André Miguel da Costa Cairrão

Bachelor's Degree in Biology

Biotechnological strategy for the exploitation of the invasive *Asparagopsis armata* as an underutilized natural source of bioactive compounds

Dissertation for the obtention of the Master's Degree in Biotechnology for Sustainability

Supervisor

Prof. Dr. Cristina Silva Pereira, Associate Professor, ITQB-NOVA

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RESUMO

A resistência dos microrganismos a antibióticos atuais aumentou a morbidez e a mortalidade, levando à pesquisa de compostos de origem natural de fontes terrestres e marinhas com potencial terapêutico. Algas são capazes de produzir uma ampla gama de metabolitos secundários bioativos, alguns dos quais exibem potencial terapêutico no tratamento de doenças humanas. Vários estudos já provaram que *Asparagopsis spp*. são fontes promissoras de compostos antimicrobianos. No entanto, até o momento, as bioatividades de extratos aquosos de *Asparagopsis* nunca foram testadas contra fungos e bactérias patogénicas. Além disso, esta espécie invasora está presente em várias zonas costeiras em todo o mundo, tornando-se um recurso natural facilmente obtivél. Neste estudo, a eficiência de vários métodos de extração à base de vapor de água para obter extratos bioativos contra microorganismos modelo foi comparada usando ensaios de atividade metabólica (MTT) e viabilidade (CFUs). O processo de otimização focou a determinação das melhores condições e parâmetros de extração para identificar os hidrolatos com maior atividade inibitória de crescimento contra os microorganismos testados. Os melhores parâmetros para os métodos testados foram determinados como extrações de 20 minutos, de *A. armata* preservada por congelamento. Concluímos que o método de Destilação a Vapor em Reator (DVR), produziu hidrolatos com atividades superiores aos dos restantes métodos, capazes de reduzir significativamente a viabilidade de bactérias (gram-positivas e negativas) e fungos (filamentosos e leveduras). Este método provou ser simples, fácil de transferir para uma escala piloto/industrial e reprodutível, permitindo recolha de hidrolatos com atividades e composições químicas consistentes, como foi confirmado por Ressonância Magnética Nuclear (RMN). Análises de RMN e cromatografia gasosa-espectrometria de massa (CG-EM) de hidrolatos produzidos por DVR revelaram a presença de ácidos gordos, indicando que estes compostos podem ser responsáveis pela atividade do hidrolato. Considerando a abundância desta alga e o seu potencial biotecnológico, o principal objetivo deste projeto foi explorar uma oportunidade de valorização dupla, que combina a remoção de algas invasivas de habitats marinhos ameaçados com a sua exploração para o desenvolvimento de novos compostos antimicrobianos.

Palavras-chave: Recursos marinhos, *Asparagopsis armata*, Destilação a vapor, atividade antimicrobiana, RMN, CG-EM

ABSTRACT

Microorganism resistance to current antibiotics has increased morbidity and mortality, prompting the search for naturally occurring candidate drugs from terrestrial and marine sources. Algae can produce a wide range of bioactive secondary metabolites, some of which display biomedical potential in the treatment of human diseases. Multiple studies have shown that *Asparagopsis spp*. are promising sources of antimicrobial compounds. However, to date, the bioactivities of *Asparagopsis* aqueous extracts have never been tested against pathogenic fungi and bacteria. Additionally, this invasive species is present in several coastlines all over the world making it a sustainable, easily obtainable, natural resource. In this study, the capacity of various steam-based extraction methods to render bioactive extracts against the model microorganisms were thoroughly compared using metabolic activity (MTT) and viability (CFUs) assays. Optimization aimed to determine the best extraction conditions and parameters for obtaining hydrolats with the highest growth inhibitory activity. The best parameters for the methods tested were determined to be 20-minute extractions, using A. armata biomass which had been stored frozen. We concluded that the Reactor Steam Distillation (RSD) method, produced hydrolats with higher activities than its counterparts, capable of significantly reducing the viability of bacteria (gram-positive and negative), and fungi (filamentous and yeasts). This method was proved to be a simple, easy to scale-up, and reproducible method that delivers hydrolats with consistent activities and chemical compositions, as it was confirmed by NMR. Nuclear magnetic resonance (NMR) and gas chromatography-mass spectrometry (GC-MS) analyses of RSD-produced hydrolats revealed primarily fatty acid (FA) signals, indicating that these compounds may be responsible for hydrolat activity. Considering the abundance of this algae and its biotechnological potential, the primary goal of this project was to explore this two-fold valorization opportunity, which combines invasive algae removal from endangered marine habitats with their exploitation for the development of novel antimicrobial compounds.

Keywords: Marine resources, *Asparagopsis armata*, Steam distillation, Antimicrobial activity, NMR, GC-MS

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ABBREVIATIONS

COSY	Correlated spectroscopy
CFU	Colony forming unit
D_2O	Deuterium oxide
DMSO	Dimethyl sulfoxide
FA	Fatty acid
GC-MS	Gas chromatography-mass spectrometry
HSA	Highest single-agent method
HSQC	Heteronuclear single quantum coherence
HMBC	Heteronuclear multiple bond correlation
HVOC	Halogenated volatile organic compounds
MIC	Minimal Inhibitory Concentration
MHA	Mueller-Hinton agar
MHB	Mueller-Hinton broth
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NMR	Nuclear magnetic resonance
OD	Optical density
PBS	Phosphate-Buffered Saline
RPMI	Roswell Park Memorial Institute Medium
RSD	Reactor Steam Distillation
RT	Retention Time
SEM	Standard Error of the Mean
SDS	Dodecyl Sulfate Solution
SSD	Standard Steam Distillation
TEM	Transmission electron microscopy

1 INTRODUCTION

1 CURRENT CHALLENGES ON MICROBIAL RESISTANCE

Infectious diseases are a serious hazard to human health and one of the leading causes of death and morbidity worldwide (1). Over the past decades, the usage of antimicrobial drugs has risen, contributing to the increase in life expectancy. However, microorganisms have adapted to the surge of antimicrobials with evolved strains that are no longer susceptible to such drugs. Antimicrobial resistance is one of the world's greatest public health challenges within the lifetime of most people living today, as we are currently dealing with multi-resistant infectious disease pathogens that are difficult, if not impossible, to treat (1,2). This issue is particularly problematic in hospitals, treating critically ill patients, whose weakened immune system is less efficient against infectious diseases without the help of antimicrobial drugs (3).

1.1 BACTERIA

Bloodstream infection is the main cause of death from bacterial infectious diseases (4). *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli (E. coli)*, in particular, are of major concern regarding healthcare-associated infections and antibiotic resistance worldwide (4).

Despite being a natural part of our microbiota the gram-positive bacterium *S. aureus* is one of the pathogens of greatest concern because of its capability to cause a wide range of life-threatening infections, its intrinsic virulence, and its ability to adapt to many environmental settings (5). It can multiply in food at room temperature and some strains can produce a wide variety of heat-stable toxins that contribute to its ability to colonize and cause disease in mammalian hosts (5,6). Moreover, pyrogenic exotoxins may be released into the bloodstream, resulting in severe bloodstream infections and toxic shock syndrome (6). Bloodstream infections caused by *S. aureus* are expected to occur in 20-35 out of 100,000 people each year leading to substantial morbidity and mortality (7). Additionally, recent studies indicate increasing incidence rates worldwide, particularly due to the emergence of methicillin-resistant *S. aureus* bloodstream infections in hospitals (7).

E. coli is a gram-negative bacterium and is an important member of the normal intestinal microbiota of humans and other mammals. Most *E. coli* strains are harmless and rarely cause disease except in immunocompromised hosts or when the normal gastrointestinal barriers are breached. There are however several highly evolved *E. coli* strains that cause a variety of severe diseases (8). *E. coli* related bloodstream infections are becoming increasingly common, with the proportion of antimicrobial-resistant *E. coli* strains having increased in the last decades. This increasing trend of antimicrobial resistance in *E. coli* is already observed against various classes of antimicrobials like carbapenem, third-generation cephalosporins, and fluoroquinolones (4). *E. coli* also accounts for the majority of community-acquired urinary tract infections. Being such a common pathology, it is prone to the abuse or misuse of antibiotics, which can lead to resistance via the emergence of mutant strains, and unresolved, relapsed urinary tract infections that tend to be resistant to previously used antibiotics (9).

Currently, over 70% of bacterial pathogens are resistant to at least one modern antibiotic treatment (10). As a result, the urgent need for the development of new antibiotics or other approaches to tackle antibiotic resistance is imminent.

1.2 FUNGI

Bacteria are not the only microorganisms responsible for infectious diseases. Fungal infections impact one-quarter of the world's population and kill around 1.5 million people every year, exceeding malaria, breast cancer, and prostate cancer in terms of mortality (11). Even though antifungal resistance is not as common as antibiotic resistance, its emergence, most notably in *Candida spp*. and *Aspergillus spp*., is becoming a growing concern due to the added impact on already limited treatment options (12).

One of the most medically relevant fungi is *Candida albicans* (*C. albicans*) as it is the most common human fungal pathogen responsible for mucosal infections. In most cases, it is a harmless commensal organism. It can, however, transform into an opportunistic pathogen in immunocompromised hosts, causing life-threatening bloodstream infections and subsequent infections of internal organs, accounting for approximately 400,000 life-threatening systemic infections each year (11,13).

C. albicans is a morphologically and physiologically highly versatile and adaptable fungus, being able to grow as unicellular yeast, in pseudohyphal, and hyphal forms and colonizing various host niches (13). Its pathogenic potential can be downregulated to maintain commensalism or increased to damage host tissue and avoid or subvert immune surveillance (14). The ability to switch its morphology and produce biofilms, in particular, is critical to its pathogenesis. *C. albicans* biofilms are naturally resistant to antimicrobial therapy, hence the treatment of such biofilms with existing drugs remains ineffective (15).

Another medically relevant fungus is *Aspergillus fumigatus (A. fumigatus)*. It is the most common airborne fungal pathogen in developed countries, causing potentially fatal invasive aspergillosis in immunocompromised patients (16). One of its characteristics is its tremendous sporulating capacity. The asexual spores of this fungus, known as conidia, are widely distributed in the environment, and it is thought that we inhale them regularly. But they rarely pose a risk since they are quickly eliminated by the innate immune system, preventing infection (16,17). However, if the immune system is unable to deal with inhaled conidia, the spores can germinate and develop elongated filamentous forms known as hyphae, causing damage to host cells by penetrating them (17). *A. fumigatus* is responsible for a variety of infections, including chronic lung disease (chronic pulmonary aspergillosis) and life-threatening systemic infection that can involve multiple organs (invasive aspergillosis) (17). Due to the increase in medical treatments involving the suppression of the immune system and therefore a growing number of vulnerable patients, *A. fumigatus* infections have become a growing concern in the last decades (16).

Moreover, the number of therapeutic options for the treatment of invasive fungal infections is quite limited compared with those available to treat bacterial infections (18). With only four antifungal drug classes in clinical practice, most of which cause severe side effects and are frequently inefficient, there is a lack of reliable treatment options for this problem (19). These kinds of limitations, coupled with increasing levels of microbial resistance, sparked the search for naturally occurring candidate drugs from natural terrestrial and marine sources (20).

2 IMPORTANCE OF MARINE NATURAL RESOURCES:

Throughout the history of mankind, people have resorted to marine resources for various reasons, for food, medicine, and even cosmetics, making them vital parts of their livelihoods. Currently, over 3 billion people rely on marine and coastal biodiversity for a living, and the annual market value of marine and coastal resources and industries is projected to be around $3 trillion, representing nearly 5 percent of the world's Gross Domestic Product (21).

The ocean spans three-quarters of the Earth's surface and accounts for 99 percent of the planet's living space by volume (21). It is home to a myriad of species, making marine environments one of the most diverse and complex ecosystems in terms of biodiversity (22). Even after 250 years of taxonomic classification, researchers estimate that 91 percent of the ocean inhabiting species have yet to be classified (23). In addition, this environment contains a large number of organisms that have no terrestrial analogues (24), making it a promising biome for the discovery of novel bioactive compounds.

In recent decades, the study of marine organisms has resulted in the discovery of multiple compounds, some of which are of high economic interest, with potential uses in the medical, and industrial sectors (22,25). Many of these compounds present a wide range of bioactive properties, with structures and characteristics distinct from terrestrial ones (26). In 2013 alone, over a thousand pharmacologically active compounds of marine origin were characterized, with potential efficacy against cancer, high cholesterol, hypertension, and other disorders, as well as pathogens such as viruses, bacteria, and fungi (25). Continuous research of these bioactive molecules is increasingly relevant to combat antimicrobial resistance.

It has been established that these bioactive compounds, some of which have chemical structures with unique mechanisms of action, are mostly produced through the secondary metabolism of marine organisms (1). Harsh physicochemical conditions in the environment have played a significant role in the development of these compounds (22). Ecological pressures such as competition for space, avoiding predation, maintenance of an unfouled surface, and being able to reproduce successfully, have influenced the evolution of these marine organisms, and their unique secondary metabolites (24).

3 MARINE ALGAE AS A SOURCE OF BIOACTIVE COMPOUNDS

Due to the sheer amount and diversity of secondary metabolites, marine algae have become a valuable resource for the pharmaceutical industry. Furthermore, recent trends in drug development from natural sources, imply that bioactive secondary metabolites derived from algae have a high potential to interfere with the pathogenesis of many human diseases (20,27). Seaweed-based antimicrobials are still a relatively untapped resource for pharmaceutical, environmental, and food applications, and they may avoid many of the negative side effects associated with synthetic antimicrobials (28) such as headache, abdominal pain, vomiting, and systemic toxicity, as is the case with Norfloxacin, a commonly used antibiotic to treat urinary tract infections (29).

This thesis focuses on algae of the genus *Asparagopsis* (Bonnemaisoniales, Rhodophyta) and the properties of its extracts. The synergistic effects of seaweed antimicrobial extracts combined with antibiotics may offer effective therapy against drug-resistant microorganisms, resulting in novel products with multiple mechanisms of action for the pharmaceutical industry (28). Also, if new compounds are discovered and proven to belong to a new class of compounds, they could serve as a basis for the development of novel antimicrobials.

4 THE GENUS *ASPARAGOPSIS* (LIFECYCLE AND INVASIVE NATURE)

Algae of the genus *Asparagopsis* are red seaweeds of the phylum Rhodophyta belonging to the Bonnemaisoniaceae family (30). Two *Asparagopsis* species are taxonomically recognized, *Asparagopsis armata* (*A. armata)* Harvey 1855 and *Asparagopsis taxiformis (A. taxiformis)* (Delile) Trevisan 1845 (30). Despite being morphologically similar, the presence of characteristic long harpoon-like hooks (Figure 1-B) in the gametophyte stage of *A. armata* distinguishes it from *A. taxiformis* (31).

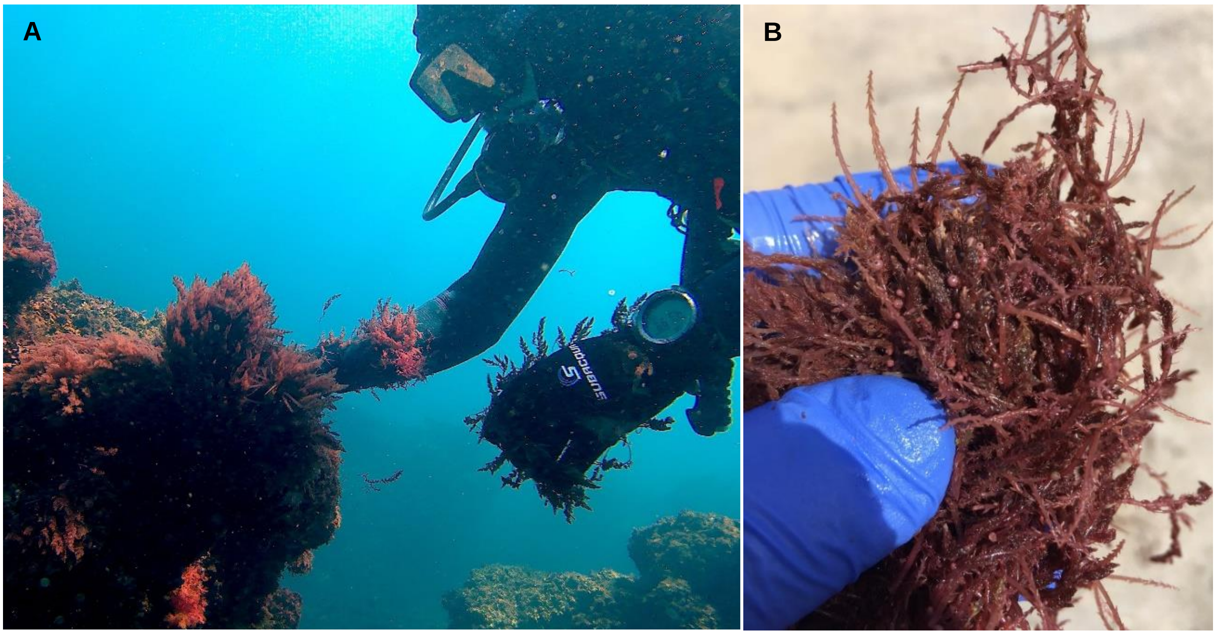


Figure 1 – *A. armata* in its natural habitat (A) and right after being collected (B). Photos taken during the collection process by Sea4Us.

A. armata is native to the southern hemisphere, more specifically from the coasts of New Zealand, Tasmania, and Australia (32), where it was first observed by the Irish botanist William H. Harvey in 1855 (33). This species exhibits a highly invasive behaviour and is currently found along several coastlines around the world. Consequently *A. armata* is one of the "Worst Invasive Alien Species Threatening Biodiversity in Europe" (EEA 2007) as is on the list of the 100 "Worst Invasives in the Mediterranean Sea." (34,35). It was introduced into the Atlantic and the Mediterranean Sea in the 1920s (36), and has since been documented at the southern and western shores of England and Ireland, as well as the Atlantic coasts of France, Spain, and Portugal, all the way down to the Canary Islands and Senegal (37), also in South Africa, the Middle East, and the Indo-Pacific region (1).

A. armata is an opportunistic species, its physical and biological traits are critical for its invasive success (38). But to better understand these traits its complex triphasic lifecycle must be taken into consideration (Figure 2). In plants and certain algae, the sexual phase (or an individual representing the phase) in the alternation of generations is referred to as a gametophyte. In this phase, which is haploid (having a single set of chromosomes), male and female organs (gametangia) develop and produce gametes through simple mitosis for sexual reproduction (39). When the gametes combine during fertilization, the zygote develops into the diploid (two sets of chromosomes) sporophyte phase. The zygote and the female gametophyte tissue around it develop into a pustule-like structure called a carposporophyte. The carposporophyte eventually produces and releases diploid carpospores, which mature into tetrasporophytes (40). Certain cells of the tetrasporophyte (diploid) undergo meiosis to produce tetraspores (haploid), and the cycle is repeated (40).

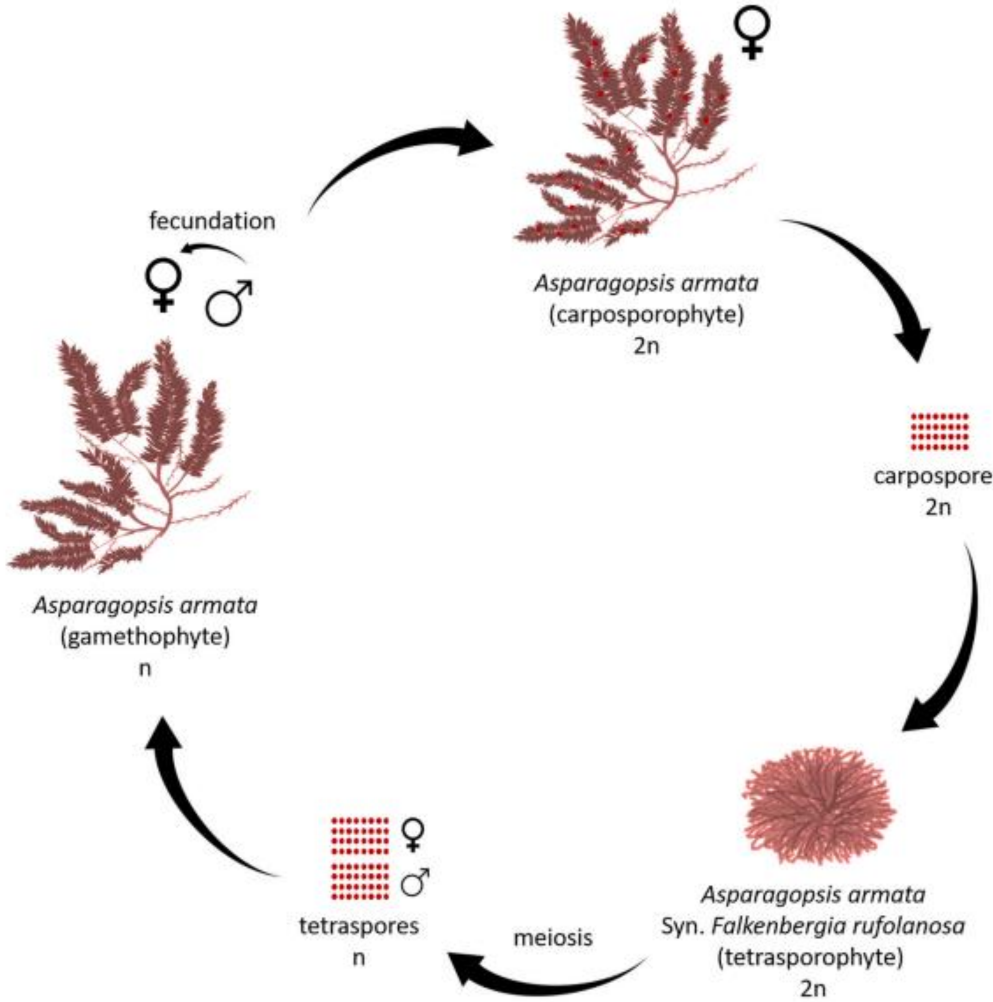


Figure 2 – *A. armata* lifecycle. Adapted from (41).

During its development, *A. armata* undergoes the gametophyte, carposporophyte and the tetrasporophyte phase (41). The gametophyte phase (haploid), the form firstly described as *A. armata,* can have either male or female gametes, has branches, vegetative growth, and is capable of sexual and vegetative reproduction. It is up to 20 cm tall, with its characteristic barbed hooks, and presents a pinkish colour, that turns red once the algae are removed from the water (41,42). The carposporophyte phase (diploid), comprises the female gametophytes after fecundation and production of multicellular carpospores (41). The tetrasporophyte phase (diploid), a free-living form of the algae, originally named *Falkenbergia rufolanosa*, can produce (via meiosis) and release tetraspores (haploid) to adhere to different surfaces and originate new gametophytes (41). It is a brownish-red, filamentous, branched algae, that forms dense cotton-wool-like tufts of 15 mm in diameter and occurs all year but it is most evident during winter (38,42).

A. armata’s invasive success is due to its free-floating tetrasporophytes, propagation by fragmentation, and its attachment to other seaweed and marine debris during the gametophyte phase via its barbed branches. *A. armata*’s tetrasporophytes are characterised by a high surface-to-volume ratio, having a higher capacity for fast nutrient absorption than other seaweeds. They can also cover other species, preventing light and nutrients from reaching those (38). Moreover, *Asparagopsis spp.* are capable of producing high quantities of halogenated compounds (35), which may be harmful to other marine species. The biologically active secondary metabolites produced by these algae, are also effective at protecting it from surface colonizing microorganisms (43) and are powerful anti-grazing agents due to their unpalatability to fish or other marine herbivores (44), promoting *Asparagopsis spp* dominance over native species. These compounds have been the subject of several studies aiming to identify and eventually apply those for the development of novel, sustainable, cost-effective, and environmentally friendly pharmaceuticals, chemicals, and cosmetics (41).

