaea*	6*	[9]

		Acacia dealbata	5.7*	[9]
		Salacia chinensis	45 mg GAE/g DW	[50]
	Methanol	*Ailanthus altissima*	41.70 mg/g DW	[57]
		Ambrosia artemisiifolia	15.00 mg/g DW	[57]
		Conyza canadensis	13.00 mg/g DW	[57]
		Dittrichia viscosa	67.78 mg/g DW	[57]
		Erigeron annuus	45.50 mg/g DW	[57]
		Xanthium strumarium	29.02 mg/g DW	[57]
	Ethanol	*Olea europaea*	4.6*	[9]
		Acacia dealbata	4.2*	[9]
		Solanum melongena	29 mg GAE/g DW	[58]
		Gleditsia triacanthos	113.62 mg GAE/g DW	[59]
	Acetone	*Olea europaea*	7.4*	[9]
		Acacia dealbata	2.8*	[9]
	Hexane	*Olea europaea*	3.2*	[9]
		Acacia dealbata	3.7*	[9]
Soxhlet	Water	*Olea europaea*	12*	[9]
		Acacia dealbata	13*	[9]
	Methanol	*Agave americana*	16.9*	[60]
Microwave	Water	*Olea europaea*	11*	[9]
		Acacia dealbata	12*	[9]
		Syzygium cumini	26.1 mg GAE/g DW	[46]
		Punica granatum	19 mg GAE/g plant	[47]
		Juglans regia	9 mg GAE/g plant	[47]
		Moringa oleifera	15 mg GAE/g plant	[47]
		Cassia fistula	12 mg GAE/g plant	[47]
	Methanol	*Salacia chinensis*	46 mg GAE/g DW	[50]

2.2.1. Extraction techniques and solvents

These bioactive compounds are extracted from the plants using different extraction techniques and different solvents, which can be more or less efficient depending on the plant and also the compound with interest to extract. The solvents can be divided into polar solvents – like water, ethanol, and methanol – and non-polar solvents – like hexane and dichloromethane – and are employed to extract polar and non-polar compounds, respectively [45]. Comparing the polar compounds, water has the highest polarity and is used to extract a wide range of polar compounds. It has also the advantages of being cheap, nontoxic, and non-flammable. Alcoholic compounds, like ethanol and methanol, can extract polar secondary metabolites, are non-toxic at low concentrations, and resulting extracts are easily concentrated with low heat requirements [45]. Regarding extraction techniques, the most common and simple method is the solid-liquid, where the plant – typically in powder – is covered by the solvent and kept under agitation for the desired duration – heating is optional [45]. In the Soxhlet method, the plant powder is imprisoned in a filter that is inserted in the Soxhlet apparatus. The solvent is heated from the bottom chamber and evaporates, passing through a condenser and accumulating in a second chamber where the plant powder is and where the extraction occurs. Consequently, when the level of solvent in the extraction chamber reaches the top of the siphon, the solvent and the extracted plant material flow back to the first chamber. This is repeated until the phytochemical is completely extracted [45]. Microwave-assisted extraction is one of the most advanced techniques and is based on the mechanism of dipole rotation and ionic transfer by displacement of charged ions present in the solvent and drug material, being especially suitable for flavonoid extraction. The object by being bombarded with microwave radiation absorbs it converting it into heat, easing the movement of solvent into the drug matrix. Ultrasound-assisted extraction applies sound energy to disrupt the vegetable cells increasing the surface area that is exposed to the solvents' action [45]. The advantages and disadvantages of each presented technique are shown in Table 2.

Table 2 – Potentialities and limitations of extraction techniques (Adapted from [45]).

Extraction technique	Advantages	Disadvantages
Solid-liquid	Suitable for thermolabile compounds Low equipment requirements	Lower extraction efficiency
Soxhlet	Extracts a large drug amount No filtration step is required	Inadequate for thermolabile compounds Regular shaking is not possible
Microwave-assisted	Low solvent requirements Low extraction time Increases the outcome	Not favors the use of nonpolar solvents Suitable only for phenolic compounds and flavonoids
Ultrasound-assisted	Reduced heat requirements Low solvent requirements Low extraction time Maximizes the yield	The material to extract should be powdered Applicable only to small samples Difficult reproducibility May degrade the phytochemicals

2.2.2. Mechanism of antimicrobial action

Contrary to conventional antibiotics, plant natural compounds (NPs) act on pathogen bacteria through different strategies that target virulence (Figure 3), potentially exerting less selective pressure for resistance since they interfere with nonessential cellular processes, as will be seen below. Antibiotics affect directly essential functions like protein and DNA synthesis, inhibiting bacterial growth as a bactericide or bacteriostatic, having as big drawback the inevitable selection of resistant microorganisms that become indifferent to a certain antibiotic mechanism of action [39]. On the other hand, phytochemicals can increase cell permeability and leakage, inhibit drug efflux pumps, obliterate resistance(R)-plasmids, inhibit enzymes, or attenuate biofilm formation by inhibiting quorum sensing activity [6].

One of the mechanisms of action of NPs is the influence on the cell's permeability and integrity. Cell walls and membranes are responsible for the support and protection. Phytochemicals can interact with N-acetylmuramic acid and N-acetylglucosamine residues inhibiting peptidoglycan synthesis, a key component of the cell wall, causing a loss in structural and chemical stability [61]. In the case of the cell membrane, some hydrophobic phytochemicals can interact with the membrane lipids, changing its permeability, leading to protection failure since bacteria will be unable to control what is uptake [62]. In both cases, the pathogen will become more susceptible to external factors, including antimicrobial compounds. For example, flavonoids such as catechin are known for their capacity to complex with the cellular wall [6]. The phytochemical 3-p-trans-coumaroyl-2-hydroxyquinic acid, a phenolic compound extracted from *Cedrus deodara*, showed antibacterial effect against *S. aureus*, by damaging the cellular membrane [63]. The terpene farnesol showed inhibition against the same pathogen by disrupting the cell membrane [64].

Many tests have proved the inhibitory role of phytochemicals on bacteria efflux pumps, one of the most common action mechanisms. The exact mechanism of action in MDR microorganisms is still unknown, but it is proposed that the compound directly interacts with the pump inactivating it. Different proposals consist in the depletion of energy – some drug efflux pumps are ATP dependent [65] – or the formation of a complex with the antibiotic easing the cell entry – the bigger size of the formed complex will confine the antibiotic inside the cell, turning it excretion harder [66], [67]. For example, the alkaloid lysergol derived from *Ipomoea muricata* showed an effect against *E. coli* by inactivating efflux pumps [68]. Resveratrol, a phenolic compound, is also known for its capacity as efflux pump inhibitors EPIs [69].

Some phytochemicals have also the R-plasmid elimination curation or elimination capacity, blocking its transmission between bacteria [70]. Some studies have demonstrated that, by the interaction of the NPs with a plasmid, causing its loss from the host or inhibiting the type IV secretion system T4SSs. There are also non-natural compounds that fulfil this duty, however, they are associated with mutation and toxicity, making them unsuitable [71], [72]. For example, the alkaloid 8-Epidiosbulbin-E-acetate obtained from *Dioscorea bulbifera* has the R-plasmid curing ability in several pathogens with relevant efficacy [65].

Another way used by NPs to combat multi-resistant bacteria is by inhibiting enzyme activity. Phytochemicals have a long spectrum of action blocking several enzymes. Related to antibacterial activity, its interaction with DNA gyrase interrupts DNA replication [73]. NPs also influence other natural catalyzers that are not linked with microbial activity like α-amylase and α-glucosidase – enzymes involved in type II diabetes disease –, and tyrosinase – involved in the aging process, protecting the skin against sunlight [74]. Enzyme inactivation by phytochemicals can also play an important role in combating cancer, inhibiting carcinogenesis by blocking phase I enzymes and induction of phase II enzymes, scavenging DNA reactive agents, suppressing the abnormal proliferation of early, preneoplastic lesions, and inhibiting certain properties of the cancer cell [75]. For example, the alkaloid kokusagine derived from *Teclea afzeli* exhibited enzymatic alteration capacity, affecting DNA synthesis [76]. Also, the organosulfur allicin extracted from *Allium sativum* showed the capacity to inhibit not only DNA synthesis but also protein synthesis [77].

Finally, phytochemicals can attenuate biofilm formation by intervention in quorum sensing – the mechanism by which bacteria communicate with each other through self-synthesized chemical signals called autoinducers (AIs) [6]. This can be done via different pathways such as inhibition of AIs synthesis, AIs receptor antagonism, inhibition of targets downstream of receptor binding, and inhibition of AI secretion/transport [78]. For example, quercetin, a well-known flavonoid, is known for its inhibitory activity against biofilm formation and quorum sensing [79]. Methyl eugenol, a terpene extracted from *Cuminum cymnium*, inhibited the biofilm formation of several MRD bacteria by attenuating the signal-based QS [80].

Some phytochemicals have also shown antimicrobial capacity working as a disruptor of the FtsZ z-ring inhibiting cytokinesis. An example is the alkaloid sanguinarine extracted from *Solidago canadensis* [81].

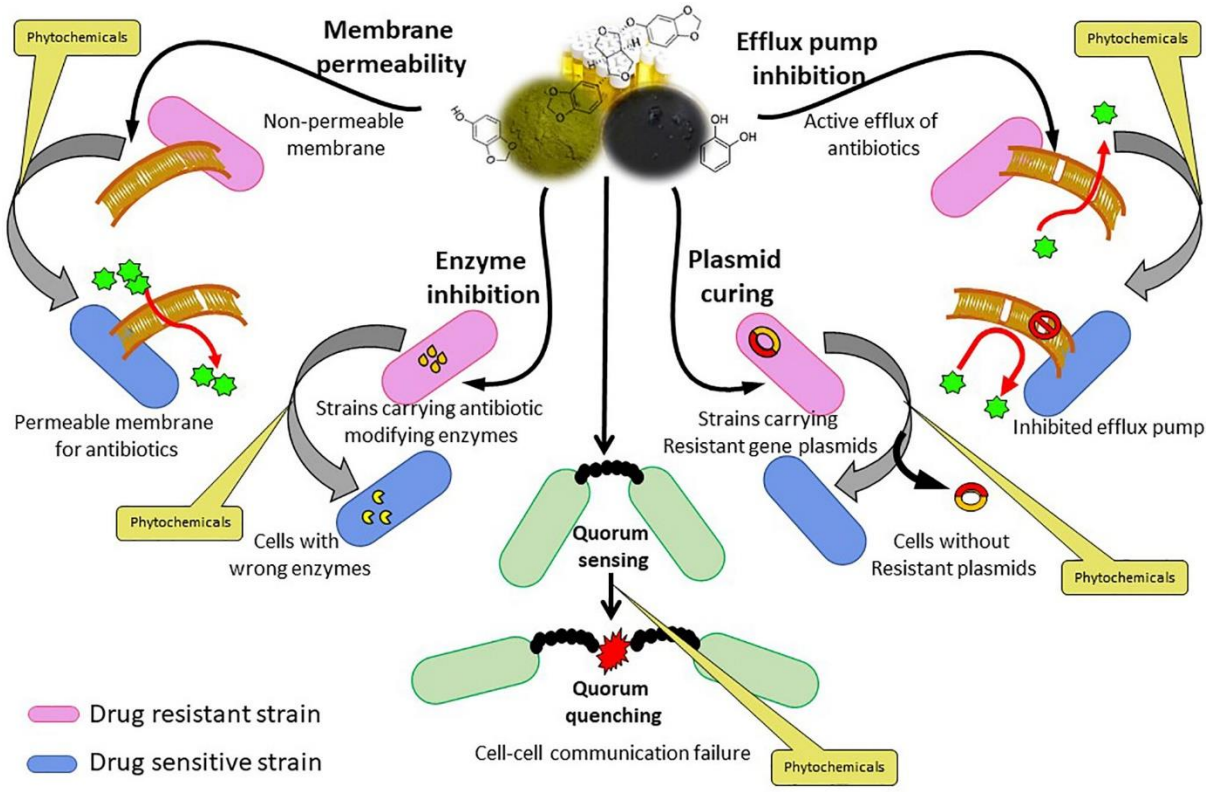


Figure 3 - Phytochemicals antibacterial mechanisms of action (From [6]).

2.3. Invasive alien plants (IAPs)

2.3.1. Impacts and control

Among all the different groups of plants that act as sources of phytochemicals or have the potential for that, invasive plant species emerge as a very interesting feature due to their known survival and growth advantage compared to the autochthonous species. However, nowadays the spotlight points to this plant group for negative reasons, namely the prejudicial effects that they have in the ecosystems which are invading but also in society in a general way. The proliferation of these plants can affect the economy (when invading crops fields), public health (in case of adverse reactions in the body), and environment/ecosystems. The environmental impacts associated to invasive plants are the ones that concern the scientific society the most. Barney *et al*. resumed and organized the different levels and respective types of ecological impacts of invasive plant species, as it is shown in Table 3 [82].

Table 3 – Levels and types of ecological impacts of invasive alien plants (Adapted from [82]).

Levels	Individual	Community	Structure	Biogeochemical	Ecosystem
Type	• Fitness • Growth	• Productivity • Diversity • Abundance • Intraspecific	• Physiognomy	• Pools • Litter • Fluxes • Moisture	• Food chain • Interaction • Fluxes • Disturbance • Geomorphology

All these impacts are a result of the competition between invasive and native plants. However, some authors have also reported that certain autochthonous species can coexist

and coevolve with the invaders, which will act as selective agents [83], [84]. This can also be seen as a negative point since these “new-native species” can turn into invasive species due to their new superior ability compared to the other autochthonous species. In Portugal, 185 plant species are signalized as invasive with different risks associated. They are listed at the *Decreto-Lei 92/2019*, alongside other types of invasive species such as insects, fishes, and birds [26]. Table 4 presents some of the most well-established alien plant species in Portugal, with their respective risk level, places of origin, and major associated impacts. The impacts were divided into economics, environment, health, and societal – which affect day-to-day, tourism, leisure, and other human activities. The risk level is obtained by a protocol adapted from Pheloung *et al*. [85], by Morais *et al.* [86], according to which a value over 13 declares that the plant has the risk to be invasive in the Portuguese territory [23], [85], [86]. These invaders can be prevenient from every continent, with a bigger expression in the American continent (Table 4). Several causes can contribute to the dispersion of these species across the globe. They can be natural, like population migrations, seeds transport by the wind, water, or animals, and geographic and climatic alterations [87]. With the growing evolution and globalization of human society, our interference in these phenomena increased, with some plants playing an important role as food, and ornament, among others, leading to their transport and commercialization to different parts of the planet. This transport can also be inadvertent when seeds are occult in vehicles of transport. The destruction of natural green spaces also contributes to the dissemination of alien plant species since it creates space for their growth and development.

Table 4 - Invasive alien plants in Portuguese territory [23].

