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Valorization of Lebanese seaweed extracts and evaluation of their potential antibiofilm activity

Maya Rima

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THÈSE

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Présentée et soutenue par

Maya RIMA

Le 8 décembre 2021

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activité antibiofilm potentielle**

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Bioingenieries**

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2nd International Symposium on Materials, Electrochemistry and Environment CIMEE'18
October 2018 – Tripoli, Lebanon.

Maya RIMA, Asma CHBANI, Christine ROQUES, Fatima EL GARAH.

Evaluation of the insecticidal activity of a green alga against *Drosophila melanogaster* fruit fly.

2nd Seaweed for Health International Conference
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ABSTRACT

The massive and often uncontrolled use of antibiotics has led to the development of multi-resistant bacterial strains (MDRs) capable of causing infectious diseases that are difficult or even untreatable. In addition, the organization of bacteria into biofilms corresponds to adaptive resistance and is involved in almost 80% of chronic infections. By definition, a biofilm is an aggregation of microorganisms attached to a biotic or abiotic surface and enclosed in an extracellular polymeric matrix (EPS). This sessile lifestyle provides a protective barrier against antimicrobial agents. In this regard, much attention has been paid to the search for anti-biofilm agents able to regulate or even inhibit biofilm formation without interfering with bacterial growth.

Natural products represent a valuable source of new molecules, including possible drug candidates. Marine organisms, in particular macroalgae, constitute a reservoir of bioactive compounds with a broad spectrum of biological activities, including insecticidal, antimicrobial and anti-biofilm activities, via different mechanisms. For example, the halogenated furanone isolated from the red alga *Delisea pulchra* is the first inhibitor molecule of Quorum Sensing, an intercellular communication system playing a major role in the formation of bacterial biofilms.

In this context, the objective of this study is to explore the potential of extracts derived from three Lebanese algae: the green alga *Ulva lactuca*, the brown alga *Stypocaulon scoparium* and the red alga *Pterocladia capillacea*, in terms of anti-biofilm activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus*, two opportunistic pathogens responsible for serious infections, particularly in immunocompromised subjects and cystic fibrosis patients. To do that, various complementary approaches (crystal violet staining method, colony-forming unit counts method, epifluorescence microscopic analysis, synergistic activity with conventional antibiotics...) were adopted. Interestingly, results showed the ability of various extracts to present a significant anti-biofilm activity against these two critical bacteria by exhibiting different mechanisms of action. At the same time, the analysis of the chemical composition of extracts was carried out in an attempt to identify compound(s) which could be responsible for their demonstrated activity.

On the other hand, in order to evaluate the potentiality of the green alga *Ulva lactuca* to present an alternative to toxic phytosanitary products, its possible insecticidal activity was studied against the *Drosophila melanogaster* fruit fly (insect pest and best model for studying the insecticidal activity) by complementary *in vivo* tests. Results showed an interesting insecticidal activity of its acetic extract as well as of its purified green pigments.

This study provides new insight into the exploration of seaweed as a valuable source of bioactive compounds that can be valorized in the agricultural area as well as in the industrial/pharmaceutical field.

Keywords: Seaweed, Anti-biofilm activity, *P. aeruginosa*, *S. aureus*, insecticidal activity.

L'utilisation massive et souvent incontrôlée des antibiotiques a conduit au développement de souches bactériennes multi-résistantes (MDR) capables de causer des maladies infectieuses difficiles et même impossibles à traiter. Par ailleurs, l'organisation des bactéries en biofilm correspond à une résistance adaptative et est impliquée dans presque 80% des infections chroniques. Par définition, un biofilm est une agrégation des microorganismes attachée à une surface biotique ou abiotique et enfermée dans une matrice polymérique extracellulaire (EPS). Ce mode de vie sessile assure une barrière de protection contre les agents antimicrobiens. À cet égard, une grande attention a été accordée à la recherche d'agents anti-biofilms dont le rôle est de réguler, voire d'inhiber, la formation de biofilm sans interférer avec la croissance bactérienne.

Les produits naturels représentent une source précieuse de nouvelles molécules dont des candidats médicaments. Les organismes marins, en particulier les macroalgues, constituent un réservoir de composés bioactifs ayant un large spectre d'activités biologiques, y compris des activités insecticide, antimicrobienne et antibiofilm, via différents mécanismes. Par exemple, la furanone halogénée isolée de l'algue rouge *Delisea pulchra* est la première molécule inhibitrice du système de Quorum Sensing, un système de communication intercellulaire jouant un rôle majeur dans la formation des biofilms bactériens.

Dans ce contexte, l'objectif de cette étude est d'explorer le potentiel des extraits issus de trois algues Libanaises : l'algue verte *Ulva lactuca*, l'algue brune *Stypocaulon scoparium* et l'algue rouge *Pterocladia capillacea*, en termes d'activité antibiofilm, contre *Pseudomonas aeruginosa* et *Staphylococcus aureus*, deux agents pathogènes opportunistes responsables d'infections graves, notamment chez les sujets immunodéprimés et les patients atteints de mucoviscidose. Pour ce faire, plusieurs approches complémentaires (méthode de marquage au crystal violet, méthode de dénombrement des unités-formant colonies, analyse microscopique à épifluorescence, activité synergique avec des antibiotiques conventionnels...) ont été adoptées. Les résultats ont montré que plusieurs extraits ont une activité antibiofilm intéressante contre ces deux bactéries critiques, avec des mécanismes d'action différents. Parallèlement, l'analyse de la composition chimique des extraits a été menée afin d'identifier le(s) composé(s) qui pourraient être à l'origine de leur activité démontrée.

D'autre part, afin d'évaluer la potentialité de l'algue verte *Ulva lactuca* à présenter une alternative aux produits phytosanitaires toxiques, son activité insecticide a été étudiée contre la mouche de fruit *Drosophila melanogaster* (insecte ravageur et le meilleur modèle d'étude de l'activité insecticide) par différents tests complémentaires. Les résultats ont montré que l'extrait acétonique ainsi que les pigments verts purifiés présentent la meilleure activité insecticide.

Cette étude fournit un nouvel aperçu de l'exploration des algues comme étant une source précieuse de composés bioactifs pouvant être valorisés dans le domaine agricole ainsi que dans le secteur industriel/pharmaceutique.

Mots clés : Macroalgues, activité antibiofilm, *P. aeruginosa*, *S. aureus*, activité insecticide.

LIST OF ABBREVIATIONS

AHLs	N-acyl homoserine lactones
AI	Autoinducers
AIP	Autoinducer peptide
ATP	Adenosine triphosphate
BAC	benzalkonium chloride
Bap	Biofilm associated protein
BB	Biofilm broth
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance
CFU	Colony forming unit
CH	Cyclohexane
Chl a	Chlorophyll a
Chl b	Chlorophyll b
CLSM	Confocal laser scanning microscopy
ConA	Concanavalin A
CV	Crystal violet
DCM	Dichloromethane
DW	Dry weight
EA	Ethyl acetate
eDNA	Extracellular DNA
EP	Eradication percentage
EPS	Extracellular polymeric matrix
FC	Fluorescence control
FDA	Food and drug administration
GC-MS	Gas chromatography–mass spectrometry
GFP	Green fluorescent protein
IDSA	Infectious Diseases Society of America
IP	Inhibition percentage
LB	Lysogeny Broth
LC-MS	Liquid chromatography–mass spectrometry
MBB	Modified Biofilm Broth
MDR	Multidrug resistant
MeOH	Methanol

MHB	Mueller Hinton Broth
MIC	Minimum inhibitory concentration
MRSA	Methicillin resistant <i>Staphylococcus aureus</i>
NO	Nitric oxide
OD	Optical density
P.c	<i>Pterocladia capillacea</i> red alga
P'	Polarity index
PI	Propidium iodide
PIA	Polysaccharide intercellular adhesin
PIPs	Plant-incorporated protectants
POPs	Persistent organic pollutants
PQS	<i>Pseudomonas</i> quinolone signal
qPCR	Quantitative polymerase chain reaction
QS	Quorum Sensing
QSI	Quorum Sensing inhibitors
ROS	Reactive oxygen species
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
S.s	<i>Stypocaulon scoparium</i> brown alga
SC	Sterility control
SDW	Sterile distilled water
SEM	Scanning electron microscopy
TA	Toxin-antitoxin system
TSA	Trypticase soy agar
U.l	<i>Ulva lactuca</i> green alga
UNEP	United Nations Environment Programme
US. EPA	United States Environmental Protection Agency
WHO	World Health Organization

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INTRODUCTION

The exploration of natural products, broadly defined as chemical compounds synthesized by living organisms, has received a tremendous interest from the scientific community in the last decades and the focus on their wide properties is consistently increasing. Indeed, the high structural and functional diversity as well as the uniqueness of natural products are the result of an evolution over millions of years. These natural chemicals are usually produced by living organisms as a natural means of countering external threats (stressful environmental conditions, competition, infections...) which explains their huge bioactivity (Sorokina & Steinbeck, 2020). Besides their prominent role in both traditional and modern pharmacology, various studies have highlighted the usefulness of natural products in food and cosmetic industries as well as in agriculture, especially in the area of biopesticides (Newman & Cragg, 2016; Sparks *et al.*, 2019).

Among the exploited living organisms, those residing in the marine environment are considered as the most recent source explored for bioactive natural products compared to terrestrial plants and nonmarine organisms (Jimenez, 2018). In fact, the marine world which accounts for approximately 70% of the Earth's surface, is the habitat of a huge diversity of species (algae, sponges, mollusks, bacteria, fungi...) (Blunt *et al.*, 2018). Interestingly, in order to survive the harsh marine conditions, marine organisms synthesize a wide variety of unique natural products with high incidence of bioactivity. However, the marine world remains under-exploited (less than 5% of its diversity has been explored) and there is still much to know about this underwater treasure in an attempt to valorize these fantastic creatures in different fields (Jimenez, 2018).

Among marine organisms, seaweed, the primary producers that occupy the base of the marine food chain, are well-known for their ability to synthesize several bioactive substances with a broad spectrum of demonstrated biological activity (antioxidant, anti-inflammatory, antimicrobial, anticancer, anti-aging...). In fact, regarding their sessile nature, algae have a strong tendency to produce bioactive metabolites and to evolve defense mechanisms in order to withstand both biotic (fungal, bacterial infections...) and abiotic (salinity, temperature, pollutants...) threats faced in the marine environment (Leandro *et al.*, 2019).