4.1 *ASPARAGOPSIS ARMATA* AS A SOURCE OF BIOACTIVE COMPOUNDS

As previously stated, the *Asparagopsis armata* seaweed has chemical defence mechanisms that are essential to its invasiveness. These mechanisms rely on the synthesis and storage of a wide range of secondary metabolites, including both halogenated and non-halogenated compounds, some of which are of high biotechnological interest (41,45). These compounds have been found to have a large variety of biological activities, including antimicrobial (antibacterial and antifungal), antiviral, antioxidant, anticancer, anti-inflammatory, antifouling, and antimethanogenic activity (41).

Within the non-halogenated compounds, hydrocarbons, fatty acids (FA), sterols, and polysaccharides, among others have been identified. FAs are organic compounds composed of carboxylic acids, generally with long aliphatic chains (46). *A. armata* has been found to contain several FAs, which may be responsible for the bioactivity of the ensuing algae extracts (1). FAs are produced by plants and algae as defensive weapons against pathogens. These compounds have long been known to have the ability to kill or inhibit the growth of bacteria, including compounds active against multidrug-resistant bacteria (46). Several FAs, including dodecanoic, tetradecanoic, hexadecanoic, octadecanoic acids, were found in the most active fractions of various bioactive crude extracts derived from *A. armata* (1).

Polysaccharides derived from sulfated galactans found in *A. armata’s* cell wall have shown remarkable antiviral activity against the human immunodeficiency virus, inhibiting its reproduction (47). Additionally, other authors reported the activity of water-soluble polysaccharides of *A. armata* against the herpes simplex virus type 1 (48). Mannitol has also been identified, it is a sugar alcohol that is often found in macroalgae with a wide range of applications, including its use as a sweetener in the food industry or as a drug in clinical practices (41).

Several halogenated volatile organic compounds (HVOC) have been found in *A. armata*. Despite representing a small part of its biomass, they are extremely important in terms of chemical ecology and biotechnological potential (41,49). Their production/ biosynthesis is one of the most well-known traits of this species. These compounds are thought to be major players in *A. armata* toxicity towards other organisms in nature and hence are also partially responsible for the bioactivities found in the ensuing extracts prepared for biotechnological applications (41). Most of these compounds are also toxic to the algae, therefore they are synthesized and stored in vacuoles within specialized glandular storage structures (43). Among these HVOCs, alkanes, alcohols, ketones, carboxylic acids, acrylic acids, and aldehydes have been identified (41,45). The major halogenated compounds with reported antimicrobial activity are tribromomethane (bromoform), dibromoacetic acid, dibromochloromethane, bromochloroacetic acid, and dibromoacrylic acid (43). Despite having antimicrobial activity, some HVOCs are not suitable pharmacological candidates due to their cytotoxicity. Large amounts of bromoform can, for example, lead to unconsciousness in adults and even to death in children, and it has been classified as a probable human carcinogen (50,51). As a result, care must be taken to ensure that HVOCs are absent, or only present at safe vestigial amounts, in any final product containing a mixture of extracted compounds.

These halogenated compounds are also responsible for the anti-methanogenic activity of *A. armata* in ruminant cattle (52,53). The inclusion of small amounts of these macroalgae in cattle feed has the potential to significantly decrease enteric methane emissions by inhibiting methanogens in the rumen (53). The bromoform concentrations in milk from lactating dairy cows subjected to this feed supplement were not significant, supporting that it does not pose any health risk for consumers (53).

The bioactivities observed in some *A. armata* extracts may be the result of synergism between the halogenated compounds, as well as more lipophilic compounds such as FA derivatives or even other compounds. This poses a limitation for pharmacological applications, where singular well-characterized compounds are usually required (54).

The majority of *A. armata* extracts studied thus far are obtained by very diverse methods that rely on complex, solvent-based strategies, as no single method of extraction and isolation can be considered entirely satisfactory (35). Solid-liquid extraction (20,35,38,55,56) is the most common extraction procedure, however, accelerated solvent extraction (57), liquid-liquid extraction (20), and Soxhlet apparatus extractions (48) have also been performed on *A. armata*. The type of solvents utilized (*e.g.* methanol, hexanes, dichloromethane, chloroform, acetone), as well as the ratios of solvent to biomass or even solvent to solvent when multiple solvents are used together for certain extractions (20,35,55,56), the state of the biomass (*e.g.* whole, fresh, dried, powdered), and the time of extraction are all important factors that influence the end result and the types of compounds present in an extract (41). Due to these variations in extraction procedures, a wide range of bioactivity-related outcomes have been recorded. Besides that, several ecological and biogeographical factors, such as the location of growth and the reproductive stage of the algae, or the season in which the samples are collected, may influence the production of bioactive compounds by the algae (35,41).

4.2 BENEFITING FROM ITS INVASIVE NATURE

Invasive algae, such as *A. armata*, rapidly dominate habitats by colonizing large areas, affecting nutrient availability, and thus displacing native species, due to their rapid growth. They have an irreversible impact on the structure and functioning of indigenous ecosystems and, along with climate change, are the most significant threats to marine biodiversity (38,58,59). On the other hand, the great quantities of biomass, that can be easily collected, make this red seaweed a readily available resource that can be biotechnologically exploited aiming at the isolation of new bioactive compounds (54). This presents a two-fold opportunity in which the need to control invasive macroalgae populations is combined with the valorization of these natural resources as a source of bioactive compounds, resulting in environmental, pharmaceutical, and economic benefits (60). This approach fits into a circular economy framework, which has grown in popularity as a result of the world's current unsustainable situation regarding limited resources (61).

5 CORE GOAL AND OBJECTIVES

Specifically, the major aim of this MSc thesis is to demonstrate that *A. armata* can be exploited as readily available raw material to produce novel antifungal and antibacterial compounds with a potentially unique mechanism of action. If true, our research will inspire the design of novel pharmaceutical compounds to counteract the rise in antimicrobial resistance. Considering *A. armata's* availability and biotechnological potential, we set out to lay the foundation for an added-value process, combining algal removal from endangered marine habitats with their exploitation for novel antimicrobial compounds. Accordingly, this meets with the Sustainable Development Goals set by the United Nations focusing on health and life below water.

This MSc thesis results from an ongoing collaborative project with Sea4Us that is a biotechnology company dedicated to marine pharmaceutical research and development that aims to discover treatments to respond to unmet clinical needs. Here, the optimization of an aqueous-based extract from *A. armata* will be described, which, unlike several previously used approaches, does not involve biomass milling or the use of harsh solvents. Despite being a greener approach, this may result in extracts of different compositions since the algal cells are not being completely destroyed by the solvents. If this approach is successful and large-scale extractions are required, this method avoids the use of large amounts of organic solvents, even if subsequent fractionation steps do require their usage.

Firstly, several hydrolats supplied by Sea4Us were tested to determine which extraction parameters or source of biomass (two algae species were tested, covering distinct collection periods throughout the year) result in hydrolats with the highest antimicrobial activity. Once a baseline of parameters had been established, the extraction method was further optimized to produce large amounts of hydrolats with higher antimicrobial potentials. The activities of these hydrolats were evaluated against model pathogenic bacteria as well as pathogenic fungi. Following this, several assays have been performed to determine if the obtained extracts show potential bactericidal/fungicidal and/or bacteriostatic/fungistatic activities.

After consistently obtaining hydrolats with suitable activities, the next step was to chemically characterize the main compounds present in the extracts using GC-MS and NMR techniques to build opening evidence of which compounds may be the source of the observed bioactivities.

Finally, preliminary tests were also performed to gather information about the extract's mode of action and how it interacts with known antibacterial drugs.

2 MATERIALS AND METHODS

1 *ASPARAGOPSIS* SPP. COLLECTION AND STORAGE

Asparagopsis armata and *Asparagopsis taxiformis* were manually collected along the southern Portuguese Atlantic coastal line by scuba diving. Samples were transported to the laboratory in cooling boxes with sea water and ice packs to keep the temperature between 10-15 ºC. Upon arrival, the biomaterial was moved into plastic buckets along with the sea water and carefully checked for any unwanted material (*e.g* other algae). The samples were subdivided and washed either in seawater (*in-situ* wash) or freshwater at room temperature (*ex-situ* wash) to remove any sands or other impurities. After this step, subsamples were either instantly frozen and stored at -20°C until further use, or they were air dried at 40 ºC for 36 hours (process done by BBKW, Azeitão, Portugal) and stored in the dark at room temperature until further use.

2 *ASPARAGOPSIS* HYDROLAT EXTRACTIONS

Hydrolats were produced via 3 different steam distillation processes. Initially distillation was outsourced to an external company (Pharmaplant, Alcoutim, Portugal) using an undisclosed steam distillation method. The remaining hydrolats were produced in-house, either at Sea4Us (standard steam distillation) or at ITQB NOVA (standard steam distillation and reactor steam distillation).

2.1 STANDARD STEAM DISTILLATION (SSD):

The extraction apparatus consists of a 1000 mL round-bottom flask (water reservoir) connected to a dual entrance 1000 mL chromatography reservoir flask (biomass flask) and a condenser ending in a collection flask (Figure. 3-A). The water reservoir is placed inside a heating mantle. The algae biomass (30 g of frozen or 10 g of dried biomass) was placed in the biomass flask onto a stainless-steel net at the bottom. The water reservoir was filled with 500 mL water and the heating mantle was switched to full power (450 W) to reach the boiling point as quickly as possible. Once the water in the flask reached boiling temperature, the power was reduced to 300 W, and the distillation performed for a specific time interval. The collected hydrolat (~ 50 mL for 20-minute extractions) was then transferred to a 50 mL falcon tube and stored at 4ºC until further use.

2.2 REACTOR STEAM DISTILLATION (RSD):

This method was developed to simulate a confined extraction method in which water vapor is re-circulated through the biomass to produce a more concentrated hydrolat using an AmAr Industries high-pressure stirred reactor (Figure 3-B). The reactor vat was filled with 100 mL of water, and the algae biomass (60 g of frozen or 20 g of dried biomass) placed in a stainless-steel net inside the reactor vat to keep it from touching the water. The vat was sealed, and the heating system switched to full power (150ºC max temperature) to reach the boiling point as fast as possible. Once the water in the reactor vat was boiling the temperature was adjusted to *c.a*. 100ºC and a bladed rotor was turned on to keep the vapor inside the vat moving. The distillation process was performed for 20 minutes. A maximum of 0.9 bar of relative pressure was reached during this process. The resulting hydrolat (~ 100 mL) was then collected from the vat, centrifuged, and filtered through a 0.45 µm Nylon membrane (Whatman) to remove any biomass particles that might have fallen from the net. It was stored in the fridge at 4ºC until further use.

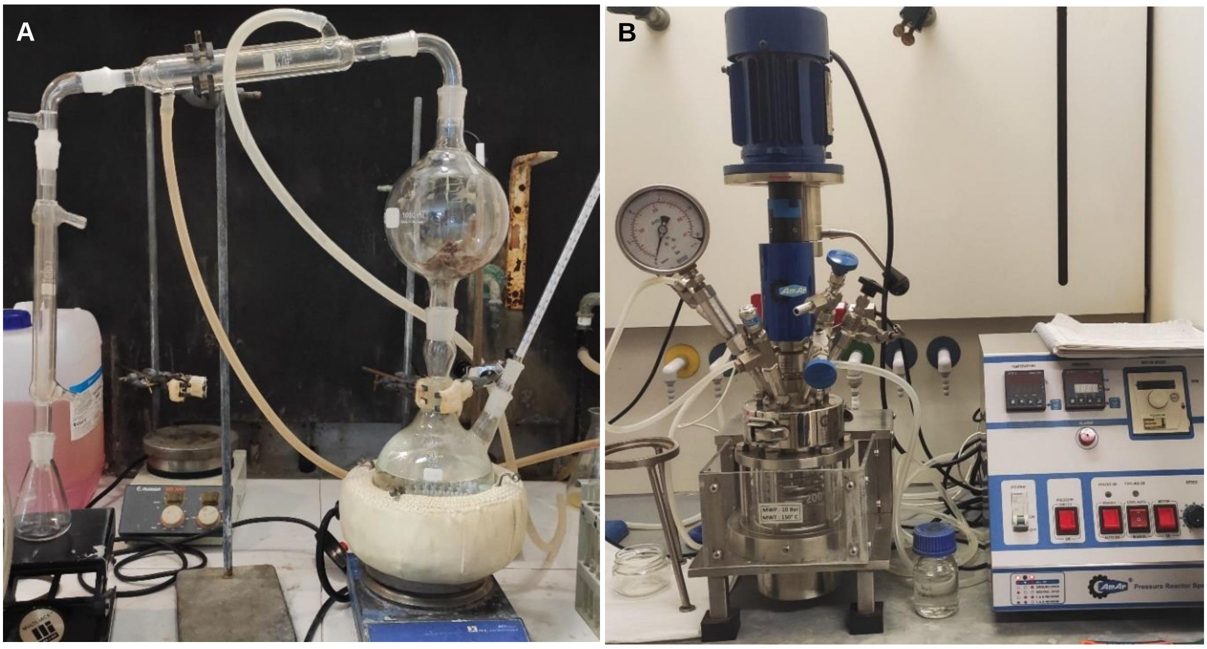


Figure 3 – Representation of the two described extraction methods. Standard Steam Distillation (A); Reactor Steam Distillation (B).

3 BIOACTIVITY ASSAYS

3.1 MICROBROTH DILUTION ASSAYS – HYDROLAT ACTIVITY TESTING

3.1.1 MICROORGANISMS AND PRECULTURES

Bacterial strains: *Staphylococcus aureus* (NCTC8325) and *Escherichia coli* (TOP 10), and fungal strains: *Candida albicans* (SC5314) and *Aspergillus fumigatus* (AF293), were chosen to test the hydrolat's bioactivity, as each represent a different class of pathogenic micro-organisms.

- Bacteria

S. aureus and *E. coli* were precultured in 20 mL of Mueller Hinton broth (MHB) (Oxoid) at 120 rpm for 24 hours. The next day, before plate inoculation, a bacterial suspension with an OD600 of 0.1 was prepared in double strength MHB and diluted 1:10 in the same medium to obtain ~5 x 10^5 CFU/mL.

- Fungi

C. albicans was precultured on PDA agar plates for 24 hours. Colonies were resuspended in MHB or RPMI (Roswell Park Memorial Institute Medium) (Sigma-Aldrich) (to assess how the organism reacts to the hydrolats when growing in its yeast or filamentous form) media and vortexed, the OD600 was adjusted to 0.1 and the inoculum was diluted 1:10 in the same media to obtain ~2.5 x 10^5 CFU/mL.

Previously, *A. fumigatus* spores were grown in t-flasks with aspergillus minimal media (62) for 96 hours before being harvested with beads and saline solution. Spores were centrifuged and resuspended in saline solution containing 15% glycerol and kept at -20ºC in cryovials. For these assays aliquots were defrosted and diluted in double strength RPMI to a final concentration of ~5 x 10^5 CFU/mL.

3.1.2 MICROBROTH DILUTION AND MTT ASSAYS

The antimicrobial effects of the produced hydrolats were evaluated using microbroth dilution assays to determine and compare the concentration-dependent activity for each sample. In 96-well plates, two-fold serial dilutions of the hydrolats were prepared. In each well 100 µL of hydrolat dilution in water were mixed with 100 µL of inoculum (or medium only for the abiotic control), and the plates incubated for 24 hours at 37ºC.

- Bacteria

100 µL of each well were transferred to a new plate and 10 µL of 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) (98%, Acros) (5 mg/mL MTT in Phosphate-Buffered Saline (PBS) (Sigma-Aldrich) were added. Bacterial plates were incubated in the dark for 30 minutes at 37ºC.

- Fungi

100 µL of medium were carefully removed from the original plate (for *C. albicans* grown in MHB, 100 µL of each well were transferred to a new plate) and 10 µL of 5 mg/mL MTT in PBS were added to the remaining 100 µL in each well. Fungal plates were incubated in the dark for 3 hours at 37ºC.

After incubation, 100 µL of fresh Sodium Dodecyl Sulfate solution (SDS) (PlusOne GE Healthcare) in 10 mM HCl (37%, Honeywell) were added to each well and the plates incubated at room temperature until all MTT crystals were dissolved (up to 2 hours for bacteria and overnight for fungi).

The plates were then read on a Tecan Infinite 200 plate reader at 560 nm and 700 nm and the values were subtracted to obtain the final results (OD 560 – OD 700). Results were analyzed in Microsoft Excel and GraphPad Prism 8.

3.2 VIABILITY ASSAYS

3.2.1 CFU VIABILITY ASSAYS ON BACTERIA AND *C. ALBICANS*

Microbroth dilution assays were prepared as described in Material and Methods, section 3.1.1 and grown for 24 hours. The following day, the OD was read on a Tecan Infinite 200 plate reader at 600 nm and wells showing different cell densities/inhibitory activities were selected (~100% inhibition, <50% inhibition, ~50% inhibition, >50% inhibition, and control). 50 µl of each well were serially diluted accordingly to their OD_{600} and plated on Mueller-Hinton agar (VWR) plates and left to grow overnight at 37ºC. The following day, colonies were counted. The data was analyzed in Microsoft Excel, and GraphPad Prism 8 was used to perform a t-test to see if there were any significant differences between the two hydrolats samples at each corresponding concentration. Statistical significance was considered when $p \leq 0.05$. 3 biological replicates were done for each microorganism.

3.2.2 VIABILITY ASSAYS ON *A. FUMIGATUS*

The hydrolat was diluted in minimal medium agar in 25%, 12.5%, 6.25%, 3.13%, 1.57% v/v concentrations. *A. fumigatus* was prepared at a concentration of 10^6 spores per milliliter of saline solution and 1 μl of inoculum was plated in the center of every plate. 3 biological replicates were done.

The spores were incubated at 37ºC for 48 hours and 96 hours, *A. fumigatus* radial growth was measured at both timepoints. Plates where no growth was observed were carefully examined under the microscope (Nikon Eclipse Ts2-FL) with 20 times magnification. Results were analyzed on Microsoft Excel and GraphPad Prism 8 was used to perform a t-test to see if there were any significant differences between the two hydrolats samples at each concentration. Statistical significance was considered when $p \leq 0.05$.

4 OPTIMIZATION OF HYDROLAT EXTRACTION TIME

Several hydrolat fractions were collected from a successive extraction over periods of 5 minutes. These hydrolats were produced and supplied by both Sea4Us (SSD method) and Pharmaplant (undisclosed method). They were all derived from the same biomass source (*A. armata*), which had been washed with both seawater and freshwater and air dried before storage. The specific details regarding each hydrolat fraction can be found in the table below.

Table 1 – List of fractions produced for the optimization of the extraction time.

	Produced by		**Extraction Time**
Hydrolat Name	Sea4Us	Pharmaplant	Fractions (min)
S4U_fraction_1	x		0 to 5
S4U_fraction_2	x		5 to 10
S4U_fraction_3	x		10 to 15
S4U_fraction_4	x		15 to 20
S4U_fraction_5	x		20 to 25
S4U_fraction_6	x		25 to 30
PHAR_fraction_1		x	0 to 5
PHAR_fraction_2		x	5 to 10
PHAR_fraction_3		x	10 to 15
PHAR_fraction_4		x	15 to 20
PHAR_fraction_5		x	20 to 25
PHAR_fraction_6		x	25 to 30
PHAR_fraction_7		x	30 to 40
PHAR_fraction_8		x	0 to 40

The antimicrobial activity of each fraction was measured via MTT assays, against each of the four model organisms to define the optimal extraction time in terms of bioactivity. All hydrolats were tested in duplicate.

5 OPTIMIZATION OF OTHER EXTRACTION PARAMETERS

The impact of several parameters, such as the source of biomass, washing procedure and storage, on the bioactivity of the derived hydrolats was evaluated. These hydrolats were derived from 20-minute extractions by Sea4Us (SSD Method). The specific details regarding each hydrolat can be found in the table below.

Table 2 – List of hydrolats produced for the optimization of the extraction time.

	Biomass source		***Storage***		***Wash***	
Hydrolat Name	*A. armata*	*A. taxiformis*	Dry	Frozen	Sea Water	Fresh Water
AA_Fro_SW_1	x			x	x	
AT_Fro_SW_1		x		x	x	
AA_Fro_FW_1	x			x		x
AA_Fro_SW_2	x			x	x	
AA_Fro_FW_2	x			x		x
AA_Fro_FW_3	x			x		x
AT_Fro_SW_2		x		x	x	
AA_Fro_FW_4	x			x		x
AA_Dry_FW_1	x		x			x
AA_Fro_SW_3	x			x	x	
AA_Dry_SW_1	x		x		x	
AA_Fro_SW_4	x			x	x	
AA_Dry_SW_2	x		x		x	
AA_Fro_FW_5	x			x		x
AA_Fro_SW_5	x			x	x	

The testing of each hydrolat was done via MTT assays. Here, several parameters were compared:

-Source of Biomass:

Hydrolats derived from *Asparagopsis armata* and *Asparagopsis taxiformis* were compared to determine which biomass source produced more bioactive hydrolats. Corresponding samples were collected at the same time and location and processed equally. Four biological replicates were tested against *S. aureus* and *C. albicans*.

-Biomass washing:

Hydrolats coming from biomass samples that were washed either in seawater or freshwater at room temperature to remove any impurities were compared. All other processing parameters were equal. They were tested against the four model organisms in three biological replicates.

-Biomass storage:

Hydrolats coming from biomass samples that were either instantly frozen and stored at -20°C or were air-dried were compared. All other processing parameters were equal. They were tested against the four model organisms in four biological replicates.

6 EVALUATION OF PRODUCTION METHODS & UPSCALING IMPACT IN THE HYDROLATS BIOACTIVITY

Hydrolats obtained using different techniques were compared to see if there was any difference in bioactivity. Hydrolats produced by RSD were done as described above. Hydrolats produced by SSD differed slightly, as several rounds of extraction were pooled to obtain greater volumes of hydrolat. To maintain the same ratio of biomass *per* milliliter of produced hydrolat, each round was done with fresh biomass. All hydrolats resulted from 20-minute extractions of *A. armata*.

Table 3 – List of hydrolats produced in larger quantities.

	Produced by			**Storage**		**Collection**	
Hydrolat Name	Sea4Us	ITQB		Dry	Frozen	May 2020	May 2021
	SSD	SSD	RSD				
S4U_SSD_Dry_1	x			x		x	
S4U_SSD_Fro_1	x				x	x	
AC01_RSD_Fro_1			x		x		x
AC02_SSD_Fro_1		x			x		x
AC03_RSD_Dry_1			x	x			x
AC04_RSD_Fro_2			x		x	x	
AC05_RSD_Dry_2			x	x		x	
AC06_RSD_Fro_3			x		x		x

The comparison of each hydrolat was done via MTT assays. They were tested against the four model organisms in three biological replicates.

7 STATISTICAL ANALYSIS OF HYDROLAT COMPARISONS

Hydrolat's bioactivities were compared for each corresponding concentration (from 12.5 - 50% vol/vol) for all optimization (except extraction time) and evaluation of production methods assays (for both metabolic activity and viability assays). Results were analyzed on Microsoft Excel and GraphPad Prism 8 was used to perform a Pairwise T-Test. Statistical significance was determined without correction for multiple comparisons, with $\alpha=0.05$. Each row was analyzed individually, without assuming a consistent SD. Three statistical significance intervals were considered for $p \leq 0.05$ (*), $p \leq 0.01$ (**).