Specie	Risk level	Origin	Major impacts
Abutilon theophrasti	27	Southeast Asia to the Centre of the Mediterranean Region	Economics
Acacia cyclops	16	Southwest Australia	Ecosystems
Acacia dealbata	31	Southeast Australia and Tasmania	Economics; Ecosystems; Health
Acacia karroo	22	South Africa to Zambia and Angola	Ecosystems; Health
Acacia longifolia	30	Southeast Australia	Economics; Ecosystems
Acacia mearnsii	27	Southeast Australia and Tasmania	Ecosystems
Acacia melanoxylon	28	Southeast Australia and Tasmania	Economics; Ecosystems
Acacia pycnantha	23	Southeast Australia	Ecosystems
Acacia retinodes	17	South Australia	Ecosystems
Acacia saligna	24	Western Australia and Tasmania	Economics; Ecosystems
Acer negundo	18	North America	Ecosystems; Society
Agave americana	18	Mexico and western USA	Economics; Ecosystems; Health
Ageratina adenophora	28	Mexico	Economics; Ecosystems; Health
Ailanthus altissima	20	China	Economics; Ecosystems; Health
Araujia sericifera	33	Southeast of South America	Economics; Ecosystems; Health; Society
Arctotheca calendula	28	South Africa	Ecosystems; Health
Arundo donax	14	Eastern Europe, Temperate and Tropical Asia	Economics; Ecosystems; Society
Aster squamatus	15	Central and South America	Economics; Ecosystems; Health
Azolla filiculoides	26	Tropical America	Economics; Ecosystems; Society
Bidens aurea	19	North America	Economics; Ecosystems
Bidens frondose	20	North America	Ecosystems
Carpobrotus edulis	23	South Africa	Economics; Ecosystems
Clethra arborea	15	Portugal (Madeira Archipelago)	Ecosystems
Conyza bonariensis	26	South America	Economics; Ecosystems; Health
Conyza canadensis	26	North America	Economics; Ecosystems; Health
Conyza sumatrensis	26	South America	Economics; Ecosystems; Health
Cortaderia selloana	26	Tropical zone of South America	Economics; Ecosystems; Health
Cotula coronopifolia	16	South Africa	Economics; Ecosystems
Datura stramonium	32	Tropical South America	Economics; Ecosystems; Health

Delairea odorata	23	South Africa	Ecosystems; Health
Egeria densa	20	South America	Ecosystems; Society
Eichhornia crassipes	30	South America	Economics; Ecosystems; Society
Elodea canadensis	25	North America	Ecosystems; Society
Erigeron karvinskianus	20	Mexico and tropical Southern America	Ecosystems
Eryngium pandanifolium	17	Subtropical zone of South America	Economics; Ecosystems
Eucalyptus globulus	21	Southeast Australia and Tasmania	Economics; Ecosystems
Fallopia japonica	17	Asia	Economics; Ecosystems; Society
Gomphocarpus fruticosus	27	East and Southern Africa, and Southern Arabian Peninsula	Economics; Ecosystems; Health
Gunnera tinctoria	27	South America	Economics; Ecosystems
Hakea salicifolia	19	Southeast Australia and Tasmania	Economics; Ecosystems
Hakea sericea	21	South Australia	Economics; Ecosystems; Health
Hedychium gardnerianum	18	Asia	Economics; Ecosystems; Health
Ipomoea indica	30	Tropical zone of South America, Asia and Hawaii	Ecosystems
Lantana camara	34	Central and South America	Ecosystems
Myriophyllum aquaticum	25	South America	Economics; Ecosystems; Society
Nicotiana glauca	23	Tropical South America	Ecosystems; Health
Opuntia ficus-indica	29	Tropical America	Economics; Ecosystems; Health
Opuntia subulate	20	South America	Economics; Ecosystems; Health
Oxalis pes-caprae	24	South Africa	Economics; Ecosystems
Paraserianthes lophantha	14	Australia	Ecosystems
Paspalum paspalodes	34	Africa and America	Ecosystems
Pennisetum setaceum	25	East and North Africa, and Southwest Asia	Economics; Ecosystems; Health
Pennisetum villosum	23	Northeast Africa and Arabian Peninsula	Economics; Ecosystems
Phytolacca americana	29	North America	Economics; Ecosystems; Health
Pittosporum undulatum	25	Southwest Australia	Economics; Ecosystems
Ricinus communis	22	Tropical Africa	Ecosystems; Health
Robinia pseudoacacia	32	North America	Economics; Ecosystems
Salpichroa origanifolia	17	South America	Ecosystems
Salvinia molesta	25	South America	Economics; Ecosystems; Health; Society
Spartina densiflora	14	South America	Economics; Ecosystems
Tradescantia fluminensis	18	South America	Economics; Ecosystems
Watsonia meriana	17	South America	Ecosystems

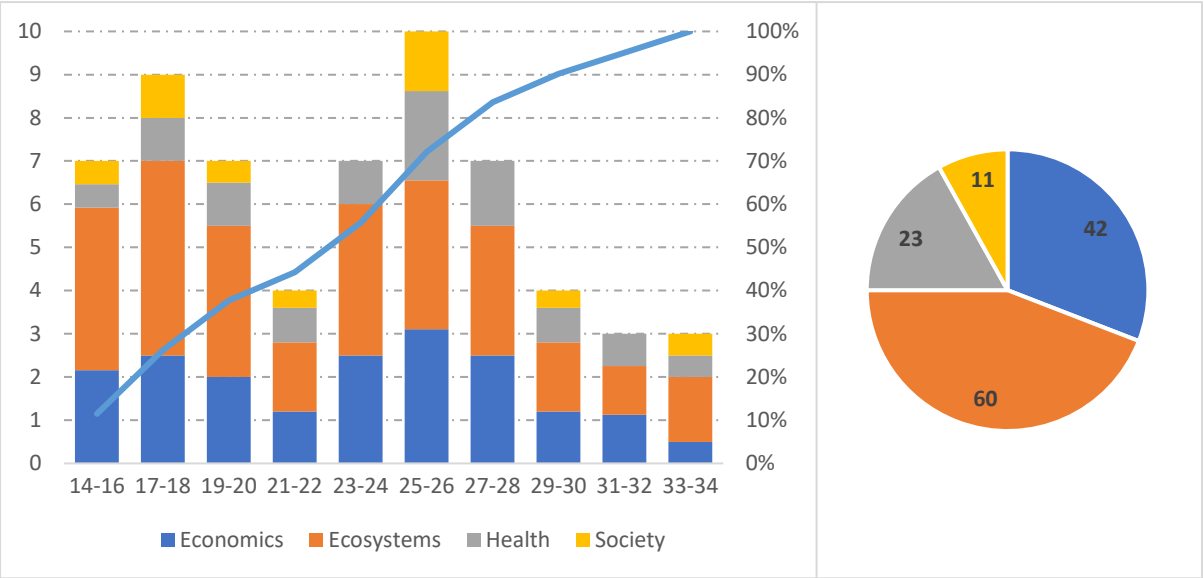


Figure 4 – Invasive alien plants distribution by risk level (left); invasive alien plants distribution by the major impacts (right).

Looking at the distribution of the species compiled in Table 4 by its risk level, it is possible to note in the Figure 4 (left) two peaks, one in the lower (17-18) and the other in the intermediate (25-26) zone. It is also observed that around 84% of the plants are classified with a 28 risk level or lower, meaning that a big part of the population represents a low to medium risk (Figure 4, left). Looking now at Figure 4 (right), 60% of the risks associated with these invaders are environmental, confirming and justifying the concerns of the scientific community [82].

2.3.2. Allelopathy: phytochemical richness

The competition between plants can be considered direct – for example nutrients, space, water – or indirect competition. Indirect competition results from the action of the invaders in a third agent that will then affect the native plants. For example, allelopathy which consists in the production of chemicals by a plant species that can affect neighboring plants – direct competition – or soil microbes – indirect competition – and may cause a relevant and significative impact, altering interactions between native plants and their natural predators and pathogens, or mutualists [83], [88]. For example, the *Centaurea* species' dominance over the native ones is attributed to their superior capacity to shift plant-plant interactions through allelochemical, microbial, and resource depletion mechanisms [89]. Allelochemicals, the compounds responsible for these interactions, are secondary metabolites and belong to diverse chemical groups such as terpenes, phenols, and flavonoids [90], [91]. They are released into the environment causing negative impacts like affecting germination and growth of neighboring plants by disrupting various physiological processes including photosynthesis, respiration, water, and hormonal balance – usually by enzyme inhibition [91]. Due to this potential, allelochemicals have been widely studied as bioherbicides since the last century [91]–[93]. In fact, the literature defines allelochemical as phytotoxic phytochemicals, being now understandable not only the chemical groups similarities but also the applications [94], [95].

Allelopathy plays an important role in the competitive advantage of invasive plant species, being these special producers of allelochemicals. Kalisz *et al.* [88] demonstrates that allelopathy is present across the plant phylogeny, present in every lineage analyzed, counting the occurrence in 51.4 % of the invasive species present on the database – not all invasive plants in the database have been tested, being the presented percentage possibly even higher – which indicates this is a prevalent trait of successful invaders. For example, in *Leucaena leucocephala*, listed among the world's 100 worst invasive alien species, several allelochemicals has been identified, such as phenolic acids, flavonoids, and the amino acid mimosine . The last one is produced in a large amount and accumulated in almost all parts of the plant like leaves and stems, in concentrations up to 6.4% of their dry weight, showing growth inhibitory activity against several plant species [91]. *Imperata cylindrica*, integrated into the same list, has also shown evidence of allelopathy that may contribute to the alteration of the microbial community, including rhizobia and mycorrhizal fungi, suppressing the regeneration process of native plant species through the inhibition of their germination and growth. Different types of allelochemicals were identified like fatty acids, terpenoids, simple phenolics, benzoic acids, phenolic acids, phenolic aldehydes, phenylpropanoids, flavonoids, quinones, and alkaloids [96].

With the ability to interact with the microbiota, allelochemicals and consequently invasive plant species reveal to be a potential source of compounds to combat microbial infections and also to become a part of the solution against bacterial resistance. Additionally to this issue, the use of these types of plants and special their valorization can motivate public and private entities to look at them with different eyes and especially increase the management and eradication of these species, with the advantage of getting residues with added value. Despite the study of phytochemicals and their antimicrobial activity being something that has been made for some time, a specific look to invasive plant species as a source of these bioactive compounds is a subject that has not received the appropriate attention resulting in the absence of studies. A quick search in *Scopus* database for the keywords "antimicrobial and plant species and phytochemicals" resulted in 72,447 document results, 17 times more results than the 4,351 document results found for "antimicrobial and invasive plant species and phytochemicals".

Kocaçalışkan *et al.* [97] have studied the antimicrobial activity of the allelochemicals catechol and pyrogallol, two phenolic compounds synthesized by the shikimate pathway in plants, on five pathogenic species – three bacteria (*Pseudomonas putida, Pseudomonas pyocyanea, Corynebacterium xerosis*) and two fungi (*Fusarium oxysporum, Penicillium italicum*) – using the disc diffusion method. Both compounds showed antibacterial effects at 5 and 10 mM concentrations, however only catechol produced effects on fungi. Alderley *et al.* [98] used a laboratory model system to compare the efficiency of different allelochemicals called isothiocyanates (ITCs) against the plant bacterial pathogen *Ralstonia solanacearum*. All three tested ITCs – allyl-ITC, sec-butyl ITC, and 2-phenylethyl ITC – showed consistent pathogen growth suppression, with the first being the most efficient compound. Also regarding allelochemicals, Jana *et al* [99]. isolated lactam nonanoic acid from *Cleome viscosa* and tested its antimicrobial activity against *Escherichia coli, P. aeruginosa, S. aureus* (bacteria) and *Aspergillus fumigatus, Aspergillus niger, Aspergillus tamarii* (fungi). At a dosage of 500

ppm and above, *P. aeruginosa* and *S. aureus* were totally inhibited. On the other hand, the growth of *A. niger* and *A. tamarii* was stimulated.

Poljuha *et al.* [57] made the phenolic characterization and determined the antimicrobial activity of the following six invasive plant species in Croatia: *Ailanthus altissima, Ambrosia artemisiifolia, Conyza canadensis, Dittrichia viscosa, Erigeron annuus,* and *Xanthium strumarium*. *X. strumarium* extract had the best activity against the tested bacteria (*S. aureus*, *Enterococcus faecalis*, *E. coli*, and *P. aeruginosa*), with an average minimum inhibitory concentration (MIC) value of 0.11 mg/mL, while *A. altissima* and *X. strumarium* extracts had the best activity against the tested fungi (*Candida albicans*, *Cryptococcus neoformans*, and *A. fumigatus*) with an average MIC value of 0.21 mg/mL in both cases. All the plant extracts studied, except *X. strumarium*, were less cytotoxic than the positive control. Marinas *et al.* [59] evaluated the extraction of cellulose from *Gleditsia triacanthos*, an aggressive invasive species in Eastern Europe, to fabricate a wound dressing that is multi-functionalized with phenolic compounds extracted from the leaves of the same species. Among other tests of the synthesized structure, the antimicrobial activity was evaluated, which occurred immediately after the contact with the tested materials and was maintained for 24 h for the clinical multi-drug-resistant strains *E. coli*, *S. aureus*, *P. aeruginosa*, *A. baumannii*, *Enterobacter cloacae*, *C. albicans*, and *Candida parapsilosis*. Still about the bioactivity of invasive plant species extracts, the compositions of the essential oils from the leaves and flowers of *Hedychium gardnerianum* and from the leaves of *Pittosporum undulatum* growing on San Miguel Island (Azores) were investigated by Medeiros *et al.* [100], alongside the antimicrobial activity against *S. aureus*, *Staphylococcus epidermis,* and *P. aeruginosa*. *H. gardnerianum* demonstrated activity over *S. aureus* (19.8 mm average inhibition zone) and *S. epidermidis* (18.8 mm average inhibition zone), while *P. undulatum* displayed no effects. Marinas *et al.* [101] tested the antimicrobial potential of *Solidago canadensis* essential oils against Gram-positive (*S. aureus*, *Bacillus subtilis*, *E. faecalis*), and Gram-negative bacteria (*P. aeruginosa*, *E. coli*, *K.pneumoniae*, *A. baumannii*), and yeasts (*Candida famata*, *Candida utilis*, *C. albicans*). The tested microbial species, with exception for *C. utilis,* proved to be susceptible to *S. canadensis* essential oil which also exhibited good antibiofilm activity, inhibiting the adhesion to the inert and cellular substrate, decreasing the *A. baumannii* adhesion index to 17.52% and changing the adherence pattern. A study made by Piccinin *et al.* [55] investigated the *in vitro* potential of the *Bidens pilosa* as an antibiofilm-antimicrobial against *S. aureus*. All extracts showed antimicrobial activity against that pathogen (MIC = 0.3 mg/mL), with special emphasis for the one obtained after 4 h maceration that at 2 mg/mL completely inhibited and eradicated the *S. aureus* biofilms after 48 h of incubation. Antimicrobial activity of aqueous (distilled water) and alcoholic (methanol) crude extracts from the leaves of the invasive alien plant species *Ageratina adenophora* was evaluated against six human pathogenic bacteria – as Gram-negative bacteria *E. coli, K. pneumoniaea*, and *Proteus mirabilis;* as Gram-positive bacteria *E. faecalis*, *B. subtilis,* and *S. aureus* – by Devkota *et al.* [102] using disc diffusion method. Plant extract in both solvents exhibited pronounced results against tested bacteria, with more evidence for the methanolic extracts. Rashmi *et al.* [103] evaluated the antifungal activity of leaf extract of five invasive plant species – *Ageratum conyzoides*, *Antigonon leptopus*, *Chromolaena odorata*, *Oxalis corniculate*, and *Passiflora foetida* – against the pathogenic

fungal *Macrophomina phaseolina*. Methanolic extract of all the plants exhibited good activity with MIC values ranging between 0.078 – 2.5 mg/mL. The leaf extract of *O. corniculata* has promising antifungal activity with a MIC value of 0.078 mg/mL, a low value compared to the other species extracts tested. Total activity was highest in *A. leptopus* (2304 mL/g).

As shown above, the potential to use invasive plant species as a source of phytochemicals active against pathogenic bacteria is real and has been proved by some authors. The most relevant bioactive compounds found in these alien species that have been reported to have action in their invasion mechanisms are: Catechins – (–)- catechin and (+)-catechin – which are present in *Centaurea* species. Catechins have demonstrated allelopathic effects in native species causing changes in gene expression and the consequent death of the roots system [104]; 8-Hydroxyquinoline, also identified in *Centaurea* species, specifically *C. diffusa*, varies biogeographically in its natural concentration and its effect as an allelochemical [105]; Cnicin, a phytotoxic compound that can be found in *Centaurea maculosa*, showed inhibition of the growth and development of larvae [106]; Deoxymikanolide, isolated from *Mikania micrantha*, play an important role on the invasion success [107]; Ocimenones, present for example in the leaves and reproductive structure of *Tagetes minuta*, delays and inhibits the germination of native species [108]; Methyl jasmonate, also a germination suppressor, can be isolated from sagebrush [109]; Emodin, present at high concentrations in the roots and fallen leaves of *Polygonum sachalinense*, causes inhibition of the seedling growth – also present in the soils where this plant community is established, especially in the autumn ; Physcion, has the same effect and provenience of emodin, with exception for the presence in the soils [110]; Antofine has inhibitory properties against bacteria, pathogenic fungi, and herbivorous insects, contributing for the invasion success of species like *Vincetoxicum rossicum* [111]. It is also believed that macronutrients like sugars, proteins, and other metabolic by-products could play an important role in soil microbial ecology [112].