In light of their valuable properties and their usefulness as pharmaceuticals, nutraceuticals, cosmeceuticals, as well as in feeding and agriculture, the cultivation of seaweed together with their value in the market are continuously rising (Market Analysis Report, 2020).

Therefore, the main objective of this thesis project consists in exploring extracts derived from three seaweed collected from the North Lebanese coast of the Mediterranean – Tripoli – Lebanon and which belong to three different groups: green alga *Ulva lactuca*, brown alga *Stypocaulon scoparium*, and red alga *Pterocliadiella capillacea*. Interestingly, this study exploits the possible ability of these seaweed to be valorized in two different fields (*Figure 1*).

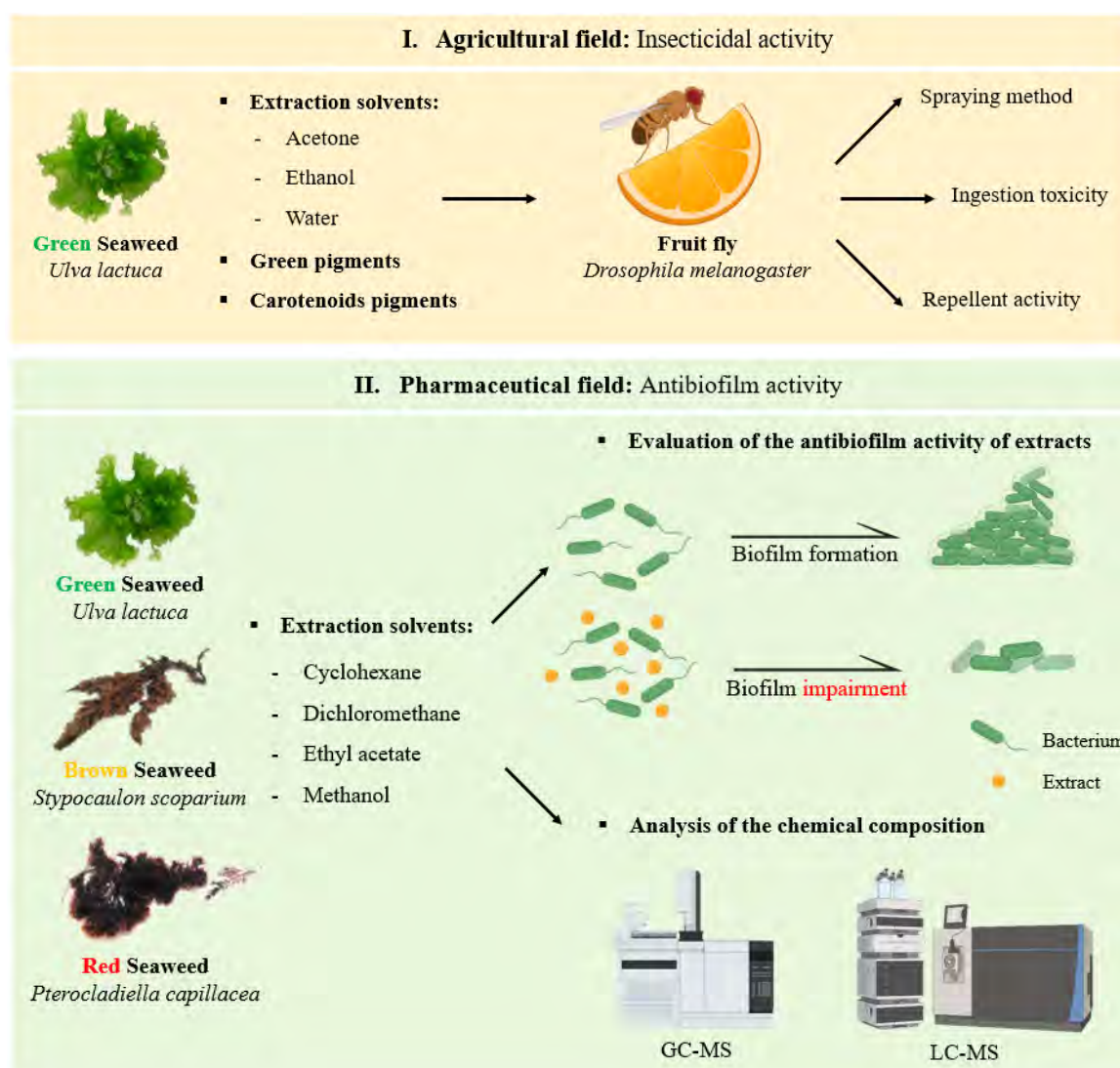


FIGURE 1 | Main objectives and steps followed in this study.

- **Agricultural field:** First, the potential capacity of extracts derived from the green alga *U. lactuca* to present a natural, eco-friendly, cost-effective, and potentially less-toxic alternative to conventional agrochemicals was assessed. In fact, the massive use of synthetic phytosanitary products undoubtedly leads to adverse effects on both public health and environment, hence the urgent need to look for new strategies (Gyawali, 2018). For this purpose, the insecticidal activity of *U. lactuca* extracts was evaluated against the fruit fly *Drosophila melanogaster*, pest insect and the best model for studying the insecticidal activity at the laboratory scale. This part of the project was carried out in the Applied Biotechnology Laboratory (LBA3B-ER032) – AZM Center for Research in Biotechnology and its Applications – Tripoli – Lebanon.
- **Pharmaceutical field:** On the other hand, various extracts derived from the three seaweed (*U. lactuca*, *S. scoparium*, and *P. capillacea*) were explored in terms of their potential antibiofilm activity against two critical bacteria known for their high ability to produce biofilms: The Gram-negative *Pseudomonas aeruginosa* and the Gram-positive *Staphylococcus aureus*. Indeed, biofilms known as “City of Microbes” and defined as an aggregation of microorganisms adhered to each other and to any kind of biotic and abiotic surfaces, embedded in an extracellular polymeric matrix “House of Biofilm Cells”, provide a strong armor for these bacteria (Flemming *et al.*, 2016). Due to the increased resilience of this bacterial association and its ability to survive harsh environmental conditions and to tolerate high concentration of antimicrobial agents as well as to escape from the host immune response, a great effort is devoted to the search for new approaches in an attempt to prevent and/or treat biofilm-associated infections (Uruen *et al.*, 2020). This approach also concerns the biopesticides concept by using anti-biofilm properties to combat plant infections. In this context, seaweed present a strong promises given their ability to control their bacterial colonization despite the abundance of bacteria in seawater, hence the conduct of this study (Shannon & Abu-Ghannam, 2016). This second part of the project was conducted in the “Laboratoire de Génie Chimique” (LGC-UMR5503) – Toulouse – France.

It is important to note that the choice of the three seaweed species examined in this study is based on their wide spectrum of demonstrated biological activity such as antimicrobial, antioxidant, antidiabetic, anti-inflammatory, and cytotoxic activity (Guner *et al.*, 2019; Salim *et al.*, 2020; Ismail *et al.*, 2020).

However, to the best of our knowledge, no previous study has assessed the insecticidal activity against the fruit fly *D. melanogaster* as well as the antibiofilm activity against *P. aeruginosa* and *S. aureus* of some extracts derived from these algae.

The present manuscript is composed of three chapters arranged as follows:

- I. The first chapter is dedicated to a literature review outlining the background of this study and divided into three distinct parts. In the first section (Part I), the benefits of algae as well as their possible applications in different fields with emphasis on the three seaweed examined in this study (green alga *U. lactuca*, brown alga *S. scoparium*, and red alga *P. capillacea*) are presented. On the other hand, the harmful effects of synthetic agrochemicals on environment and public health as well as the importance of biopesticides in the search for novel alternatives with focus on those derived from marine organisms, especially from seaweed, are reported in the second part (Part II). Then, in the third part (Part III), an overview on biofilms, their various resilience mechanisms as well as the different therapeutic approaches developed in order to control biofilms formation are outlined. The promising role of natural medicine in the search for novel and effective antibiofilm agents is also highlighted.
- II. The evaluation of the insecticidal activity of extracts derived from the green alga *U. lactuca* against the fruit fly *D. melanogaster* which resulted in a published article (Rima *et al.*, 2021) is presented in the second chapter of this manuscript.
- III. The third chapter which is devoted to the evaluation of the potential antibiofilm activity of various extracts derived from the three seaweed (green alga *U. lactuca*, brown alga *S. scoparium*, and red alga *P. capillacea*) is divided into three parts. The first section (Part I) groups all materials and methods used in this chapter. Then, in the second part (Part II), results obtained upon the evaluation of the antibiofilm activity of extracts against *P. aeruginosa* and which resulted in a submitted article, are presented. The promising antibiofilm activity of extracts against *S. aureus* and which also resulted in an article (to be submitted), are described in the third part (Part III) of this chapter.

At the end of the manuscript, a general conclusion with some perspectives are outlined.

Part I:

Seaweed: an underwater treasure trove of multiple benefits

“Focus on the three algae explored in this study”

▪ PREVIEW

Although most people do not imagine it, seaweed extracts are part of the composition of many products that we use or consume daily such as toothpaste, deodorizer, ice cream as well as bottled chocolate drinks. Interestingly, the possible applications of algae are not restricted to a particular field, but various studies have documented their amazing properties to use in pharmaceutical, cosmetical, nutraceutical and even in agricultural sectors. Among their broad spectrum of demonstrated biological activities, a wide variety of compounds derived from seaweed have exhibited interesting antimicrobial activities. Thus, seaweed offer a natural resource of unique bioactive products to maintain and preserve.

In this first part of chapter I, overview of algae as well as their potential benefits in different fields are introduced with a focus on pharmaceuticals. As the seaweeds evaluated in this study are collected from a Lebanese coast, the actual exploitation of Lebanese algae and their demonstrated biological activities are reviewed.

On the other hand, the green *Ulva lactuca*, the brown *Stypocaulon scoparium* and the red *Pterocladia capillacea* algae involved in this study are presented along with a summary of the previous studies showing potential biological activities especially regarding different types of extracts. The region of sample collection is also indicated given the high impact of environmental conditions related to the location of harvesting on the chemical composition of algae and thus their activity.