8 LYOPHILIZATION OF HYDROLATS

Three hydrolat samples with similar bioactivity profiles were chosen, two derived from SSD (provided by Sea4Us) and one from the undisclosed method (provided by Pharmaplant). 10 mL of each hydrolat sample were lyophilized on a Labconco FreeZone 4.5, weighed, and resuspended in methanol (99.8%, Sigma-Aldrich) in a 1:50 ratio. These extracts were diluted and plated in the same concentration as the hydrolats. Their activities were then assessed via MTT assays, tested in triplicate against *C. albicans* and *S. aureus*. Methanol was used in the control wells at concentrations equal to the maximum concentration used in this assay. Hydrolats PHAR_fraction_5; AA_Fro_FW_5; AA_Fro_SW_5 were used for this experiment.

9 LIQUID-LIQUID EXTRACTION ASSAYS

Hydrolats were extracted with diethyl ether (99.5% Fisher) at a 1:5 ratio of solvent to sample. The samples were left incubating overnight on a tilting platform shaker (VWR Signature™ 3-D Rotator Waver) to promote the contact between phases. The following day the solvent phase was collected, new solvent was added to the hydrolat and left in the shaker overnight to be collected the day after.

The activities of the diethyl ether extracts were compared to that of the original hydrolat sample via MTT assays against *A. fumigatus* and *S. aureus,* all tested in triplicate. Diethyl ether was used in the control wells at concentrations equal to the maximum concentration used in this assay. Hydrolats S4U_SSD_Dry_1 and S4U_SSD_Fro_1 were used for this experiment.

10 NMR ANALYSES

10.1 BROMOFORM PRESENCE IN THE HYDROLATS

10.1.1 QUANTIFICATION OF BROMOFORM BY NMR

To quantify bromoform, 500 µL of selected hydrolat samples were mixed with 50 µL deuterium oxide (D_2O) (Acros). A known amount of sodium formate (99%, Sigma-Aldrich) was added to the mixture as an internal concentration standard. Spectra were acquired at 25°C on a Bruker Avance III 800 spectrometer (Bruker, Rheinstetten, Germany) working at a proton operating frequency of 800.33 MHz, equipped with a 5 mm, three channel, inverse detection cryoprobe TCI-z H&F/C/N with pulse-field gradients. A 3 second soft pulse before the excitation pulse was applied to pre-saturate the water signal. For quantification purposes, an additional delay of 60 seconds was applied to ensure full relaxation of the spins in the sample. Integration of the signals was performed using the tools available in the TopSpin software (Bruker, Rheinstetten, Germany) version 3.6.2.

Bromoform peaks were confirmed by spiking the samples with bromoform solution (96%, containing 1-3% ethanol as stabilizer, Sigma-Aldrich). Hydrolats AA_Fro_SW_1; AA_Fro_FW_1; AA_Fro_SW_2; AA_Fro_FW_2; AA_Fro_SW_3; AA_Dry_SW_1; AA_Fro_SW_4; AA_Dry_SW_2; AC01_RSD_Fro_1; AC02_SSD_Fro_1; AC03_RSD_Dry_1; AC06_RSD_Fro_3; and PHAR_fraction_5 were used for this experiment.

10.1.2 EVALUATION OF EXTANT BROMOFORM CONTRIBUTION TO BIOACTIVITY OF THE HYDROLATS

MTT assays were done to test various bromoform concentrations against the four model organisms in three biological replicates. The highest concentration tested was also the highest concentration detected by NMR in the hydrolats samples.

10.2 NMR ANALYSIS OF THE HYDROLATS

10 mL samples of S4U_SSD_Fro_1, S4U_SSD_Dry_1, AC01_RSD_Fro_1, and AC06_RSD_Fro_3 were lyophilized on a Labconco FreeZone 4.5 and resuspended in 2 mL of deuterated DMSO (Sigma-Aldrich). A 500 µL aliquot was added to an NMR tube. ^{1}H, ^{1}H-^{13}C HSQC, ^{1}H-

^{1}H COSY, and ^{1}H-^{13}C HMBC spectra were acquired. PHAR_fraction_5 was dried under an N_2 flow and resuspended in 300 µL of deuterated DMSO. ^{1}H, ^{1}H-^{13}C HSQC, ^{1}H-^{1}H COSY, and ^{1}H-^{13}C HMBC spectra were acquired.

1 µL of a 1:10 Dioxane solution in DMSO was used as an internal standard for all samples. All spectra were acquired at 25°C on a Bruker Avance III 800 spectrometer (Bruker, Rheinstetten, Germany) working at a proton operating frequency of 800.33 MHz, equipped with a 5 mm, three-channel, inverse detection cryoprobe TCI-z H&F/C/N with pulse-field gradients. The analysis of the obtained spectra was performed using MestreNova, version 11.0.4.

11 GC-MS ANALYSIS OF THE HYDROLATS

Hydrolats AC02_SSD_Fro_1, AC03_RSD_Dry_1, and AC06_RSD_Fro_3 were extracted once with diethyl ether in a 1:2 solvent to sample ratio as explained above. The solvent phase was collected, filtered through sodium sulphate anhydrous to remove any traces of water, concentrated under an N_2 flow sample concentrator (Stuart Sample Concentrator, SBHCONC/1) and spiked with a known concentration of hexadecane (99%, Sigma-Aldrich) as an internal standard. Additionally, the lyophilized PHAR_fraction_5 sample, resuspended in methanol, was analyzed.

An aliquot of 100 µL was taken from each sample and directly analysed by GC-MS. The remaining sample was further dried and derivatized with 250 µL N,O-bis(trimethylsilyl)trifluoroacetamide (99%, Sigma-Aldrich) containing 1% (v/v) of trimethylchlorosilane in pyridine (5:1) (99.8%, Sigma-Aldrich) for 30 min at 90°C.

Both derivatized and non-derivatized samples were analysed by GC-MS (Agilent: 7820A GC and 5977B quadrupole MS; HP-5MS column) operated as follows: 60 °C (hold 2 min), 10 $°C \cdot min^{-1}$ until 300 °C; 300 °C during 15 min. Data were acquired using an MSD ChemStation (Agilent); compounds were identified based on EI-MS fragmentation patterns, including the Wiley-NIST reference library, and quantified using external standards of some classes of aliphatic compounds (heptadecanoic acid and pentadecanol).

12 CHECKERBOARD ASSAYS

The evaluation of the interaction between AC06_RSD_Fro_3 and two antibiotics (Ciprofloxacin and Polymyxin B) (AlfaAesar and Sigma-Aldrich, respectively) was carried out via microdilution checkerboard assays in 96 well plates (Columns stand for numbers 1 to 10; lines for capital letters, starting in A). Eight hydrolat dilutions were prepared in water by sequential ½ dilutions and 100 µL were added to columns 3-10 with final concentrations ranging from 50% v/v (column 10) to 0.39% v/v (column 3). In the same manner, six antibiotic dilutions were prepared in water, at decreasing concentrations, ranging from twice the minimal inhibitory concentration - MIC (row B) to MIC/16 (row G) and 8 µL were pipetted to rows B-G (Figure 4). MIC values for each antibiotic/microorganism combination can be observed in Table 4. Finally, 100 µL of inoculum, containing ~5 x 10^5 CFU/mL of bacterial suspensions in MHB were added to each well. The plates were incubated for 24 hours at 37°C. Each plate was then subjected to an MTT assay to measure cell viability (as described in 3.2).

The expected drug combination responses were calculated based on the HSA reference model the SynergyFinder R package (63). 2 biological replicates were done for each bacterium.

Table 4 – MIC values for each antibiotic/microorganism combination (64).

MIC µg/mL	***S. aureus***	***E. coli***
Ciprofloxacin	0.25	0.0075
Polymyxin B	512	0.125

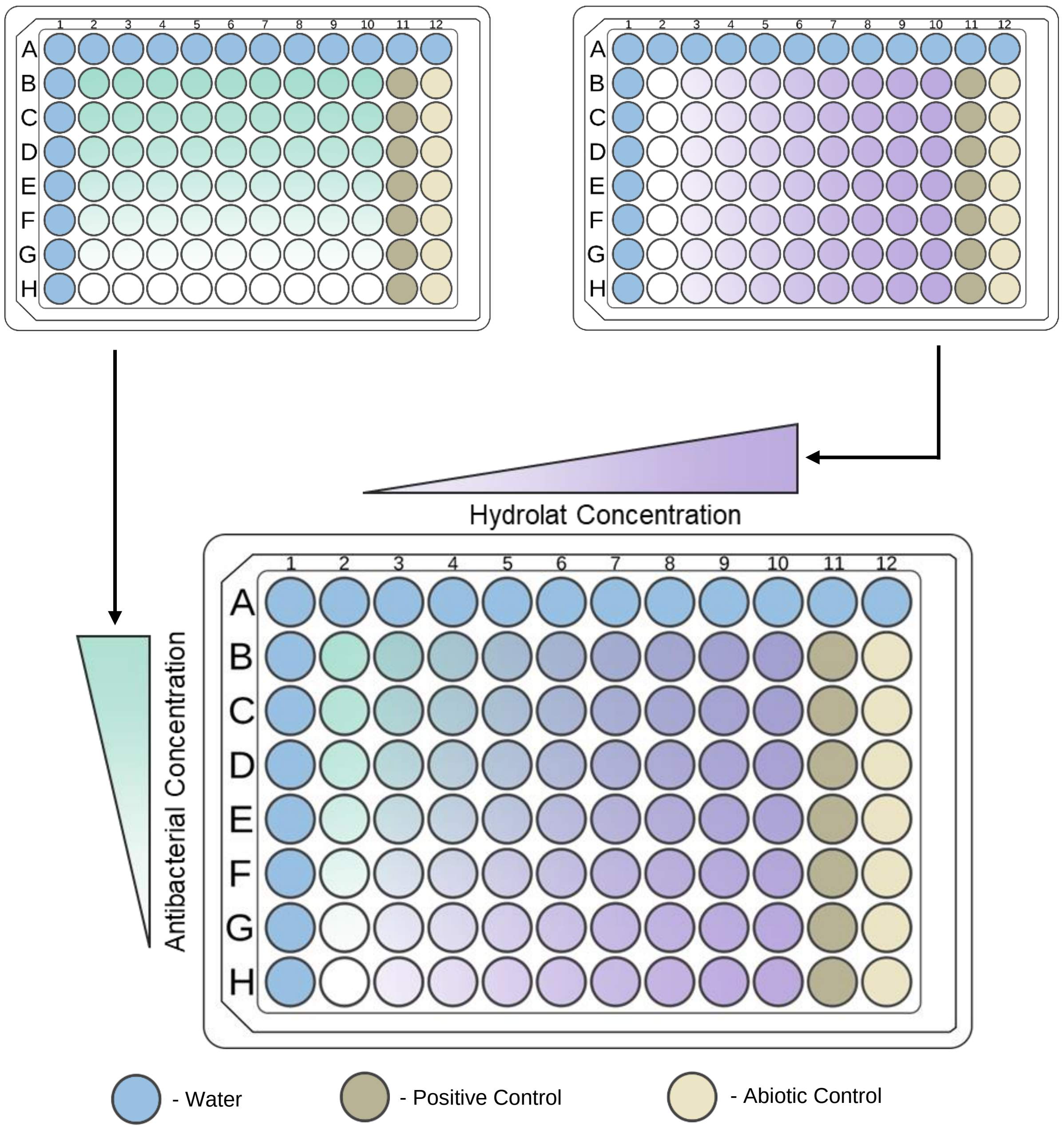


Figure 4 – 96 well plate schematic for the checkerboard assays. Wells filled with water to keep the plate's humidity levels stable. Control wells do not contain any hydrolat or antibacterial drug. Positive control – Growth medium with bacterial inoculum; Abiotic – Growth medium only.

3 RESULTS AND DISCUSSION

1 OPTIMIZATION OF HYDROLAT EXTRACTION TIME

Several hydrolat fractions resulting from various timed extractions were tested against the four model organisms (*S. aureus*, *E. coli*, *C. albicans* and *A. fumigatus*) using microbroth dilution assays followed by MTT to determine the optimal extraction times to obtain hydrolats with the highest antimicrobial activity. Specific details on the following hydrolat fractions can be found in Table 1 (Materials and Methods, section 4)

1.1 PHARMAPLANT FRACTIONS

During the first phase of this project, hydrolat fractions were outsourced to Pharmaplant, which used an undisclosed extraction method to obtain the hydrolat fractions shown in Figure 5.

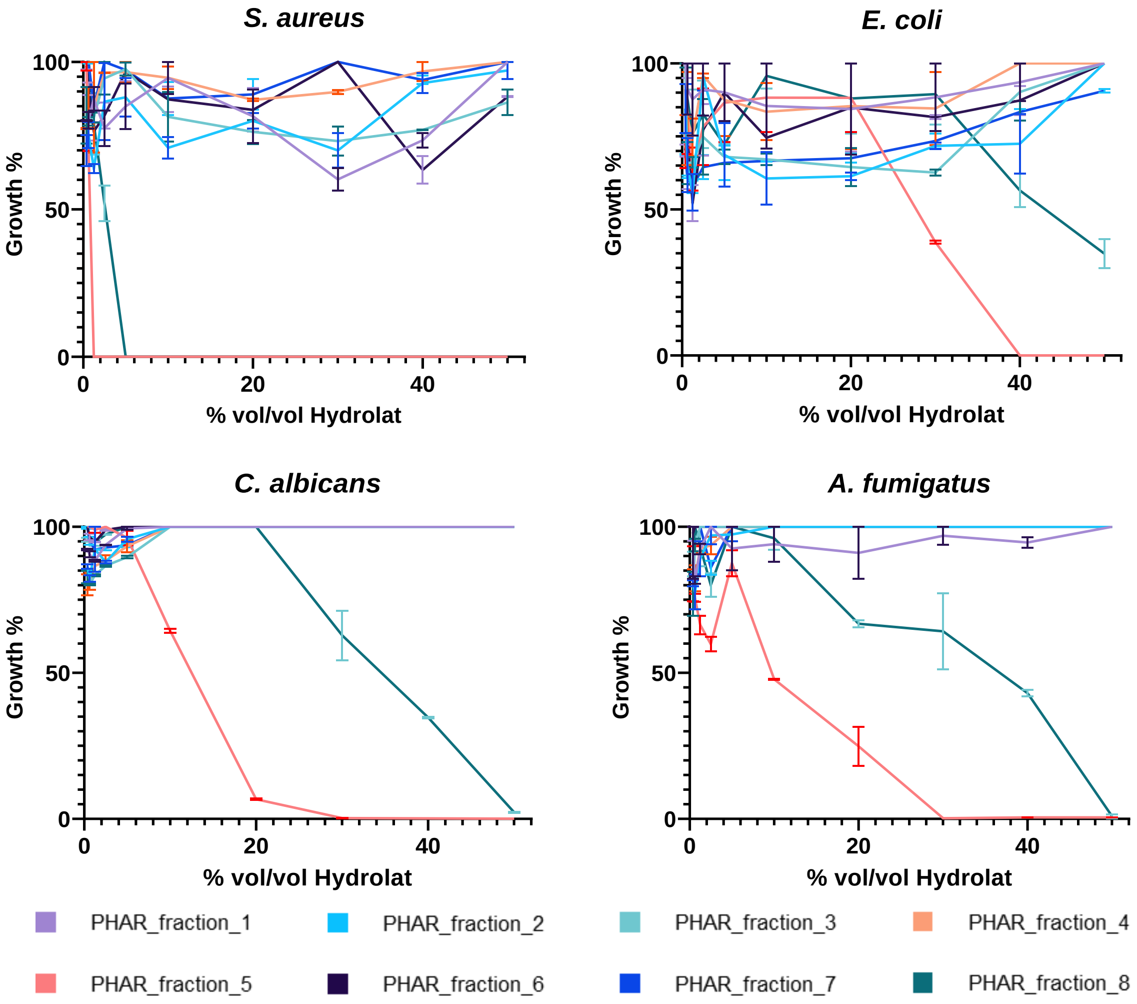


Figure 5 – Antimicrobial activities of hydrolat fractions produced by Pharmaplant. The activity of each fraction was tested against the four microorganisms by microbroth dilution followed by a MTT assay. Growth was calculated relative to the control (without any hydrolat). PHAR_fraction_5 (red) and 8 (dark green) show the highest activity against all 4 organisms. The mean and SEM (Standard Error of the Mean) of two replicates are shown.

With Pharmaplant's extraction method, the fifth hydrolat fraction (from 20 to 25 minutes) (PHAR_fraction_5 in red) was the most active against the four model organisms tested (Figure 5). The only other sample that exhibited bioactivity was the one that was collected concurrently throughout the entire 40-minute period (PHAR_fraction_8 in Dark Green), which is essentially a diluted version of the fifth fraction. No major bioactivity could be found in any other fraction.

1.2 SEA4US FRACTIONS (SSD)

Sea4Us hydrolat-fractions were then tested for their ability to produce bioactive hydrolats and to determine the optimal time of extraction using their method.

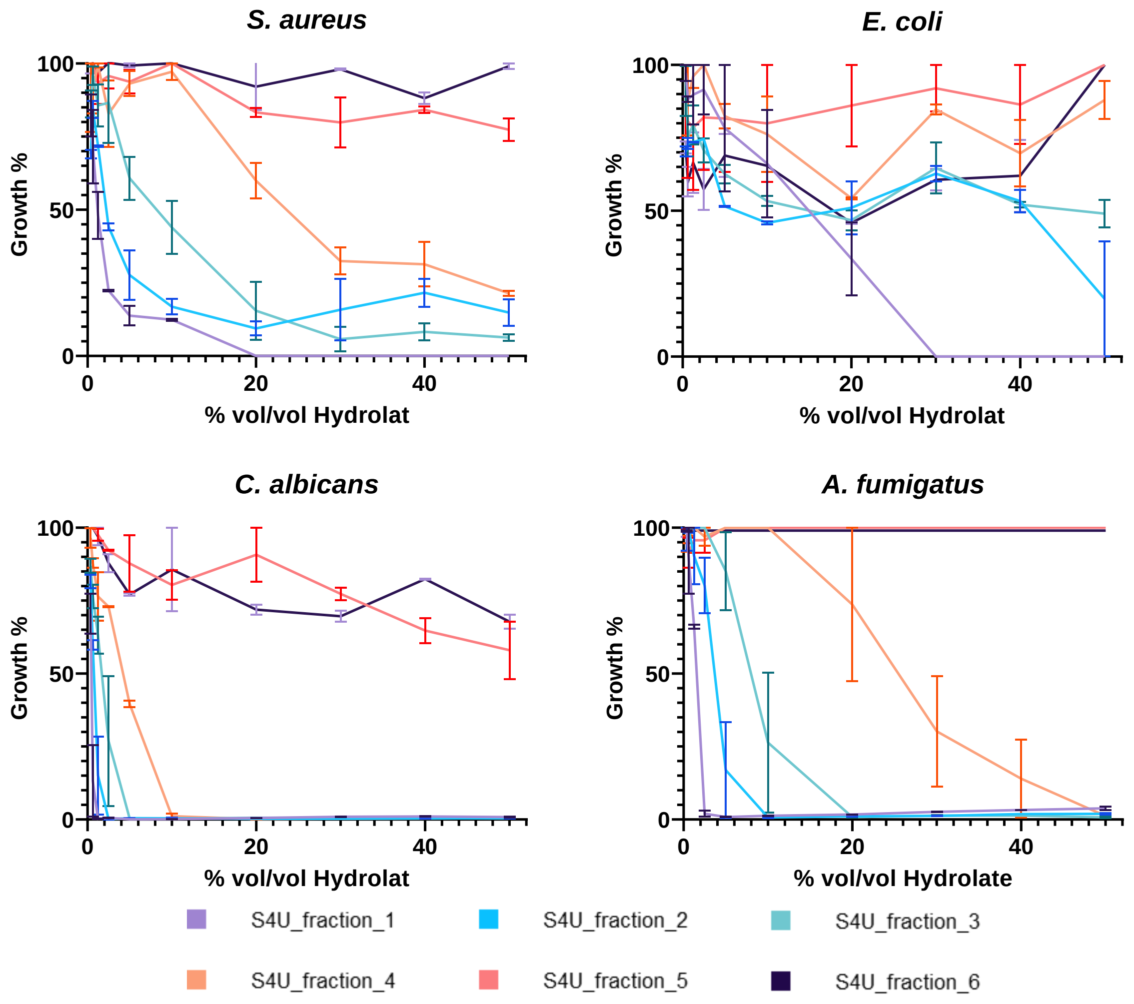


Figure 6 – Antimicrobial activities of hydrolat fractions produced by Sea4Us using the SSD method. The activity of each fraction was tested against the four microorganisms by microbroth dilution followed by a MTT assay. Growth was calculated relative to the control (without any hydrolat). No activity is detected for hydrolats extracted for more than 20 minutes (S4U_fraction_4 in orange). The mean and SEM of two replicates are shown.

The first hydrolat fraction (0-5 min) (S4U_fraction_1 in purple) was the most active one against the four tested microorganisms, with activity decreasing over time until no bioactivity was detected in fractions that were extracted for more than 20 minutes (Figure 6). Based on these results all following extractions were performed for 20 minutes. In this preliminary stage, it was also observed that the inhibitory activity of the hydrolat-fractions produced by SSD was greater against fungi than bacteria.

We know that both extraction methods (Pharmaplant and SSD) are steam-based. Unfortunately, Pharmaplant did not disclose how their hydrolats are made, so we cannot pinpoint why their hydrolat fractions only display such high activity at a specific timepoint (from 20 to 25 minutes). We can only speculate that Pharmaplant's hydrolat fractions result in the simultaneous extraction of compounds with antimicrobial activity against fungi and bacteria, whereas SSD favors the extraction of antifungal compounds only.

2 OPTIMIZATION OF OTHER EXTRACTION PARAMETERS

After determining the optimal extraction time, several parameters had to be evaluated to determine which had the greatest impact on the final product in terms of bioactivity, with the goal of streamlining production parameters. Specific details on the following hydrolats can be found in Table 2 (Materials and Methods, section 5)

2.1 SOURCE OF BIOMASS

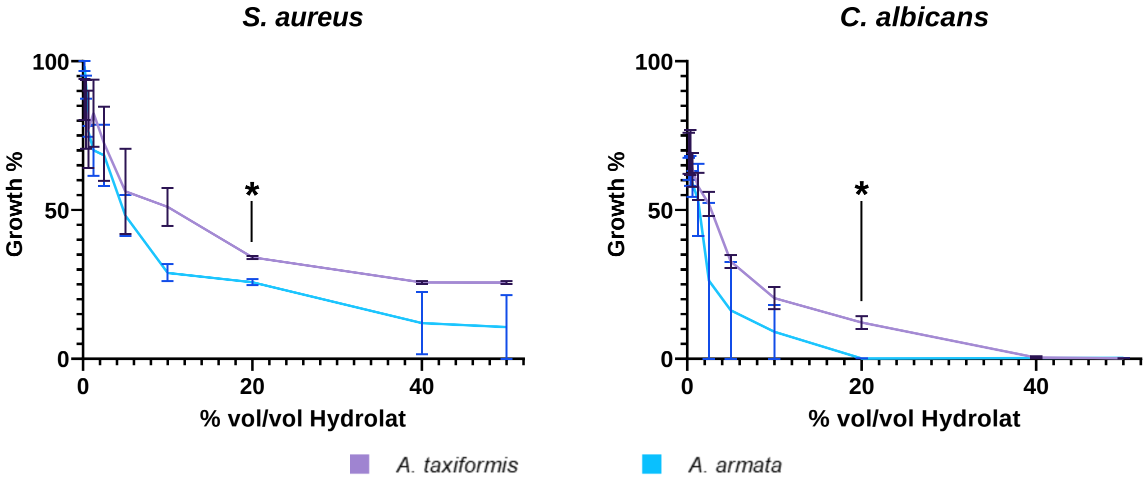


Figure 7 – The antimicrobial activities of hydrolats derived from two different biomass sources: *A. taxiformis* (purple) and *A. armata* (light blue); were tested by microbroth dilution assays followed by MTT against *S. aureus* and *C. albicans*. Growth was calculated relative to the control (without any hydrolat). The mean and SEM of four biological replicates are shown. * p-value≤0.05.