2.3.3. Challenges of using IAPs as phytochemicals source

Like every solution to a problem, the use of invasive plant species as a source of antimicrobial phytochemicals, or as an antibiotic potentiator, has challenges and eventual limitations associated with it. The first to enunciate is the toxicity of some of those alien plants. As listed in Table 4, a considerable amount of species cause health impacts in humans and also in animals. *In vitro* cytotoxicity tests are indispensable to evaluate, whether the compounds will cause any undesired cell death or not – in classifying the *in vitro* cytotoxicity of plant extracts, values of lethal concentration 50 (LC50) below 20 µg/mL are considered cytotoxic [112], [113]. Poljuha *et al*. [57] determined the cytotoxicity of six extracts obtained from invasive alien species (*A. altissima, A. artemisiifolia, C. canadensis, D. viscosa, E. annuus,* and *X. strumarium*) in an assay against Vero monkey kidney cells. *A. altissima* and *C. canadensis* had the highest LC50 (lowest toxicity) of 0.17 mg/mL. The selectivity index (SI), a ratio that measures the window between cytotoxicity and antimicrobial activity by dividing the given LC50 by the MIC value, was also calculate. The higher the SI ratio, the more effective and safe an extract would be. *A. altissima* presented the highest value against both bacterial and fungi strains. However, caution must be taken in making conclusions based on *in vitro* cytotoxic data only. *In vitro* toxicity data may not always agree with *in vivo* toxicity tests owing

to many factors, such as gut interactions, solubility, bioavailability, and other pharmacokinetic/dynamic parameters. Therefore, further toxicity testing in *in vivo* systems will be useful [57]. Liu *et al.* [114] performed a safety evaluation of extracts from *Eupatorium adenophorum*. *In vitro* tests in human hepatic L02 cells and HepG2 human hepatoma cell culture revealed that the products had no obvious negative effect on cell viability when the concentration was increased to 400 μg/mL in the culture medium. The *in vivo* acute toxicity evaluation in ICR mice demonstrated the safety of the three products at a high dose of up to 15 g/kg bw of mice. Besides cytotoxicity, chronic toxicity, mutagenicity, carcinogenicity, and teratogenicity studies are desired to further support the safe use of these plants. However, the toxicity of the plants does not automatically restrain the use of the plant. In the previously cited work, the researchers were able to extract chlorogenic acid from *Eupatorium adenophorum* and then purify that bio-compound of interest with a macroporous resin, reducing the concentration of the toxin 9-oxo-10,11-dehydroageraphorone in 94% and completely removing 10Hα-9-oxo-ageraphorone and 10Hβ-9-oxo-ageraphorone [114]. Thus, in some cases is still possible to use the plant despite its toxicity, by purifying the target-bioactive compound.

Another inherent challenge associated to the use of phytochemicals is the unwanted invasiveness of the species from which they derive. The worldwide governmental goal, which motivates this work, is to eradicate or at least reduce and control the population of these invasive species. If eventually, phytochemicals from alien species become a worldwide accepted strategy to fill the lack of antibiotics against multi-resistant strains and its exploitation grows until an industrial level there is a risk that comes a time when there are no more invasive plants available for this purpose because after collection they should not be replanted – or another strategy should be implemented – turning this into a non-renewable source. On one hand, this is positive because the goal of eradication is achieved, but it turns this antibiotic solution temporary. But is the total eradication of all invasive plant species populations a real scenario? Unfortunately no, due to several reasons like their ability to reproduce, difficulty in containing the spread of seeds and roots, globalization, and as climate change pushes more species out of their home ranges and into new areas, the number of so-called invaders is likely to multiply exponentially. All these factors contribute to make impossible the complete eradication of invasive species [114]. Thus, the scarcity of phytochemical sources should never be a problem.

Still related to the source of bioactive compounds, there is the risk that the transport and use/manipulation of invasive plant species may contribute to their spread. To mitigate and go around this challenge it should be avoided the use of invasive species that are not spread in the place where they are going to be processed. For example, *A. artemisiifolia* is considered to be invasive and it is disseminated in Croatia, but not in Portugal, so it should not be brought to Portugal to extract their phytochemicals even though it has the potential as a source of them. Instead, the process should be done in Croatia, or another species considered as an invader in Portugal should be chosen. In the Alinea a) from the no. 3 of the article 4th from the *Decreto-Lei 92/2019* it is created an exception to the transport of alien species for exclusive scientific endings, however, all the cautions should be taken [26]. One strategy is by inactivating the germination or reproduction of the plants. This can be done for example by

exposing the vegetal material to temperature and moisture after collection. Fenollosa *et al.* studied the effect of temperature and relative humidity on the seed longevity of the invasive plant species *Carpobrotus edulis*. It was found that at 55 °C under 87% relative humidity the seed's viability was lost within a working week (L50 = 42 h and L95 = 76 h) [115].

Additionally, the risk of the development of resistance strategies by pathogens to the new bioactive compounds may also be a challenge. In fact, the chance of the target-bacteria to develop resistance by any of the strategies like decreasing its ability to penetrate the cell, expelling it from the cell through the efflux pump mechanism, inactivating it via modification or degradation, or modification in its target within the microorganism has been reported when any other new antibiotics are introduced in the market [34]. However, as already referred, one of the biggest drawbacks of antibiotics currently in the clinic compared to phytochemicals is that they only work by inhibiting bacterial growth – as bactericidal or bacteriostatic –, which is achieved by drug-mediated perturbation of an essential cellular process, causing the inevitable selection for resistance due to the rapid evolution of the bacterial genome under selective pressure on essential cellular processes. Thus, since natural active compounds act as antibacterial targeting the bacterial virulence – antivirulence – under different mechanisms like membrane and cell synthesis inhibitors, EPIs, R-plasmid obliterator, enzyme inhibitors, or biofilm formation attenuators, they exert less selective pressure for resistance, minimizing the appearance and spread of MDR bacteria [39].

2.3.4. Selected IAPs: *A americana* and *T. fluminensis*

Thus, this work targets to evaluate the potential of *A. americana* and *T. fluminensis*, two invasive plant species in the Portuguese territory, as potential source of bioactive compounds with antimicrobial and antioxidant activities.



Figure 5 - T. fluminensis (left) [116], and A. americana (right) [117].

A. americana is in all the Portuguese territory, including the islands. As major impacts: the big size of the leaves causes a considerable shadow, avoiding the growth of native vegetation in its surroundings; high costs to apply control measures, that are hard to employ due to the spines included in the leaves – that also keep away predators – and its toxicity. Due to these impacts, *A. americana* has been associated with a risk level of 18 [23]. In its phytochemical composition are known to be present alkaloids, tannins, phenolic compounds, flavonoids, and saponins, having also a recognized antimicrobial activity [53], [60]. *T*

fluminensis is especially spread in the coastal districts – except Faro – and in Madeira. As major impacts: formation of dense continuous carpets that, due to its persistency, avoid the development of other vegetation in the zone; high costs to apply control measures. Due to these impacts, *T. fluminensis* has been associated with the same risk level. Alkaloids, flavonoids, tannins, and phenolic compounds are some of the groups present in these plant specie, however, no antimicrobial activity is yet reported [49]. The plants were collected and the extracts were obtained with two extraction techniques (solid-liquid and Soxhlet) using two different solvents (ethanol and methanol). The remaining biomass was then hydrolyzed to obtain a liquid fermentative culture medium, that was tested for *Saccharomyces cerevisiae* growth. This process aims to reach the two problems referred to at the beginning of the present document: find a solution to the lack of antibiotics due to MDR bacteria and contribute to sustainable management of the spread of invasive plant species by valuing the vegetal residue generated.

3. Materials and methods

3.1. Plants origin and collection

The selected plants for this work were *A. americana* and *T. fluminensis*. They were collected fresh in Vila Nova de Gaia Municipality (Porto, Portugal) in February 2023 and immediately dried at 60 °C for 2-3 days – depending on the amount of plant – and milled (Comercial model 38BL41; Dynamics Corporation of America, Connecticut, United States of America) to preserve from microbial contamination and facilitate storage. For *T. fluminensis*, leaves and stem were separated and all the following procedures were done for the two parts of the plant individually. All plant residues were destroyed by autoclaving to avoid propagation problems after discarding the plant.

3.2. Extracts preparation

Two extractions methods adapted from Borges *et al.* [9] were performed (solid-liquid and Soxhlet) using two different solvents (ethanol (VWR, Pennsylvania, United States of America) and methanol(VWR, Pennsylvania, United States of America)) for both plants. Briefly, the plants material was used as a powder after the drying and milling processes, and the resultant extract was then evaporated in a rotary evaporator (Büchi Rotavapor R-200; Büchi Labortechnik AG, Flawil, Switzerland) at a 50 °C bath temperature, with a pressure of 142 mbar for ethanol and 300 mbar for methanol – the pressure was optimized during the experiments being the values present the final ones. The dry extracts were finally dissolved in dimethyl sulfoxide (DMSO; VWR, Pennsylvania, United States of America) for further use on the antimicrobial and antioxidant tests. Samples were protected from light during all the extraction process and further extract storage at 4 °C.

To choose the best solvents to test, a preliminary solid-liquid extraction was made with two additional solvents (water and hexane).

3.2.1. Preliminary solid-liquid extraction

A mass 2.5 g of plant material was placed in a flask with 50 mL of solvent (ethanol, hexane (VWR, Pennsylvania, United States of America), methanol, or water) under extraction at room temperature with agitation (Multistirrer 14; VELP Scientifica, Monza, Italty) for 1 h. The reaction occurred inside an extraction hood in the dark. After the extraction was completed, the suspension was filtrated with a glass filter (Whatman 1; Cytiva, Marlborough, United States of America) and the liquid phase was stored in a clean round-bottom flask at 4 °C, protected from light exposure. The extracts were evaporated as described in section 3.2, except aqueous extracts, which were lyophilized (VirTis BenchTop 6K Freeze Dryer – Model # 6KBTEL – 85; VirTis) with a minimum pressure of 10 mTorr reaching a temperature of -80 °C.

This extraction process was used for solvent screening for further optimizations and extraction scale-up.

3.2.2. Definitive solid-liquid extraction

A mass of 10 g of plant material was placed in a flask with 200 mL of solvent (ethanol or methanol) under extraction at room temperature with agitation (Multistirrer 14; VELP

Scientifica, Monza, Italy) for 1 h. The resulting extracts were saved and evaporated as referred to in the previous section.

3.2.3. Soxhlet extraction

A mass of 5 g of plant material was placed in a filter paper thimble (10-20 µm of pore size; VWR, Pennsylvania, United States of America) inside the main chamber of a 100 mL Soxhlet. 125 mL of solvent was put in a distillation balloon connected to the bottom part of the Soxhlet apparatus, and heated with a heating mantle (Model 22; Fisatom, Brazil). A condenser was connected to the top of the Soxhlet apparatus with cooling water in circulation at 8 °C. The extraction was left ongoing until the solvent recirculated three times. The effective extraction volume was 100 mL, but to prevent the degradation of the extract by the absence of solvent in the distillation balloon because of its total evaporation, 25 mL of extra solvent was added. The solvent evaporation was performed as mentioned in Section 3.2.

3.2.4. Extraction efficiency determination

The extraction efficiency for each method and solvent was calculated using Equation 1:

$$Extraction\ efficiency\ (\%) = \frac{m_e}{m_p} \times 100, \quad (1)$$

with m_e representing the mass of extract after evaporation and m_p the mass of dried plant used for extraction (dry-weight). Only for the Soxhlet extraction was obtained extraction efficiency in triplicate.

3.3. Antibacterial activity evaluation tests

3.3.1. Bacterial strains and growth conditions

The bacteria used in this work were obtained from the American Type Culture Collection (ATCC): *E. coli* ATCC 25922 and *S. epidermidis* ATCC 35984. Before their use for antibiograms, the bacteria were inoculated overnight – from 16 to 18 hours – in Mueller-Hinton broth (MHB; Pennsylvania, United States of America), at 37 °C for *E. coli* and 30 °C for *S. epidermidis*, under agitation at 160 rpm (KS130; IKA, Staufen, Germany).

3.3.2. Crude extracts antibacterial activity evaluation

For the evaluation of the antibacterial activity of the three plant extracts, it was followed the disc diffusion method, originally described by Bauer *et al.* [118], wit modifications. From an overnight culture previously prepared (section 3.3.1), a bacterial suspension with 0.5 McFarland standards (Genesys 10uv; Thermo Scientific, Massachusetts, United States of America) was prepared in a sterile saline solution (NaCl 8.5 g/L; Merck, Darmstadt, Germany). Then, the cell suspension was seeded in plates with Mueller-Hinton agar (MHA; VWR, Pennsylvania, United States of America), using a sterile swab (Deltalab, Barcelona, Spain), and sterile blank discs(Labbox, Barcelona, Spain) were placed over the bacterial culture. The discs were impregnated with 10 µL of the antibacterial compound/solution. Two different tests were conducted: antimicrobial activity of the crude extracts and crude extract's potentiation of antibiotic effects. For both, DMSO was used as the negative control. Finally, the plates were incubated at 37 °C for 24 h, the moment when the inhibition diameter (mm) was measured.

3.3.3. Crude extract's combination with antibiotics - antibiotics potentiation test

For this test, the extracts were mixed in a 1:1 proportion with five different antibiotics. The antibiotic selection and concentration were based on the reported antimicrobial action in both *E. coli* and *S. epidermidis* and the disk content was defined according to the describe on *Performance Standards for Antimicrobial Susceptibility Testing* [119]. As the positive control, the discs were impregnated with the antibiotics isolated, having the same antibiotic mass as the crude extract/antibiotic mixture. The antibiotic solutions were also prepared within DMSO, except ciprofloxacin which was prepared in water and hydrochloric acid.

Table 5 – Selected antibiotics and respective disk content.

Antibiotic	Disk content (µg)	Suplier
Chloramphenicol	30	Fisher Scientific, Massachusetts, United States of America
Ciprofloxacin	5	Fluka, Steinheim, Germany
Levofloxacin	5	Alfa Aesar, Massachusetts, United States of America
Tetracycline	30	Sigma -Aldrich, Missouri, United States of America
Trimethoprim	5	Alfa Aesar, Massachusetts, United States of America

The effect of the combination of the extracts with the antibiotics was classified following Abreu *et al.* [120]:

- Potentiation (++): $(IZD_{a+p} - IZD_a) \geq 6$ mm;
- Additive (+): 6 mm > $(IZD_{a+p} - IZD_a) \geq 4$ mm;
- Indifferent: 4 mm > $(IZD_{a+p} - IZD_a) > -6$ mm;
- Negative (-): $(IZD_{a+p} - IZD_a) \leq -6$ mm,

where IZD corresponds to the inhibition zone diameter, a = antibiotic, and p = phytochemicals (extracts).

3.4. Phytochemicals characterization

3.4.1. Total phenolic content

The quantification of the total phenolic content (TPC) was estimated by the method of Folin-Ciocalteu (FC) adapted from H. Abramovič et *al.* [121]. The extracts solutions were firstly diluted by 1:200 and then 96 µL were mixed with 736 µL of ultrapure water (Milli-Q) and 48 µL of Folin-Ciocalteu reagent (Sigma-Aldrich, Missouri, United States of America). After 5 minutes of reaction, 120 µL of a solution of sodium carbonate (20%; VWR, Pennsylvania, United States of America) was added. The mixture was left for 1 h reacting in the dark and the absorbance was read in a microplate reader (Synergy HT; BioTek, Vermont, United States of America) at 765 nm. The calibration curve (Table A.1, Annex A) was obtained using gallic acid (Sigma-Aldrich, Missouri, United States of America) solutions with concentrations ranging from 169.3 µM to 3386 µM – the gallic acid solutions were only diluted by 1:20.

The TPC was expressed in mg of gallic acid equivalents per gram of extract (mg GAE/g extract).

3.4.2. Total flavonoids content

The quantification of the total flavonoids content (TFC) was estimated by adaptation of the protocol proposed by Derakhshan *et al.* [122]. The extracts solutions were diluted by 1:20 and 100 µL was mixed with 30 µL of $NaNO_2$ (Sigma-Aldrich, Missouri, United States of America) and 400 µL of ultrapure water. After 6 minutes, 30 µL of $AlCl_3$ (10%; Thermo Scientific Massachusetts, United States of America) was added, followed by 400 µL of NaOH (4%) and 40 µL of ultrapure water after another 6 minutes. The absorbance was read in a microplate reader at 510 nm after 15 minutes of reaction. The catechin (TCI, Tokyo, Japan) calibration curve (Table A.2, Annex A) was obtained by using concentrations ranging from 500 µM to 10000 µM.

The TFC was expressed as mg of catechin equivalents per gram of extract (mg CE/g extract).