I. SEAWEED: MYRIAD OF BENEFITS IN VARIOUS FIELDS

I.1 Initiation of marine resources exploitation

The initiation of marine world exploitation as a valuable source of natural products with high pharmaceutical relevance was first launched in 1967 during a conference named “Drugs from the Sea” held in Rhode Island, USA. Since then, the search for primary and secondary metabolites derived from marine organisms has received worldwide attention in view of new drug discovery (Nogueira & Teixeira, 2016). After extensive efforts of many researchers from around the world who were dedicated to the isolation and identification of novel marine natural products as well as to the evaluation of their potential bioactivity, approximately 28,500 bioactive products derived from marine organisms were characterized by the end of 2016 (Jimenez, 2018; Blunt *et al.*, 2018).

Interestingly, in 2004, the Food and Drug administration (FDA) authorized the first drug directly derived from a marine organism particularly from a cone snail and that is used for the treatment of chronic pain. At present, there are six therapeutic structures based on natural marine products that have been approved by the FDA (Jimenez, 2018).

In this context, the marine world, which hosts a huge species diversity producing a variety of bioactive metabolites, ensures a promising gateway in the search for novel cost-effective and highly efficient drugs. Among prokaryotic as well as eukaryotic creatures, seaweed, the primary producers occupying the base of the marine food chain, are known as a valuable reservoir of bioactive products already used for different purposes ranging from food applications to medicine (Leandro *et al.*, 2019).

I.2 What are seaweed?!

Seaweed also named “macroalgae” are macroscopic, multicellular, autotrophic, ubiquitous organisms that can be found in any wet environment as well as in fresh and salt-water. In seawater, they often inhabit shallow coastal areas by growing on rocks, pebbles, shells and even on aquatic plants. Based on the color of their thallus provided by their distinctive pigments, macroalgae are taxonomically classified into three large groups: Chlorophyceae (green algae), Phaeophyceae (brown algae) and Rhodophyceae (red algae) (Leandro *et al.*, 2019; Nakhate & van der Meer, 2021). The typical characteristics as well as the pigments associated with each group are summarized in the table below (*Table 1*).

TABLE 1 | Typical characteristics of the three algae groups (Leandro *et al.*, 2019; Salehi *et al.*, 2019; Nakhate & van der Meer, 2021).

Components	Green seaweed (Chlorophyceae)	Brown seaweed (Phaeophyceae)	Red seaweed (Rhodophyceae)
Pigments	– Chlorophylls <i>a</i> and <i>b</i> – Carotene – Xanthophylls	– Chlorophylls <i>a</i> and <i>c</i> – Carotenoids – Fucoxanthin (brown color)	– Chlorophylls <i>a</i> and <i>d</i> – Carotenoids – Phycoerythrin (red color)
Water content	60 – 80%	50 – 75%	60 – 88%
Total carbohydrates	29.8 – 58.1%	12.2 – 56.4%	34.6 – 71.2%
Proteins	15 – 25%	4 – 10%	8 – 40%
Total lipids	0.2 – 4.1%	0.3 – 4.5%	0.12 – 3.8%
Minerals	11 – 73%	17 – 44%	7 – 37%
Other	Xylan 30 – 40%	Fuoidan 4 – 10%	Xylan 20 – 40%

Besides the primary metabolites (proteins, polysaccharides...) essential for their growth and reproduction, algae possess an extended ability to produce a wide variety of unique and bioactive compounds (phenolic compounds, sterols...) that are not found in terrestrial organisms. In fact, being sessile organisms raises the risk of encountering biotic (predators, bacteria, virus, or fungal infections) and abiotic (salinity, environmental pollutants, temperature changes...) threats. For this reason, seaweed have evolved powerful defensive mechanisms that require the synthesis of a heterogeneous group of bioactive compounds in order to sustain their versatile nature (Leandro *et al.*, 2019).

I.3 Seaweed applications

Even if there is still a lot to investigate and explore about these marine organisms, many studies have revealed the ability of their naturally synthesized molecules to offer a wide array of applications such as in human food, animal feed, pharmacy, cosmetic, agriculture, biofuels and other chemical industries (*Figure 2*) (Leandro *et al.*, 2019; Nakhate & van der Meer, 2021).

In fact, given their high nutritional value thanks to their richness in vitamins, minerals and dietary fibers along with their low caloric intake, seaweed known as “Sea vegetables” are widely consumed as a healthy meal especially in Asian countries such as China and Japan. Not only for human but seaweed have also been used for longtime in animal feed providing digestive and immune benefits. In addition, they have traditionally been used in agriculture to fertilize the fields and thus promote the growth and the productivity of plants (Leandro *et al.*, 2019).

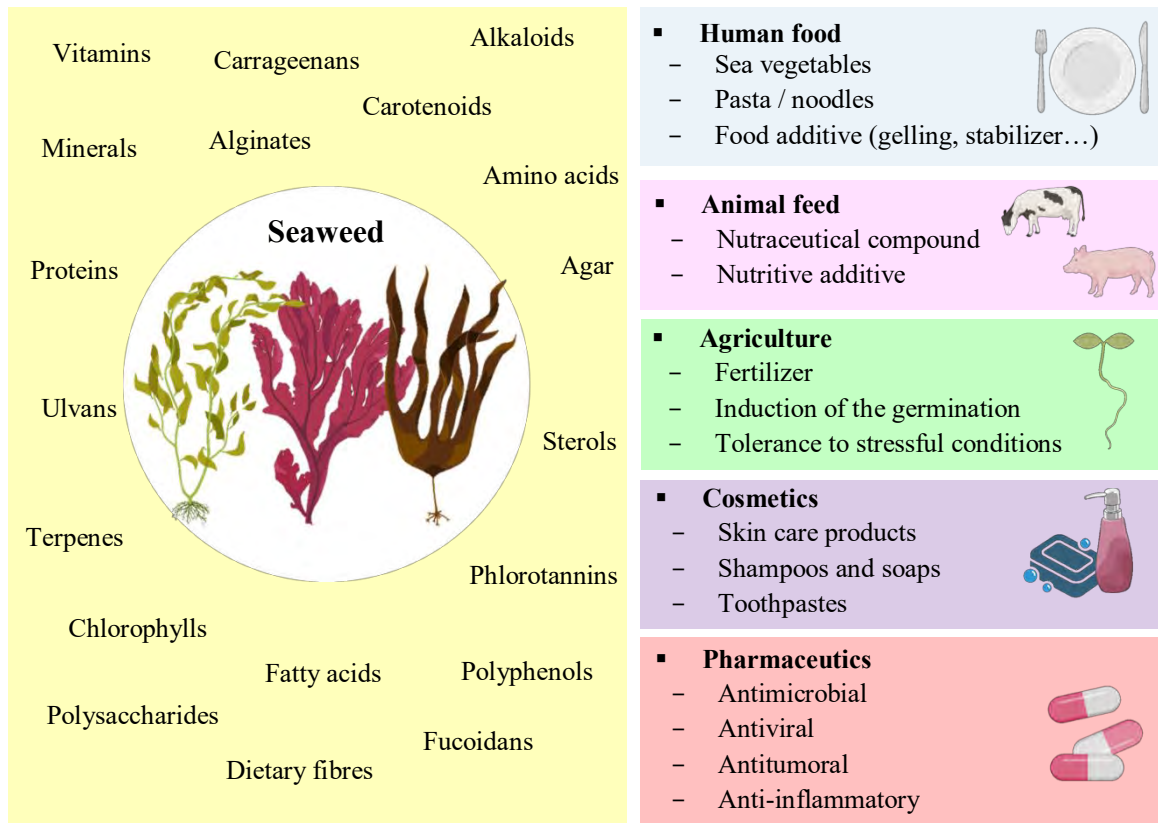


FIGURE 2 | Non-exhaustive summary of the possible seaweed applications.

On the other hand, algae are known as a valuable source of active-based natural ingredients widely exploited by the cosmetic industries in an effort to respond to the increasing demand for “natural” cosmetic products with reduced chemical toxicity in comparison with conventional cosmetics products (“synthetic” chemical agents) (Leandro *et al.*, 2019).

Interestingly, the incredible properties of algae do not stop here but they extend to cover medical and pharmaceutical fields as well. In fact, extensive researches carried out over the past few decades have reported a large spectrum of biological activities (anti-oxidant, antimicrobial, anti-inflammatory, antitumoral...) exhibited by several compounds derived from seaweed such as polysaccharides, fatty acids, polyphenols and pigments...(Michalak & Chojnacka, 2015; Silva *et al.*, 2020).

I.4 Seaweed of the Lebanese coasts: an endless richness

The exploitation of the Mediterranean Lebanese coast has evidenced its huge richness in a large diversity of algae widely distributed from the northern coasts to the southern ones. In fact, the discovery mission conducted by (Kanaan *et al.*, 2014) throughout the Lebanese coastline has led to the identification of 94 species of algae belonging to the different groups with nearly the half of them are red seaweed.

Despite their diversity, studies that focus on the investigation of Lebanese seaweed in view of their potential capacity to be valorized in different fields remain limited. Nevertheless, some studies have evaluated the potential biological activities (in-vitro) of extracts and/or compounds (especially polysaccharides) derived from some Lebanese algae (*Table 2*). However, there is a lot to be achieved in an attempt to advance towards a real application of these seaweed products in the pharmaceutical field.

TABLE 2 | Summary of the previous *in vitro* studies conducted on Lebanese seaweed with the demonstrated biological activities of their extracts/compounds.