Several factors, such as the season in which the samples were collected, may influence the production of bioactive compounds by algae (35,41). Therefore, corresponding samples were collected at the same time and location and processed equally (One pair of samples used in this assay was subjected to different washing methods; however, since the comparison of the washing conditions revealed that it had no effect on bioactivity, these samples were included in the comparison of the two species). It was observed that hydrolats originating from *A. armata* tend to have better bioactivity against the tested microorganisms (Figure 7). At the concentration of 20% vol/vol, there was a statistically significant difference between *A. taxiformis* and, *A. armata* hydrolats for both tested microorganisms. Taking these results into consideration, *A. armata* was used to produce the subsequent hydrolats.

2.2 BIOMASS STORAGE

For a large-scale production, biomass storage is one of the most important factors to consider. From a logistical standpoint, dried biomass would be preferred over frozen biomass because it would take up far less space and avoid the need for large freezers to keep the frozen biomass inside. Therefore, both conditions were tested for the bioactivity of the resulting hydrolat.

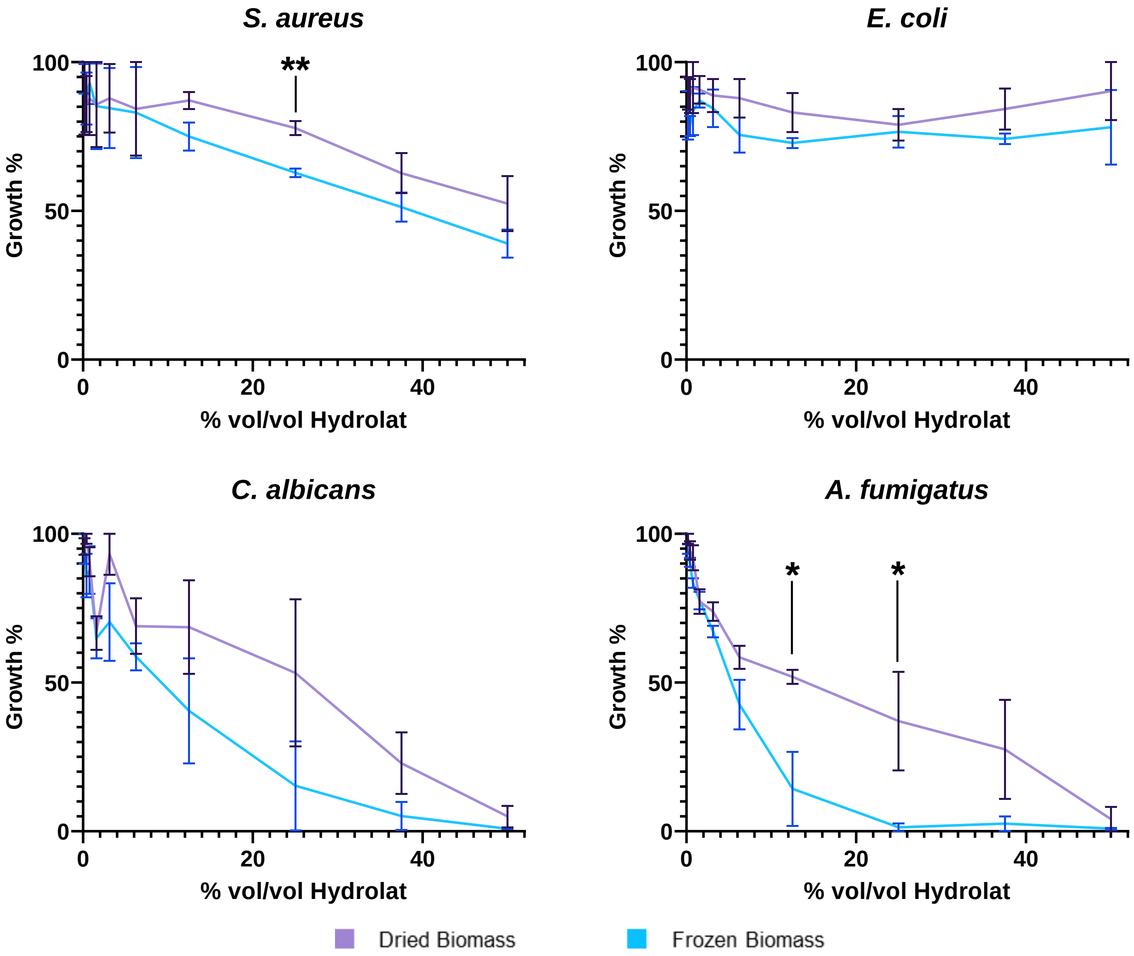


Figure 8 – Biomass storage effects on the bioactivity of hydrolats that were produced with dried biomass (purple) or frozen biomass (light blue). The activities of the hydrolats were tested against the four microorganisms by microbroth dilution assays followed by MTT. Growth was calculated relative to the control (without any hydrolat). The mean and SEM of three biological replicates are shown. * p-value≤0.05, ** p-value≤0.01.

MTT results revealed that the inhibitory activity of hydrolats derived from dried biomass are lower than that of hydrolats derived from frozen biomaterial (Figure 8). There was a statistically significant difference between dried biomass and frozen biomass hydrolats for *S. aureus* at a concentration of 20% v/v and *A. fumigatus* at a concentration of 20% and 12.5% v/v, indicating that frozen biomass is preferred for hydrolat production to maintain bioactivity. Since the error in these assays is relatively large, storage conditions should be tested once more to determine whether or not storage conditions affect bioactivity.

The difference in activity is more pronounced when tested against fungi than bacteria. One explanation for the diminished activity is, that the conglomeration of the biomass reduced the overall efficiency of the extraction of the bioactive compound(s). Even though it is unlikely, it can’t be ruled out that some compounds may be lost during the drying process of biomass due to their volatility. Additionally, the antifungal compounds appear to be distinct from the antibacterial compounds, since the loss of activity was not equally observed for these organisms.

2.3 BIOMASS WASHING

Preliminary tests in Sea4Us suggested that washing the biomass with fresh water (*ex-situ*) or salt water (*in-situ*) could change the composition of compounds in the sample, which could in turn change the hydrolats' efficiency at inhibiting microbial growth. Therefore, the different washing conditions were tested.

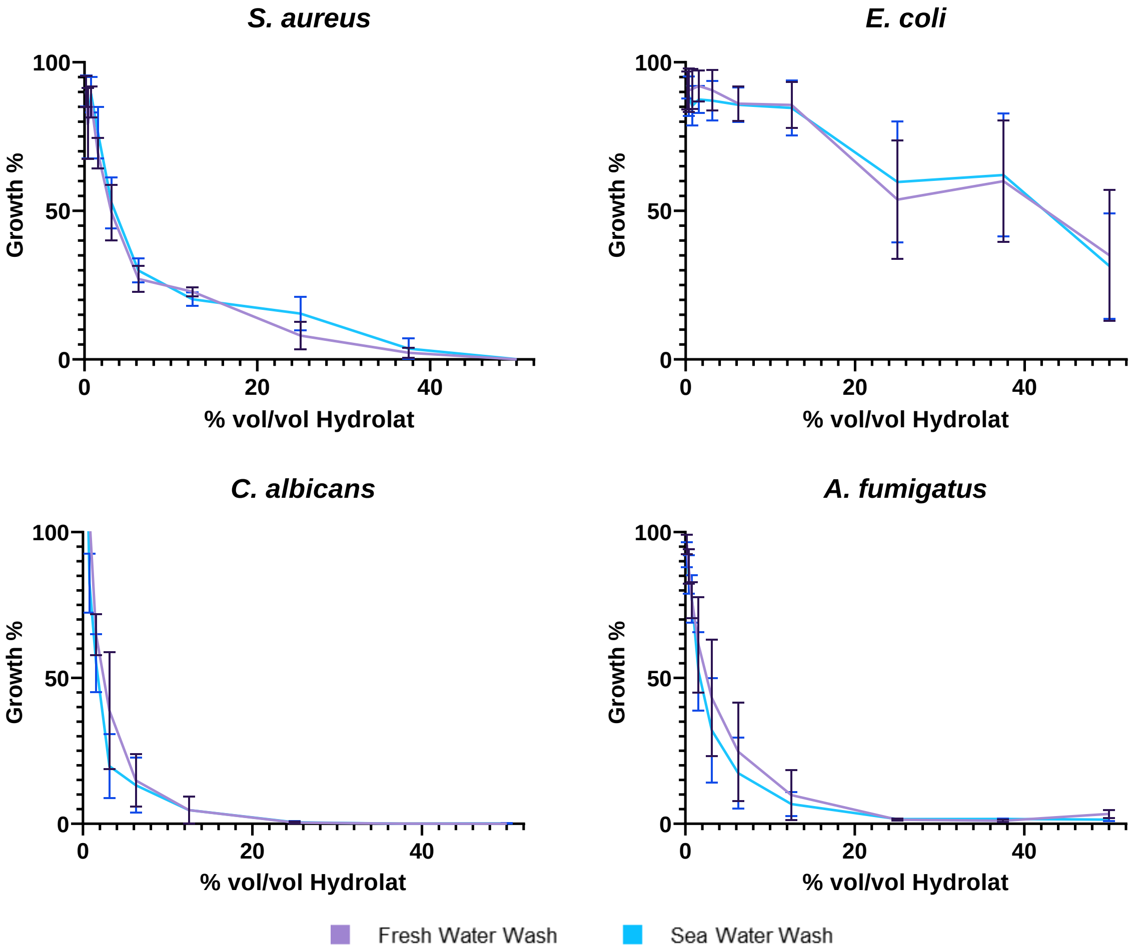


Figure 9 – Effects on the bioactivity of hydrolats that were produced from biomass washed with fresh water (purple) or sea water (light blue). The activities of the hydrolats were tested against the four microorganisms by microbroth dilution assays followed by MTT. Growth was calculated relative to the control (without any hydrolat). The mean and SEM of three biological replicates are shown.

No statistically significant differences were observed in these assays. Washing the biomass with either seawater or freshwater after collection had no effect on the hydrolat's inhibitory activity (Figure 9). This implies that the extraction of the bioactive compound(s) was unaffected, and no active compounds were washed out with either method or that, to some extent, both washing method impacted the biomass equally. Nevertheless, this is a necessary step to remove any impurities from the biomass after it is collected.

3 EVALUATION OF PRODUCTION METHODS & UPSCALING IMPACT ON THE HYDROLATS BIOACTIVITY

Specific details on the following hydrolats can be found in Table 3 (materials and methods, section 6).

3.1 SEA4US SSD UPSCALING

Based on the optimization of the extraction parameters, the SSD method was used to produce large amounts of hydrolats. Since assays regarding the biomass storage were not completely clear, hydrolats of both storage conditions were produced (frozen and dried). These hydrolats were made from the same biomass that was used to produce PHAR_fraction_5, as this biomass was known to give origin to hydrolats with high bioactivity levels.

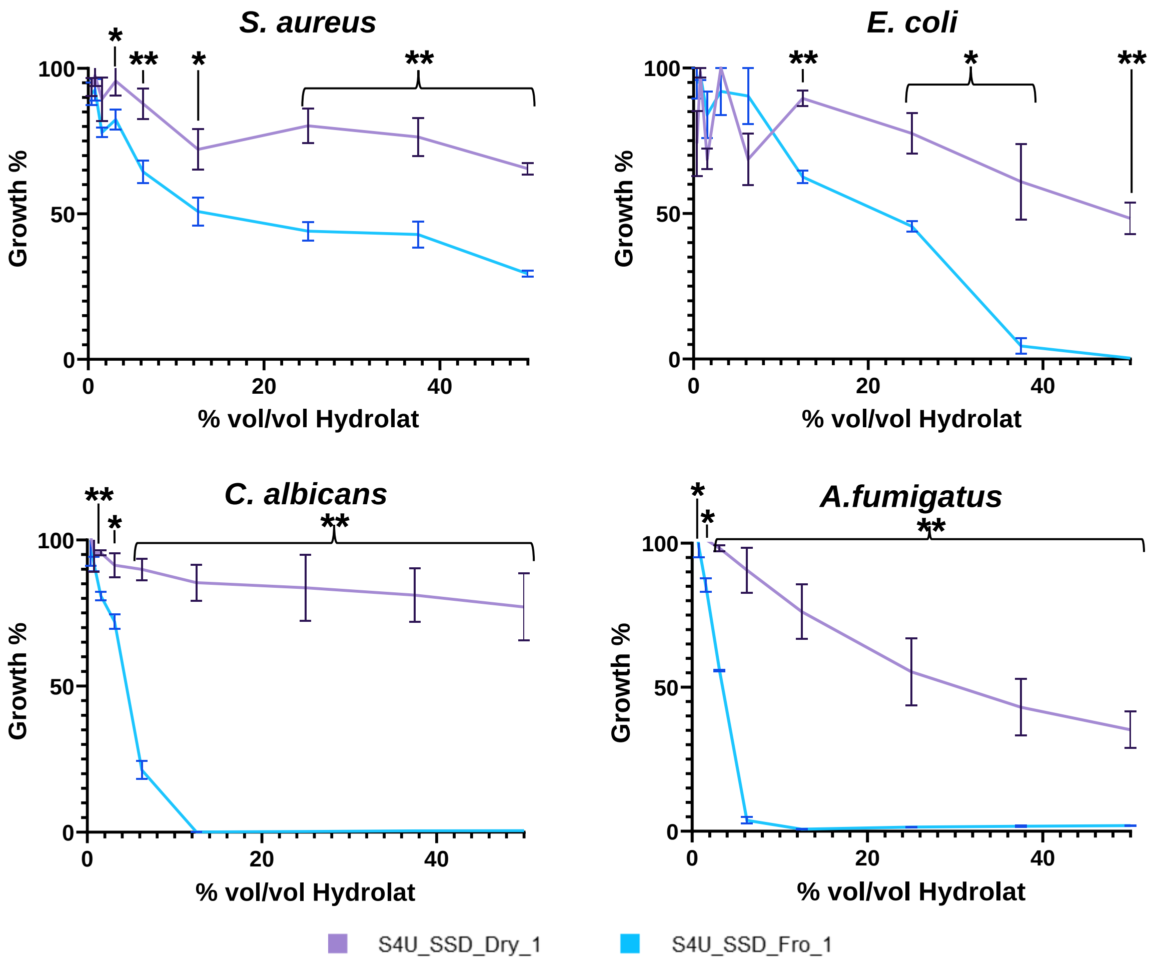


Figure 10 – Biomass storage effects on the bioactivity of hydrolats that were produced in large volumes with dried biomass (purple) or frozen biomass (light blue). The antimicrobial activities of the hydrolats were tested against the four microorganisms by microbroth dilution assays followed by MTT. Growth was calculated relative to the control (without any hydrolat). The mean and SEM of three biological replicates are shown. * p-value≤0.05, ** p-value≤0.01.

In accordance with previous MTT results (Figure 8), hydrolats derived from dried biomass showed lower activity than their frozen biomass counterparts (Figure 10). Both hydrolats showed statistically significant bioactivity differences compared to each other against all four organisms at several of the tested concentrations. Once again suggesting that aggregation of the biomass affected the extraction efficiency, particularly for antibacterial compounds.

3.2 RSD METHOD WITH FROZEN BIOMASS

The RSD method was attempted to produce a more concentrated hydrolat while also producing higher volumes of hydrolat per batch (100 mL). Two hydrolats were made with frozen biomass that were collected at the same place in the same season.

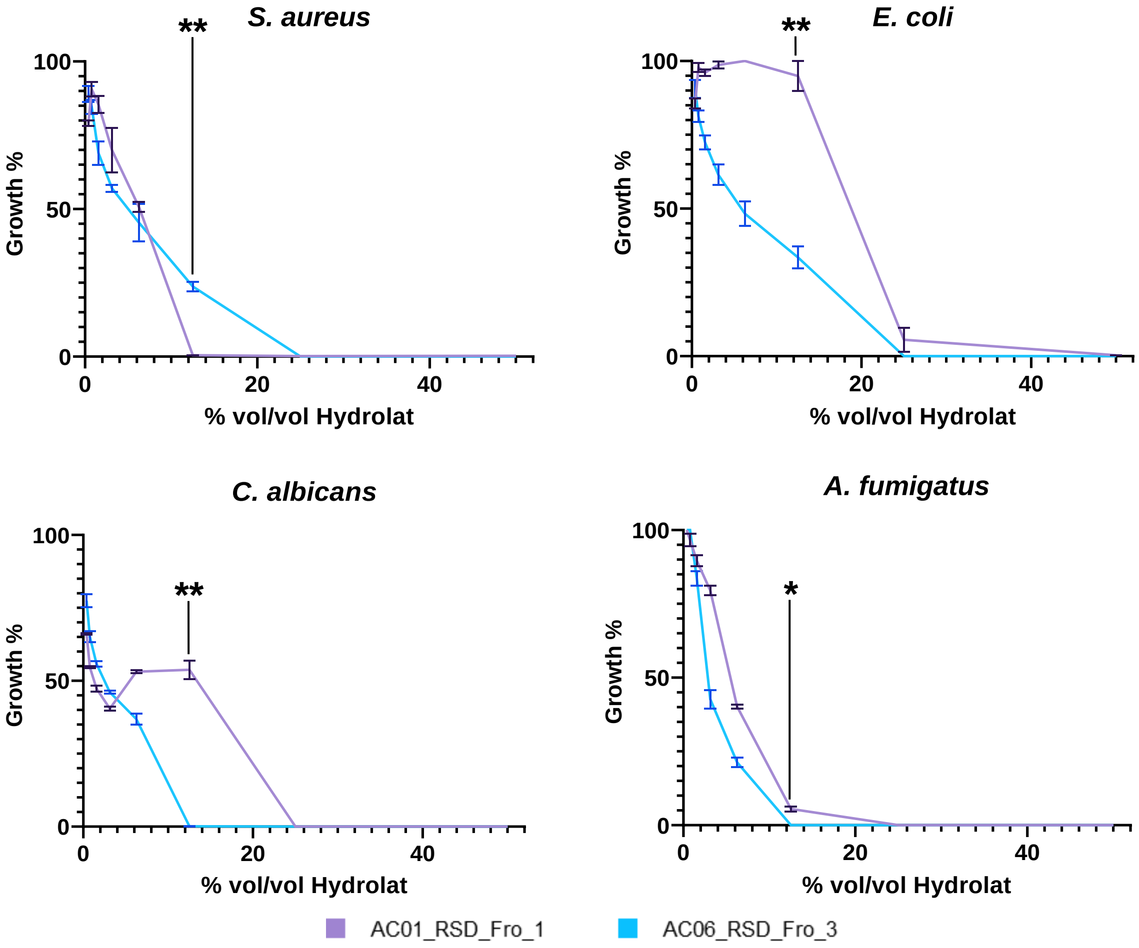


Figure 11 – Comparison, by microbroth dilution assays followed by MTT, of the antimicrobial activities of two hydrolats produced via RSD with frozen biomass (with the same collection point) against the four model organisms. Growth was calculated relative to the control (without any hydrolat). The mean and SEM of three biological replicates are shown. * p-value≤0.05, ** p-value≤0.01.

MTT results showed good inhibition against both fungi and bacteria (Figure 11), in contrast to the SSD method. These two hydrolats derived from the same biomass exhibited consistent activity (not showing statistically significant bioactivity differences) down to a concentration of 25% vol/vol for all organisms (which is the minimal concentration of hydrolat that completely inhibits microorganism growth), implying that this is a reliable method for producing hydrolats with consistent minimal inhibitory potentials. This method will be investigated further in the following paragraphs.

3.3 REPEATED BIOMASS DEFROSTING MAY LEAD TO LOSS OF ACTIVITY

The RSD extraction method was used to produce more hydrolats from the same biomass that was used to make PHAR_fraction_5, S4U_SSD_Dry_1, and S4U_SSD_Fro_1, to compare extraction methods. Unfortunately, no inhibition was observed for these hydrolats (data not shown).

The lack of activity could be explained by biomass deterioration during transportation (from Sagres to ITQB) and/or because the samples were relatively old (collected in May 2020), and their properties may have deteriorated after being frozen for long periods of time and defrosted multiple times, as these biomass samples had already been used several times for hydrolat production. This hints that when using biomass samples, they should not be defrosted frequently or stored indefinitely.

3.4 COMPARISON OF THE TWO EXTRACTION METHODS (SSD VERSUS RSD)

Since the results of the previous assay (section 3.3) were unsatisfactory, and we suspected that the biomass used to produce them was not in the best condition, we turned to the fresh biomass sample that was used to produce AC01_RSD_Fro_1 to produce all hydrolats displayed in this assay. This allowed for a valid comparison between extraction methods as well as testing whether dried biomass could be used in the RSD method, providing a full picture of this method's capabilities.

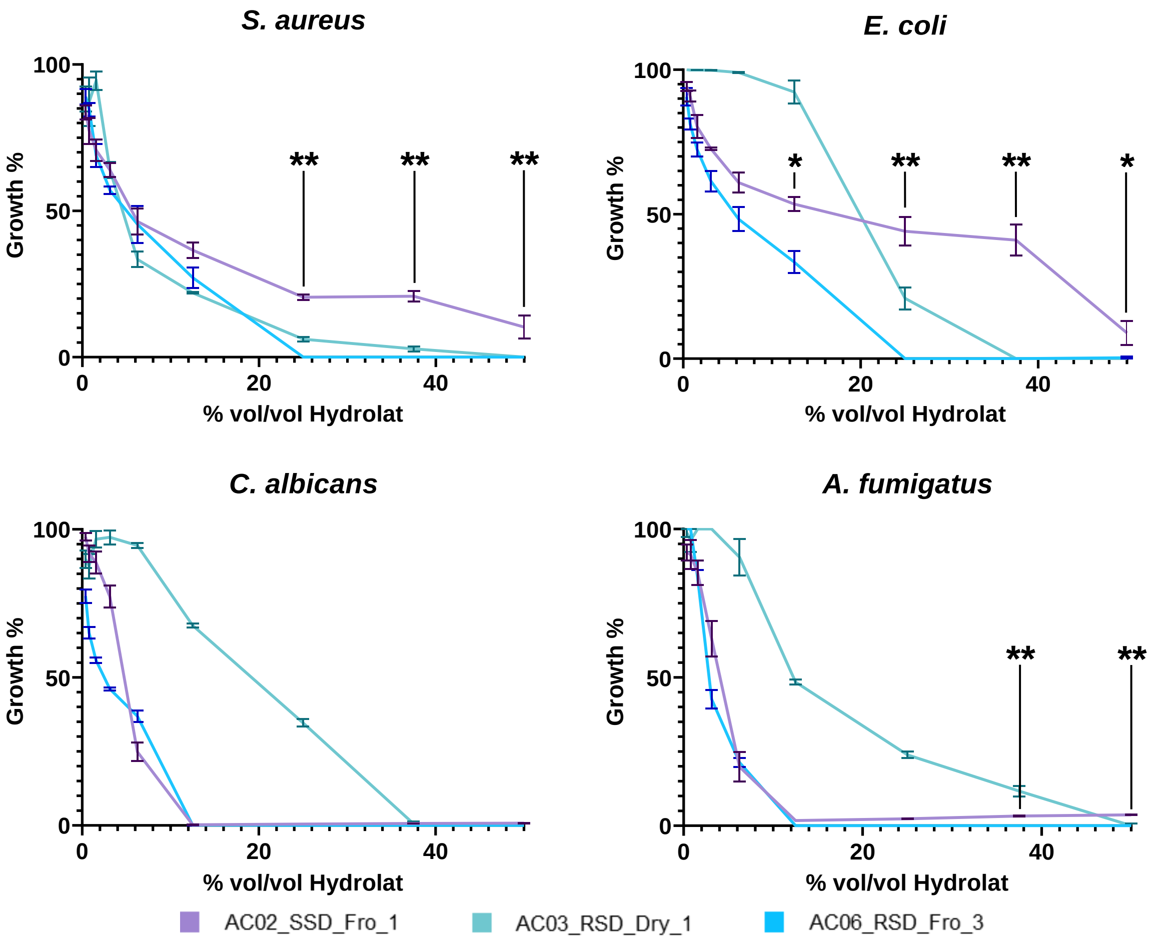


Figure 12 – Comparison by microbroth dilution assays followed by MTT, of the antimicrobial activities of three hydrolats produced via different extraction methods (SSD versus RSD) with either frozen or dried biomass against the four model organisms. Growth was calculated relative to the control (without any hydrolat). The mean and SEM of three biological replicates are shown. Statistical analysis shown between AC02_SSD_Fro_1 and AC06_RSD_Fro3 (* p-value≤0.05, ** p-value≤0.01).