3.5. Antioxidant capacity determination

The antioxidant capacity was determined using two different methods: DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2-azinobis (3-ethyl-benzothiazolina-6-sulfonic acid)). For both, the antioxidant activity was expressed in mg of Trolox equivalents (TE) per gram extract (mg TE /g extract) and scavenging activity (%) by using equation 2. The absorbance of the control corresponds to the absorbance of the DPPH or ABTS reactive solution, depending on the method in use, with the same dilution of the samples.

$$Scavenging\ Activity\ (\%) = \frac{Abs_{Control} - Abs_{Sample}}{Abs_{Control}} \times 100 \qquad (2)$$

3.5.1. DPPH method

The antioxidant capacity by the DPPH method was determined following a protocol adapted from Magalhães *et al.* [123] and [124], Xiao *et al.* [125], and Marinova *et al.* [126]. DPPH (0.06 mM; TCI, Tokyo, Japan) was dissolved in glacial ethanol (VWR, Pennsylvania, United States of America) and set to an absorbance of 0.700±0.02 at a wavelength of 517 nm. The Trolox (Sigma-Aldrich, Missouri, United States of America) calibration curve (Table A.3, Annex A) was made with concentrations ranging from 0.25 mM and 0.0078 mM.

In a 96 wells microplate, the samples and Trolox were mixed with DPPH 1:100 v/v in quadruplicates. The absorbance was read, in the microplate reader, after 30 minutes at a wavelength of 517 nm.

3.5.2. ABTS method

The antioxidant capacity by the ABTS method was determined following a protocol adapted from the methods proposed by Xiao *et al.* [125]. ABTS (7 mM; Sigma-Aldrich, Missouri, United States of America) and potassium persulfate (2.45 mM; Sigma-Aldrich, Missouri, United States of America) were mixed 1:1 v/v and allowed to react for 12-16 hours in the dark at room temperature. After, the solution was diluted with acetate buffer (50 mM) at pH 4.6, 1:40 v/v. The absorbance of the ABTS solution was set to 0.700±0.02 in a

spectrophotometer at a wavelength of 734 nm. A Trolox calibration curve (Table A.4, Annex A) was made by dissolving Trolox in glacial ethanol with concentrations ranging from 0.25 mM and 0.0078 mM.

In a 96 wells microplate, the samples and Trolox were mixed with reactive ABTS 1:10 v/v in triplicates. The absorbance was read in the microplate reader after 15 minutes at a wavelength of 734 nm.

3.6. Extraction by-product fermentation

3.6.1. First fermentation round

3.6.1.1. Biomass pre-processing

Biomass was processed to produce five different growth media, described in Table 6. For the growth media 1 to 4, solid-liquid extraction of 10 g of *A. americana* biomass with 200 mL distilled water was made, according to the described in section 3.2. After centrifugation at 3202 g for 10 minutes (5810 R; Eppendorf, Hamburg, Germany), the liquid phase was kept and the plant's biomass passed through another solid-liquid extraction, using methanol as solvent. The first aqueous extraction aimed to keep the soluble sugars and use them further in the fermentation, avoiding their loss in the methanolic extraction, which was run simply to have the *A. americana* biomass with post-extraction characteristics.

A mass of 7.5 g of *A. americana* previously used in the methanolic extraction was mixed with 50 mL of the obtained aqueous extract and hydrolyzed via thermal treatment in an autoclave (Uniclave 88; AJC, Cacém, Portugal) at 121 °C for 30 minutes. The resulting mixture was then centrifuged for 10 minutes at 3202 g, keeping the liquid phase. The supplementation of the growth media 2 and 4 were made with yeast extract (3g/L) and peptone (5g/L) following Shirish *et al.* [127]. The growth media 3 and 4 were diluted in distilled water.

Table 6 - Different growth media descriptions.

Medium	Description
1	Aqueous extract (autoclaved at 121 °C for 15 minutes)
2	Protein-supplemented aqueous extract (autoclaved at 121 °C for 15 minutes)
3	1:1 diluted hydrolysate (extraction biomass with aqueous extract)
4	Protein-supplemented 1:1 diluted hydrolysate (extraction biomass with aqueous extract)
5	Protein-supplemented diluted hydrolysate (biomass with distilled water)

For growth medium 5 (Table 6), 75 g *A. americana* biomass was mixed with 500 mL of distilled water and hydrolyzed via thermal treatment in an autoclave at 121 °C for 30 minutes. The resulting mixture was then centrifuged for 10 minutes at 3202 g, keeping the liquid phase that was immediately filtered with a cellulose nitrate filter (0.45 µm pore size; Sartorius, Gottingen, Germany). Both steps were conducted under sterile conditions. Finally, a 250 mL yeast extract (3g/L) and peptone (5g/L) solution was mixed with the hydrolysate.

3.6.1.2. Inoculum preparation

The yeast used for the fermentation was *Saccharomyces cerevisiae* DSM 1333, obtained from the German Collection of Microorganisms and Cell Cultures. Before the fermentation, *S. cerevisiae* was inoculated overnight – from 16 to 18 h – in yeast growth medium composed of glucose (5 g/L; Sigma-Aldrich, Missouri, United States of America), bacto-peptone (2.5 g/L; Sigma-Aldrich, Missouri, United States of America), yeast extract (1.25 g/L; Sigma-Aldrich, Missouri, United States of America), disodium hydrogen phosphate monohydrate (3.56 g/L; VWR, Pennsylvania, United States of America), and potassium dihydrogen phosphate monohydrate (2.76 g/L; VWR, Pennsylvania, United States of America), at 30 °C under agitation at 160 rpm.

3.6.1.3. Fermentation

The growth media 1-4 were fermented in parallel. Inoculum was added in a volume that guaranteed an initial optical density on the fermentative broths of 0.04 (approximately 1.5×10^5 CFU/mL). The biological processes occurred at 30 °C under agitation at 120 rpm (I26; New Brunswick Scientific, New Jersey, United States of America). The yeast growth was monitored by CFU counting in plate count agar (PCA) after 24 h of incubation at 30 °C.

Two fermentations of growth medium 5 were conducted in parallel – one in 200 mL and the other in 100 mL using the same growth medium. Inoculum was added in a volume that guaranteed an initial optical density on the fermentative broths of 0.04 (approximately 1.5×10^5 CFU/mL). The biological processes occurred at 30 °C under agitation at 120 rpm. The yeast growth was monitored by optical density reading at 620 nm.

3.7. Antifungal activity evaluation test

For this step, a similar procedure as the one described in subsection 3.3 was followed. The main difference was the microorganism studied. In this case, *S. cerevisiae* DSM 1333 was used instead of the two mentioned pathogenic bacteria. The antifungal activity of the three extracts was assessed as mentioned in 3.3, however, PCA plates were used instead of MHA. Thus, the inoculum preparation followed the same conditions as described in subsection 3.6.3 Inoculum preparation. The plates were incubated at 30 °C for 24 h, the moment when the inhibition diameter (mm) was measured.

3.8. Statistical analysis

Data were analyzed using Microsoft Excel (Version 2305). The t-test with the significance level for the difference set at $p < 0.05$ was used.

Regarding the antibacterial activity evaluation tests (section 3.3), independent assays were conducted. For statistic calculations, four replicates were considered. Regarding the antifungal activity evaluation tests, only one assay was conducted, obtaining two replicates that were both considered for statistic calculations.

4. Results and Discussion

The main goal of the present work was to extract and evaluate the bioactive potential of phytochemicals extracted from two invasive plant species. As a secondary objective, the valorization of the remaining plant biomass for bioethanol production was studied, which would increase the sustainability of the process by the generation of green energy – second-generation fuel. *Tradescantia fluminensis* was the first plant species to test due to its widespread presence in the Porto district. *Agave americana* was chosen as an additional species to test because of its already documented antimicrobial activity [53], [60], [128], and also its fermentative potential due to the relevant percentage of water-soluble sugars [129]. However, a lack of information on the biomolecules and mechanisms responsible for the antibacterial effect of these species extracts was noted in the literature, being the gap that this work aims to fill.

4.1. Preliminary solid-liquid extraction for solvent selection

For the extraction of the bioactive compounds present in the collected plant material of *A. americana* and *T. fluminensis*, the first goal was to select, among ethanol, hexane, methanol, and water the best solvents to proceed with extractions. For that, a preliminary single series of solid-liquid extraction was conducted to access qualitatively what were the solvents which promoted higher extraction efficiencies (Table B.1, Annex B) and with higher potential to extract bioactive compounds. Table 7 presents the results of the antimicrobial effects of each extract.

There is no information regarding the aqueous extracts of *A. americana*. Despite a successful solid-liquid extraction of *A. americana* in water, the following lyophilization lead to the caramelization of the extract, possibly due to the high sugar content in this plant [129], turning it unusable – its dissolution in DMSO was impossible. The caramelization of solutions or solids with high soluble sugar contents is recurrent, however, the caramelized extract is usually still soluble in DMSO [130], [131]. Grigoras *et al.* [132] recommended to repeat three times the freezing and lyophilization steps for high sugar amount substrate. The reason for the impossibility to solubilize the lyophilized aqueous *A. americana* extract remains to access, but it may be due to a second or third compound that reacted together with the sugars.

Table 7 - Antibacterial activity results of the extracts obtained in the preliminary solid-liquid extraction (mm). Results are presented as the average and standard deviation of two replicates (one independent assay).

Solvent	*A. americana*		*T. fluminensis* (leaves)		*T. fluminensis* (stem)	
	E. coli	*S. epidermidis*	*E. coli*	*S. epidermidis*	*E. coli*	*S. epidermidis*
EtOH	2	1	2	1	2	2
MetOH	3	0	0	1	2.5	0
H_2O	-	-	0	0	0	0
Hexane	0	0	0	0	0	0

Analyzing the results obtained, only the ethanolic and methanolic extracts produced slight inhibitory effects on the seeded bacteria. Integrating those with the extraction efficiencies (Table B.1, Annex B), methanolic extracts produced by far the highest yield,

followed by the other solvents with similar results between each other. Thus, the solvents that revealed more antimicrobial potential were methanol and ethanol. Therefore, those were the selected solvents for further studies. Despite having similar extraction efficiency compared to hexane and water, ethanol showed antimicrobial potential while the others did not.

4.2. Definitive solid-liquid extraction

With the solvents selected, a new solid-liquid extraction was conducted, with bigger amounts of plant biomass and solvent to have enough quantity of extracts for all the subsequent experiments. Also, from the preliminary solid-liquid extraction to the definitive one, the extraction technique and especially the use of the evaporator were improved, namely the pressure applied during the evaporation. This lead to a better evaporation process causing less solvent to be present in the final extract, resulting in a lower extraction efficiency – the lower the mass of extract after evaporation, the lower the extraction efficiency (Equation 1) – as it is possible to verify in Table 8. It is unlikely that this difference is real or at least so pronounced since the used method was the same. To avoid degradation of the bioactive compounds by excessive temperature or loss of extract because of its attachment to the walls due to over-drying, it was decided to do not let the extracts completely evaporation causing, in a general way, an overestimation of the extract efficiency due to the presence of solvent in the mass of the extract after evaporation (Equation 1).

Table 8 - Definitive solid-liquid extraction efficiencies (%). The average and standard deviation results of the efficiency of the three extracts obtained for each solvent.

Solvents	*A. americana*	*T. fluminensis* (leaves)	*T. fluminensis* (stem)	Average
EtOH	4.7	1.5	1.4	2.5 ± 1.5
MetOH	14.0	4.4	9.8	9.4 ± 3.9

Comparing the ethanolic and methanolic extraction efficiencies, there were not found significant differences ($p > 0.05$). Míguez *et al.* [49] obtained 14% and 8% of extraction efficiency from *T. fluminensis* leaves and stem, respectively, using ethanol as the solvent in a solid-liquid extraction method. However, note that the extraction was performed at 25 °C for 4 h (quadruple the time). Shegute *et al.* obtained an extraction yield of 15.11% with *A. americana* leaves methanolic extracts using the same method, a result that is not far from the one obtained in the present work [53].

4.3. Soxhlet extraction

Soxhlet method was also tested to access if it could provide higher extraction efficiencies. As shown in Table 9, using the Soxhlet method the extraction efficiency increased for both solvents globally ($p < 0.05$). This was an expected result since it is widely reported in the literature the higher extraction efficiency using Soxhlet compared to the use of the solid-liquid technique. One of the advantages of the Soxhlet method over the others is the extraction of larger amounts of molecules with the same or smaller volume of solvent [45]. Borges *et al.* [9] compared the extraction efficiencies of different techniques for obtaining extracts from *Acacia dealbata* and *Olea europaea*. The results showed that the efficiency of the extraction using the Soxhlet technique was 2 times higher in *O. europaea* extracts and 1.6 times higher in *A.*

dealbata, compared to the extraction efficiency using the solid-liquid method. However, the Soxhlet technique is usually not suitable for the extraction of thermolabile compounds, such as some phytochemicals, so the option for this method is questionable as is discussed below [133]. Pandey *et al.* [60] obtained an extraction efficiency of 16.9% from *A. americana* with methanol as solvent by the Soxhlet method. The higher efficiency reached in the present work might be due to the not complete evaporation of the solvent, causing an overestimation of the extraction yield, as already discussed.

Table 9 - Soxhlet extraction efficiencies (%). Results are presented as the average and standard deviation of three replicates.

Solvents	*A. americana*	*T. fluminensis* (leaves)	*T. fluminensis* (stem)	Average
EtOH	20.4 ± 1.2	19.6 ± 1.6	13.1 ± 1.3	17.7 ± 3.3
MetOH	40.6 ± 3.3	35.5 ± 11.9	55.2 ± 28.5	43.8 ± 8.4

Among the ethanolic extractions with the Soxhlet technique, the extract obtained from the stem of *T. fluminensis* revealed less efficiency than the others ($p < 0.05$). Kaneria *et al.* [134] studied the effect of extraction techniques and solvents on the antioxidant activity of *Punica granatum* leaf and stem, obtaining for all the tested conditions higher extractions yields for the extracts from leaves. Míguez *et al*. [49] performed extractions with *T. fluminensis* leaves and stem using ethanol as solvent, and the extract obtained from the leaves was also associated with a higher yield. Moreover, reported yields were similar to the ones obtained in this work. One possible justification is the higher content of natural pigments in the leaves, like chlorophyll. These pigments are easily extracted increasing the number of compounds present in the final extract, which may result in a positive impact on the mass of the extract after evaporation/extraction efficiency. This is revealed in the more intense coloration of the leaves extracts (Figure C.1, Annex C). For the methanolic extractions, no significant differences were registered between different extracts ($p > 0.05$).

Comparing both ethanol and methanol as solvents in the Soxhlet technique, methanol was globally more efficient ($p < 0.05$). Once again, this was an expected result since methanol is the solvent that can extract a higher diversity of compounds. Borges *et al.* [9] obtained higher extraction efficiency with methanol compared to ethanol using different extraction methods for both *O. europaea* and *A. dealbata*. Zeroual *et al*. [135] studied the effects of extraction technique and solvent on phytochemicals, antioxidant, and antimicrobial activities from *R. officinalis*, and also achieved a higher extraction efficiency for methanol than ethanol.

Qualitatively, the Soxhlet method was more difficult to perform, especially the temperature and boiling control, a critical parameter when dealing with possible thermolabile phytochemicals. In some cases, it was possible to note the attachment of burned extract to the walls of the round-bottom flask, being these parts unrecoverable. This leads to extract material loss and variances between replicates, as observed for the methanolic extract from the leaves of *T. fluminensis*. The same issue happened during evaporation, but it was transversal for both techniques. Another verified disadvantage of this method was the time required for the extraction, even though only making three reflux cycles – between 1.5 to 2 h of extraction. In Borges *et al.* [9] the plant material was under extraction for 16 h, and in

Zeroual *et al.* [135] for 6 h. A bigger extraction time could eventually lead to an increase in efficiency, however, longer extractions could also cause the loss of compounds like those referred to above for the *T. fluminensis* leaves extracts.

After the extraction step, all the obtained extracts were diluted in DMSO achieving different concentrations depending on the volume of DMSO needed to dissolve as much evaporated extract as possible (Table 10). For some extracts, especially the ones from *T. fluminensis* leaves, it was harder to recover the total extraction mass due to the attachment of the extract to the flask walls and consequent mass loss, as already discussed above. Thus, in some cases, the concentration of the extract may be overestimated because the mass of the extract diluted in DMSO may not be the same as was weighted after the evaporation.

Table 10 - Extracts concentration after dilution in DMSO (mg/mL).

Solvent	*A. americana*		*T. fluminensis* (leaves)		*T. fluminensis* (stem)	
	Solid-liquid	Soxhlet	Solid-liquid	Soxhlet	Solid-liquid	Soxhlet
EtOH	234.95	550.45	49.333	130.85	136.20	141.68
MetOH	931.77	663.83	145.78	259.26	243.69	855.23

Regarding the extractions with the Soxhlet method, it was only presented the concentrations of the extracts used for the following tests – despite were obtained extracts in triplicate for the three plants' biomass, only one extract for each type was used since the replicates had theoretically the same composition.