Seaweed species	Evaluated fraction	Demonstrated biological activities	Reference
<i>Laurencia obtusa</i> (Red alga)	Protein fraction	<ul style="list-style-type: none"> - Antioxidant activity - Antiproliferative activity (Human colorectal cancer cells HCT 116) 	(Al Monla <i>et al.</i> , 2021)
<i>Padina pavonica</i> (Brown alga)	<u>Organic extracts:</u> <ul style="list-style-type: none"> - Petroleum ether - Chloroform - Methanol 	<ul style="list-style-type: none"> - Antibacterial activity against <i>E.coli</i>, <i>P. aeruginosa</i>, <i>K. pneumonia</i>, <i>P. vulgaris</i> and <i>E. faecalis</i> 	(Chbani <i>et al.</i> , 2011)
<i>Colpomenia sinuosa</i> (Brown alga)	<u>Organic extracts:</u> <ul style="list-style-type: none"> - DCM : MeOH - MeOH 	<ul style="list-style-type: none"> - Antiproliferative activity (Human colorectal cancer HCT 116 and breast cancer cells MCF-7) - Antioxidant activity - Anti-inflammatory activity - Antibacterial activity (<i>P. aeruginosa</i>, <i>S. aureus</i>, <i>B. subtilis</i>, <i>P. vulgaris</i> and <i>E. faecalis</i>) 	(Al Monla, Dassouki, Kouzayha, <i>et al.</i> , 2020; Al Monla, Dassouki, Gali-Muhtasib, <i>et al.</i> , 2020)
<i>Dictyopteris polypodioides</i> (Brown alga)	<u>Polysaccharides:</u> <ul style="list-style-type: none"> - Fucoidan - Laminaran - Mannuronan 	<ul style="list-style-type: none"> - Antioxidant activity - Anticoagulant activity - Antiproliferative activity (Human melanoma cells RPMI-7951) 	(Sokolova <i>et al.</i> , 2011; Karaki <i>et al.</i> , 2013)
<i>Corallina</i> (Red alga)	<u>Polysaccharides:</u> <ul style="list-style-type: none"> - Sulfated galactans - Carrageenan 	<ul style="list-style-type: none"> - Anticoagulant activity - Antibacterial activity (<i>S. epidermidis</i> and <i>E. faecalis</i>) 	(Sebaaly <i>et al.</i> , 2014)
<i>Stypodium schimperi</i> (Brown alga)	<u>Polysaccharides:</u> <ul style="list-style-type: none"> - Fucoidan - Sodium alginate 	<ul style="list-style-type: none"> - Antioxidant activity - Antiproliferative activity (Human colorectal cancer cells HCT 116) 	(Haddad <i>et al.</i> , 2017)
<i>Pterocladia</i> (Red alga)	<u>Polysaccharides:</u> <ul style="list-style-type: none"> - Sulfated galactans - Carrageenan 	<ul style="list-style-type: none"> - Antioxidant activity - Anticoagulant activity 	(Sebaaly <i>et al.</i> , 2012)

I.5. The “Sea Lettuce” *Ulva lactuca*: wide range of potential applications

I.5.1 Overview

Ulva lactuca commonly known as “Sea Lettuce” is a green macroalga that belongs to the family of Ulvaceae and was described in 1753 by Linnaeus in the Baltic sea (Figure 3). This cosmopolitan green seaweed is widely distributed throughout the world and it usually inhabits rocky shores in the littoral and sublittoral zones of coastal areas. Besides its ability to grow attached to a substratum, *U. lactuca* can also live as free-floating alga (Dominguez & Loret, 2019).



FIGURE 3 | Classification of the green alga *Ulva lactuca* (AlgaeBase, 2021).

As an edible species with a fruitful taste, *U. lactuca* is extensively consumed in salads and soups especially in Asian countries. Moreover, it is used in Chinese medicine as a food supplement with a prehistoric record in the treatment of urinary diseases as well as of hyperlipidemia and sunstroke (Yu-Qing *et al.*, 2016).

On the Lebanese coast, *U. lactuca* was first found in 1991 at Beirut. Nowadays, it invades the whole Lebanese shores by growing on vermetid reefs and in shallow habitats (Bitar *et al.*, 2017).

I.5.2 Chemical composition and potential riches of *U. lactuca*

The chemical composition and the nutritional properties of the green alga *U. lactuca* have been documented in several studies based on algae harvested from different regions. Although this constitution varies according to the geographical origin, the seaweed physiological maturity as well as to the variation of environmental conditions and collecting season, the reported data have highlighted the nutritional value of this alga (Yu-Qing *et al.*, 2016).

In fact, the richness of *U. lactuca* green alga in essential minerals (magnesium, iron, calcium, potassium...), in vitamins (B1, B2, B12, C...), in good unsaturated fatty acids, in dietary fiber, as well as in proteins, make it an excellent food with high nutritional value along with a low-fat intake (Yu-Qing *et al.*, 2016; Dominguez & Loret, 2019).

On the other hand, algae belonging to the *Ulva* genus including *U. lactuca* are mostly exploited for their high content in ulvan, sulfated heteropolysaccharide, that accounts to almost 30% of *Ulva* dry weight. Owing to its antiviral, antitumor, anticoagulant, antioxidant and even antidepressant proprieties demonstrated in various studies, this polysaccharide is increasingly requested for pharmaceutical and food purposes (Kidgell *et al.*, 2019). Despite its widely documented biological activities (*in vitro* and *in vivo*) as well as its demonstrated ability to be used in pharmaceutical formulations (polymers, excipients...) and in bone tissue engineering (ulvan-based hydrogels...), further clinical studies are required prior to its real application (Cindana Mo'o *et al.*, 2020).

In addition, *U. lactuca* contains phenolic compounds as well as chlorophylls and carotenoids pigments that can serve as free-radical scavengers (Dominguez & Loret, 2019). Not only for human, but *U. lactuca* can also be valued in animal feed, in agriculture as well as in biofuels production (Dominguez & Loret, 2019).

1.5.3 Review of previous studies conducted on *U. lactuca* seaweed

Various studies have highlighted the considerable bioactivity of extracts derived from the green alga *U. lactuca* collected from different regions. *Table 3* presents a summary of studies conducted on this green alga with emphasis on the region of sample collection, the type of extracts as well as on the demonstrated biological activities (antibacterial, antifungal, antiviral, antioxidant, cytotoxic...). It should be noted that in some studies, the chemical composition of the active extracts has been elucidated particularly by GC-MS analysis.

However, the exploitation of this green alga in terms of its capacity to be valorized in the pharmaceutical field requires more efforts mainly by going further in studies (elucidation of mechanisms of action, implementation of in-vivo assays...) in order to pave the way towards concrete applications. On the other hand, if it is done, the identification of compounds involved in the bioactivity of extracts is mostly based on supposition with no confirmation of the real implication of these identified molecules in the demonstrated biological activity.

TABLE 3 | Non-exhaustive summary of the previous *in vitro* studies conducted on the green alga *U. lactuca*. DCM and MeOH are dichloromethane and methanol, respectively.

Origin	Extraction method	Extraction solvent	Demonstrated biological activities	Compounds identified in extracts by analytical methods	Reference
Lebanon	Protein fraction		<ul style="list-style-type: none"> - Antioxidant activity - Cytotoxic activity against two cancer cells lines (human colorectal cancer cells HCT-116 and epithelioid carcinoma Hela) 	-	(Al Monla <i>et al.</i> , 2021)
Lebanon	Maceration	<ul style="list-style-type: none"> - Acetone - Ethanol - Ethyl acetate - Water 	<ul style="list-style-type: none"> - Antifungal activity against <i>Penicillium digitatum</i> 	-	(Salim <i>et al.</i> , 2020)
Egypt	Maceration	<ul style="list-style-type: none"> - Chloroform 	<ul style="list-style-type: none"> - Antibacterial activity against <i>K. pneumoniae</i> and <i>P. mirabilis</i> - Antifungal activity against <i>Aspergillus</i> species - Antioxidant activity - Cytotoxic activity against different human cancer cells lines (breast cancer MCF-7, prostate cancer PC3, hepatocellular carcinoma HepG2 and epithelioid carcinoma Hela) 	<u>Identified compounds by GC-MS:</u> <ul style="list-style-type: none"> - 2-Allyl-2-methyl-1,3-cyclopentanedione - Cyclododecanemethanol - Diisooctyl phthalate 	(Saeed <i>et al.</i> , 2019)
Oman	Maceration	<ul style="list-style-type: none"> - Methanol - Water 	<ul style="list-style-type: none"> - Antioxidant activity - Antibacterial activity against <i>E. coli</i> and <i>S. typhi</i> 	GC-MS analysis of MeOH extract	(Anjali <i>et al.</i> , 2019)
India	Soxhlet apparatus	<ul style="list-style-type: none"> - Hexane - Ethyl acetate - Chloroform - Methanol 	<ul style="list-style-type: none"> - Antifungal activity against <i>Aspergillus niger</i> and <i>Penicillium janthinellum</i> 	GC-MS analysis of all prepared extracts	(Barot <i>et al.</i> , 2016)
India	Hot maceration	<ul style="list-style-type: none"> - Water 	<ul style="list-style-type: none"> - Antibacterial and antibiofilm activities against <i>Bacillus</i> species, <i>E. coli</i> and <i>P. vulgaris</i> - Larvicidal activity against <i>Aedes aegypti</i> 	-	(Ishwarya <i>et al.</i> , 2018)
Indonesia	Maceration	<ul style="list-style-type: none"> - Ethyl acetate - Hexane - Ethanol 	<ul style="list-style-type: none"> - Cytotoxic activity against two cancer cells line (human colorectal cancer cells HCT-116 and breast cancer cells MCF-7) 	-	(Arsianti <i>et al.</i> , 2016)
Egypt	Maceration	<ul style="list-style-type: none"> - Ethanol 	<ul style="list-style-type: none"> - Antibacterial activity against <i>E. coli</i>, <i>K. pneumonia</i> and <i>P. mirabilis</i> 	Di-(2-ethylhexyl)-phthalate	(El Shouny <i>et al.</i> , 2017)

TABLE 3 | Continued.