It was observed that while the bioactivities against fungi are comparable for AC02_SSD_Fro_1 and AC06_RSD_Fro_3, the RSD hydrolat has considerably higher activities against bacteria than the SSD hydrolat, as the latter does not completely inhibit bacterial growth (Figure 12).

Regarding the use of dried biomass in RSD, despite the loss of some bioactivity, a part of it is still retained at higher concentrations of AC03_RSD_Dry_1 (Figure 12). This method outperforms the SSD method when dealing with dried biomass, which could not produce hydrolats that achieve full inhibitory activity even at the highest concentrations (Figure 10). If the use of dried biomass is required in the future due to storage logistics, this method is an excellent alternative for producing bioactive extracts. However, frozen biomass is preferred, as it produces more active hydrolats, mainly against fungi, as shown in figure 8. A few variations of the RSD method can still be tested to optimize the extraction for better activity results, such as humidifying the dried sample prior to extraction with agitation and/or sonication, or simply to test longer extraction times.

We think that the higher activity rates observed in RSD-produced hydrolats are due to the recirculation of steam and the pressure reached during extraction, which promotes a greater degree of steam diffusion through the biomass resulting in the simultaneous extraction of both antibacterial and antifungal compounds in an efficient way. In terms of bioactivity, the resulting hydrolats are more similar to those produced by Pharmaplant. Additionally, the closed system of the RSD method ensures a constant amount of water and therefore of the final hydrolat, whereas the SSD is subject to fluctuations of the final hydrolat amount, influencing its concentration.

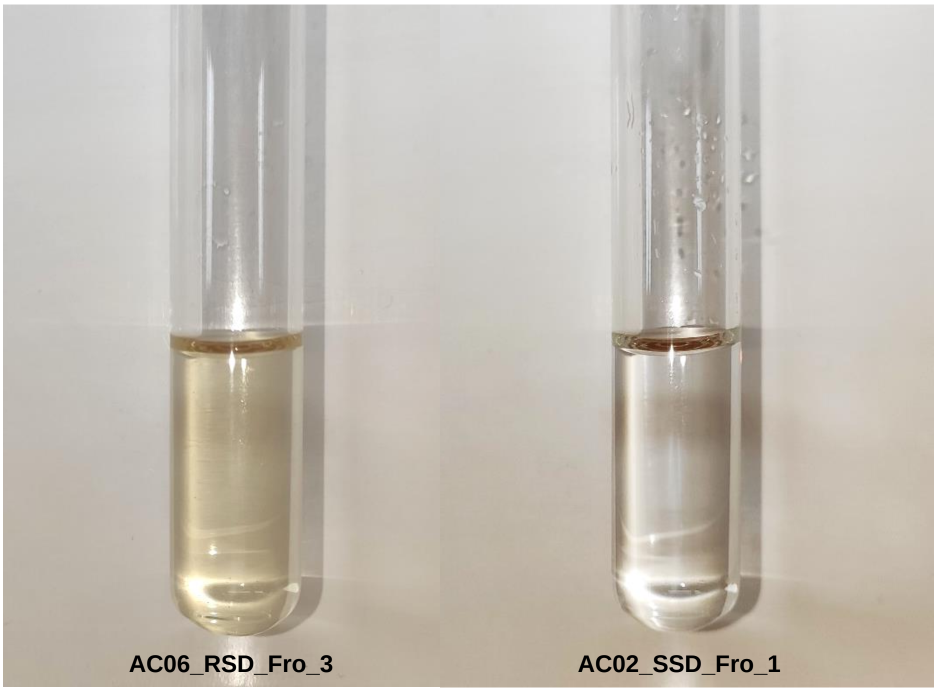


Figure 13 – Comparison between hydrolats produced by RSD (left) and SSD (right).

Unlike the transparent SSD-produced hydrolats, RSD-produced hydrolats exhibited a slight yellow tint (Figure 13). This could suggest the presence of aromatics in RSD-produced hydrolats or that they might be more concentrated.

4 CFU VIABILITY ASSAYS ON BACTERIA AND *C. ALBICANS*

4.1 BACTERIA

The purpose of these assays was to validate the MTT results and compare the effectiveness of both extraction methods against the tested microorganisms, determining whether the hydrolats have the ability to kill both model bacteria. The concentrations tested for each hydrolat were selected based on OD_{600} values following the criteria described on Material and Methods, section 3.2.1.

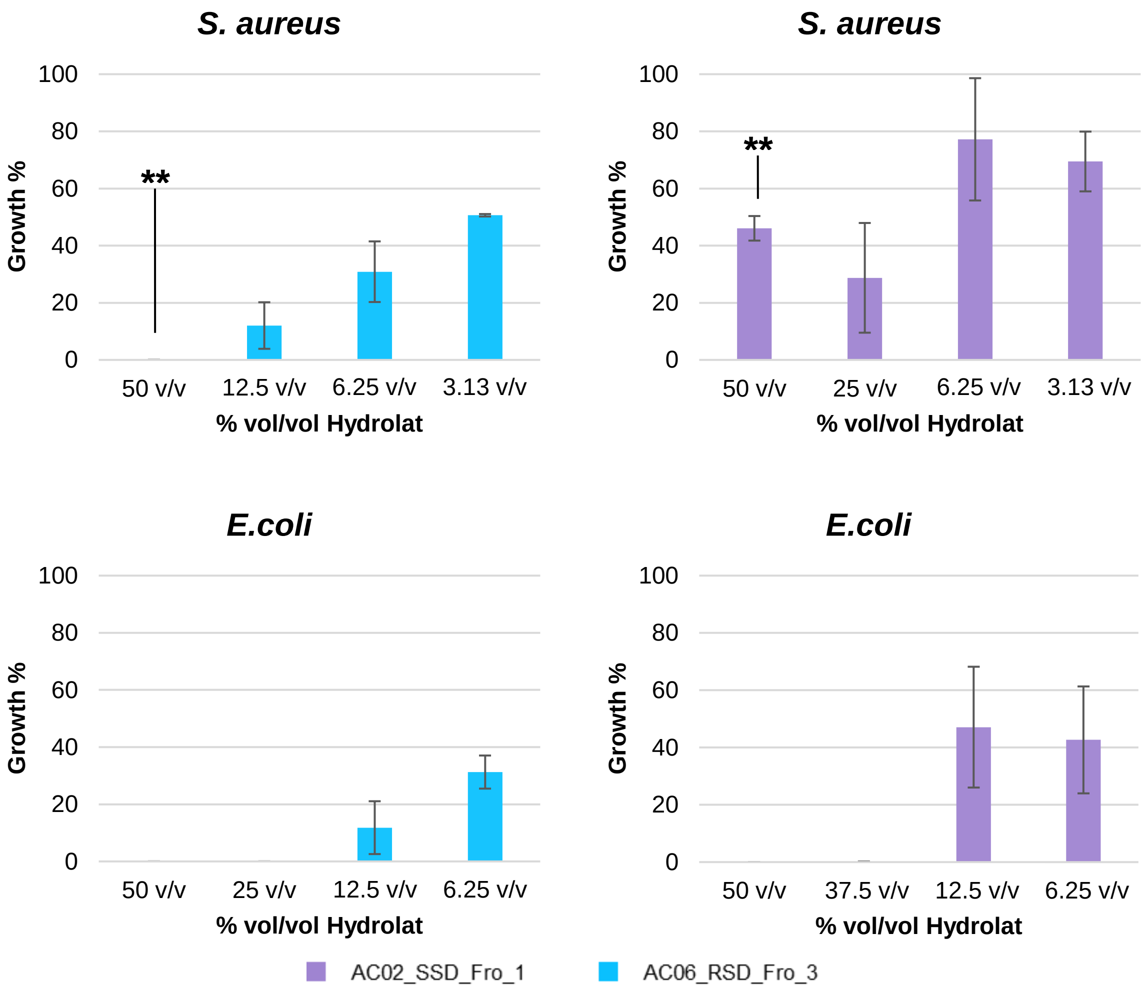


Figure 14 – Comparison, by microbroth dilution followed by CFU assays, of hydrolats coming from different extraction methods (SSD versus RSD), resulting from 3 biological replicates of *E. coli* and *S. aureus*. Growth was calculated relative to the control (without any hydrolat). The mean and SEM are shown for each microorganism. ** p-value≤0.01.

No statistical differences were found when comparing both hydrolats at the same concentrations except for *S. aureus* at the concentration of 50 % v/v.

AC06_RSD_Fro_3 reduced the viability of both *S. aureus* and *E. coli* when the bacteria were grown in the presence of 25% v/v of hydrolat or more (Figure 14). For the rest of the concentrations tested, the results obtained are consistent with the MTT results (Figure 12), meaning that the metabolic activity is in line with the number of viable cells observed.

Regarding AC02_SSD_Fro_1 there is a killing effect against *E. coli* when the bacteria were grown in the presence of 37.5% v/v of hydrolat or more (Figure 14). Additionally, its effectiveness against *S. aureus* is lower compared to AC06_RSD_Fro_3. The results obtained here at 50% v/v are notably higher than MTT results (Figure 12) (around 10% growth relative to the control), indicating that *S. aureus* cells might be metabolically impaired, despite of being 50% viable.

4.2 C. ALBICANS

To facilitate this assay and allow for the counting of colonies, *C. albicans* had to be grown in its yeast form. This assay also allowed us to see how this highly adaptable microorganism grew in the presence of the two different hydrolats in its yeast form. The concentrations tested for each hydrolat were selected based on OD_{600} values following the criteria described on the methodology section (Methods **3.2.1**).

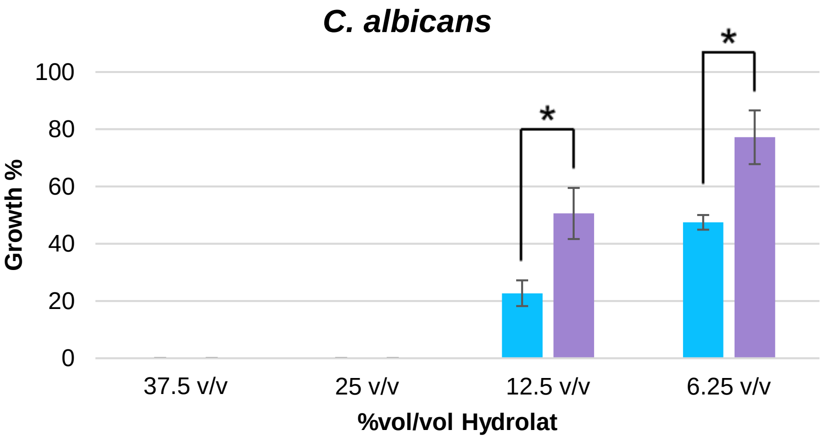


Figure 15 – Comparison, by microbroth dilution followed by CFU assays, of hydrolats coming from different extraction methods (SSD versus RSD), resulting from 3 biological replicates of *C. albicans*. Growth was calculated relative to the control (without any hydrolat). The mean and SEM are shown. * p-value≤0.05.

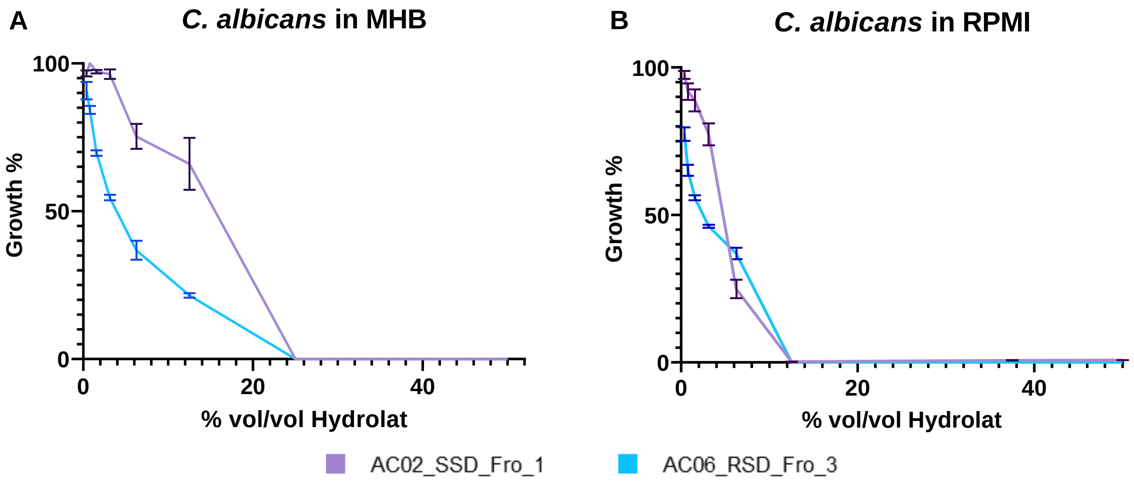


Figure 16 – (A) Comparison by microbroth dilution assays followed by MTT, of the antimicrobial activities of two hydrolats produced via different extraction methods (SSD versus RSD) with frozen biomass against *C. albicans*. Growth was calculated relative to the control (without any hydrolat). The mean and SEM of three biological replicates are shown. (B) Depicts the same assay but *C. albicans* was grown in RPMI.

In contrast of *C. albicans* grown in RPMI (inducing filamentation) where both hydrolats had comparable activities (Figure 16-B), lower inhibitory activities were observed against *C. albicans* when grown in MHB (as a yeast) in the presence of AC02_SSD_Fro_1 at concentrations below 25% v/v (Figure 16-A).

Despite that, hydrolats produced by both methods (SSD and RSD) seem to have an impact in the viability *C. albicans* when the fungus was grown in the presence of 25% v/v of hydrolat or more (Figure 15). These results are in line with values obtained by the MTT assays for *C. albicans* when grown in MHB. (Figure 16-A)

5 VIABILITY ASSAYS ON *A. FUMIGATUS*

The purpose of this assay was to validate the data obtained with the MTT assays and compare the effectiveness of both extraction methods (SSD versus RSD) against *A. fumigatus*, determining whether the hydrolats have the ability to kill the fungal spores or just delay their growth.

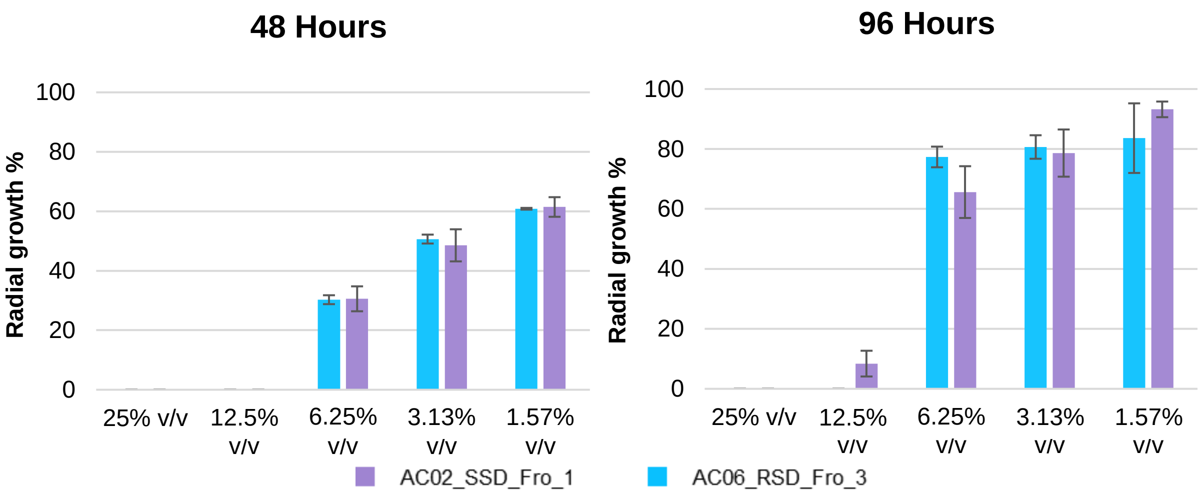


Figure 17 - *A. fumigatus* spores' radial growth relative to the control (without any hydrolat) after 48 and 96 hours in media supplemented with AC02_SSD_Fro_1 (purple) or AC06_RSD_Fro_3 (light blue). The mean and SEM of 3 biological replicates is shown.

The results (Figure 17) show the same trend observed before in the MTT assay (Figure 12): the growth inhibiting effect of the hydrolats is concentration dependent, and from 12.5% v/v upwards there was no growth observed (Figure 18-A) after 48h. The hydrolats appears to have a killing effect on the fungal spores for concentrations higher that 12.5% v/v, as no signs of fungal growth were detected even after 96 hours at higher concentrations.

There were no statistically significant differences between the two hydrolats; however, the fact that there was some slight growth in the plates supplemented with 12.5% v/v AC02_SSD_Fro_1 cannot be overlooked, since some spores were not completely killed by this concentration of hydrolat in the growth medium in one of the biological triplicates as seen in Figure 18-B.

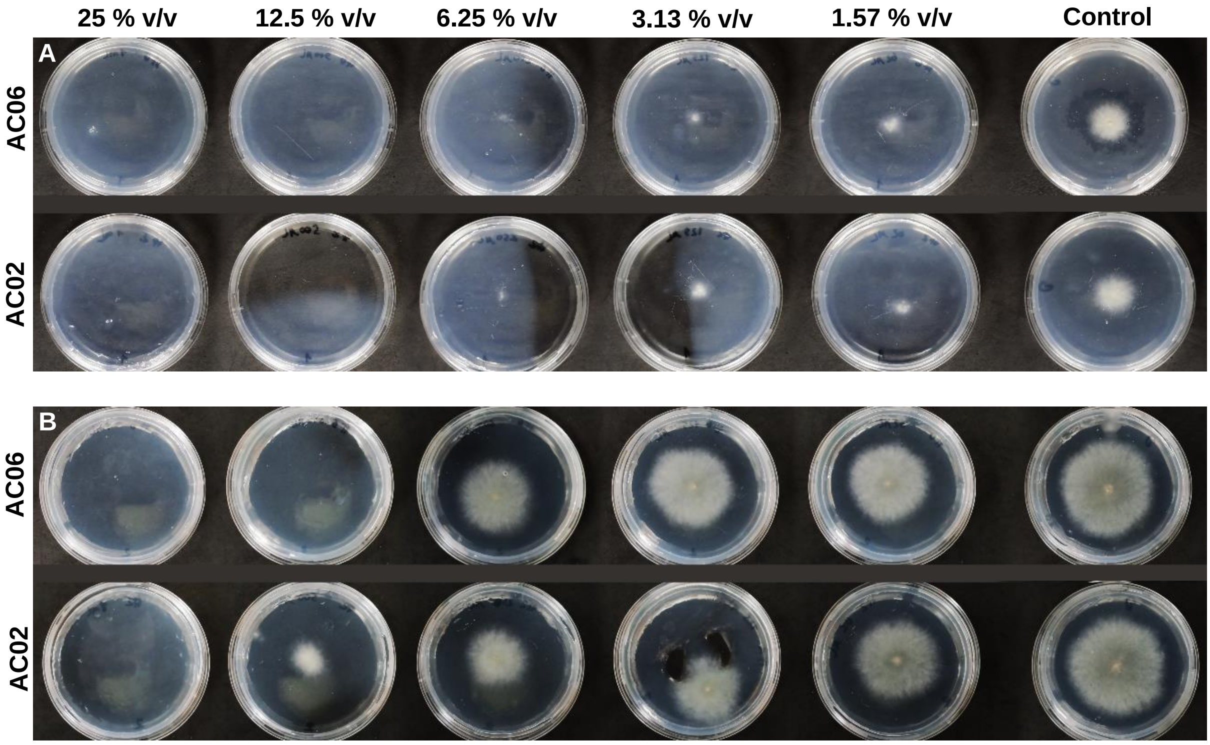


Figure 18 – *A. fumigatus* radial growth after 48 (A) and 96 (B) hours in contact with hydrolats AC06 and AC02 at concentrations ranging from 1.57%-25% v/v. Control growth, in the absence of hydrolat, is also shown for comparison

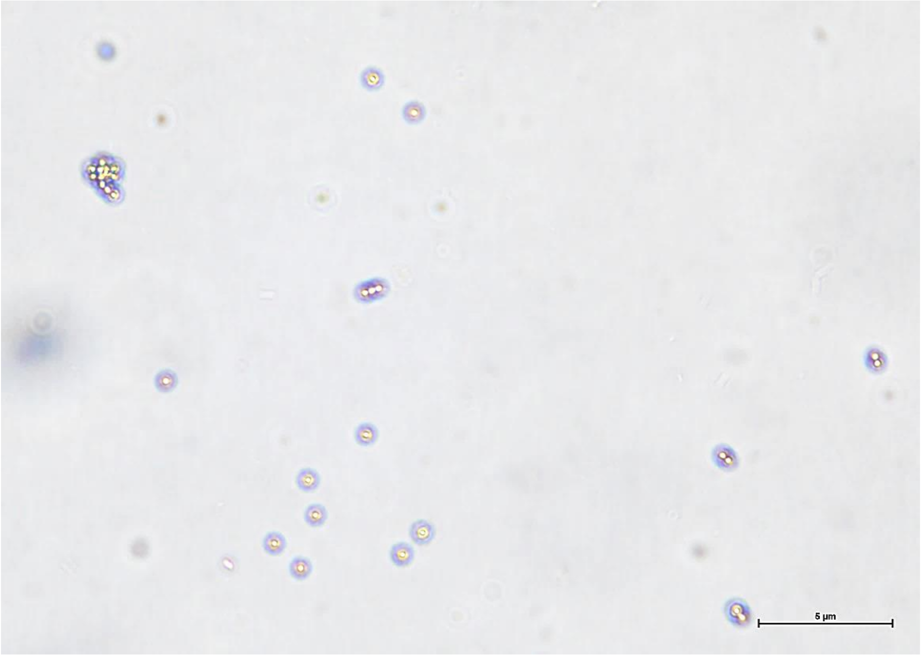


Figure 19 – *A. fumigatus* spores grown on a plate supplemented with 12.5% v/v of AC06_RSD_Fro_3 after 96 hours (20x magnification).