4.4. Crude extracts antibacterial activity evaluation

The first approach to evaluate the antimicrobial potential of the plant extracts was to test them as antibacterial agents against *E. coli* and *S. epidermidis*, which respectively represent Gram-negative and Gram-positive bacteria. However, in this step, small inhibition hallos like the ones obtained for the extracts from the preliminary solid-liquid extraction for solvent selection (Table B.1, Annex B) were not considered because it was hard to distinguish between inhibition hallos and hallos caused by the diffusion of the extract. Thus, globally, the obtained plant extracts did not reveal any direct effect on both bacteria strains. Shegute *et al.* [53] studied the antibacterial activity and phytochemical components of leaf extracts of *A. americana*, obtaining growth inhibition from 10 mg/mL and 2.5 mg/mL to higher extract concentrations in *E. coli* and *S. epidermidis*, respectively. Pandey *et al*. [60] evaluated the phytochemical, antimicrobial, antioxidant activity, and cytotoxic potentials of *A. americana*, reporting also growth inhibition but with considerably smaller halos compared to the previous study. Comparing the extraction methods, both experiments were run using methanol as solvent, but in Shegute *et al*. was employed the solid-liquid method, and in Pandey *et al.* the Soxhlet. As already discussed, despite the Soxhlet technique guaranteeing higher extraction efficiency, it might not be the most suitable method to extract phytochemicals that are potentially thermolabile. A study comparing Soxhlet and solid-liquid extraction methods discovered that the amounts of both polyphenols and alkaloid extracts decreased for the Soxhlet method [136]. For the antimicrobial capacity screening, Pandey *et al.* used a similar disk diffusion method to the present work, while Shegute *et al.* used the agar diffusion

method, which is also associated with the use of a higher extract volume (100 µL). Mirtaghi *et al.* [137] evaluated the antibacterial activity of *Urtica dioica* leaf ethanolic extract using agar well diffusion and disc diffusion methods. The results revealed that the susceptibility of bacteria to the extract was significantly higher (61%) in the well diffusion method than that in the disc diffusion method. This difference may be because, in the well diffusion method, the extract is poured directly into the well, while in the disk diffusion method, the disks create additional resistance to the diffusion of the bioactive compounds. Thus besides the extraction technique, the method selected to evaluate the antimicrobial effect also plays a crucial role in the results, understanding the differences between the inhibition halos reported in the two studies about *A. americana* as a potential source of bioactive compounds.

Looking at the study of Pandey *et al.* [60], since it is the one with similar methods, for an identical concentration of extract to the ones obtained in this work overall (200 mg/mL), the zones of inhibition are 9 mm for both *E. coli* and *S. aureus*, being classified as "mildly susceptible to the extracts" by the authors. The reason presented for this weak effect is the low concentration of extracts, arguing that identification and purification of the bioactive compound with antibacterial activity would probably increase the level of inhibitory action. The same argument could also be used for the presented results: as said before, the concentration of the extract may be overestimated, meaning that the real concentrations could be significantly lower than the ones presented in Table 10 – if 200 mg/mL is considered to be a low concentration to produce relevant results, lower concentrations even more.

Regarding *T. fluminensis*, no studies on antibacterial activity with the values of the inhibition halos were found, unfortunately turning comparisons difficult to be made. The most similar work found was the determination of antioxidant and antibacterial properties of *Tradescantia spathacea* root extracts by Lim *et al.* [138]. The extracts were obtained using the Soxhlet method with hexane and ethyl-acetate as solvents. For an extract concentration of 40 mg/mL, an inhibition zone of 10 and 8 mm was verified for hexane and ethyl-acetate extracts, respectively, against *S. aureus*. For *E. coli*, no antibacterial effects were registered. Comparing the results of the cited work with the ones obtained might lead to erroneous conclusions for several reasons: comparison of extracts obtained from different plant species that, despite sharing the same genera, are very distinct morphologically; different solvents used – ethanol and methanol are polar while hexane and ethyl-acetate are non-polar [45]; considerably different TPC of the plant extracts – 130 mg GAE/g DW and 243 mg GAE/g DW for the extracts obtained with hexane and ethyl acetate as the solvents, respectively, from *T. spathacea* by Lim *et al.*, compared to 3.01 mg GAE/g DW and 1.92 mg GAE/g DW in ethanolic extracts from *T. fluminensis* leaves and stem, respectively, obtained by the solid-liquid method by Míguez *et al.* [49].

4.5. Evaluation of crude extracts as potentiators of antibiotic activity

After the unsuccess of the crude extracts as antibacterial agents, it was decided to combine them with five different antibiotics (chloramphenicol, ciprofloxacin, levofloxacin, tetracycline, trimethoprim). Antibiotics were selected attending their activity against Gram-positive and Gram-negative bacteria and to have representativity of different classes of antibiotics: tetracyclines, fluoroquinolones, phenicols, and folate pathway antagonists. This

procedure aims to test a possible potentiation effect improving the action of the antibiotics. Phytochemicals' positive influence on antibiotics' action is known and can happen in several ways. For example, the formation of a complex with the antibiotic eases the cell entry – the bigger size of the formed complex will confine the antibiotic inside the cell, turning its excretion harder by EPIs [66], [67]. For this, the extracts and the antibiotics were mixed in a 1:1 proportion, having in mind that the antibiotic disk content should be the same described in *Performance Standards for Antimicrobial Susceptibility Testing* [119] for each antibiotic. As positive control was used pure antibiotics with the same disk content.

Both *E. coli* and *S. epidermidis* were supposed to be affected by the selected antibiotics – susceptible to some like levofloxacin and resistant to others like tetracycline against *E. coli*. However, no inhibition halo was ever verified for trimethoprim against *S. epidermidis*.

Looking at the potentiation results on the antibiotic action against *E. coli* (Table 11), the antibiotics that benefited from the combination of the bioactive compounds were tetracycline and chloramphenicol, with special evidence for the first. Tetracycline belongs to an antibiotic group with the same name, and its action is based on the binding to the 30s ribosomal subunit and blocking the binding of aminoacyl-tRNA to the A site, thereby preventing the addition of new amino acids to the growing peptide chain. This ultimately leads to the inhibition of bacterial growth and proliferation [139]. Chloramphenicol belongs to the phenicol antibiotics group, and its action is also on the ribosome affecting the protein synthesis by inhibiting its elongation cycle, resulting in the formation of incomplete and non-functional proteins, leading to bacterial cell death. However, instead of binding to the 30s ribosomal subunit, it binds to the 50s [140]. Regarding *E. coli*, the main resistance mechanism that affects the action of both antibiotics is the presence of efflux pumps that expel the antibiotics outside of the cell – chloramphenicol action is also affected by enzymatic inactivation by chloramphenicol acetyltransferases. In this turn, ciprofloxacin, trimethoprim, and levofloxacin are susceptible to a wider range of resistance mechanisms, including EP, target modification, and others, making harder to combat resistance mechanisms against these antibiotics since there might be several mechanisms to stop/attenuate [139]–[144]. Ciprofloxacin and levofloxacin are part of the fluoroquinolones antibiotics groups and their action targets bacterial DNA replication by inhibiting the activity of bacterial DNA gyrase and topoisomerase IV, which are essential enzymes involved in the synthesis and maintenance of bacterial DNA [142], [144]. Trimethoprim belongs to the antibiotic group of folate pathway antagonists that targets the folate synthesis pathway, which is essential for the synthesis of DNA, RNA, and protein by inhibiting the bacterial enzyme dihydrofolate reductase [143].

Table 11 - Inhibition zone diameter (mm) for the selected antibiotics combined with the obtained extracts against E. coli. Results are presented as the average and standard deviation of four replicates (three independent assays).
Tetra: tetracycline, Cipro: ciprofloxacin, Chlo: chloramphenicol, Trime: trimethoprim, Levo: levofloxacin; S: soxhlet extraction, S/L: solid-liquid extraction; ++: potentiation effect, +: addictive effect.

Bacterium		*Escherichia coli*						
Plant	Antibiotic	*A. americana*		*T. fluminensis* (leaves)		*T. fluminensis* (stem)		Positive controls
Technique		S	S/L	S	S/L	S	S/L	
EtOH	Tetra	4.8 ± 0.4	5.3 ± 0.8	2.8 ± 0.8	8.3 ± 2.9	12.8 ± 1.6 $^{++}$	2.8 ± 0.4	5.3 ± 0.4
	Cipro	23.5 ± 0.5	24.5 ± 0.5	25.5 ± 1.5	24.3 ± 0.4	24.0 ± 0.7	24.8 ± 1.3	24.5 ± 0.5
	Chlo	13.8 ± 1.8	15.5 ± 1.5 $^{+}$	12.3 ± 1.3	12.3 ± 1.8	11.5 ± 1.5	14.5 ± 1.1 $^{+}$	10.6 ± 1.5
	Trime	25.3 ± 1.1	28.0 ± 1.8	24.0 ± 1.2	25.3 ± 1.8	22.8 ± 0.4	21.5 ± 1.1	26.5 ± 2.6
	Levo	24.3 ± 0.8	23.5 ± 1.1	23.5 ± 1.5	23.3 ± 1.8	26.3 ± 2.1	22.3 ± 0.8	24.3 ± 1.1
MetOH	Tetra	11.5 ± 0.9 $^{++}$	13.5 ± 1.9 $^{++}$	9.5 ± 1.4 $^{+}$	9.2 ± 0.7 $^{+}$	10.0 ± 0.6 $^{+}$	8.8 ± 1.3	5.3 ± 0.4
	Cipro	22.7 ± 1.0	23.3 ± 2.2	23.1 ± 1.3	25.0 ± 1.6	23.6 ± 2.8	23.0 ± 2.8	24.4 ± 2.2
	Chlo	16.8 ± 2.0 $^{++}$	14.0 ± 0.8	12.5 ± 1.1	11.0 ± 1.3	11.5 ± 1.5	10.9 ± 1.4	10.6 ± 1.5
	Trime	21.2 ± 2.7	23.3 ± 2.5	22.0 ± 2.1	23.5 ± 2.1	23.7 ± 2.8	21.8 ± 2.2	25.9 ± 2.5
	Levo	20.2 ± 2.5	20.0 ± 2.6	24.5 ± 2.6	24.0 ± 2.9	22.8 ± 2.7	23.4 ± 2.7	25.3 ± 1.9

Thus, both tetracycline and chloramphenicol, the antibiotics whose effect was potentiated by the combination with the obtained extracts, are mainly affected by the same resistance mechanism (efflux pumps) and also have the same target of action in the MDR bacteria (ribosomal subunits). So, the effect of increasing the susceptibility to both pharmaceuticals is understandable since the same type of phytochemicals is applied to stop/attenuate the resistance mechanism in action. Also, despite not being the most common resistance mechanism over these two antibiotics, it should not be discarded the action of these phytochemicals on the ribosomal subunits, increasing the bond antibiotic-ribosome strength, and consequently its effect [139], [140]. It is important to reinforce the idea that the bioactive compounds present in the extracts may not have the same mechanism of action as tetracycline and chloramphenicol because isolated they did not cause any growth inhibition in the tested strains. Instead, they are potentiating the effect on the ribosomal subunits or decreasing the resistance of these bacteria by inhibiting EP mechanisms. Alkaloids, flavonoids, and tannins are some examples of compounds that have a known potentiation on tetracycline, more specifically the case of the phytochemicals carnosol and baicalein [120], [145], [146]. Seebaluck-Sandoram *et al.* [147] evaluated the antibiotic-potentiating activity, phytochemical profile, and cytotoxicity of *Acalypha integrifolia* extracts. Methanolic extracts containing terpenes, tannins, and flavonoids showed synergistic activity combined with chloramphenicol against *E. coli*.

Considering the ethanolic extracts against *E.coli*, the one obtained from the *T. fluminensis* stem with the Soxhlet technique was the one that produced a higher effect, potentiating the tetracycline effect around 2.4 times. The effect on chloramphenicol was considerably lower – around 40% – but still relevant, despite being only additive. For the methanolic extracts, the pattern was similar: the highest effect was observed for tetracycline, but this time combined with the extract from *A. americana* obtained with solid-liquid technique (increase on the inhibition halo by 2.5 times). The *A. americana* extract by the Soxhlet method also produced potentiation of over 2 times more effect of tetracycline. Potentiation of chloramphenicol occurred only when combined with *A. americana* obtained with the solid-liquid technique, increasing its activity by 66%. Overall, the methanolic extracts produced positive results in a wider range of combinations, which was expected since methanol can extract a more diversity of different compounds [9]. Despite that, the antibiotic action increase for each extract was quantitatively similar between ethanolic and methanolic extracts, however, the extracts with higher potentiation are different. For example, the ethanolic extract that provided higher potentiation was the one from *T. fluminensis* stem obtained by Soxhlet extraction, while the methanolic was the one from *A. Americana* obtained by solid-liquid extraction. Ethanol and methanol are used to extract some tannins, polyphenols, and terpenoids, however, despite being both alcohols, they have different polarities thus different affinities to the compounds [45]. Ethanol is also used to extract polyacetylenes, flavonols, sterols, and alkaloids, while methanol extracts a big variety of different molecules such as saponins, lactones, and flavones [148]. So, the difference observed in the extracts with higher potentiation between ethanolic and methanolic ones could mean that the most relevant bioactive compounds belong to those groups of molecules that are not equally extracted using the two solvents. Some of these cited

compounds are alkaloids, flavonols, and saponins [149]–[151]. This argument will be explored deeper in the next section.

Finally, the higher potentiation of tetracycline over chloramphenicol may indicate that, despite being affected by the same resistance mechanism and having the same target, the active phytochemicals have higher activity in the specific tetracycline efflux-pumps or the 30s ribosomal subunits, or that the active phytochemicals for both antibiotics are different and the one(s) active in chloramphenicol potentiation is in a lower concentration. Despite being affected by the same resistance mechanism, the efflux pumps are specific for each antibiotic type, meaning that the active sites for the binding of phytochemicals might be different [152]. The same could be said about the two ribosomal subunits.

Looking now at the results of the combination extracts-antibiotic against *S. epidermidis* (Table 12), only tetracycline was positively influenced by the presence of the bioactive compounds. Compared to the results obtained against *E. coli* that was previously discussed, there were fewer extracts active, and also the effects were less notable. The best combination resulted in an increase of tetracycline action of 53% by the combination with the *T. fluminensis* stem ethanolic extract obtained with the Soxhlet extraction. The remaining extracts with positive effects were only considered addictive. The differences between ethanolic and methanolic extracts are similar to the ones observed in *E. coli* tests, being the considerations presented above about those applicable here for *S. epidermidis* too.

Regarding the differences between the behavior of the two strains, there are two important points to explore. The first is the lower effect on the Gram-positive bacteria. This result was contrary to what was expected and is reported in the literature. Gram-negative bacteria possess an outer membrane (OM) that makes them less susceptible to antimicrobials than Gram-positive bacteria. Indeed, Gram-negative bacteria have lipopolysaccharide (LPS) in their OM, which acts as a barrier that slows the penetration of antimicrobials. In these bacteria, the passage through the OM is regulated by the presence of hydrophilic channels (porins) that usually exclude the entry of hydrophobic compounds. Moreover, the OM of these bacteria lacks phosphoglycerides and, hence, the effective channels for hydrophobic diffusion [153]. However, there are some natural compounds extracted from plants that increase OM permeability. Farrag *et al.* [154] concluded that some natural outer membrane permeabilizers like thymol and gallic acid, boost antibiotic action against resistant bacteria. Since this work tested crude extracts, there is a chance of being present some of those natural permeabilizers that are part of compound groups like phenolics and terpenes, which are usually extracted using ethanol and methanol, as already discussed. Thus the eventual presence of these molecules could be the reason for the higher potentiation verified for *E. coli*. Nirosha *et al.* [155] also reported that Gram-negative were more susceptible to the *Carica papaya* tested extracts

Table 12 - Inhibition zone diameter (mm) for the selected antibiotics combined with the obtained extracts against S. epidermidis. Results are presented as the average and standard deviation of four replicates (three independent assays).
Tetra: tetracycline, Cipro: ciprofloxacin, Chlo: chloramphenicol, Trime: trimethoprim, Levo: levofloxacin; S: soxhlet extraction, S/L: solid-liquid extraction; ++: potentiation effect, +: addictive effect.