India	Maceration	- Methanol	- Antibiofilm activity against <i>Vibrio</i> species, <i>P. aeruginosa</i> , <i>S. aureus</i> and other pathogenic bacteria	-	(Yuvaraj & Arul, 2014)
Morocco	Maceration	- DCM : MeOH	- Larvicidal activity against <i>Artemia salina</i>	-	(Oumaskour <i>et al.</i> , 2017)
Egypt	Soxhlet apparatus	- Petroleum ether - Chloroform - Acetone - Ethanol - Methanol	- Insecticidal activity against <i>Culex pipiens</i> and <i>Spodoptera littoralis</i> - Antifungal activity against <i>Aspergillus niger</i> , <i>Penicillium digitatum</i> , and <i>Rhizoctonia solani</i>	<u>Identified compounds by GC-MS analysis of MeOH extract:</u> - 1,2-Benzenedicarboxylic acid, bis (2-ethylhexyl) ester - Palmitic acid - Benzene, 1,2,4-trimethyl - 8-Octadecanoic acid methyl ester - Benzene, 1-ethyl 2-methyl	(Abbassy <i>et al.</i> , 2014)
India	Soxhlet apparatus	- Hexane - Chloroform - Ethyl acetate - Acetone - Methanol	- Antifungal activity against <i>Candida</i> species.	-	(Raj <i>et al.</i> , 2017)
India	Maceration	- Methanol	- Antioxidant activity - Antibacterial activity against <i>P. aeruginosa</i> , <i>S. aureus</i> , <i>E. coli</i> ... - Antifungal activity against <i>Aspergillus</i> species and <i>C. albicans</i>	GC-MS analysis	(Alagan <i>et al.</i> , 2017)
India	Maceration	- Ethanol - Water	- Antidiabetic activity	-	(Reka <i>et al.</i> , 2017)
Saudi Arabia	Maceration	- Methanol	- Antibacterial activity against <i>P. aeruginosa</i> and <i>S. aureus</i>	-	(Al-Zahrani <i>et al.</i> , 2017)
South Africa	Sulfated polysaccharides		- Antioxidant activity - Cholinesterase inhibitory activity	-	(Olasehinde <i>et al.</i> , 2019)
Egypt	Maceration	- Ethyl acetate - Methanol	- Antifungal activity against <i>Fusarium</i> species, <i>Trichoderma hamatum</i> , <i>Aspergillus flavipes</i> , and <i>Candida albicans</i>	GC-MS analysis	(Shobier <i>et al.</i> , 2016)
Taiwan	Sulfated polysaccharides		- Antiviral activity against Japanese Encephalitis Virus	-	(Chiu <i>et al.</i> , 2012)

I.6 The “Sea broom” *Stypocaulon scoparium*: insufficiently explored benefits

I.6.1 Overview

Stypocaulon scoparium (Linnaeus) Kützing, 1843 known as “Sea broom” is a brown macroalga that belongs to the family of Stypocaulaceae (Figure 4). This seaweed presents a rigid thallus covered with filamentous branches. It usually grows attached to rocks and forms a beautiful fluffy clumps in shallow water. The sea broom is found in the Mediterranean, in the Black sea as well as in the Atlantic (MACOI, 2008). According to our knowledge, the distribution of *S. scoparium* brown alga throughout the Lebanese coasts as well as the date of its first recognition in Lebanon are not communicated.



- **Phylum:** Ochrophyta
- **Class:** Phaeophyceae
- **Order:** Sphacelariales
- **Family:** Stypocaulaceae
- **Genus:** *Stypocaulon*
- **Species:** *scoparium*

FIGURE 4 | Classification of the brown alga *Stypocaulon scoparium* (AlgaeBase, 2021).

I.6.2 Chemical composition

The analysis of the chemical composition of *S. scoparium* brown alga is not well documented in the literature due to the scarcity of studies that focus on this seaweed. Nevertheless, basic algal constituents such as carbohydrates, proteins, phenolic compounds and pigments have been detected and quantified in *S. scoparium* collected from the Northeastern Mediterranean coast of Turkey (Ozgun & Turan, 2015). On the other hand, the study conducted by (Ragonese *et al.*, 2014) has highlighted the great variety of fatty acids and triacylglycerols structures contained in this brown seaweed collected from Italy. Phospholipids such as phosphatidylserine and phosphatidylcholine have been detected as well.

1.6.3 Review of previous studies conducted on S. scoparium seaweed

Although the number of studies that have evaluated the potential bioactivity of this brown alga is limited, some biological proprieties such as antioxidant, antibacterial as well as cytotoxic activities have been demonstrated for its extracts (*Table 4*).

However, the exploitation of this brown seaweed deserves more consideration since there are only a few studies that have focused on the evaluation of its potential bioactivity. In addition, advanced experiments are required in an attempt to include this alga in medical applications.

TABLE 4 | Non-exhaustive summary of the previous *in vitro* studies conducted on the brown alga *S. scoparium*. MeOH, CHCl₃, H₂O are methanol, chloroform and water, respectively.

Origin	Extraction method	Extraction solvent	Demonstrated biological activities	Compounds identified in extracts by analytical methods	Reference
Turkey	Maceration	- MeOH : CHCl ₃ - Hexane	- Acetylcholinesterase inhibitory activity - Antiprotozoal activity against <i>Trypanosoma</i> species	-	(Cinar <i>et al.</i> , 2019)
Turkey	Ultrasound assisted maceration	- Hexane - Chloroform - Methanol	- Antioxidant activity - Cytotoxic activity against human cancer cells lines (breast cancer cells MCF-7 and colorectal cancer cells CaCo-2)	<u>HPLC profiles using standards:</u> - <u>Hexane</u> : caffeic acid, p-coumaric acid and quercetin - <u>Chloroform</u> : p-coumaric acid - <u>Methanol</u> : gallic acid	(Guner <i>et al.</i> , 2019)
Spain	Maceration	- Methanol - Ethanol - Water - MeOH : H ₂ O	- Antioxidant activity	Quantification of several polyphenols by HPLC using standards	(López <i>et al.</i> , 2011)
Algeria	Maceration	- Methanol	- β-lactamase inhibitory activity	<u>Identified compound by HPLC-ESI-MS:</u> - α-linolenic acid - Linoleic acid - Oleic acid - Arachidonic acid	(Houchi <i>et al.</i> , 2019)
Portugal	Hot maceration	- Ethanol - Water	- Antioxidant activity - Anti-inflammatory activity	-	(Campos <i>et al.</i> , 2018)
Turkey	Soxhlet apparatus	- Methanol	- Antibacterial activity against <i>S. aureus</i> , <i>P. aeruginosa</i> , <i>E. coli</i> , <i>K. pneumonia</i> ... - Antifungal activity against <i>Candida albicans</i> , <i>Cryptococcus neoformans</i> ...	-	(Dulger <i>et al.</i> , 2009; Taskin <i>et al.</i> , 2011)

I.7 The “Wing weed” *Pterocladia capillacea*: a valuable agarophyte

I.7.1 Overview

Pterocladia capillacea known as “Wing weed” or “Small agar weed” is a red macroalga that belongs to the family of Pterocladaceae (Figure 5). This seaweed described by S.G. Gmelin in 1768 (initially named *Fucus capillaceus*) appears as a reddish-brown filamentous clumps. *P. capillacea* is a worldwide distributed species found in tropical as well as in temperate waters. It usually grows attached to rocks in the low intertidal and shallow subtidal areas (Patarra *et al.*, 2019).



- **Phylum:** Rhodophyta
- **Class:** Florideophyceae
- **Order:** Gelidiales
- **Family:** Pterocladaceae
- **Genus:** *Pterocladia*
- **Species:** *capillacea*

FIGURE 5 | Classification of the red alga *Pterocladia capillacea* (AlgaeBase, 2021).

Interestingly, this seaweed has traditionally been used in the production of edible jellies especially in Asian countries such as Japan, China and Korea. Moreover, according to our knowledge, *P. capillacea* is the only *Pterocladia* species that is harvested for commercial purposes. In fact, this red alga is well known as natural source of high quality agar and agarose used in biomedical, biotechnological and pharmacological applications, hence the name of “agarophyte” (Patarra *et al.*, 2019).

Regarding its distribution throughout the Lebanese coasts, *P. capillacea* red alga has been detected on the northern as well as on the southern shores (Kanaan *et al.*, 2014).

I.7.2 Chemical composition

The chemical composition of the red alga *P. capillacea* has been reported in various studies (Patarra *et al.*, 2019). Indeed, its considerable content in proteins, dietary fibers, vitamins, and in essential minerals especially in calcium, emphasizes the relevance of this seaweed in the nutraceutical industry (Patarra *et al.*, 2019; Penalver *et al.*, 2020).

In addition, polyunsaturated fatty acids, chlorophylls and carotenoids pigments, as well as phenolic compounds have also been detected and quantified in this alga (Mohy El-Din & El-Ahwany, 2018).

As mentioned above, the red alga *P. capillacea* is mostly exploited for its agar content which is characterized by a high gelling strength along with low gelling temperatures. In fact, analyzes performed on algae samples collected from different regions have revealed that the quality as well as the quantity of the extracted agar which varies between 5 and 34% are site and harvest season specific (Patarra *et al.*, 2019).

1.7.3 Review of previous studies conducted on P. capillacea seaweed

Some studies have investigated the potential biological activities (antioxidant, anti-inflammatory, antimicrobial, ...) of *P. capillacea* red alga. As shown in the table below (*Table 5*) most studies have focused on the evaluation of polysaccharide fractions or polar extracts of which the chemical composition has sometimes been elucidated. As for the two algae presented below, it is essential to go further in the experiments.

TABLE 5 | Non-exhaustive summary of the previous *in vitro* studies conducted on the red alga *P. capillacea*. MeOH and DCM are methanol and dichloromethane, respectively.