After 96 hours of incubation with 12.5 % v/v of AC06_RSD_Fro_3, observed spores (Figure 19) show no signs of germination (contrarily to what was observed for the control where hyphae growth was observed – data not shown), highlighting the killing potential of this hydrolat at this concentration.

6 LYOPHILIZATION OF HYDROLATS

Hydrolat samples were lyophilized, weighed, and resuspended in methanol to see if it was possible to normalize and moreover to determine a MIC. Since we didn´t know the concentration of the hydrolat, weighing and resuspending in methanol would give us the possibility not to work with % v/v but with µg/mL or similar. Because the weight of the resulting products after lyophilization was extremely low, we decided to resuspend it in a volume ten times smaller than the original volume that was lyophilized (10 mL). These resuspensions were then tested via microbroth dilution assays followed by MTT against *S. aureus* and *C. albicans* at concentrations equal to the original hydrolat to check if they remained active.

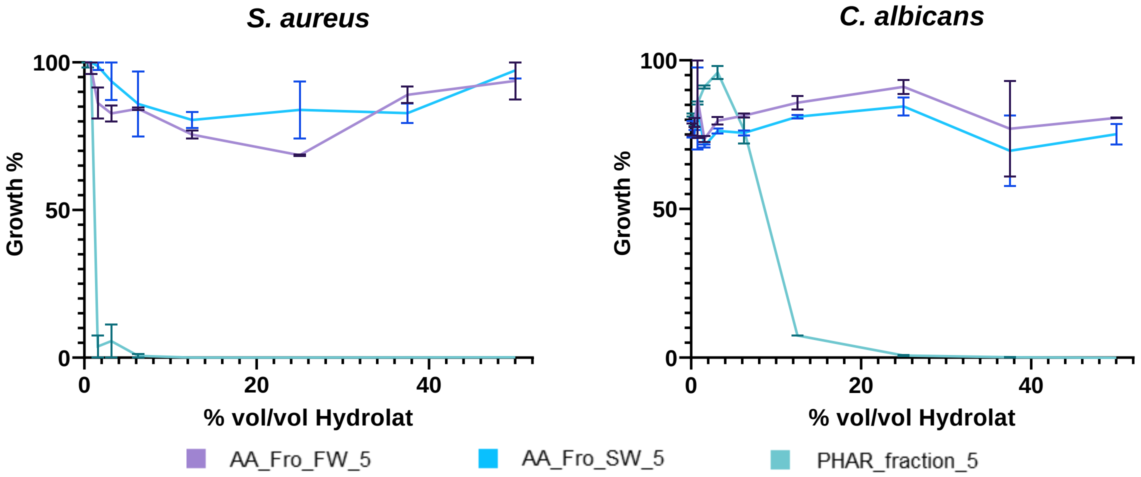


Figure 20 – The antimicrobial activities of resuspended methanolic extracts of each hydrolat were compared using microbroth dilution assays followed by MTT in the same concentration as the untreated hydrolat. Growth was calculated relative to the control (without any hydrolat). PHAR_fraction_5 (green) kept the same levels of activity before the treatment. The mean and SEM of three biological replicates are shown for each microorganism.

Hydrolats produced via Pharmaplant’s extraction method retain similar levels of bioactivity after being lyophilized and resuspended in methanol (Figure 20). The same cannot be said for the SSD-produced hydrolats shown in Figure 20. Unfortunately, this issue also persists with RSD-produced hydrolats as they cannot to be lyophilized and resuspended in the same concentration as untreated hydrolats without losing activity (data not shown).

Since these hydrolats originated from different extraction methods and have different activities after being subjected to lyophilization, it could be speculated that the Pharmaplant method extracts different compounds than SSD. However, in future assays, the lyophilized product will be resuspended with heat and/or sonication and other solvents might be tested, since this lack of activity could be explained by crystallization reactions with subsequent loss of bioavailability.

7 LIQUID-LIQUID EXTRACTION ASSAYS

This method was chosen as an attempt to concentrate and fractionate the samples to determine whether there was differential activity in the various fractions resulting from a liquid-liquid extraction with diethyl ether. Which could indicate whether the compound(s) were water soluble or organic solvent soluble.

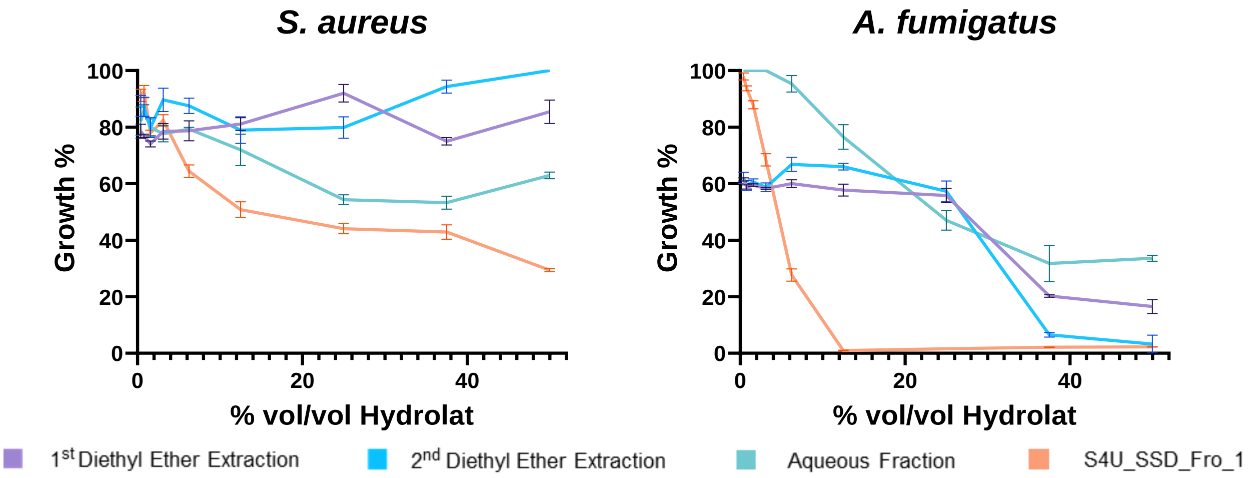


Figure 21 – Antimicrobial activity of fractions resulting from the liquid-liquid extraction with diethyl ether of S4U_SSD_Fro_1, compared using microbroth dilution assays followed by MTT. Growth was calculated relative to the control (without any hydrolat). The mean and SEM of three biological replicates are shown for each microorganism.

A faint yellow tint was visible in the diethyl ether fractions and some degree of activity was maintained in those fractions compared to the untreated hydrolat (S4U_SSD_Fro_1 in orange) (Figure 21). The solvent fractions appear to be more active against fungi, while the aqueous fraction appears to be more active against bacteria, further suggesting that the compounds responsible for antifungal activity might not be the same as the ones responsible for antibacterial activity. It is also worth noting that the solvent fractions were not pooled together after extraction, so the activity present on both solvent fractions could be additive if the fractions were pooled together. Besides that, both fractions exhibit similar levels of activity, indicating that both had the active compound(s) in its composition; additional extraction steps may be required in the future.

8 NUCLEAR MAGNETIC RESONANCE (NMR) ANALYSES

8.1.1 BROMOFORM PRESENCE IN THE HYDROLATS

A. armata is known for containing large amounts of bromoform (43). Despite their antimicrobial activity, some HVOCs are not suitable pharmacological candidates. Bromoform, classified as a probable human carcinogen, can be a harmful compound if ingested in large amounts (50,51). As a result, any final product containing a mixture of extracted compounds must be carefully monitored to ensure that Bromoform are either absent or present in safe vestigial quantities.

The presence of bromoform was detected by NMR in a variety of hydrolats at concentrations ranging from 0.13 to 0.72 mM. There were also hydrolats where no bromoform was detected.

8.1.2 EVALUATION OF EXTANT BROMOFORM CONTRIBUTION TO BIOACTIVITY OF THE HYDROLATS

To understand if bromoform was responsible for microbial growth inhibition, its activity has been tested at concentrations equimolar to those determined by NMR in the hydrolat samples via microbroth dilution assays followed by MTT (Material and methods, Section 3.1.2).

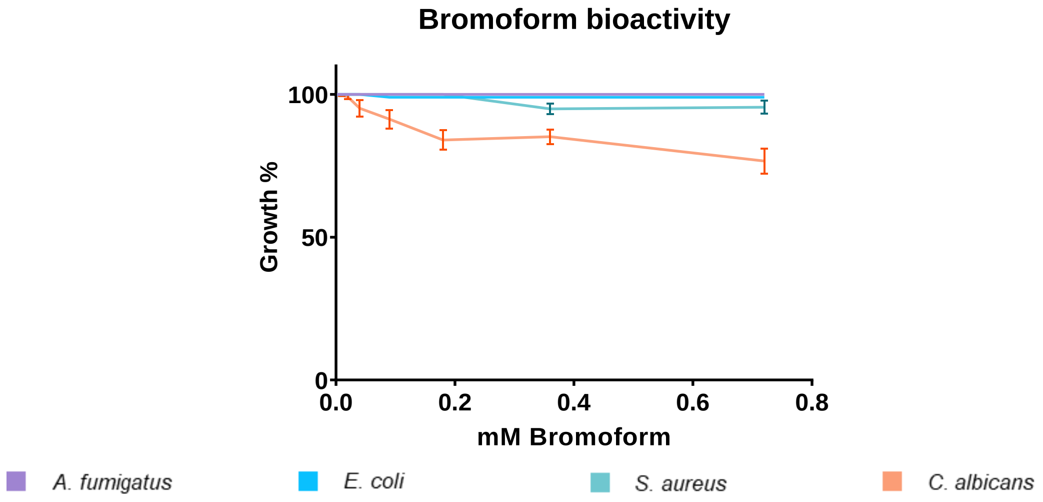


Figure 22 – Bromoform inhibition activity against the four model organisms tested via microbroth dilution assays followed by MTT. Growth was calculated relative to the control (without bromoform). The mean and SEM of three biological replicates are shown for each microorganism.

Minor inhibitory activity was detected against *C. albicans* and *S. aureus*, but not to the level of the corresponding hydrolat's inhibitory efficacy (Figure 22). Results imply that the amount of bromoform present in the sample is not solely responsible for the antimicrobial activity detected in the hydrolat samples, and that the concentration of bromoform in a hydrolat sample is not directly related to its antimicrobial activity.

If the presence of these already low concentrations of bromoform becomes an issue in the future, the RSD method appears to be a reliable way to reduce bromoform presence in hydrolats to a bare minimum. This technique produced hydrolats with no or vestigial levels of bromoform detected in their NMR spectra, values so low that they could not be quantified by this method.

8.2 NMR ANALYSIS OF THE HYDROLATS; RSD METHOD CONSISTENCY

AC01_RSD_Fro_1 and AC06_RSD_Fro_3 were analyzed by NMR and their spectra were compared to identify the main chemical groups present in both samples and ensure that the RSD method was consistent in producing hydrolats with comparable chemical structures in their composition.

These hydrolats were produced in the same manner with biomass with the same collection period and location.

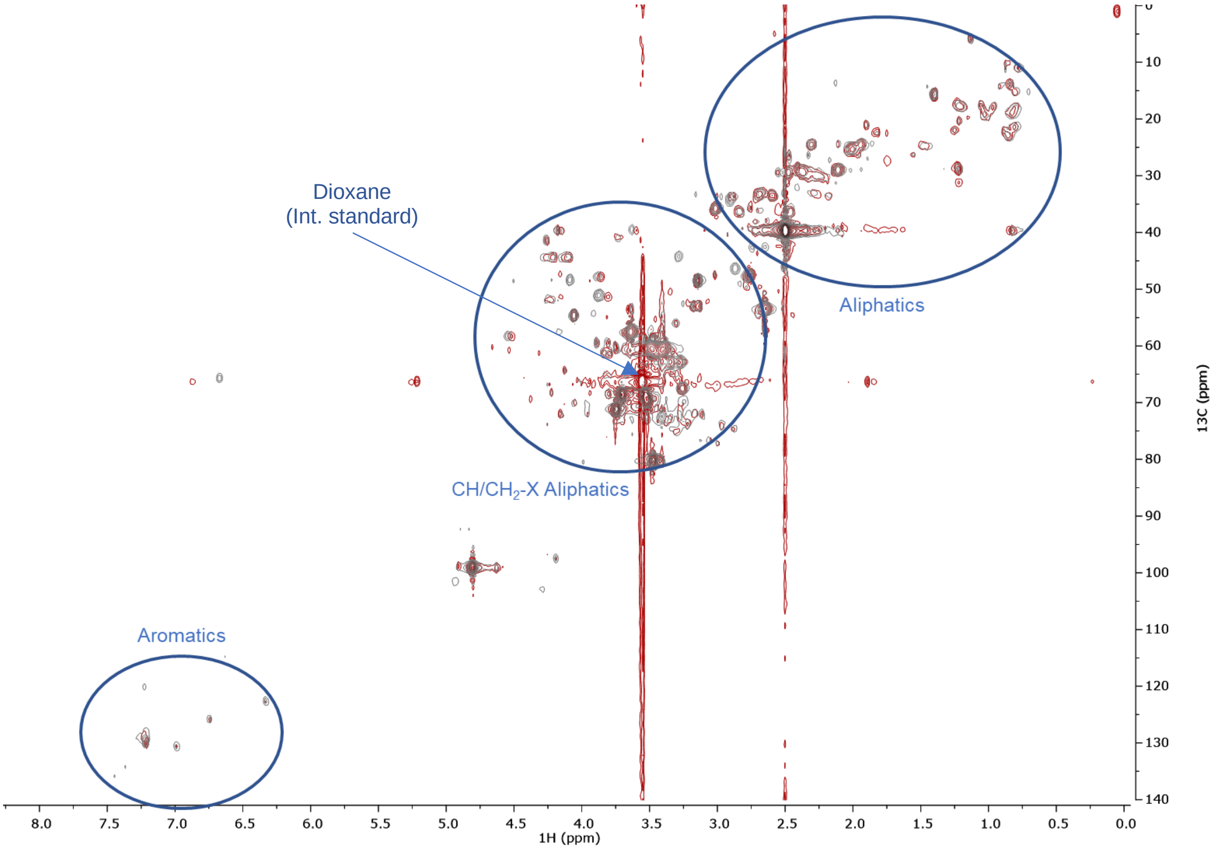


Figure 23 – Comparison of HSQC spectra AC06_RSD_Fro_3 (Red) and AC01_RSD_Fro_1 (Gray).

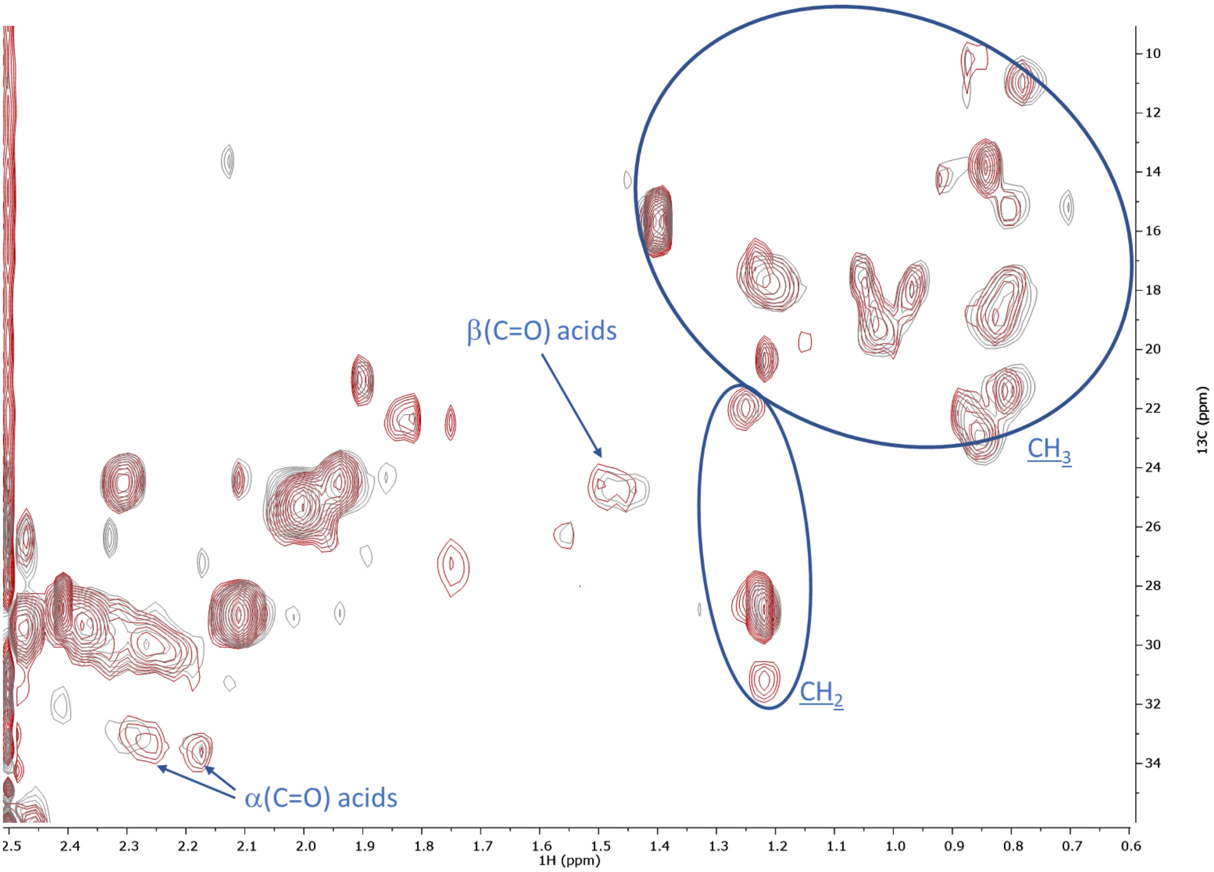


Figure 24 – Detail on the Aliphatic region of the comparison of HSQC spectra AC06_RSD_Fro_3 (Red) and AC01_RSD_Fro_1 (Gray).

The analyzed hydrolats displayed very similar spectra (Figure 23), indicating that the reactor extraction method is reproducible. Based on the presence of the signals assigned to $\underline{CH_2}$ and $\underline{CH_3}$ aliphatics and to the α and β carbonyl groups related to acids (Figure 24), the obtained HSQC spectra suggest that the main compounds present in these hydrolats are FAs, which corresponds to GC-MS data (Table 6). Some signals were also detected in the Aromatic region although no specific compounds could be identified. No specific class of compounds could be identified in the CH/CH2-X Aliphatic region either.

8.3 NMR ANALYSIS OF THE HYDROLATS COMING FROM DIFFERENT EXTRACTION METHODS

Spectra from the Hydrolats S4U_SSD_Fro_1 and S4U_SSD_Dry_1 were compared as well but had very faint signals and are thus not shown here. This section focuses on the comparison and identification of major chemical groups present on the hydrolats with the highest inhibition rates, AC06_RSD_Fro_3 and PHAR_fraction_5.

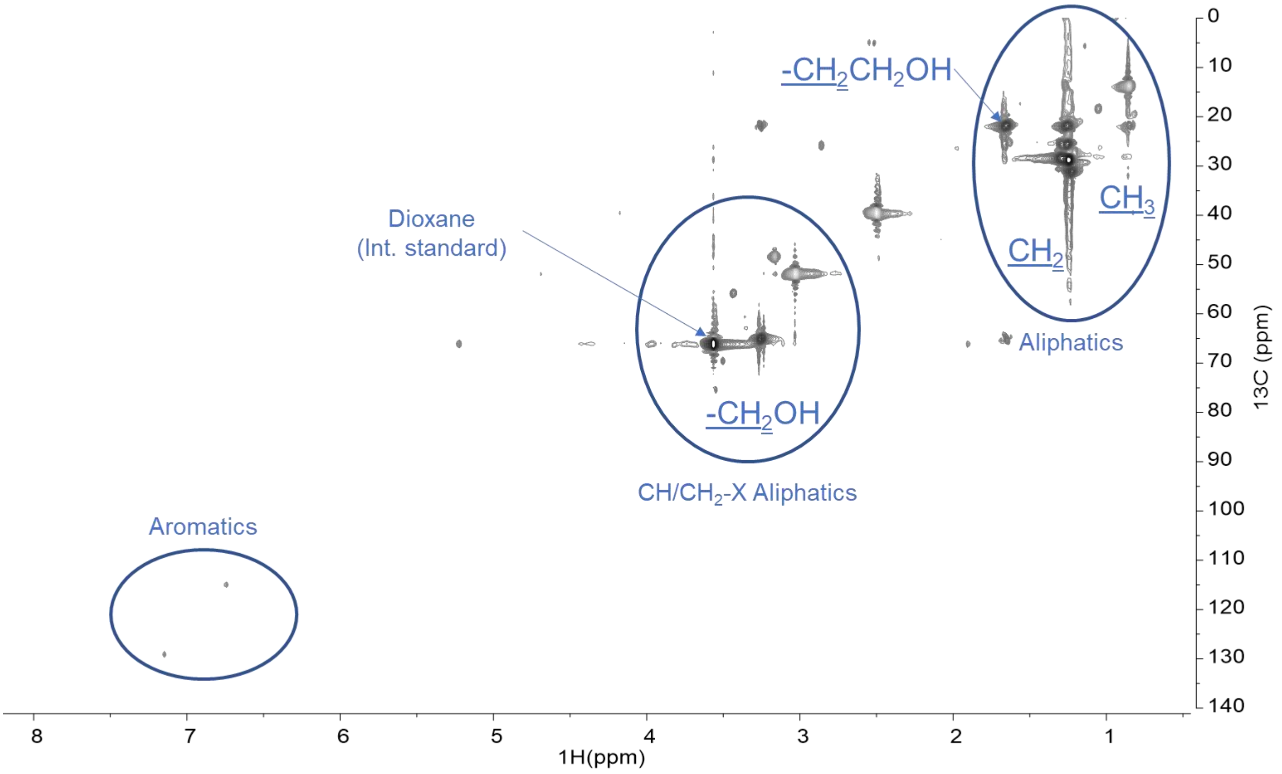


Figure 25 – HSQC spectra of PHAR_fraction_5.

NMR was used to identify the main chemical groups present in Pharmaplant's most active hydrolat (PHAR_Fraction_5) and to compare it to AC06_RSD_Fro_3 (most active RSD hydrolat). Based on the presence of the signals assigned to $\underline{CH_2}$ and $\underline{CH_3}$ aliphatics, and to -$\underline{CH_2}$OH and -$\underline{CH_2}CH_2$OH groups, the obtained HSQC spectrum (Figure 25) suggests that the main compounds present in PHAR_fraction_5 are fatty alcohols. This hydrolat appears to be less chemically diverse than AC06_RSD_Fro_3 shown in Figure 23.