Bacterium		*Staphylococcus epidermidis*						
Plant	Antibiotic	*A. americana*		*T. fluminensis* (leaves)		*T. fluminensis* (stem)		Positive controls
Technique		S	S/L	S	S/L	S	S/L	
EtOH	Tetra	20.3 ± 1.3 $^{+}$	18.5 ± 0.5	16.3 ± 0.4	17.8 ± 0.8	24.5 ± 0.9 $^{++}$	13.8 ± 1.1	16 ± 2.0
	Cipro	25.3 ± 0.8	28.8 ± 1.5	26.8 ± 0.4	28.0 ± 2.9	30.2 ± 3.6	27.0 ± 2.0	26.8 ± 0.8
	Chlo	22.0 ± 0.0	19.3 ± 0.4	22.3 ± 1.6	16.0 ± 0.7	19.3 ± 1.1	19.3 ± 0.8	19.5 ± 1.5
	Trime	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	Levo	28.3 ± 0.8	27.8 ± 0.4	26.8 ± 0.8	29.0 ± 0.7	28.3 ± 0.8	28.5 ± 0.5	29.0 ± 2.0
MetOH	Tetra	25.2 ± 2.3 $^{+}$	23.4 ± 1.7	24.3 ± 2.2	23.7 ± 1.9	21.8 ± 2.0 $^{+}$	24.5 ± 2.1 $^{+}$	20.2 ± 0.7
	Cipro	26.8 ± 0.4	26.0 ± 0.8	26.5 ± 1.7	27.3 ± 1.1	27.5 ± 1.9	27.2 ± 1.8	25.0 ± 2.2
	Chlo	19.0 ± 0.0	20.5 ± 3.5	21.2 ± 2.0	20.7 ± 2.5	22.0 ± 1.7	19.7 ± 2.5	22.8 ± 0.4
	Trime	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	Levo	25.7 ± 1.9	25.3 ± 1.7	26.2 ± 2.0	26.2 ± 1.3	27.6 ± 1.6	27.3 ± 1.4	25.5 ± 3.0

The second point is the absence of chloramphenicol potentiation against *S. epidermidis*. This might be due to the same reason discussed in the last paragraph about the structural differences between Gram-positive and Gram-negative bacteria but can be also related to mechanistic differences. For *S. aureus*, the resistance mechanisms with more relevance against chloramphenicol are enzymatic inactivation by chloramphenicol acetyltransferases and alterations of the drug target in the ribosomal subunit [156], [157]. This could reinforce the hypothesis that the present active phytochemicals are acting as EPI, keeping the positive effects on tetracycline action since it is still mainly affected by efflux pumps in Gram-positive bacteria [158], [159].

Considering the cases where positive effects were registered by the combination of extracts obtained with the same solvent from the same plant biomass, these were more significant when using the Soxhlet method for the extraction in comparison to the solid-liquid method ($p < 0.05$). This is following the fact that Soxhlet can extract more drug quantity [45]. The only exception is for the *Agave americana* methanolic extract combined with tetracycline against *E. coli*, where the opposite is verified ($p < 0.05$). This can be due to one of the disadvantages of the Soxhlet technique: the thermal degradation of the thermolabile compounds [45]. Thus, a possible mistake during the extraction in managing the temperature could lead to the degradation of the active compounds against *E. coli*, affecting the global potential activity of the extract. Also, the high content of sugar could have increased the phytochemical degradation. It is suggested by some authors that sugars and their degradation products tend to accelerate the degradation of some bioactive compounds like anthocyanins [160].

Finally, the different plant biomass shows different antimicrobial potentials. Extracts from *T. fluminensis* leaves have only produced a positive effect in tetracycline against *E. coli* (methanolic extract by the Soxhlet method), while *T. fluminensis* stem and *A. americana* extracts gave both relevant results against the two pathogens. The first reason for this is the considerably lower concentration of the *T. fluminensis* leaves extracts due to being harder to recover the dried extract with DMSO, as already discussed above. There are only two cases where the extraction conditions are the same and the concentrations between two extracts from different plants' biomass are similar. The first case is for the methanolic extracts of *T. fluminensis* stem and *A. americana* obtained by the Soxhlet method, where at a concentration of 663.83 mg/mL and 855.23 mg/mL, respectively, *A. americana* extracts were more active. The second case is for the ethanolic extracts of *T. fluminensis* leaves and *T. fluminensis* stem obtained by the same method as the previous, where at a concentration of 130.85 mg/mL and 141.68 mg/mL, respectively, *T. fluminensis* stem extracts were more active. The last case is especially curious since it is observed a big difference in the potentiation effect from different plant biomass that after all belong to the same plant. The activity differences between stem and leaf extract seem to be species-dependent since both cases of higher activity appear in the literature. Adedapo *et al.* [159] and Beevi *et al.* [160] reported in methanolic extracts of *Calpurnia aurea* and extracts of *Raphanus sativus*, respectively, that the leaves extract had superior antibacterial activity than the stem[161], [162]. On the other hand, Piccirillo *et al.* [163] work shows that stem ethanolic extracts from *Prunus cerasus* cause higher bacterial inhibition than the ones from the leaves. The registered differences might be

globally justified by the presence of different bioactive compounds or in different concentrations, as will be explored in the next section.

During the elaboration of this dissertation, it was noted a clear tendency for the biggest part of the studies about the approached theme to focus on leaf extracts instead of other parts of the plant like the stem and roots. Since the idea of understanding and using plants as sources of antimicrobial compounds come from traditional medicine where leaves were widely used, it makes sense to use the already existent knowledge, focusing more on that plant part. Also, leaves (and fruits) are easily harvested and processed, which makes their selection and study more practical – it was probably the same reason for this plant part to be more used in traditional medicine over the others. Lastly, some plants' stems and roots are toxic, making it harder to handle and study them [163]. However, behind human toxicity can also be bacterial toxicity, since are both defensive mechanisms against predators and pathogens. Also, a big part of the allelopathy interactions happens at the soil level where the big part of the microbiota is, thus stem and especially roots might also have allelochemicals in their composition [88]. This reflection aims to say that a big part of the plant is being neglected in antimicrobial studies and might be interesting and fruitful to fill this research gap.

4.6. Phytochemical characterization

To understand some of the obtained results on the antibacterial activity of the extracts, two tests to quantitatively characterize the composition of the extracts were conducted. The total phenolic content test by the Folin-Ciocalteu method was chosen since this is a large group of compounds with the widest spectrum of different bioactivities and this is a well-established method. The total flavonoid content method is also commonly used and quantifies one of the families of phenolic compounds.

Table 13 - TPC (mg GAE/g extract) and TFC (mg CE/g extract) of the obtained extracts. Results are presented as the average and standard deviation of three replicates.
S: Soxhlet extraction S/L: solid-liquid extraction.

	Total phenolic content			
	EtOH		MetOH	
	S	S/L	S	S/L
A. americana	$1.73 \pm 4.23 \times 10^{-3}$	$1.06 \pm 1.12 \times 10^{-2}$	$2.27 \pm 1.53 \times 10^{-2}$	$2.67 \pm 4.23 \times 10^{-3}$
T. fluminensis (leaves)	$1.15 \pm 1.27 \times 10^{-2}$	$0.880 \pm 1.12 \times 10^{-2}$	1.69 ± 0.00	$1.31 \pm 1.27 \times 10^{-2}$
T. fluminensis (stem)	$1.61 \pm 7.33 \times 10^{-3}$	$1.12 \pm 1.53 \times 10^{-2}$	$2.75 \pm 4.23 \times 10^{-3}$	$2.28 \pm 8.46 \times 10^{-3}$
	Total flavonoids content			
	EtOH		MetOH	
	S	S/L	S	S/L
A. americana	$2.54 \pm 7.40 \times 10^{-2}$	$2.31 \pm 3.70 \times 10^{-2}$	$2.72 \pm 2.05 \times 10^{-2}$	$3.67 \pm 2.05 \times 10^{-2}$
T. fluminensis (leaves)	$3.25 \pm 3.70 \times 10^{-2}$	$2.52 \pm 1.78 \times 10^{-2}$	$3.32 \pm 2.05 \times 10^{-2}$	$2.67 \pm 6.41 \times 10^{-2}$
T. fluminensis (stem)	$1.79 \pm 1.03 \times 10^{-2}$	$1.40 \pm 2.72 \times 10^{-2}$	$3.48 \pm 3.22 \times 10^{-1}$	$2.64 \pm 3.53 \times 10^{-1}$

For all the cases presented in Table 13, methanolic extracts presented higher total phenolic and flavonoid content ($p < 0.05$), as was expected since methanol extracts a higher variety of different compounds, including inside the large group of phenolic compounds. Overall, the Soxhlet technique also showed to produce extracts with more concentration of these two groups ($p < 0.05$) which was also an expected result because this method can extract a higher quantity of drugs [45]. There were only two exceptions: the first is for the methanolic extracts of *A. americana*, whose higher TPC was measured for the extracts obtained with the solid-liquid method ($p < 005$). This is also the extract type that exhibited higher antibacterial activity for the one obtained by the solid-liquid method than the Soxhlet method, contrary to the statistical fashion. The same hypothesis proposed before could be applied here. Additionally, phenolic compounds seem to be more sensitive to the temperature than flavonoids, especially polyphenols, explaining the still normal higher content of flavonoids in the extracts by the Soxhlet method despite the contrary for TPC [164]. The second exception is for the methanolic extract of *T. fluminensis* stem, whose higher TPC was measured for the one obtained with the solid-liquid method ($p < 0.05$). This is an unexpected result, thus a more exact validation of the extract composition would be advantageous to confirm this result.

Comparing the different extracts, unfortunately, no pattern shows a higher content of this group of compounds in a plant biomass over another, not giving a possible explanation for the higher effects of *A. americana* and *T. fluminensis* stem in the antimicrobial tests, not even correlating with the results of those tests: for example, *T. fluminensis* stem shows the higher TPC in methanolic extracts obtained by the Soxhlet method, but are the *A. americana* extracts that have high potentiation activity. The used methods might not be the most sensitive ones, for example, the weak selectivity of the Folin-Ciocalteu reagent, as it reacts positively with different antioxidant compounds, could lead to erroneous results [16]. As an alternative, a method like reversed-phase high-performance liquid chromatography (RP-HPLC) could have been used for the same purpose providing more accurate results. The verified lack of pattern could also mean that the active molecules or combination of molecules that increased the activity of the antibiotic do not make part of these groups of compounds. As referred before, the extracts that provided higher potentiation on the effect of the antibiotic were different, comparing ethanolic and methanolic extracts. However, considering the overall effect of ethanolic and methanolic extracts, this potentiation was quantitatively similar. This could mean that the most relevant bioactive compounds belong to groups of molecules that are not equally extracted using the two solvents, like alkaloids, flavonols, and saponins. Thus it would be relevant, as future work, to also perform tests about the presence and amount of other types of phytochemicals or, ideally, identify and purify the specific bioactive molecules.

The only relation that might be interesting to refer is the higher TPC for stem extracts compared to leaf extracts from *T. fluminensis*, and the opposite regarding flavonoids ($p< 0.05$). The same relative differences were reported by Parvin *et al.* [165] in a study about the antibacterial potential of *Crescentia cujete* leaves (TPC of 28.08 mg of GAE/g and TFC of 139.58 mg of QE/gm of extract) and stem bark (TPC of 111.43 mg of GAE/g of extract and TFC of 20.77 mg of QE/g of extract) ethanolic extracts. However, Míguez *et al.* [49] reported that *T. fluminensis* leaves (3.01 mg GAE/g) extracts have higher TPC than the stem (1.58 mg GAE/g) [49]. However, in that work, the temperature (25-45 °C) and time (2-6 h) of extraction were

considerably different, which could have altered the results. There is a big fluctuation in the total phenolic and total flavonoid content, as is possible to observe in the Table 1. This value is very dependent on the quantification method and also on the extraction conditions, explaining the big variance observed in the literature [166].

Wawer *et al.* [167] performed the phytochemical characterization of *T. fluminensis* leaves founding the presence of alkaloids, flavonoids, tannins, and phenolic compounds in the obtained ethanolic extract. Boonprasert *et al.* [168], with a similar study but using the total biomass of the plant, identified the same compounds with tannins and phenicol compounds in more relevant amounts but also identified saponins in the aqueous extract. Note that alongside methanol, water is the other most commonly chosen solvent when the active compounds of interest to extract are saponins [148]. Shegute *et al.* [53] characterized the methanolic extract obtained from *Agave americana* by the Soxhlet method and reported the existence of alkaloids, tannins, phenolic compounds, flavonoids, and saponins. Pandey *et al.* [60] used the solid-liquid technique to obtain an ethanolic extract from the same plant and identified alkaloids, flavonoids, and saponins. Both *A. americana* and *T. fluminensis* (total plant biomass) extracts are shown to have the additional presence of saponins compared to the *T. fluminensis* leaves extract. Despite the difference in the solvents used for the extraction, this may indicate the potential of this group of molecules to be one of the relevant compounds potentiating the action of tetracycline and chloramphenicol in this work. However, this is only a hypothesis with no experimental data to support it, so information should be acquired to confirm or deny it.

4.7. Antioxidant activity

Antioxidant activity from a plant extract refers to the ability of the extract to neutralize or prevent oxidative damage caused by free radicals in the body. Free radicals are unstable molecules that can damage cells, proteins, and DNA, leading to various chronic diseases such as cancer, arthritis, and cardiovascular diseases [169]. Antioxidant-rich plant extracts can help in reducing inflammation, slow down aging, and prevent chronic diseases. In addition to their application as therapeutics, they are also commonly employed in the food industry to help reduce food deterioration by oxidation, causing alteration of the color, flavor, texture, and safety of the food products [169]. The loss of the original properties of the food usually leads to its discard, causing profit losses by the merchants.

The antioxidant activity of the obtained extracts was evaluated by two different methods: the DPPH and the ABTS method. For both, the results are presented in mg TE/g extract (Table 14) and scavenging activity (Table 15). Looking globally at the results, *T. fluminensis* leaves extracts show to have a higher antioxidant capacity and, generally, *T. fluminensis* biomass extracts have a higher scavenging capacity than the *A. americana* ones ($p < 0.05$). However, comparing the results from the two methods, they do not match completely. Both measurements were highly affected by the intense natural color of the extracts, especially DPPH where a big part of the samples maintained the natural green color instead of acquiring a purple tone (Figure D.1, Annex D) – darker or lighter depending on the antioxidant activity. In some cases, it was also observed the crystallization of some samples, affecting the final results by excess. This issue was already reported in the literature and is caused by the reaction

products of DPPH with antioxidants like phenolic compounds, flavonoids, and carotenoids. The crystalization occurs as a result of the reduction of DPPH's free radical electron from its reactions with the antioxidant, resulting in a reduction in solubility [169].

The results obtained by the DPPH method were less trustable than ABTS: no pattern was observed for extracts prevenient from the same plants biomass. Globally, no differences were observed between the two solvents, but for the solid-liquid extractions methanol showed to be better, and the opposite was observed for the Soxhlet extractions ($p < 0.05$). Considering Soxhlet extraction, methanol was the best solvent to extract antioxidants and the opposite was observed for the solid-liquid technique. This data also does not correlate with the TPC or TFC.

Considering now the data from the ABTS test, *T. fluminensis* was the plant with higher antioxidant activity for all four scenarios of extraction technique/solvent, with especially relevance for the stem extracts. While the extraction of antioxidants was higher in *T. fluminensis* leaves with the Soxhlet method, for the other two plant biomasses was higher with solid-liquid. Regarding the solvents, ethanol was globally the most efficient, with special relevance in the Soxhlet method ($p < 0.05$), while solid-liquid was the best method regarding both solvents. This goes in contrast to the antimicrobial, TPC, and TFC results which were higher for methanolic extracts and using the Soxhlet method. It could be argued that antioxidants are more susceptible to temperature than antimicrobial agents, but in the literature, it is exactly the opposite that is reported. Mphahlele *et al.* [170] studied the effects of different drying temperatures on the bioactive compounds, antioxidant, antibacterial and antityrosinase activities of *Punica granatum* extracts, and results revealed no significant antioxidant differences despite the big negative influence that the temperature increase had in the antimicrobial activity. Once again, the interference of the extract's color might have been crucial: both methanol as solvent and the Soxhlet method produced higher extraction efficiencies, leading to a higher pigmentation and extract color intensity which may interfere with colorimetric methods resulting in the measurement of higher OD and consequently being interpreted as reduced antioxidant activity.