Origin	Extraction method	Extraction solvent	Demonstrated biological activities	Compounds identified in extracts by analytical methods	Reference
Egypt	Extraction of sulfated polysaccharides		<ul style="list-style-type: none"> - Antioxidant activity - Anti-inflammatory activity - Anticoagulant activity - Antibacterial activity against <i>B. subtilis</i> and <i>S. aureus</i> - Antifungal activity against <i>Aspergillus niger</i>, <i>Penicillium decubens</i>... - Antifouling activity 	-	(Ismail & Amer, 2020)
Tunisia	Maceration	- Methanol	<ul style="list-style-type: none"> - Antibacterial activity against <i>Pseudomonas cepacia</i> and <i>Streptococcus B</i> - Antifungal activity against <i>Candida albicans</i> 	-	(Hmani <i>et al.</i> , 2021)
Egypt	Maceration	<ul style="list-style-type: none"> - Acetone - Ethanol - Methanol - Water 	<ul style="list-style-type: none"> - Antioxidant activity - α-amylase inhibitory activity - α-glucosidase inhibitory activity 	-	(Ismail <i>et al.</i> , 2020)
Egypt	Maceration	- Methanol	- Antifungal activity against <i>Fusarium</i> species, <i>Trichoderma hamatum</i> , <i>Aspergillus flavipes</i> , and <i>Candida albicans</i>	GC-MS analysis	(Shobier <i>et al.</i> , 2016)
Brazil	Maceration	- DCM : MeOH	- Antifungal activity against <i>Colletotrichum</i> species	<u>Identified compounds by GC-MS analysis:</u> <ul style="list-style-type: none"> - Hexadecanoic acid - Cholesterol - Quercetin 	(Machado <i>et al.</i> , 2014)
Egypt	Maceration	<ul style="list-style-type: none"> - Chloroform - Acetone - Ethanol - Methanol 	<ul style="list-style-type: none"> - Antioxidant activity - Antibacterial activity against <i>Vibrio fluvialis</i> 	GC-MS analysis of ethyl acetate extract	(Mohy El-Din & El-Ahwany, 2018)

TABLE 5 | Continued.

Egypt	Polysaccharides		- Antiviral activity against Hepatitis C virus	-	(Gheda <i>et al.</i> , 2016)
Egypt	Sulfated polysaccharides		- Antioxidant activity	-	(Fleita <i>et al.</i> , 2015)
Egypt	Water soluble polysaccharides		- Antibacterial activity against <i>S. aureus</i> , <i>B. cereus</i> , <i>P. flouresens</i> and <i>S. pyogenes</i> - Cytotoxic activity against human cancer cells lines (breast cancer cells MCF-7 and epithelioid carcinoma Hela) - Anticoagulant activity	-	(Abou Zeid <i>et al.</i> , 2014)
Brazil	Maceration	- DCM : MeOH	- Antiviral activity against <i>Herpes simplex</i>	-	(Soares <i>et al.</i> , 2012)
Egypt	Maceration	- Ethanol	- Antioxidant activity - Anti-inflammatory activity - Antibacterial activity against <i>S. aureus</i>	<u>Identified compounds by UV, IR, ¹H-NMR and ¹³C-NMR:</u> - Diisooctyl phthalate - 24-Norcholest-5-en-3,7-dione - Cholesterol - Stigmasterol - Linoleic acid - Isodomoic acid	(Aboutabl <i>et al.</i> , 2010)

Part II:

Biopesticides: an urgent need for a sustainable and safe agriculture

▪ PREVIEW

The continuous and rapid growth of world population, which is expected to reach approximately 9.7 billion by 2050, is associated with a dramatic increase in food demand (UNDESA, 2015). Therefore, the consumption of chemical fertilizers and pesticides has increased in the past decades in an effort to respond to these growing needs by improving crop yields (Liu *et al.*, 2014). However, the massive use of these chemicals, especially the pesticides applied to protect crops, leads to long-term threats that are posed both to environment and living beings (J. Kumar *et al.*, 2021). In this context, the need for novel, safe, and eco-friendly alternatives to these hazardous chemicals has become a necessity.

In this second part of chapter I, we present an overview of currently used pesticides, the evolution of their worldwide consumption, as well as their harmful effects on environment and public health. In this context, the relevance of biopesticides as an effective alternative to chemical pesticides is emphasized by presenting their various types, as well as their benefits, in comparison with conventional agrochemicals.

II. PESTICIDES: A DOUBLE-EDGED SWORD

II.1.1 History of pesticides consumption

For many thousands of years, the protection of farmed crops against all kinds of pests and diseases has posed a main concern to ancient peoples who have focused on the use of easily obtained and available remedies. The first records of the use of sulphur-containing compounds to combat insects and mites date back over 4500 years. At that time, the phytosanitary products used were mostly of natural origin such as the pyrethrum, derived from the dried flowers of *Chrysanthemum cinerariaefolium*, used for over 2000 years (Unsworth, 2010).

In 1939, the discovery of the insecticidal effect of dichloro-diphenyl-trichloroethane (DDT) by the Swiss chemist Paul Hermann Müller, who was awarded a Nobel Prize for this finding, has presented a remarkable shift in this field. In 1940 and during World War II (1939 - 1945), the use of synthetic chemical pesticides such as DDT, aldrin, parathion..., has peaked in order to boost food production (Gyawali, 2018).

For some decades, the application of pesticides in agriculture was considered beneficial and no interest was shown for the eventual risks of these agrochemicals on public health and environment. However, the book “Silent Spring”, published by the American marine biologist Rachel Carson in 1962, in which she outlined the harmful effects of the indiscriminate use of synthetic pesticides, has reversed the international policy leading to the banning of DDT use in 1972 in the United States, and later in other countries (Carson, 1962 ; Gyawali, 2018). This paved the way for the research of novel safer and eco-friendly products for agriculture and for other uses including domestic one.

II.1.2 Pesticides

A pesticide is defined as a substance, or mixture of compounds, employed to prevent, eradicate and/or repel pests. This term comprises insecticides (insect pest control), herbicides (parasitic plants control), fungicides (harmful microbes control) and any other substance used to control pests (Gyawali, 2018). According to the Food and Agricultural Organization of the United Nations (FAO), the worldwide use of pesticides is continuously increasing over the years, especially in Asian countries (*Figure 6*) (FAO, 2021).

Although 85% of the world’s pesticide consumption is devoted to agriculture, these chemicals are also applied in public health activities to control vector-borne diseases (malaria, dengue...), in gardens and houses in order to avoid the proliferation of undesirable plants and insects, as well as in agro-food sector as a food preservation tool (Kim *et al.*, 2017).

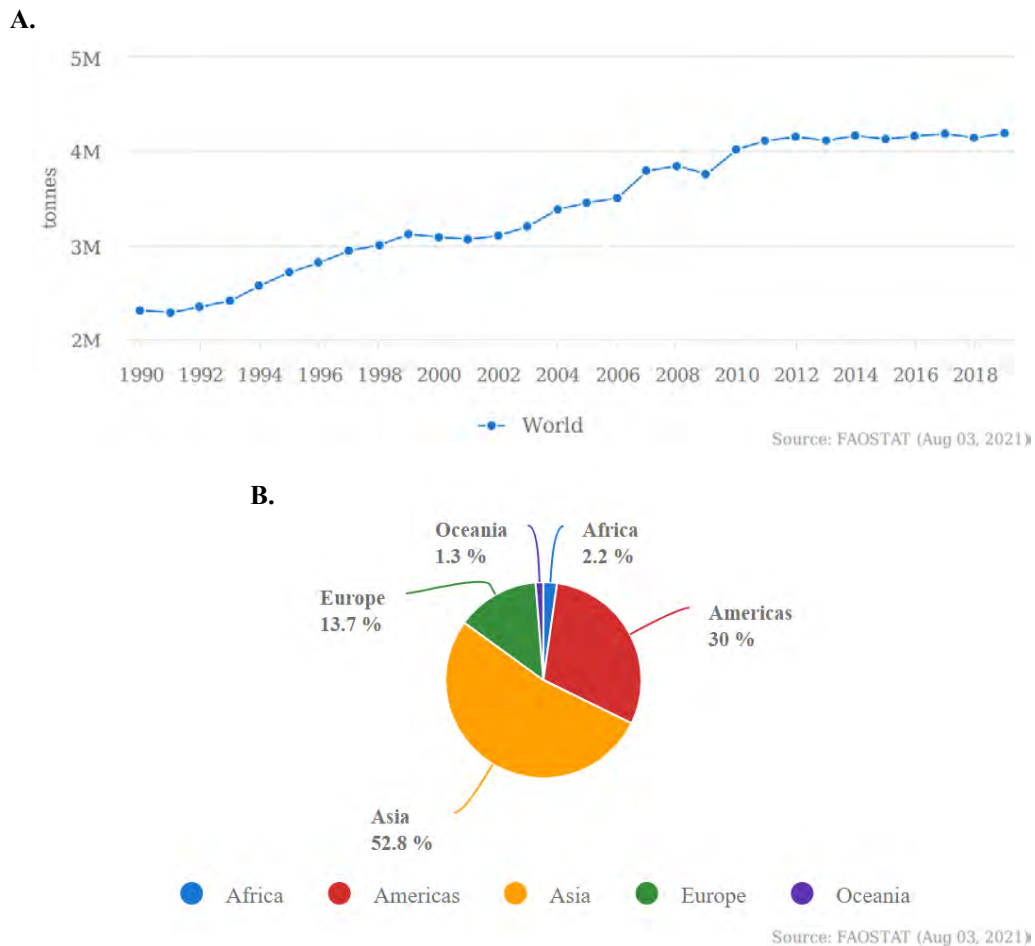


FIGURE 6 | Quantity (in tonnes) of pesticides used in the world from 1990 to 2019 (A). The repartition of the quantity of pesticides used between the different continents (B).

Besides their classification based on the type of pest to be managed (insecticides, herbicides, fungicides, nematocides...), pesticides can be classified according to various criteria such as their level of toxicity (classification recommended by the World Health Organization WHO), their chemical classes (organochlorines, organophosphorus...), and their mode of action (direct contact, oral and/or respiratory entry...) (WHO, 2010; Kim *et al.*, 2017).

II.1.3 Pesticides: undeniable harmful effects

Despite the beneficial outcomes of the use of pesticides in terms of productivity, agriculture commodity, control of vector-borne diseases..., their excessive and unconscious application has led to serious repercussions on the environment and consequently on public health (Gyawali, 2018). Among pesticides, insecticides are known to be the most intensely toxic which has prompted the banning of many of these agrochemicals, especially those belonging to organochlorines class (Rani *et al.*, 2021).

II.1.3.1 Adverse effects of pesticides use on the environment

While pesticides are designed to target a particular group of pests, harmful effects on non-targeted fauna and flora including birds, fish, and beneficial insects, as well as on the various environmental media (soil, water, and air) are known to occur (Rani *et al.*, 2021). In fact, it has been estimated that only 5% of the applied pesticides reach the targeted pests, while more than 95% of these used agrochemicals are able to end up in non-targeted organisms and to disperse and accumulate in the environment, which significantly affects the biodiversity (Farcas *et al.*, 2013).