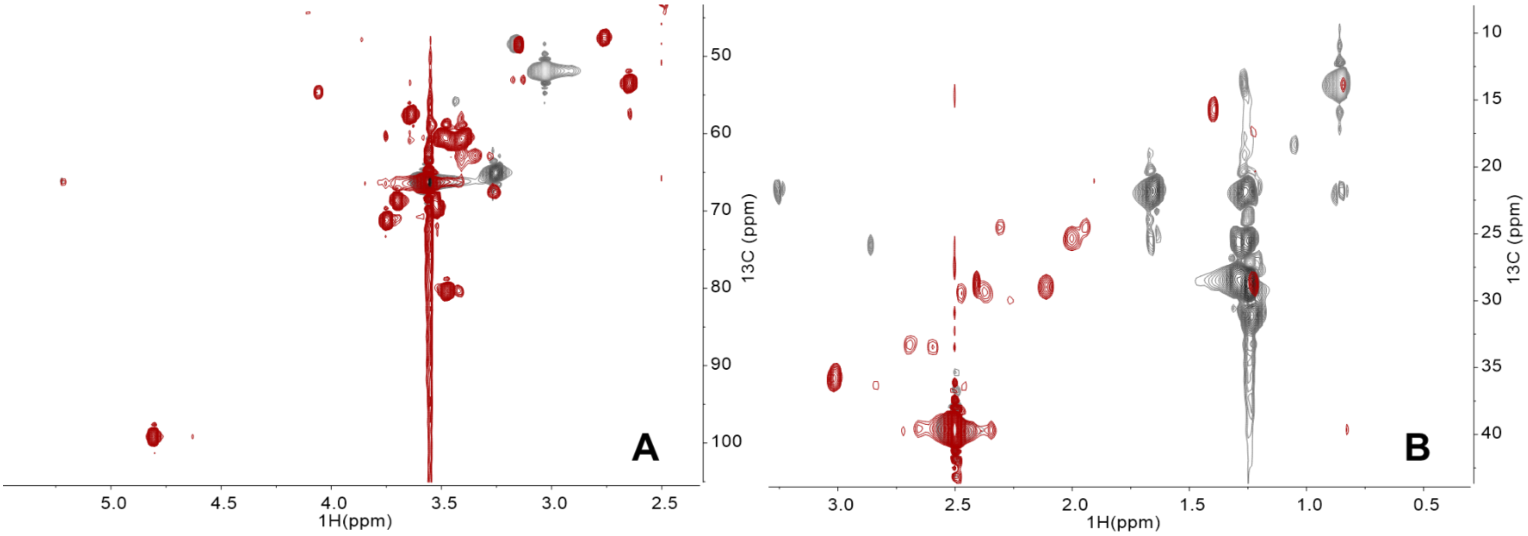


Figure 26 - Comparison of the HSQC spectra of AC06_RSD_Fro_3 in red, and PHAR_fraction_5 in gray. A) Highlight of the CH/CH2-X aliphatics region. B) Highlight of the aliphatic region.

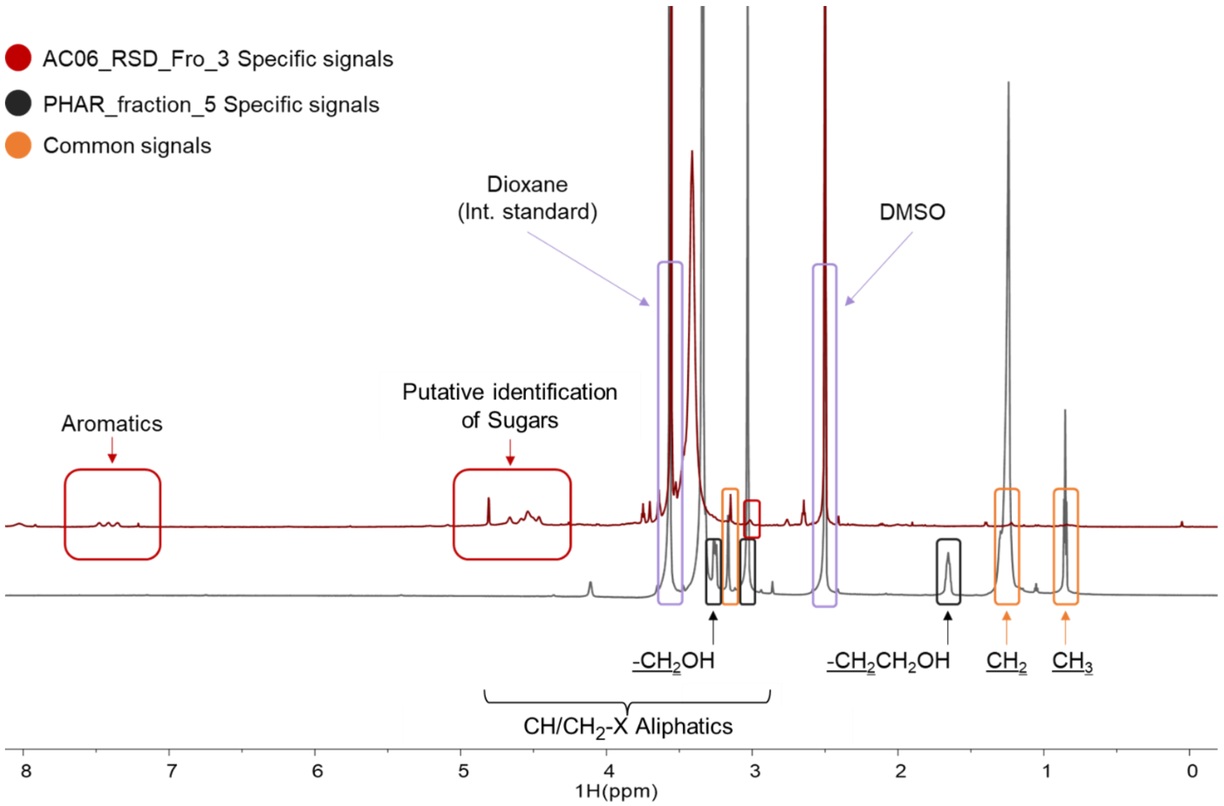


Figure 27 - Comparison of the 1H spectra of AC06_RSD_Fro_3 in red, and PHAR_fraction_5 in gray.

Both extraction methods give result to hydrolats with very distinct chemical profiles (Figure 26 and 27), there are however common signals in both samples, mainly in the aliphatic region, making them good candidates for the active compound(s). This analysis did, however, generate a new hypothesis: the active compound(s) could be present in low concentrations below the NMR detection threshold which prompted the analysis of some hydrolats via GC-MS.

9 GC-MS ANALYSIS OF THE HYDROLATS

Table 5 – Main common compounds identified via GC-MS in derivatized samples

With derivatization		Relative %			
RT (min)	**Compound**	**AC02**	**AC03**	**AC06**	**PHAR5**
19.4	1-octadecanol	x	20.85	9.92	5.26
22.6	1-docosanol	x	22.62	15.97	24.09
18.4	Hexadecanoic acid	34.01	21.47	26.94	26.28
19.3	Heptadecanoic acid	31.32	13.26	21.89	28.16
20.1	Octadecanoic acid	34.67	21.81	25.29	16.20
Identification yield (%)		2.57	9.12	10.35	25.74

Table 6 – Other compounds identified via GC-MS in derivatized and non-derivatized samples

With derivatization		Presence of other compounds			
RT (min)	**Compound**	**AC02**	**AC03**	**AC06**	**PHAR5**
6.5	Lactic Acid	x	x	x	yes
9.7	Glycerol	x	x	x	yes
14.5	D-Arabinopyranose	x	x	x	yes
14.9	1-Tetradecanamine, N,N-dimethyl-	x	x	x	yes
17.2	D-Glucose	x	x	x	yes
18.1	B-D-Glucopyranose	x	x	x	yes
18.7	9-Octadecenenitrile, (Z)-	x	x	x	yes
21.1	9-Octadecenamide, (Z)-	x	x	x	yes
25.7	22-hydroxydocosanoic acid	x	x	x	yes
Identification yield (%)		2.57	9.12	10.35	25.74

Without derivatization

3.7	Bromoform	yes	x	yes	x
4.5	trans-B-Chloroacrylic acid	x	yes	x	x
5.1	Phenol	x	x	x	yes
8.7	Dibromoacetic acid	x	x	yes	x
14.9	1-Tetradecanamine, N,N-dimethyl-	x	x	x	yes
15.5	1-Bromotridecane	x	x	x	yes

To get a better understanding of which specific compounds were present in each sample, GC-MS analysis was performed on AC02_SSD_Fro_1, AC03_RSD_Dry_1, AC06_RSD_Fro_3, and PHAR_fraction_5. Taking the NMR results into account, it was expected to find FAs and fatty alcohols, so calibration curves were created to potentially quantify these compounds. The identification yields represent the ratio between the identified peak area and the total peak area in the chromatogram.

Except for AC02_SSD_Fro_1, which lacks fatty alcohols, all tested samples have relatively similar levels of the detected FA and fatty alcohol compounds (Table 6). Most of these compounds have already been found in other *A. armata* extracts (1) and have been shown to have antibiotic activity (46).

If these compounds are the ones responsible for bioactivity, since they are distributed similarly in the samples, we can speculate that differences in activity are caused by differences in the concentration of those compounds between samples. AC06_RSD_Fro_3, for example, has a chemical profile similar to AC03_RSD_Dry_1 (Table 6), but the reactor-extraction method applied to frozen biomass may have resulted in a more concentrated hydrolat. However, it is important to note that the identification yield from these assays is low, implying that there is still a large number of compounds to be identified in these samples. Based on these identifications, new calibration curves for other compounds may be done, so their quantitative analysis might be expanded.

Based on previous NMR results, it was hypothesized that PHAR_fraction_5 was less chemically complex than the other tested samples, being primarily composed of fatty alcohols. However, both the derivatized and non-derivatized samples show that it contains a wide range of compounds that are not observable in the reactor-produced hydrolats (Table 7). This difference could be explained by the fact that those compounds are present in low concentrations in PHAR_fraction_5 and thus fall below the NMR detection threshold since it is a less sensitive technique than GC-MS. Another possibility is that the samples (AC02_SSD_Fro_1, AC03_RSD_Dry_1, AC06_RSD_Fro_3) that were subjected to liquid-liquid extraction prior to derivatization lost some of these compounds to the aqueous fraction and thus were not present in the fraction analyzed by GC-MS.

It can also be seen that PHAR_fraction_5 contains compounds known to have cryo-preservative functions, such as glycerol. This raises the question of whether they play any role in keeping the compounds active after lyophilization, as other compounds in the sample may not crystallize, making them easier to solubilize when resuspended in methanol after being lyophilized.

Additionally, it is confirmed once again that AC03_RSD_Dry_1 and PHAR_fraction_5 do not contain any trace of bromoform, as previously seen by NMR.

Moreover, it can’t be ruled out that, the biomass used to make RSD hydrolats might not contain the same active compound(s) as the one used to produce PHAR_fraction_5 as they had different collection periods.

10 CHECKERBOARD ASSAYS

Finally, the interactions of AC06_RSD_Fro_3 with two well characterized antibiotics (Polymyxin B, that is active against Gram-negative bacteria by damaging the bacterial cell membranes (65); and Ciprofloxacin, a fluoroquinolone with a broad spectrum of activity against aerobic Gram-negative and Gram-positive bacteria by inhibiting the synthesis of nucleic acids (65,66)) was tested by microdilution checkerboard assays. Checkerboard assays enabled sensitive interaction measurements between the hydrolat and the tested drugs against *S. aureus* and *E. coli*, as well as determining the effect of each individual antimicrobial agent, but most importantly, the effect produced by their combination. The main goal of these assays was to understand how AC06_RSD_Fro_3 inhibited the tested microorganisms and gather some hints about the hydrolat's mechanism of action.

Interactions were evaluated using both OD and MTT measurements for each tested condition, with biological duplicates. There is no consensus on which reference model should be used to analyze these data in an unbiased and statistically robust manner due to inherent differences in model assumptions (67–69). For this preliminary assay, the highest single-agent (HSA) method was chosen, due to its simplicity, to get an overview of the hydrolats synergistic effects with the tested antibacterial drugs. The HSA model assumes that the expected combination's effect is either equal to the higher individual drug effect or if exceeding the effect of the single components, it implies some synergistic interaction. Therefore, any additional effect over the higher single drug will be considered as HSA synergy (67,69). The FDA has adopted the HSA model's synergy as a criterion for approving drug combinations because of its ease of calculation and interpretation as clear clinical benefits. However, since the HSA model does not account for the dose-additive effect of a drug combined with itself, it may result in a high rate of false discovery in preclinical drug screening studies (69).

One of the outputs from SynergyFinder is the HSA synergy score (Table 8). These scores can be interpreted as the average excess response due to drug interactions (*e.g*. a synergy score of 15 corresponds to 15 percent of a response beyond the individual components effect). Since combinatorial synergy is highly context-specific, there is no specific threshold for defining a good synergy score. However, it has been found that synergy scores near 0 indicate a lack of synergy or antagonism. If the score is below -10, the drug interaction is likely to be antagonistic; between -10 and 10, the interaction is most likely non-interactive and above 10, the interaction is likely to be synergistic (63).

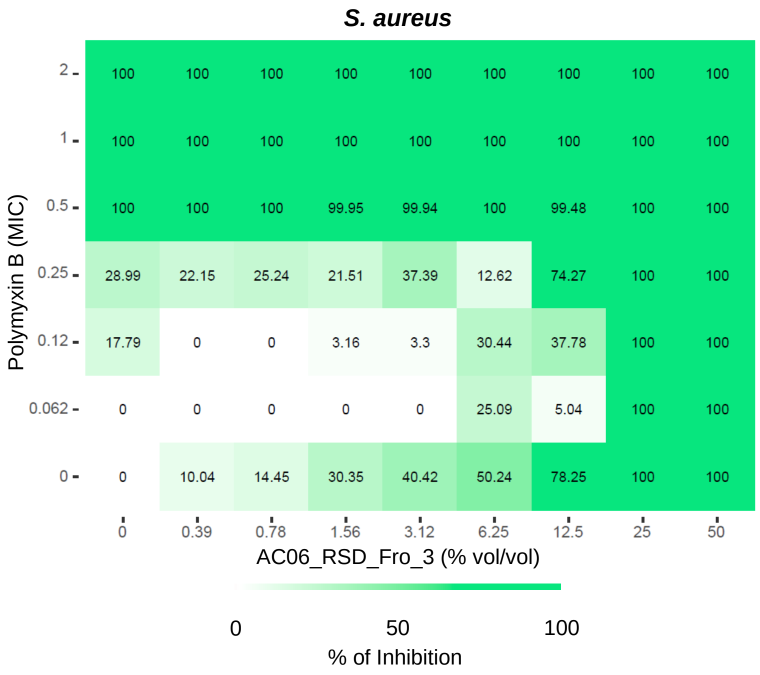
Following these guidelines, the results depicted in Table 8 show that Polymyxin B combined with the hydrolat has an overall antagonistic effect, whereas the combination of hydrolat with Ciprofloxacin has a non-interactive effect against both tested microorganisms. However, some divergent results can be observed for specific concentrations (Figures 31 and 33).

Table 7 – HSA synergy score for AC06_RSD_Fro_3 combined with each antibiotic, calculated using the SynergyFinder R package.

S. aureus		***E. coli***	
Polymyxin	Ciprofloxacin	Polymyxin	Ciprofloxacin
-20.12	-8.34	-10.27	-6.44

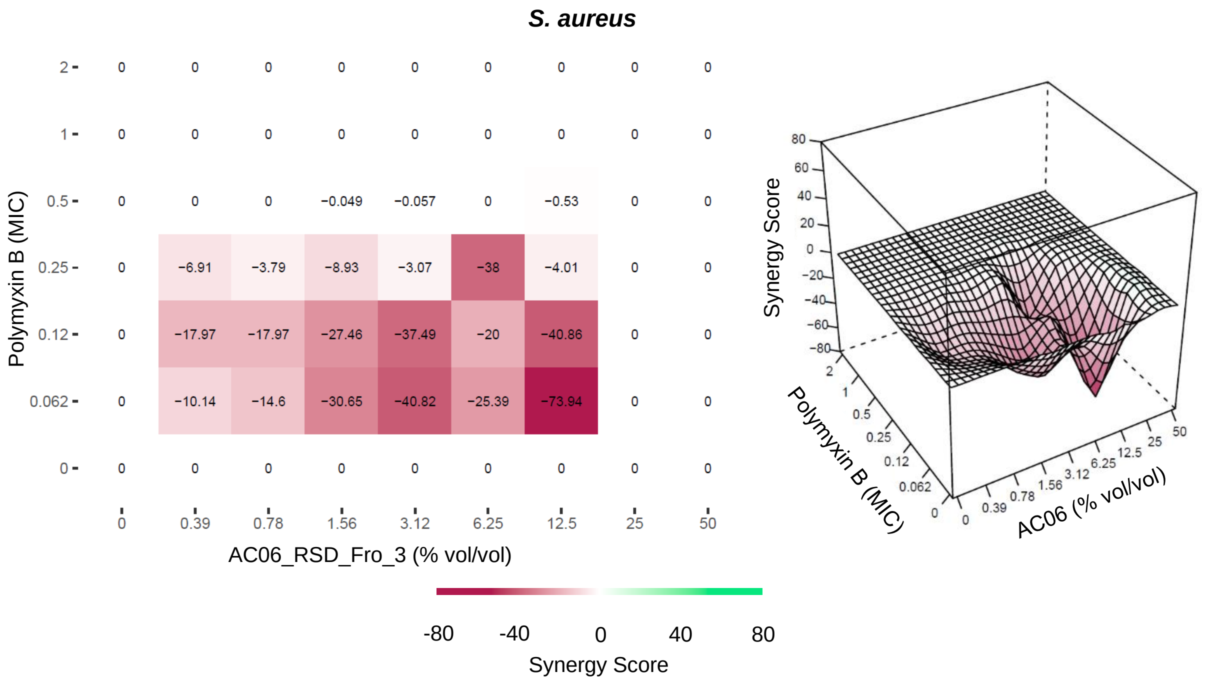
10.1 INTERACTION BETWEEN AC06_RSD_FRO_3 AND POLYMYXIN B AGAINST *S. AUREUS*

A previous preliminary study in the AEM lab team estimated that the MIC value for Polymyxin B against the utilized *S. aureus* strain was 512 µg/mL (64). Differences in these results (first column of Figure 28) could be attributed to the fact that the previous tests were performed with agitation.



Polymyxin B (MIC) \ AC06_RSD_Fro_3 (% vol/vol)	0	0.39	0.78	1.56	3.12	6.25	12.5	25	50
2	100	100	100	100	100	100	100	100	100
1	100	100	100	100	100	100	100	100	100
0.5	100	100	100	99.95	99.94	100	99.48	100	100
0.25	28.99	22.15	25.24	21.51	37.39	12.62	74.27	100	100
0.12	17.79	0	0	3.16	3.3	30.44	37.78	100	100
0.062	0	0	0	0	0	25.09	5.04	100	100
0	0	10.04	14.45	30.35	40.42	50.24	78.25	100	100

Figure 28 – Dose response matrix for the % of inhibition measured by MTT assays of each individual concentration of AC06_RSD_Fro_3 /Polymyxin B combination against *S. aureus*. Estimated MIC of Polymyxin B of 512 µg/mL.

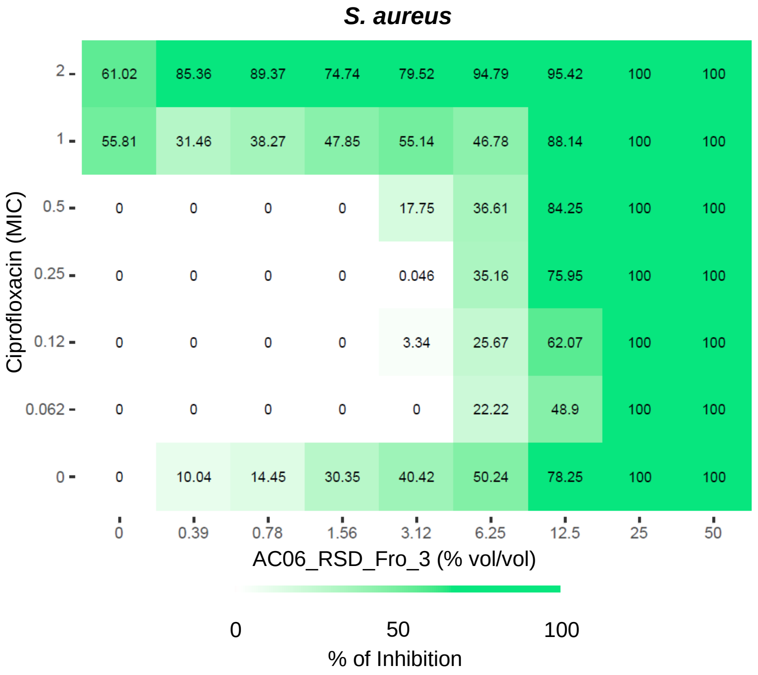


Polymyxin B (MIC) \ AC06_RSD_Fro_3 (% vol/vol)	0	0.39	0.78	1.56	3.12	6.25	12.5	25	50
2	0	0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0	0	0
0.5	0	0	0	−0.049	−0.057	0	−0.53	0	0
0.25	0	−6.91	−3.79	−8.93	−3.07	−38	−4.01	0	0
0.12	0	−17.97	−17.97	−27.46	−37.49	−20	−40.86	0	0
0.062	0	−10.14	−14.6	−30.65	−40.82	−25.39	−73.94	0	0
0	0	0	0	0	0	0	0	0	0

Figure 29 – Heatmap (left) and 3D surface synergy map (right) showing the synergy scores of each individual concentration of the AC06_RSD_Fro_3 /Polymyxin B combinations against *S. aureus* calculated by the HSA method. Estimated MIC of Polymyxin B of 512 µg/mL.

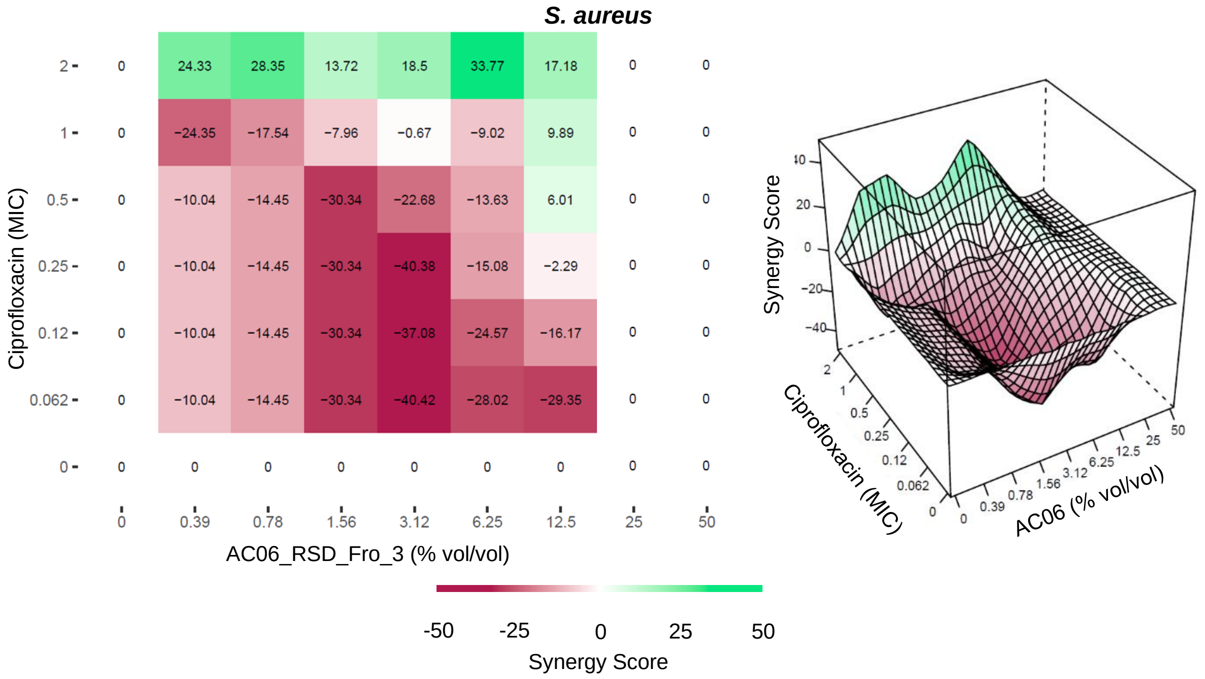
10.2 INTERACTION BETWEEN AC06_RSD_FRO_3 AND CIPROFLOXACIN AGAINST *S. AUREUS*

A previous preliminary study in the AEM lab team estimated that the MIC value for Ciprofloxacin against the utilized *S. aureus* strain was 0.25 µg/mL (64). Differences in these results (first column of Figure 30) could be attributed to the fact that the previous tests were performed with agitation.