Amensour *et al*. [171] studied the antioxidant activity and chemical content of methanol and ethanol extracts from leaves of *Cistus ladaniferus*, concluding that methanolic extracts in comparison to ethanolic ones, had higher TPC, TPF, and antioxidant activity. On the other hand, Haq *et al.* [172] reported the opposite: a TPC and antioxidant activity higher for ethanolic extracts from *Bruguiera gymnorrhiza*. It was also tested the leaves and stem, separately, with the extracts from the stem revealing high TPC and antioxidant activity. So, the efficiency of the solvents in extracting phenolic and antioxidant compounds – phenolic and others like flavonoids – seems to be dependent on the plant specie. However, the relative difference should be concordant – higher TPC, and higher antioxidant activity. Once again, the used methods showed some vulnerabilities regarding the natural color of the extracts in analysis. In the future, additional protocol changes should be studied as other techniques to evaluate the antioxidant activity that is not based on colorimetry, like oxygen radical absorbance capacity (ORAC), which is based on fluorescence [173].

Table 14 - Antioxidant activity (mg TE/g extract) of the obtained extracts. Results are presented as the average and standard deviation of three replicates.
S: Soxhlet extraction S/L: solid-liquid extraction.

	DPPH			
	EtOH		MetOH	
	S	S/L	S	S/L
A. americana	1.70 × 10-3 ± 1.30 × 10-4	1.37×10-3 ± 4.09 × 10-5	8.97×10-4 ± 3.50 × 10-5	9.32×10-4 ± 3.43 × 10-4
T. fluminensis (leaves)	1.64×10-3 ± 3.73 × 10-5	7.58×10-4 ± 9.00 × 10-5	1.67×10-3 ± 5.91 × 10-5	1.81×10-3 ± 4.94 × 10-5
T. fluminensis (stem)	2.12×10-3 ± 3.54 × 10-5	1.21×10-3 ± 3.26 × 10-5	6.16×10-4 ± 4.28 × 10-5	1.73×10-3 ± 8.81 × 10-5
	ABTS			
	EtOH		MetOH	
	S	S/L	S	S/L
A. americana	1.71×10-3 ± 3.64 × 10-6	2.09×10-3 ± 9.62 × 10-6	5.00×10-4 ± 5.60 × 10-5	1.30×10-3 ± 4.19 × 10-5
T. fluminensis (leaves)	2.08×10-3 ± 2.28 × 10-5	1.93×10-3 ± 3.15 × 10-5	2.03×10-3 ± 4.29 × 10-5	1.93×10-3 ± 1.20 × 10-4
T. fluminensis (stem)	2.14×10-3 ± 2.75 × 10-5	2.26×10-3 ± 3.47 × 10-5	1.61×10-3 ± 2.98 × 10-5	2.32×10-3 ± 2.38 × 10-5

Table 15 - Scavenging activity (%) of the obtained extracts. Results are presented as the average and standard deviation of three replicates.
S: Soxhlet extraction S/L: solid-liquid extraction.

	DPPH			
	EtOH		MetOH	
	S	S/L	S	S/L
A. americana	56 ± 4.3	45 ± 1.4	29 ± 1.2	30 ± 11
T. fluminensis (leaves)	54 ± 1.2	24 ± 3.0	55 ± 2.0	59 ± 1.6
T. fluminensis (stem)	69 ± 1.2	40 ± 1.1	20 ± 1.4	57 ± 2.9
	ABTS			
	EtOH		MetOH	
	S	S/L	S	S/L
A. americana	63 ± 0.12	75 ± 0.31	24 ± 1.8	50 ± 1.3
T. fluminensis (leaves)	75 ± 0.76	70 ± 1.0	73 ± 1.4	70 ± 3.8
T. fluminensis (stem)	77 ± 0.87	80 ± 1.1	60 ± 0.95	82 ± 0.76

4.8. Fermentation

After the extractions, most of the biomass remained as process residue. Thus the use of plant leftovers in a downstream process continuing to extract other biological value-added products should be explored to assess the possibility of circularity of the process. So, it was decided to use and test that vegetal material as a fermentative substrate, by converting it into a liquid growth medium for *S. cerevisiae*. The approach based on the saccharification of vegetal structures for the obtention of a broth rich in reducing sugars is not new being widely studied, especially with the aim of bioethanol obtention [174]. However, the use of invasive plant species as raw-material and the coupling to up-stream phytochemical extraction process is a new perspective, increasing the circularity of both steps.

For this final step, only *A. americana* was studied due to its known high concentration of soluble sugars (44%), being the aim to reproduce the process for *T. fluminensis* in case of success. The steps described in the methods section are based on acquired previous experience and several works on saccharification of lignocellulosic biomass [175]–[179].

Despite the high content of soluble sugars, there was also a considerable part of the plant biomass (31%) composed of recalcitrant polysaccharides such as cellulose, hemicellulose, holocellulose, and lignin [129]. Thus a hydrolysis step was carried out exactly to break the glycosidic bonds between these polymers and the β-type bonds that are established between the monomers of each of them (β-1,4, in the case of cellulose), which generates a complex structure that is extremely difficult to degrade and break [180]. Usually, besides the temperature and pressure conditions created by the autoclave, strong acids like sulfuric acid and hydrochloric acid are added. However, since *A. americana* has a higher content of soluble sugars (44%) compared to other vegetal raw materials like *Miscanthus x giganteus* (0.25%), *Sorghum bicolor* (1.14%), and *Panicum virgatum* (0.28%) [129], it was decided not to add any acid for the hydrolysis step, avoiding a pH adjustment – and a consequent salt concentration increase due to the addition of an alkali compound like sodium hydroxide or potassium hydroxide – and the formation of toxic compounds like hydroxymethylfurfural (HMF) – formed by the dehydration of the acid and the fermentable monomers like fructose and glucose [173], [177]. Usually, the hydrolysate coming from vegetal material is deficient in nitrogen, limiting the growth of the fermenter microorganisms [183]. Thus, nitrogen supplementation like peptone and yeast extract is usually employed in this case. After the hydrolysis, it was added 250 mL of sterile distilled water – estimated volume to maintain the concentration of sugars after some water evaporation during the hydrolysis [182].

In the first approach, before the extraction with methanol, an aqueous extraction was performed to keep the soluble sugars and use them further in the fermentation, avoiding their loss in the methanolic extraction. Four different growth media were tested: aqueous extract, supplemented aqueous extract, hydrolysate, and supplemented hydrolysate. The goal was to compare two variables. The first was the need for supplementation by testing supplemented *versus* non-supplemented growth media. The second was the degradation of sugars and the formation of toxic compounds during hydrolysis. For this, it was tested aqueous extract (autoclaved for the conventional 15 minutes) *versus* hydrolysate growth medium (autoclaved for 30 minutes). The different media were inoculated with *S. cerevisiae*, ensuring an initial

concentration of approximately 0.09 g/L. However, after 45 minutes the culture was already not viable. The results allowed to conclude that supplementation and toxic compound formation were not the main cause for the negative results.

In the second approach, it was only prepared two equal hydrolysate-based growth media with supplementation. Also, this time it was used plant biomass that have not passed through an extraction step to try to eliminate possible sources of failure coming from the extraction step. Still, the addition of the nitrogen supplement was done after the hydrolysis to avoid the possible formation of compounds with antimicrobial activity like melanoidins [184]. Thus, after the hydrolysis it was added 250 mL of a sterile solution with yeast extract and peptone – estimated volume to maintain the optimum concentration of sugars after some water evaporation during the hydrolysis. Another change was the filtration step of the hydrolysate after centrifugation to reduce the number of particles in suspension, allowing the quantification by measuring the OD density. However, no growth was verified for 72 h.

4.9. Antifungal activity evaluation

Since none of the variables tested – major sources of failure or efficiency loss in the fermentation of plant/lignocellulosic-based substrates – was revealed to be the major cause of the absence of growth, the hypothesis of the presence of antifungal phytochemicals extracted during the hydrolysis arose. However, as said before, some phytochemicals are thermolabile, especially at the autoclave extreme temperature and pressure conditions. Usually, phenolic compounds are associated with destruction or inactivation by heat, consequently causing the partial or total loss of important properties of the extract. Surprisingly, the use of an autoclave to sterilize extracts is not as harmful as expected. Harjanti *et al.* [185] studied the effect of different sterilization methods on herbal extracts, concluding that the TPC reduction after autoclavation was only 7.7-11.2%, much less compared to syringe filter sterilization (18.6-23.1%). Faraone *et al.* [186] also tested autoclavation at 121 °C for 20 minutes as an extraction technique compared to maceration, ultrasound, and accelerated solvent extraction, revealing even higher TPC in *O. europaea* extracts (143.00 mg GAE g^{-1}) than the other more conventional techniques.

Thus, the presence of phytochemicals in the obtained growth medium after autoclavation has to be considered and might be the reason for the absence of growth and immediate loss of cell viability. Benarba *et al.* [187] tested the antifungal activity of *Aristolochia longa* aqueous extracts against *S. cerevisiae*. The experiment was run under similar conditions as the one described in the present work – liquid growth medium, 37 °C – and various concentrations of extract were tested. At 500 µg/mL, the maximal inhibitory dose on the cell growth, the extract almost abolished the proliferation of *S. cerevisiae* cells after 72 h of treatment (97.06% inhibition of proliferation). For this work, it is unknown what the phytochemical concentration in the fermentative broth is, however, having into consideration the extracted mass obtained with ethanol as a solvent – in the preliminary solid-liquid assay produced similar extraction efficiency as distilled water – it is possible to estimate an extract concentration of 2349.50 µg/mL and, following the same logic, a total phenolic concentration of 129.33 µg/mL. It would be interesting, in a future experiment, to access these contents.

To confirm the surprising results in the fermentation, an antimicrobial activity test was performed, this time against *S. cerevisiae*, by the disk diffusion method using the previously obtained ethanolic and methanolic crude extracts.

Table 16 - Inhibition zone diameter (mm) for obtained crude extracts against S. cerevisiae. Results are presented as the average and standard deviation of two replicates (one independent assay).

	A. americana		*T. fluminensis* (leaves)		*T. fluminensis* (stem)	
	Soxhlet	Solid-liquid	Soxhlet	Solid-liquid	Soxhlet	Solid-liquid
EtOH	8.5 ± 0.5	5 ± 1.0	0	0	0	0
MetOH	11 ± 0.0	16 ± 0.0	0	0	0	0

The data obtained from the disk diffusion method is presented in Table 16 and confirms the hypothesis for the failure in the fermentation. The mechanisms thought to be responsible for phenolic toxicity to yeasts include enzyme inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups or through more nonspecific interactions with the proteins [187]. Besides phenolic compounds, flavonoids and saponins may also be responsible for antifungal activity [187]. These results are especially relevant compared to the ones obtained in the antibacterial tests because the registered inhibition of *S. cerevisiae* growth was verified for the crude extracts alone, while *E. coli* and *S. epidermidis* were only inhibited by the extracts combined with antibiotics. Thus, the antifungal potential of the obtained and tested *A. americana* extracts might be higher than the antibacterial potential. Regarding the extracts from *T. fluminensis*, no inhibition was observed. It would be relevant to also combine the extracts with commonly used antifungals against *S. cerevisiae* to access if there is any potentiation activity, like for bacteria.

Patel *et al.* [188] performed a phytochemical analysis and antifungal activity assessment of *Moringa oleifera* ethanolic extracts (solid-liquid extraction) obtaining a 15 mm inhibition zone against *S. cerevisiae* with the addiction o 300 µL of extract – 30 times more volume than in this study. Awang-Jamil *et al.* [189] tested the antifungal activity of *Merremia borneensis* methanolic extracts (Soxhlet extraction) against the same yeast, revealing an inhibition zone between 5.5-15.5 mm, depending on the extract concentration, for a volume of 40 µL of extract – 4 times more volume than in this study.

The application of plant extracts and specifically their phytochemicals as antifungal agents is as important as for antibacterial uses. Against the backdrop of this major global health threat, invasive fungal diseases are rising overall and particularly among immunocompromised populations. Despite the growing concern, fungal infections receive very little attention and resources, leading to a paucity of quality data on fungal disease distribution and antifungal resistance patterns. Consequently, it is impossible to estimate their exact burden. In response to this threat, WHO released the *WHO fungal priority pathogens list to guide research, development and public health action* [190]. Among all the microorganisms listed, *C. neoformans*, *Candida auris*, *A. fumigatus,* and *C. albicans* are the ones that deserve more attention, being included in the critical group. Despite not representing the same danger, *S. cerevisiae* is considered to have a close relationship to *C. albicans* [191], which increases the interest to extend the antifungal activity tests of especially

A. americana extracts to pathogenic fungi. This becomes even more attractive since it is a natural product, passing around some problems already cited for antibiotic/therapeutics currently in the market like the limited selection of medications, their adverse side effects, and the emergence of resistance to them by fungal pathogens [187].

In addition, to the applications in the health sector, natural antifungal agents can also be employed in other industries. For example, wood products are very susceptible to biodegradation by fungi. Wood is treated with preservatives, often with inorganic compounds or synthetic pesticides that harm the environment. Therefore, the development of new, environmentally friendly wood preservatives derived from plants as well as obtaining synthetic compounds that will be safe for humans and do not pollute the environment, while at the same time present biological activity is crucial in terms of environmental protection [192].

5. Conclusions

Overall, this work allowed to conclude that both invasive plant species *T. fluminensis* and *A. americana* have phytochemicals with relevant antimicrobial activity, confirming the biotechnological potential of this group of plants.

Regarding the extractions, Soxhlet showed to be a better extraction technique than solid-liquid extraction, producing significantly higher extractions efficiencies compared to the second. Comparing methanol and ethanol, the two solvents that were deeply studied, methanolic extracts were always associated wither higher extractions efficiencies, with an average value of 43.8 ± 8.4 % compared to 17.7 ± 3.3 % for ethanolic extracts – values obtained by the Soxhlet extraction.

More methanolic extracts were active than ethanolic extracts combined with the selected antibiotics, however, potentiation effects were globally similar – the antibiotic action increase for each extract was quantitatively similar between ethanolic and methanolic extracts. Tetracycline was the antibiotic whose antibacterial capacity was highly potentiated by the presence of the extracts, especially against *E. coli*, with the extract from *A. americana* producing the strongest positive effect of 2.5 times. Chloramphenicol was also potentiated against the Gram-negative strain with the highest potentiation effect of 66 % being caused by *A. americana* extract. The extracts from the *T. fluminensis* stem also produced relevant effects on the action of the antibiotics in both bacteria, with the maximum potentiation of 2.4 times of tetracycline registered against *E. coli*. *A. americana* crude extract was the only one that revealed antifungal activity against *S. cerevisiae* (11 ± 0.0 mm). Regarding the phytochemical characterization, the extract from *T. fluminensis* leave showed both the highest TPC and TFC of $2.75 \pm 4.23 \times 10^{-3}$ mg GAE/g extract and $3.48 \pm 3.22 \times 10^{-1}$ mg CE/g extract, respectively. *T. fluminenesis* also revealed higher antioxidant activity.

Thus, the hypothesis of the potential of invasive plant species as a source of phytochemicals with antimicrobial activity was validated, opening several windows of applications where these plant extracts can be used beyond the health sector, like natural preservatives that can be applied in several industries such as the wood sector.

6. Future work

A. americana and *T. fluminensis* have proven to be a source of phytochemicals with antibacterial – combined with antibiotics – and/or antifungal activity. However, some questions remain to answer like which specific phytochemicals are responsible for the observed results and which mechanism of action they are using against bacteria and fungi. The easiest and most direct strategy would be to identify and purify the bioactive compound(s). Still, it may be also interesting to access what groups of compounds beyond phenolic compounds are present in the extracts, like alkaloids, terpenes, and so on, using preferably color-independent methods to avoid the problems faced during this work. It would also be interesting to research further the differences observed between stem and leaves phytochemical content.

Considering the antifungal activity, it is relevant to understand if the inhibition caused in *S. cerevisiae* is reproducible in other independent assays and if it prevails in pathogen yeasts like *Candida albicans*.

Finally, regarding the fermentation of the remaining biomass from the extractions, the optimization of the extraction process – for example, using different solvents in a cascade and sequential process – beyond increasing the possible amount of phytochemicals, could also benefit the down-stream fermentation since it would reduce the concentration of phytochemicals in the biomass after the extractions. Thus, despite also reducing the concentration of soluble sugars, this could reduce the toxicity of the hydrolysate growth medium, enabling *S. cerevisiae* growth. Additionally, the use of a more adapted strain to lignocellulosic substrates could also enhance the chances of success. It would be also interesting to test the potential of *T. fluminensis* biomass for fermentation since its extracts did not cause any inhibition of *S. cerevisiae*.