The accumulation of some pesticides in the environment, such as aldrin and chlordane which belong to the organochlorines class, is due to their content in persistent organic pollutants (POPs). These compounds resist degradation and therefore persist for many years in soils and sediments and can bioconcentrate by up to 70 000-fold relative to their initial concentration (Kim *et al.*, 2017).

Soil contamination by pesticide residues is associated with a considerable decrease in beneficial soil microorganisms leading to a decline in its quality, fertility and productivity (Rani *et al.*, 2021). On the other hand, the high ability of volatile pesticides (around 80 - 90% of the used pesticides) to spread rapidly in the atmosphere leads to the disruption of the whole ecosystem (Gyawali, 2018). In addition, the hazard of the excessive use of pesticides also occurs in the aquatic environment contaminated through runoff, drift and draining. Indeed, the contamination of surface waters by these harmful agrochemicals affects aquatic species at different trophic levels, mainly by decreasing the concentrations of dissolved oxygen (Rani *et al.*, 2021). Moreover, groundwater poisoning by pesticides is known to be a worldwide issue once contaminated, it can require many years for contamination to dissipate or to be cleaned up by expensive and complex techniques (Gyawali, 2018).

II.1.3.2 Adverse effects of pesticides use on human health

Due to their long-term persistence in the environment as well as to their high ability to accumulate in the food chain, the continuous application of conventional chemical pesticides poses a serious threat to public health (Rani *et al.*, 2021). Unfortunately, according to the report of the United Nations Environment Programme (UNEP), approximately 385 million cases of accidental pesticides-related intoxication occur every year, with almost 11,000 deaths (UNEP, 2021).

Exposure to pesticides can happen via various routes such as (Kim *et al.*, 2017):

- (1) Inhalation of volatile components of pesticides,
- (2) Penetration of pesticides into the organism through ingestion, known as the most severe way of pesticides poisoning,
- (3) Dermal absorption of chemical pesticides, the most common and effective route of exposure especially for pesticide applicators.

Exposure to pesticides can lead to both acute and chronic illnesses, whose severity depends not only on the toxicity of agrochemicals, but also on the intake dose, the route of exposure, the duration of exposure as well as on the age since children and elderly people are more sensitive than others (Kim *et al.*, 2017). In fact, several studies have highlighted the relation between pesticides exposure and various health disorders such as cancer, diabetes, respiratory (asthma, bronchitis...) and neurological (Parkinson, Alzheimer...) issues, as well as reproductive syndromes (Rani *et al.*, 2021). In this context, Shah *et al.*, have demonstrated the link between pesticide exposure and the increased risk of Epithelial Ovarian Cancer by showing the ability of some organochlorine pesticides such as β -hexachlorocyclohexane and Dieldrin to stimulate the production of cellular ROS (reactive oxygen species), to induce an inflammatory response, as well as to damage the DNA of human ovary surface epithelial cells (Shah *et al.*, 2020). In addition, pesticides can create critical problems for pregnant women due to their ability to cross the placenta resulting in structural and functional defects in the foetus (Woodruff *et al.*, 2008).

II.2 BIOPESTICIDES: AN INTERESTING ALTERNATIVE TO CHEMICAL PESTICIDES

II.2.1 Biopesticides definition

Biopesticides or biological pesticides are defined as natural products derived from living organisms such as plants, animals, nematodes, microorganisms, ... and which are used in order to control agricultural pests (arthropods, nematodes, mollusks), and plant diseases (Figure 7) (Samada & Tambunan, 2020).

In view of the effectiveness of these natural products in controlling pests as well as in generating sustainable agricultural products, the production of biopesticides is rising at an annual rate of 20% (Leng *et al.*, 2011). Indeed, it is estimated that the market size of biopesticides will equalize with that of chemical pesticides between the late 2040s and the early 2050s (Damalas & Koutroubas, 2018).






The different categories of biopesticides			
Microorganisms <ul style="list-style-type: none"> - <i>Bacillus</i> spp. - <i>Pseudomonas</i> spp. - <i>Streptomyces</i> spp. - <i>Chromobacterium subtsugae</i> - <i>Trichoderma</i> spp. - <i>Burkholderia rinojensis</i> - <i>Metarhizium brunneum</i> - <i>Beauveria bassiana</i> 	Botanicals <ul style="list-style-type: none"> - Azadirachtin - Essential oils - Terpenes - Plant extracts - Pyrethrins 	Toxins <ul style="list-style-type: none"> - Avermectins - Spinosad - Spider venom peptide 	Others <ul style="list-style-type: none"> - Potassium salts of fatty acids - Acetic and citric acids - Minerals
Target pests			
Arthropods <ul style="list-style-type: none"> - Borers - Defoliators - Gall-makers - Root feeders - Miners - Webbers - Sucking pests 	Diseases <ul style="list-style-type: none"> - Mold - Rot - Rust - Spot - Wilt - Smut - Mildew 	Nematodes <ul style="list-style-type: none"> - Root knot nematode - Bulb and stem nematode - Dagger nematode - Spiral nematode - Sting nematode - Reniform nematode - Lesion nematode 	Molluscs <ul style="list-style-type: none"> - Snails - Slugs 
			Weeds <ul style="list-style-type: none"> - Annual weeds - Biennial weeds - Perennial weeds 

FIGURE 7 | Different categories of biopesticides and their target pests. Adapted from (Dara, 2021).

II.2.2 Biopesticides vs chemical pesticides

Compared to conventional chemical pesticides, biopesticides are expected provide various merits that occur at different levels (*Table 6*) (J. Kumar *et al.*, 2021). Interestingly, biopesticides are formulated to affect a target species which minimizes their risk on non-target organisms such as mammals, birds or beneficial insects (Prabha *et al.*, 2016; Samada & Tambunan, 2020). Furthermore, the rapid decomposition of some biopesticides, without releasing problematic residues, renders them an eco-friendly alternative that has no, or very little, detrimental impact on the environment (J. Kumar *et al.*, 2021). Additionally, these natural biodegradable agents are usually effective in small amounts (Prabha *et al.*, 2016).

TABLE 6 | Advantages of biopesticides over chemical pesticides. Adapted from (J. Kumar *et al.*, 2021).

Conventional chemical pesticides	Biopesticides
- Synthetic origin	- Natural origin
- Hazardous to non-target organisms	- Target specific
- Adverse effect on environment	- Eco-friendly
- Development of resistant pests	- No reported development of resistant pests so far

II.2.3 Types of biopesticides

Biopesticides are classified into three major categories (J. Kumar *et al.*, 2021):

- (1) Microbial biopesticides consisting of microorganisms such as bacteria (the insect pathogenic bacterium *Bacillus thuringiensis*...), fungi, and viruses or of their produced toxins (spinosad, avermectins...) (*Figure 7*).
- (2) Biochemical biopesticides comprising mainly of plant-derived extracts or insect pheromones.
- (3) Plant-incorporated protectants (PIPs) consisting in the production of pesticides by genetically modified plants.

Interestingly, according to the United States Environmental Protection Agency (U.S EPA), 299 active ingredients, as well as 1401 active biopesticide products belonging to different categories, have been registered (EPA, 2021).

II.2.3.1 Microbial biopesticides

The search for biopesticides of microbial origin, in which microorganisms act as active ingredients used to control plant diseases and pests, has attracted an extensive attention over the last decades.

Different kinds of pests can be controlled by microbial biopesticides, although each active ingredient is relatively specific to a particular species (Kesho, 2020; Thakura *et al.*, 2020). Owing to their high efficiency, specificity, as well as to their environmental friendliness, microbial biopesticides market accounts for 90% of total biopesticides (Kesho, 2020).

The most widely known microorganism in the development of microbial biopesticides, and the one that paved the way for novel discoveries is the insect pathogenic bacterium *Bacillus thuringiensis*. The insecticidal activity of this bacterium is expressed by its ability to produce toxins (crystal proteins), which once ingested by the target insect, induces its death through lysis of its gut cells. Interestingly, *B. thuringiensis*-based biopesticide is characterized by its host-specificity with a limited chances to affect non-target organisms (P. Kumar *et al.*, 2021).

II.2.3.2 Biochemical biopesticides

Biochemical pesticides include chemical compounds recovered from living organisms such as those extracted from plants, as well as pheromones produced by insects (Thakura *et al.*, 2020).

Plants are naturally able to produce a wide variety of secondary metabolites (flavonoids, phenols, alkaloids, terpenes...) in order to protect themselves against pests and microbial attacks (Prabha *et al.*, 2016). Therefore, in the last few years, plant-based extracts have occupied a prominent place in the search for alternatives to conventional chemical pesticides (J. Kumar *et al.*, 2021). In this context, about 2400 plant species have been reported for their wide range of action against pests. These exhibit various mechanisms of action: repellents, antifeedants, ovicidal, larvicidal effects,... (Thakura *et al.*, 2020; J. Kumar *et al.*, 2021). *Azadirachta indica*, commonly called neem and with azadirachtin as the most important constituent, is a well-known example of plant possessing considerable insecticidal proprieties (Chaudhary *et al.*, 2017). Indeed, nearly 195 insect species, even those that have developed a resistance to conventional pesticides, have shown a sensibility towards neem-based products. Interestingly, beneficial insects such as pollinator insects are not harmed by the application of neem-based biopesticides (Thakura *et al.*, 2020).

On the other hand, pheromones, chemical compounds produced and dispersed by insects as a chemical signal that induce a sexual response, provide a promising strategy for controlling insect pests (Rizvi *et al.*, 2021). These chemical signals are not considered as true “insecticides” since they do not kill pests, but their mode of action is based on the perturbation of insect’s behavior (attract and capture insects, mating disruption, mass trapping...) by acting on their olfactory system (J. Kumar *et al.*, 2021).

The application of insect pheromones in pests control is recognized as a new ecological concepts (respectful approach to the environment, as well as to the public health) with recent applications in mosquito control (Wooding *et al.*, 2020; Rizvi *et al.*, 2021).

II.2.3.3 Plant-incorporated protectants (PIPs)

PIPs are a category of biopesticides that are expressed and produced by genetically modified plants through the incorporation of an exogenous genetic material. Thus, the genetically modified crops are able to protect themselves from harmful pests by releasing toxic compounds. The integration of transgenes, encoding for toxic crystal proteins, from the insect pathogenic bacterium *Bacillus thuringiensis* into the modified plants is well-known as the first generation insecticidal PIPs (Parker & Sander, 2017).