S. aureus

Ciprofloxacin (MIC) \ AC06_RSD_Fro_3 (% vol/vol)	0	0.39	0.78	1.56	3.12	6.25	12.5	25	50
2	61.02	85.36	89.37	74.74	79.52	94.79	95.42	100	100
1	55.81	31.46	38.27	47.85	55.14	46.78	88.14	100	100
0.5	0	0	0	0	17.75	36.61	84.25	100	100
0.25	0	0	0	0	0.046	35.16	75.95	100	100
0.12	0	0	0	0	3.34	25.67	62.07	100	100
0.062	0	0	0	0	0	22.22	48.9	100	100
0	0	10.04	14.45	30.35	40.42	50.24	78.25	100	100

Figure 30 – Dose response matrix for the % of inhibition measured by MTT assays of each individual concentration of AC06_RSD_Fro_3 /Ciprofloxacin combination against *S. aureus.* Estimated MIC of Ciprofloxacin of 0.25 µg/mL.

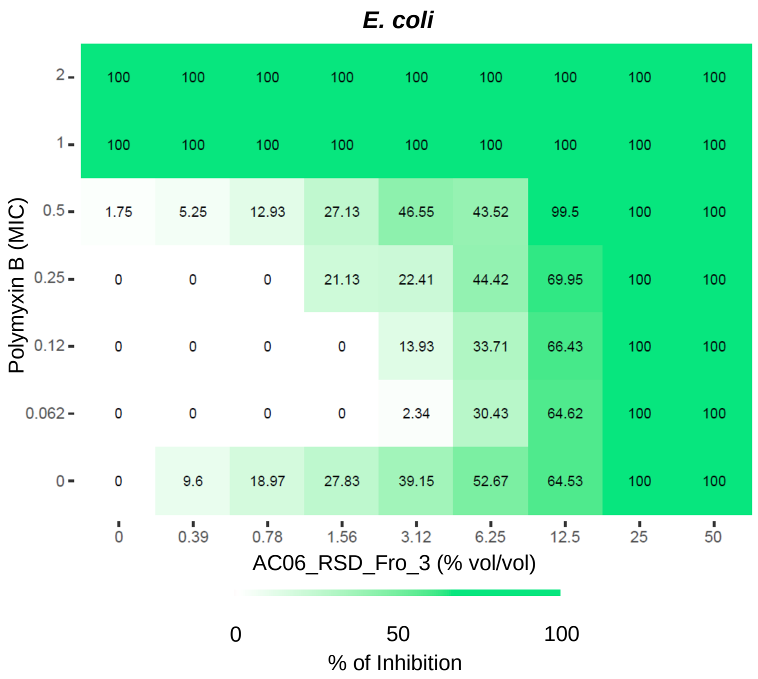

S. aureus

Ciprofloxacin (MIC) \ AC06_RSD_Fro_3 (% vol/vol)	0	0.39	0.78	1.56	3.12	6.25	12.5	25	50
2	0	24.33	28.35	13.72	18.5	33.77	17.18	0	0
1	0	−24.35	−17.54	−7.96	−0.67	−9.02	9.89	0	0
0.5	0	−10.04	−14.45	−30.34	−22.68	−13.63	6.01	0	0
0.25	0	−10.04	−14.45	−30.34	−40.38	−15.08	−2.29	0	0
0.12	0	−10.04	−14.45	−30.34	−37.08	−24.57	−16.17	0	0
0.062	0	−10.04	−14.45	−30.34	−40.42	−28.02	−29.35	0	0
0	0	0	0	0	0	0	0	0	0

Figure 31 – Heatmap (left) and 3D surface synergy map (right) showing the synergy scores of each individual concentration of AC06_RSD_Fro_3 /Ciprofloxacin combination against *S. aureus* calculated by the HSA method. Estimated MIC of Ciprofloxacin of 0.25 µg/mL.

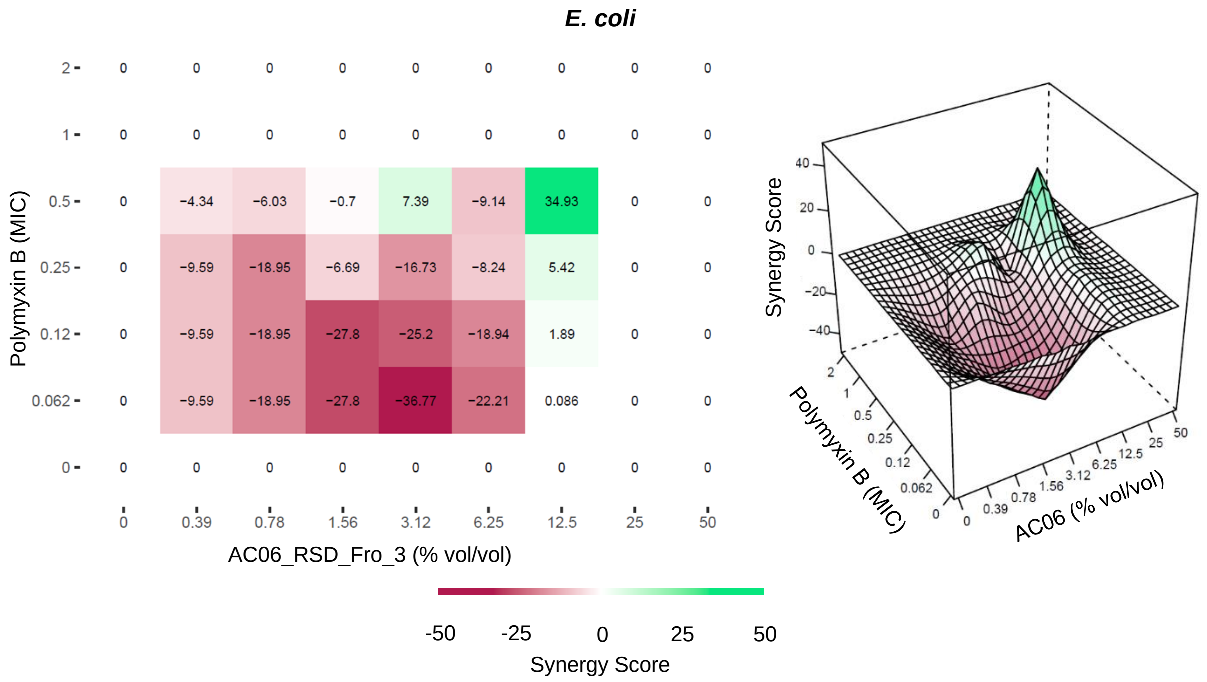
10.3 INTERACTION BETWEEN AC06_RSD_FRO_3 AND POLYMYXIN B AGAINST *E. COLI*

A previous preliminary study in the AEM lab team estimated that the MIC value for Polymyxin B against the utilized *E. coli* strain was 0.125 µg/mL (64). Differences in these results (first column of Figure 32) could be attributed to the fact that the previous tests were performed with agitation.



Polymyxin B (MIC)	0	0.39	0.78	1.56	3.12	6.25	12.5	25	50
2	100	100	100	100	100	100	100	100	100
1	100	100	100	100	100	100	100	100	100
0.5	1.75	5.25	12.93	27.13	46.55	43.52	99.5	100	100
0.25	0	0	0	21.13	22.41	44.42	69.95	100	100
0.12	0	0	0	0	13.93	33.71	66.43	100	100
0.062	0	0	0	0	2.34	30.43	64.62	100	100
0	0	9.6	18.97	27.83	39.15	52.67	64.53	100	100

Figure 32 – Dose response matrix for the % of inhibition measured by MTT assays of each individual concentration of AC06_RSD_Fro_3 /Polymyxin B combination against *E. coli*. Estimated MIC of Polymyxin B of 0.125 µg/mL.

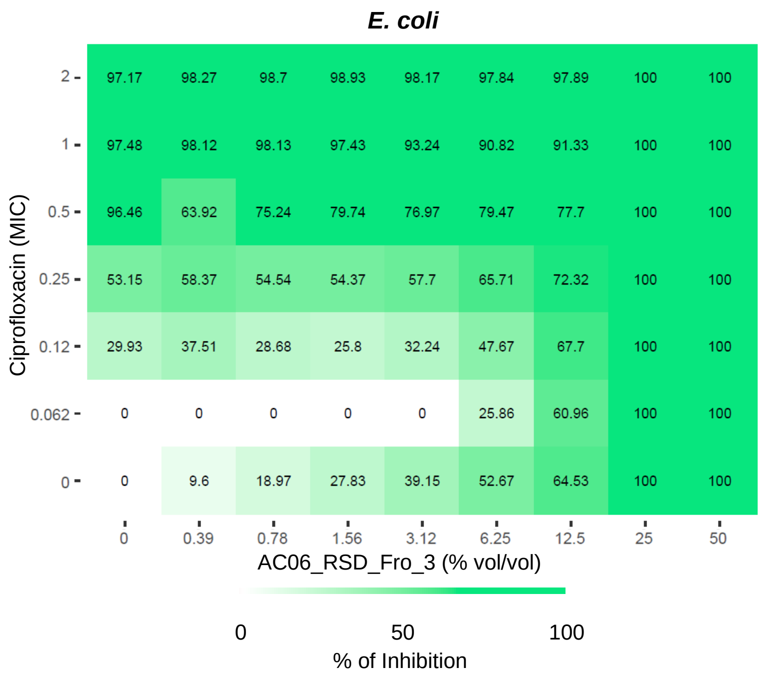


Polymyxin B (MIC)	0	0.39	0.78	1.56	3.12	6.25	12.5	25	50
2	0	0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0	0	0
0.5	0	−4.34	−6.03	−0.7	7.39	−9.14	34.93	0	0
0.25	0	−9.59	−18.95	−6.69	−16.73	−8.24	5.42	0	0
0.12	0	−9.59	−18.95	−27.8	−25.2	−18.94	1.89	0	0
0.062	0	−9.59	−18.95	−27.8	−36.77	−22.21	0.086	0	0
0	0	0	0	0	0	0	0	0	0

Figure 33 – Heatmap (left) and 3D surface synergy map (right) showing the synergy scores of each individual concentration of AC06_RSD_Fro_3 /Polymyxin combination against *E. coli* calculated by the HSA method. Estimated MIC of Polymyxin B of 0.125 µg/mL.

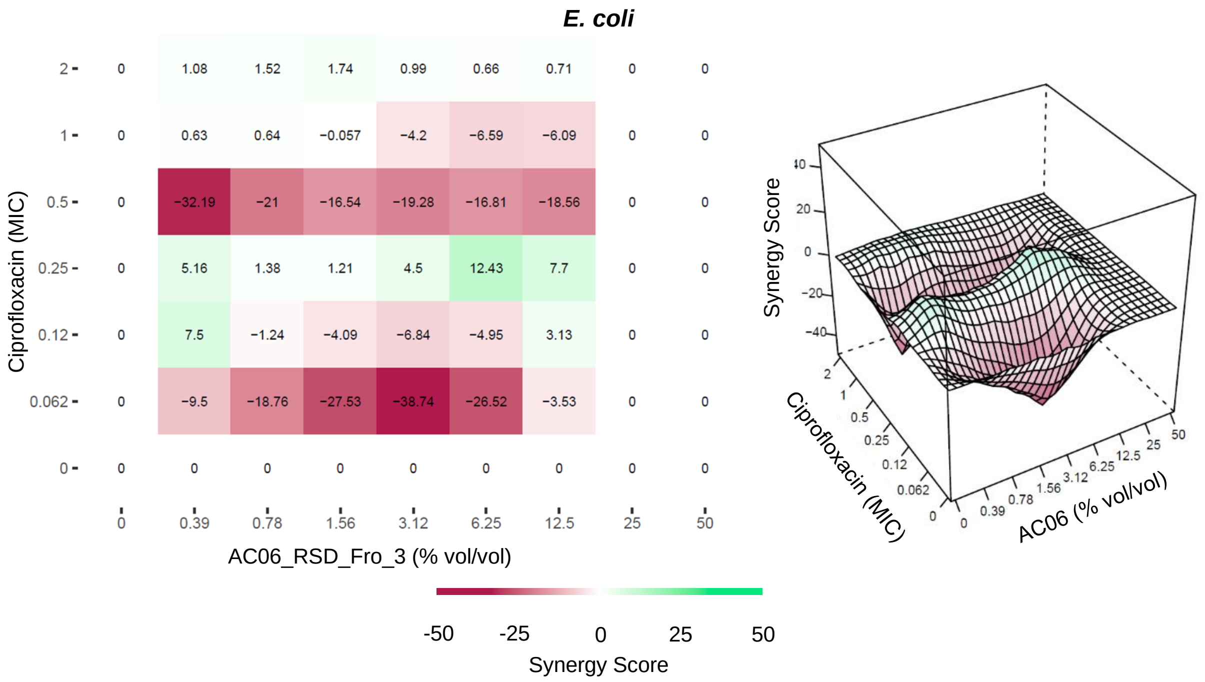
10.4 INTERACTION BETWEEN AC06_RSD_FRO_3 AND CIPROFLOXACIN AGAINST *E. COLI*

A previous preliminary study in the AEM lab team estimated that the MIC value for Ciprofloxacin against the utilized *E. coli* strain was 0.0075 µg/mL (64). Differences in these results (first column of Figure 34) could be attributed to the fact that the previous tests were performed with agitation.



	0	0.39	0.78	1.56	3.12	6.25	12.5	25	50
2	97.17	98.27	98.7	98.93	98.17	97.84	97.89	100	100
1	97.48	98.12	98.13	97.43	93.24	90.82	91.33	100	100
0.5	96.46	63.92	75.24	79.74	76.97	79.47	77.7	100	100
0.25	53.15	58.37	54.54	54.37	57.7	65.71	72.32	100	100
0.12	29.93	37.51	28.68	25.8	32.24	47.67	67.7	100	100
0.062	0	0	0	0	0	25.86	60.96	100	100
0	0	9.6	18.97	27.83	39.15	52.67	64.53	100	100

Figure 34 – Dose response matrix for the % of inhibition measured by MTT assays of each individual concentration of AC06_RSD_Fro_3 /Ciprofloxacin combination against *E. coli*. Estimated MIC of Ciprofloxacin of 0.0075 µg/mL.



	0	0.39	0.78	1.56	3.12	6.25	12.5	25	50
2	0	1.08	1.52	1.74	0.99	0.66	0.71	0	0
1	0	0.63	0.64	−0.057	−4.2	−6.59	−6.09	0	0
0.5	0	−32.19	−21	−16.54	−19.28	−16.81	−18.56	0	0
0.25	0	5.16	1.38	1.21	4.5	12.43	7.7	0	0
0.12	0	7.5	−1.24	−4.09	−6.84	−4.95	3.13	0	0
0.062	0	−9.5	−18.76	−27.53	−38.74	−26.52	−3.53	0	0
0	0	0	0	0	0	0	0	0	0

Figure 35 – Heatmap (left) and 3D surface synergy map (right) showing the synergy scores of each individual concentration of AC06_RSD_Fro_3 /Ciprofloxacin combination against *E. coli* calculated by the HSA method. Estimated MIC of Ciprofloxacin of 0.0075 µg/mL.

Strong synergistic effects were detected when ciprofloxacin was combined with the hydrolat against *S. aureus* (Figure 31) at the concentration of 2 times the MIC of the tested drug (although it was expected that ciprofloxacin alone would be able to completely inhibit *S. aureus* cell growth at this concentration). The presence of the hydrolat, even at low concentrations, seemed to potentiate the effect of ciprofloxacin, and the combined effect had higher inhibitory activity than either drug alone at the respective concentrations.

A synergy peak was also detected when polymyxin B was combined with the hydrolat against *E.coli* (Figure 33) at the concentrations of 0.5 times the MIC and 12.5 % v/v respectively. These correspond to the concentrations of the antimicrobial and the hydrolat, where bacterial growth inhibition is not complete anymore. When used together, they achieve a higher inhibitory activity than either drug alone at the respective concentrations.

The Checkerboard assay was used to demonstrate the synergistic effect of combinations of antibiotics with the hydrolat. At this moment, we cannot infer a precise mechanism of action from these datasets. As observed by NMR and GC-MS, the hydrolat is mainly composed by FAs, some of which have been shown to have antibiotic activity (46). The hydrolat's synergistic effect when combined with ciprofloxacin was more pronounced in *S. aureus*, most likely due to structural differences between *S. aureus* and *E. coli* cells. One hypothesis is that the FAs present in the hydrolat would facilitate the action of ciprofloxacin, by permeating the peptidoglycan layer of *S. aureus* cells. Additionally, synergism could be explored for the development of novel therapeutic options since this approach could be used to reduce the required dosage of a compound and thus reduce its toxicity for the patient (*e.g*. Ciprofloxaxin for *S. aureus*).

Future work on this topic will include a larger number of replicates with adjusted antimicrobial MICs for a range of concentrations where synergy was detected, and results from various reference models (63) will be compared to identify more informative interaction patterns over the dose-response matrices tested. In addition, other types of antibiotics might also be tested to understand and possibly correlate with the mode of action of the hydrolat. The hydrolat will also be tested against the two fungal organisms combined with antifungal drugs (to be determined). Nevertheless, we are aware that additional techniques, such as microscopy (Fluorescence microscopy and/or Transmission electron microscopy (TEM)), are required to fully disclose the mechanisms of actions in play.

4 CONCLUSIONS AND FUTURE PERSPECTIVES

The main purpose of this research project was to demonstrate that *A. armata* was a readily available raw material that could be exploited as a source of antibacterial and antifungal compounds, using a simple and environmentally friendly approach. Typically, *A. armata* and other natural products are extracted using harsh chemical solvents. To break away from already established methods and fully embrace a sustainable approach, it was decided to test the efficacy of *A. armata* water-based extracts (hydrolat), as this is the most sustainable solvent available. Besides, to the best of our knowledge, the bioactivities of *A. armata*'s aqueous extracts have never been tested against pathogenic fungi and bacteria, providing an excellent opportunity for novel findings. Moreover, given *A. armata*'s invasive nature, the collection of this species would benefit not only the pharmaceutical industry in its research for new sources of bioactive compounds but also the marine environment, as an invasive algal species would be reduced.

Three different extraction procedures were thoroughly compared and optimized to determine the best extraction conditions and parameters to obtain the hydrolats with the highest growth inhibitory activity against the four model organisms *S. aureus, E. coli, C. albicans*, and *A. fumigatus*. It was determined that the best parameters for this approach were a 20-minutes extraction, using *A. armata* biomass which had been stored frozen. Differences in biomass washing had no effect on the bioactivity of the obtained hydrolats. Drying the biomass prior to extraction severely limited the activity of hydrolats produced by the SSD method, in contrast to the RSD and Pharmaplant methods. The results suggest that biomass drying does not result in the loss of active compounds (volatiles for example), but rather the conglomeration/clotting of biomass hinders the SSD extraction efficiency. However, as RSD-produced hydrolats made from dried biomass did not perform as well as their frozen biomass counterparts, this method can still be optimized.

After establishing a baseline of parameters, we compared the extraction efficiency of two distinct methods (SSD versus RSD). It was concluded that while both methods originate hydrolats with comparable bioactivities against fungi, RSD-produced hydrolats have significantly higher bioactivities against bacteria than the SSD-produced hydrolats. We suppose that the higher inhibitory activities found in RSD-produced hydrolats are due to the recirculation of steam and the pressure reached during extraction, which promotes steam diffusion in the biomass to a greater extent, leading to an efficient extraction of both antibacterial and antifungal compounds. It was also shown that RSD-produced hydrolats significantly reduced the viability of the four tested microorganisms, whereas SSD-produced hydrolats were less active, even at higher concentrations, especially against *S. aureus*. In addition, the RSD method has the potential to be used as a simple, easy to scale-up, and reproducible method that delivers hydrolats with consistent activities and chemical compositions, as it was confirmed by NMR. Despite the advantages listed above, the RSD method has one limitation in common with SSD: the produced hydrolats cannot be lyophilized and resuspended at the same concentration as untreated hydrolats without losing activity. However, efforts are being made to understand and circumvent this setback, such as applying sonication, heat or resuspension in other solvents.

According to NMR analysis, bromoform is not the main compound responsible for the bioactivity found in *A. armata* extracts. When tested alone at concentrations equimolar to those found in the hydrolats, its inhibitory effects were much lower compared to the hydrolats. Nevertheless, to keep bromoform levels as low as possible, the RSD method is preferred as it produces hydrolats that contain no or only trace amounts of bromoform. NMR chemical analyses of the hydrolats also revealed that the main groups of compounds found in the most active RSD-produced hydrolat are FAs. This information was confirmed by GC-MS, which identified a mix of FAs such as hexadecanoic (palmitic), heptadecanoic (margaric), and octadecanoic (stearic) acids. Some of the FAs identified were consistent with previous research and have been linked to antimicrobial activity. However, because our identification yields were low, it is possible that other active minor compound(s) are still unidentified. It is critical to remember that the observed activity could also be the result of a mixture of compounds. Therefore, additional analyses will be required to fully characterize the chemical landscape of our extracts. For example, LC/GC-MS of more concentrated hydrolats or chromatographic techniques such as Thin Layer Chromatography to fractionate the hydrolat prior to GC-MS and NMR analyses. Further identification and quantification of compounds will allow to pin-point potential candidates for the observed activities.

Finally, checkerboard assays revealed that the hydrolat has an overall antagonistic effect with both antibiotics tested for both bacteria. However, some divergent results can be observed for specific concentrations of AC06_RSD_Fro_3. This synergistic potential could be used to reduce the required dosage of the tested antimicrobial drugs and thus their toxicity for patients when used in therapeutics. Nonetheless, further research is needed to validate this hypothesis as well as to test the hydrolat's capabilities when combined with other antimicrobials to understand the possible mechanisms of action involved. This task can be bolstered by the use of fluorescence microscopy and/or TEM.

Beyond the goals already stated, future research will also focus on ways to further valorize biomass after extraction. For example, it will be explored whether valuable cell wall polymers could be extracted and the hydrolats' potential as surface cleaners/disinfectants will be evaluated.

A. armata has long been recognized as a valuable source of antimicrobial compounds. This study, however, revealed the potential of natural, water-based extracts with high growth-inhibitory potentials against various pathogens including Gram-positive, Gram-negative bacteria, and fungi (filamentous and yeasts). The ultimate goal of this project is to achieve a sustainable method for developing new drugs to combat challenging human pathogens and the rise of antimicrobial resistance. It advocates for resource sustainability, which is essential for achieving a bioeconomy that contributes to the long-term development and longevity of the planet. Accordingly, this resonates with the 2030 Agenda for Sustainable Development, focusing specifically on Goals 3 (Good Health and Well-Being) and 14 (Life below water).

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