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Annexes

A. Calibration curves

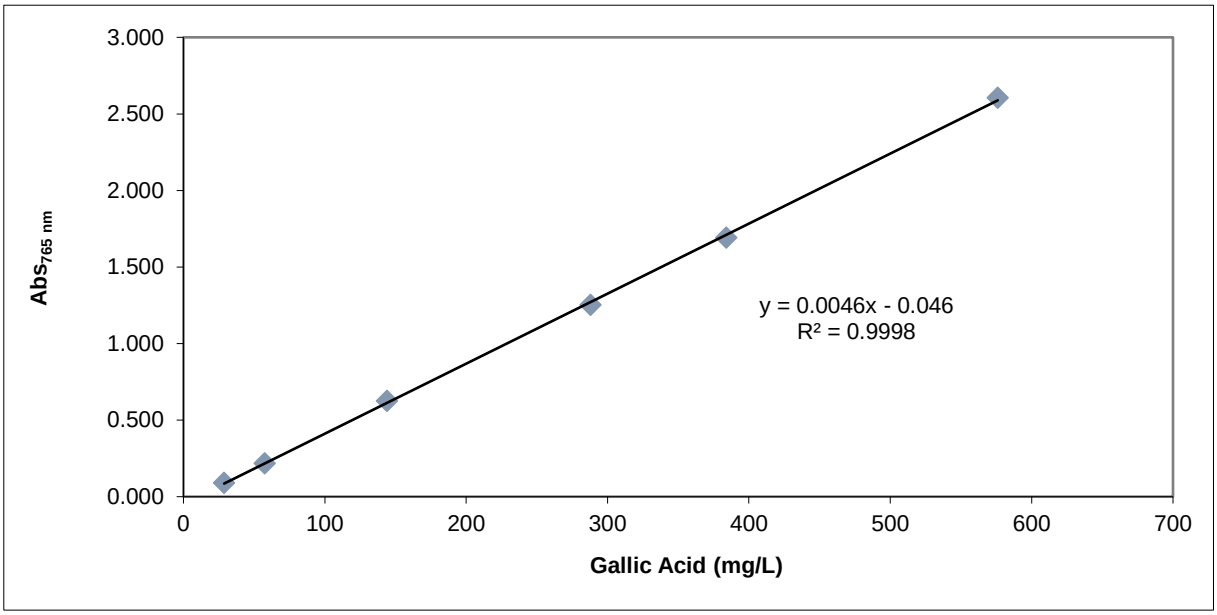


Figure A.1 - Total phenolic content calibration curve.

Acid gallic concentration *versus* Absorbance equation (TPC):

$$Abs_{765\,nm} = (4.57 \times 10^{-3} \pm 9.86 \times 10^{-5}) \times TPC\ (mg\ GAE/L) - (4.60 \times 10^{-2} \pm 3.08 \times 10^{-2}) \quad (A.1)$$

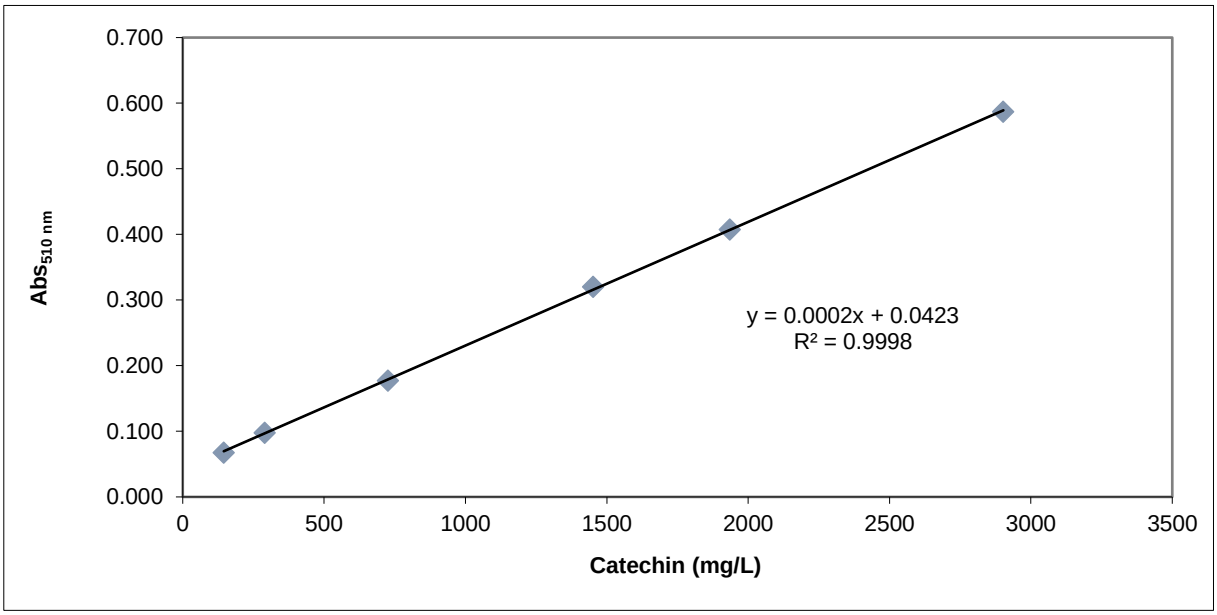


Figure A.2 - Total flavonoids content calibration curve.

Catechin concentration *versus* Absorbance (TFC):

$$Abs_{510\,nm} = (1.88 \times 10^{-4} \pm 3.37 \times 10^{-6}) \times TFC\ (mg\ CE/L) - (4.23 \times 10^{-2} \pm 5.31 \times 10^{-3}) \quad (A.2)$$

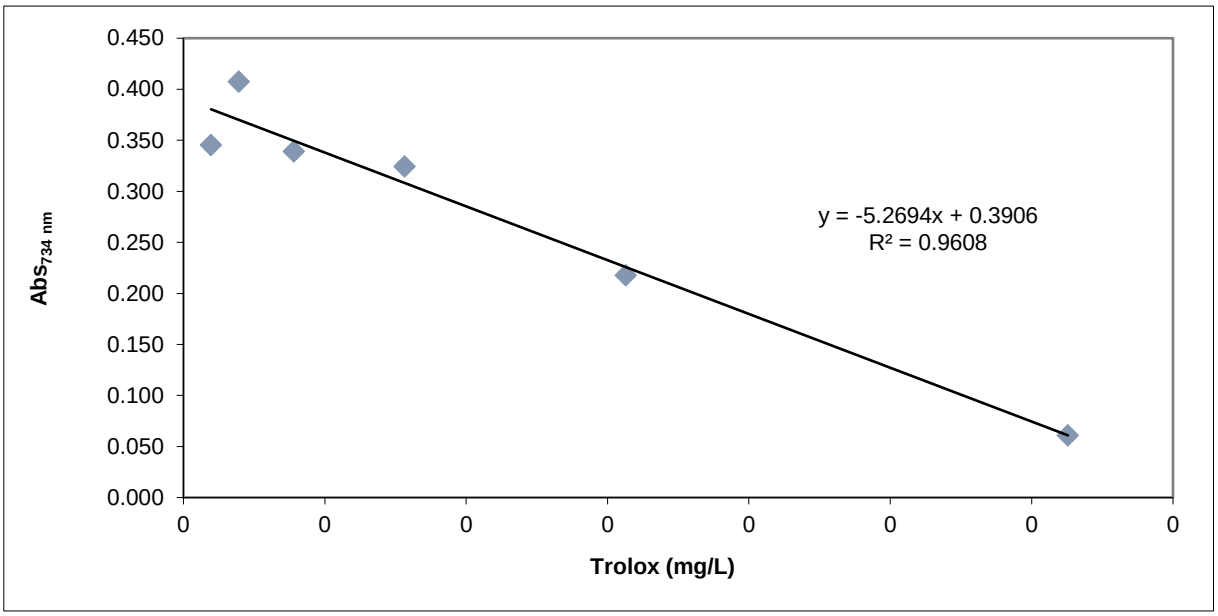


Figure A.3 - DPPH method calibration curve for antioxidant activity determination.

Trolox concentration versus Absorbance (DPPH):

$$Abs_{517\,nm} = -(5.27 \pm 1.48) \times AC\ (mg\ TE/L) + (3.91 \times 10^{-1} \pm 4.36 \times 10^{-2}) \quad \textit{(A.3)}$$

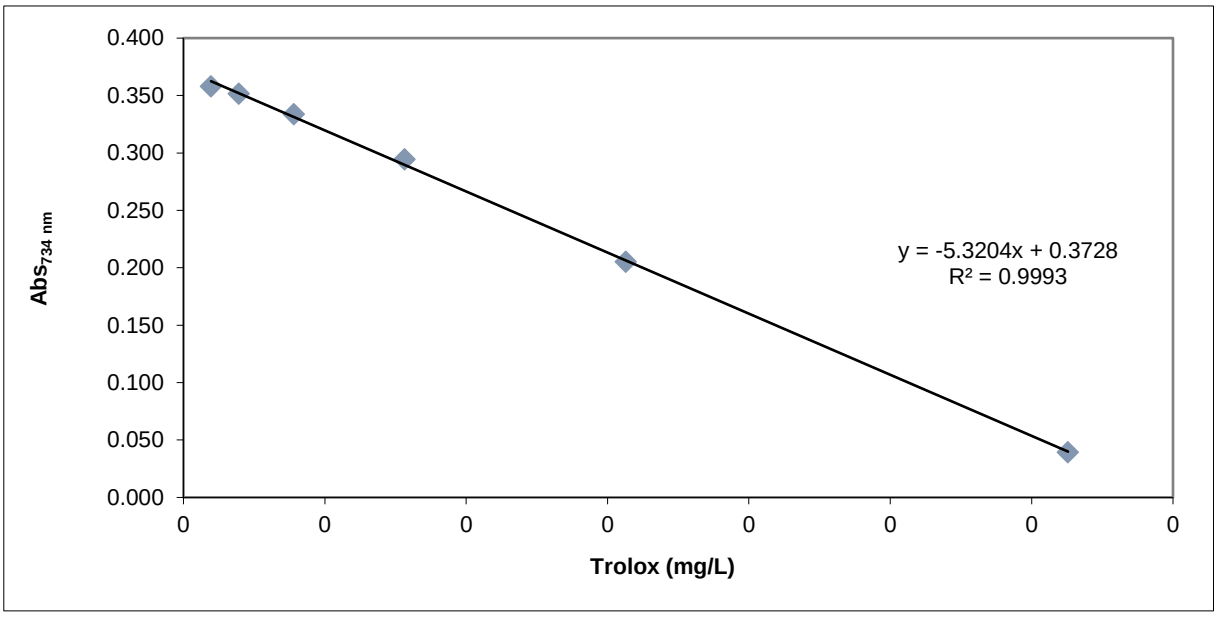


Figure A.4 - ABTS method calibration curve for antioxidant activity determination.

Trolox concentration *versus* Absorbance (ABTS):

$$Abs_{734\,nm} = -(5.32 \pm 1.91 \times 10^{-1}) \times AC\ (mg\ TE/L) + (3.73 \times 10^{-1} \pm 5.62 \times 10^{-3}) \quad \textit{(A.4)}$$

B. Preliminary solid-liquid extraction efficiencies

Table B.1 - Preliminary solid-liquid extraction efficiencies (%).

Solvent	*A. americana*	*T. fluminensis* (leaves)	*T. fluminensis* (stem)
EtOH	9.34	15.8	3.89
MetOH	48.7	47.5	49.3
H_2O	-	11.7	18.6
Hexane	8.16	26.3	8.30

C. Solid-liquid extracts

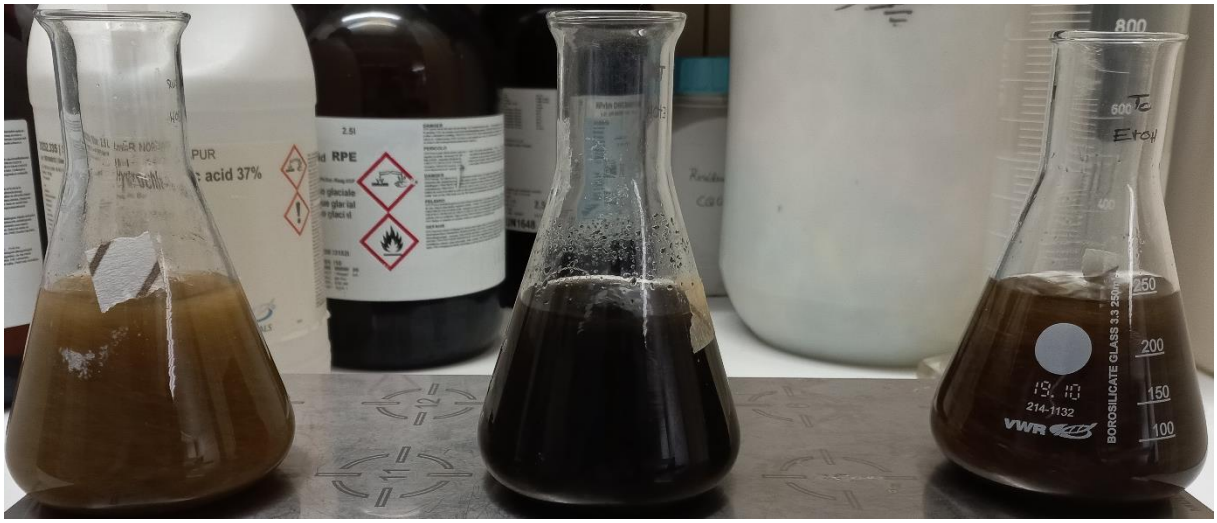


Figure C.1 - Ethanolic extracts before filtration: A. americana (left), T. fluminensis leaves (center), and T. fluminensis (right).

D. DPPH method

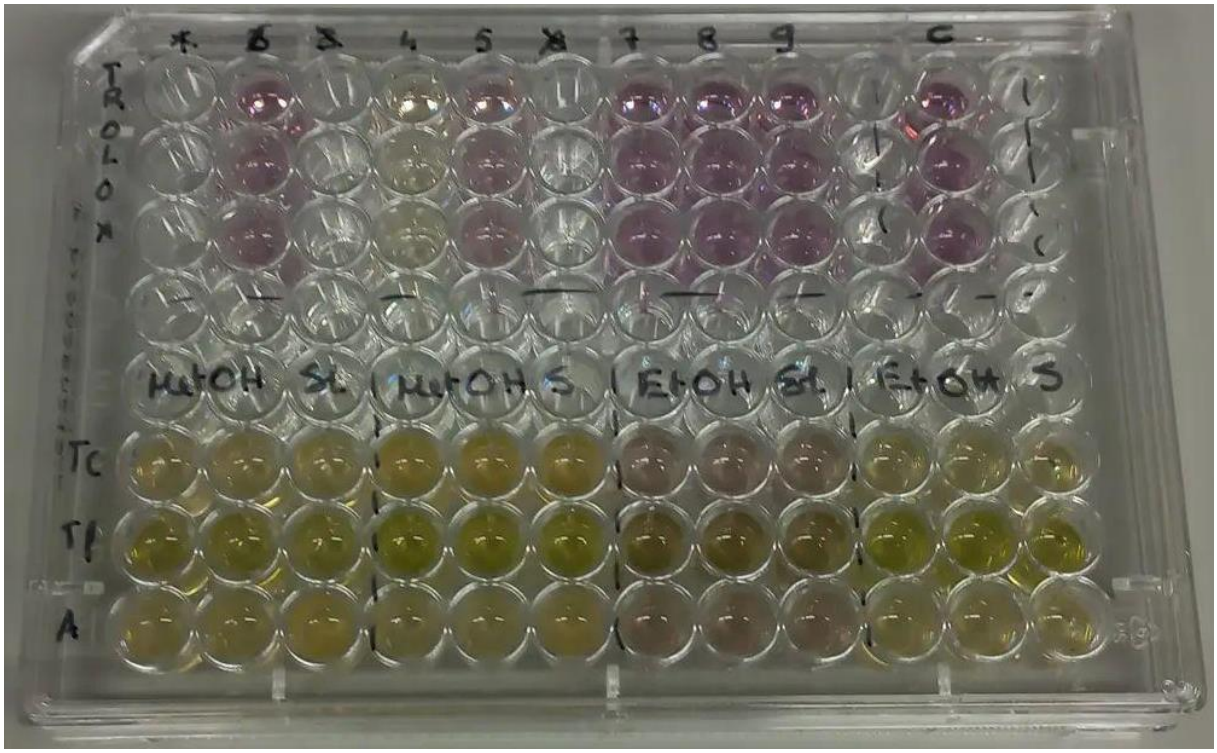


Figure D.1 - 96 wells microplate to measure the antioxidant activity by the DPPH method; Superior half (purple solutions): Trolox standard solutions inferior half (green solutions): samples.