II.2.4 Marine world: a valuable and promising source of biopesticides

The marine world, characterized by unique environmental conditions harbors a wide variety of organisms that are considered as a valuable source of unique and bioactive natural products (Hamed *et al.*, 2015). Besides the great interest given to marine natural products in the search for novel drugs, various studies have revealed their expanded capacity to present alternatives to conventional chemical pesticides (Jimenez, 2018; Song *et al.*, 2021).

II.2.4.1 Seaweed as a potential source of biopesticides

Although most researches focused on the possible pharmaceutical applications of seaweeds, several studies have highlighted the ability of these marine organisms to be valorized in different ways in the agricultural field. Indeed, in view of their richness in mineral substances, vitamins, amino acids, as well as in plant growth regulators (cytokinin, auxin, gibberellins...), various seaweed derived extracts have exhibited a high capacity to stimulate plant growth and productivity (Hamed *et al.*, 2018). Moreover, an improvement in soil chemical and physical properties has also been attributed to algae which can be used as biofertilizers as well as soil stabilizers (Nabti *et al.*, 2016).

On the other hand, biocidal properties such as microbicidal, virucidal, nematocidal, and insecticidal activities of seaweed derived crude extracts and/or purified compounds have been documented in several studies paving the way for their potential application in the control of plant pathogens and pests (Hamed *et al.*, 2018). In this context, Esserti *et al.* (2016) have demonstrated the ability of pulverized aqueous extracts derived from the two brown algae, *Fucus spiralis* and *Cystoseira myriophylloides*, to reduce the damage caused on tomato plants by two phytopathogenes: *Agrobacterium tumefaciens* bacterium and *Verticillium dahlia* fungus (Esserti *et al.*, 2016). In addition, Baloch *et al.* (2013) showed that mixing the soil with the powder of *Spatoglossum variabile* (brown alga), *Stokeyia indica* (brown alga), or of *Melanothamnus afaqhusainii* (red alga) significantly reduced the infection of watermelon and eggplant roots by the root knot nematode *Meloidogyne incognita* (Baloch *et al.*, 2013).

II.2.4.1.1 Seaweed derived extracts/compounds with insecticidal activity

The promising role of seaweed as a valuable source of bioactive molecules with interesting insecticidal activity has been evidenced in various studies (Yu *et al.*, 2014; Hamed *et al.*, 2018). However, for the moment, there are no commercial algae-based products used in the control of phytopathogenic insects (Machado *et al.*, 2019). This can be explained in part by the fact that most studies are limited to laboratory scale *in vitro* screenings, without proceeding to field trials (Hamed *et al.*, 2018; Machado *et al.*, 2019). Therefore, advanced investigations in the research of seaweed-based insecticidal agents are still needed in order to benefit from these marine organisms in the formulation of eco-friendly alternatives to conventional insecticides. Furthermore, the target specificity of these potential bioinsecticides must be assessed so as to avoid any possible adverse effects on beneficial and non-target organisms including humans (Yu *et al.*, 2014).

A non-exhaustive summary of extracts and compounds derived from seaweed that exhibited an insecticidal activity (through various modes of application) against phytopathogenic insects as well as disease vectors, are listed in the table below (*Table 7*). Interestingly, a synergistic insecticidal activity between seaweed extracts and synthetic insecticides was also documented in an early study (Thangam & Kathiresan, 1991). Although these potential synergistic effects between seaweed-derived products and conventional synthetic insecticides are not sufficiently exploited, their combination will undoubtedly lead to a reduction in insecticides consumption and consequently in their adverse effects.

TABLE 7 | Non-exhaustive summary of seaweed derived extract/compound with their demonstrated insecticidal activity.

Seaweed species	Active extract/compound	Chemical family	Target insect	Target growth phase	Mechanism of action	References
<i>Turbinaria turbinata</i> (Brown alga)	- Ethanolic extract	-	<i>Spodoptera littoralis</i> (Cotton leaf worm)	- Larvae	- Contact toxicity - Ingestion toxicity	(Elbrense & Gheda, 2021)
<i>Ceramium siliquosum</i> (Red alga)	- Ethanolic extract	-	<i>Aedes aegypti</i> (mosquito)	- Larvae - Adult	- Contact toxicity	(Kilic <i>et al.</i> , 2021)
<i>Chondria capillaris</i> (Red algae)						
<i>Prasiola crispa</i> (Green alga)	- Hexane extract with campesterol, β -sitosterol and stigmasterol as expected active compounds	Steroids	<i>Nauphoeta cinerea</i> (cockroach)	- Adult	- Sub-cutaneous injection - Effect on muscles and heart activities	(Holken Lorensi <i>et al.</i> , 2019)
	- Methanolic extract	-	<i>Drosophila melanogaster</i> (fruit fly)	- Adult	- Ingestion toxicity	(Zemolin <i>et al.</i> , 2014)
<i>Laurencia johnstonii</i> (Red alga)	- Ethanolic extract with debromolaurinterol, isolaurinterol, and laurinterol as expected active compounds	Terpenes	<i>Diaphorina citri</i> (Asian citrus psyllid)	- Adult	- Ingestion toxicity - Repellent activity	(González-Castro <i>et al.</i> , 2019)
<i>Caulerpa racemosa</i> (Green alga)	- Caulerpin - Caulerpinic acid	Alkaloids	<i>Culex pipiens</i> (mosquito)	- Larvae	- Ingestion toxicity	(Alarif <i>et al.</i> , 2010)
<i>Sargassum tenerrimum</i> (Brown alga)	- Benzene extract - Chloroform extract - Benzene : chloroform extract	-	<i>Dysdercus cingulatus</i> (Cotton stainer)	- Nymph	- Ingestion toxicity - Reduction in total body protein	(Sahayaraj & Jeeva, 2012)
<i>Laurencia brandenii</i> (Red alga)	Octadecadienoic acid and n-hexadecanoic acid (expected active compounds identified in methanolic extract)	Fatty acids	<i>Sitophilus oryzae</i> (rice weevil)	- Adult	-	(Manilal <i>et al.</i> , 2011)

TABLE 7 | Continued.

<i>Chondria armata</i> (Red alga)	- Domoic acid - Palytoxin-like CA II	Fatty acids	<i>Periplaneta Americana</i> (American cockroach)	- Adult	- Sub-cutaneous injection	(Mori <i>et al.</i> , 2016)
<i>Parachlorella kessleri</i> (Green alga)	- Ethanolic extract	Rich in fatty acids	<i>Spodoptera littoralis</i> (Cotton leaf-worm)	- Larvae	- Contact toxicity	(Saber <i>et al.</i> , 2018)
<i>Sargassum wightii</i> (Brown alga)	- Ethanolic extract	-	- <i>Anopheles stephensi</i> (malaria vector) - <i>Aedes aegypti</i> (Zika virus vector) - <i>Culex tritaeniorhynchus</i> (Japanese encephalitis vector)	- Larvae	- Contact toxicity - Disintegration of the epithelial layers	(Suganya <i>et al.</i> , 2019)
<i>Halimeda gracilis</i> (Green alga)						
<i>Caulerpa scalpelliformis</i> (Green alga)	- Acetonic extract	-	<i>Culex pipiens</i> (mosquito)	- Larvae	- Contact toxicity	(Cetin <i>et al.</i> , 2010)
<i>Laurencia papillosa</i> (Red alga)	- (12E)-cis-maneonene-E	C ₁₅ acetongenin	- <i>Tribolium confusum</i> (Flour beetle)	- Larvae	- Contact toxicity	(Abou-Elnaga <i>et al.</i> , 2011)
			- <i>Culex pipiens</i> (mosquito)		- Ingestion toxicity	
<i>Caulerpa scalpelliformis</i> (Green alga)	- Acetonic extract in combination with benzene hexachloride chemical insecticide	-	- <i>Aedes aegypti</i> (mosquito)	- Larvae	- Contact toxicity	(Thangam & Kathiresan, 1991)

Part III:

Biofilms: a microbial assemblage of scientific significance

“Focus on two highly critical pathogenic bacteria”

▪ PREVIEW

It is obvious that the microbial community known as “Biofilm” is an ubiquitous and complex structure widely distributed on all kinds of imaginable biotic and abiotic surfaces: metal, plastic, natural materials (rocks...), medical devices, living tissues, kitchen counters, contact lenses... Although some microbial communities can be beneficial in particular fields, some biofilms, especially those formed by pathogenic microorganisms, present a veritable public health issue, hence the necessity to look for strategies for overcoming them.

In this third part of chapter I, an overview of biofilm, particularly bacterial biofilms, including its development stages and its major components is presented with emphasis on two well-known biofilm former opportunistic/pathogenic bacteria: *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The Quorum Sensing (QS) cell-to-cell communication system, which is strongly implicated in biofilm formation and maintenance, is also described. Mechanisms responsible for biofilm resilience are then detailed along with the main biofilms-associated diseases.

On the other hand, the therapeutic approaches, as well as the place occupied by natural medicine in the search for effective antibiofilm agents are discussed. At the end of this part, experimental techniques frequently adopted in the evaluation of the antibiofilm activity are discussed.

P. aeruginosa, bacterium of interest, is a Gram-negative bacillus, aerobic, motile and non-spore forming rods, isolated for the first time in 1882 from green pus. This bacterium belonging to the order of Pseudomonadales and to the family of Pseudomonadaceae is

widely distributed in nature, soil, water and it is often associated with plant, animal and human infections. This ubiquitous feature is mainly due to its minimal nutritional requirements and to its ability to survive in stressful conditions not tolerated by other microorganisms (Pachori *et al.*, 2019). This “superbug” is known as an opportunistic pathogen associated with chronic infections that frequently infects patients with immunocompromising or underlying conditions by exploiting their weakness, hence its association with nosocomial infections (Pang *et al.*, 2019). It is reported by the International Nosocomial Infection Control Consortium as a serious worldwide healthcare threat (Rosenthal *et al.*, 2016).

On the other hand, *S. aureus* is a Gram-positive coccus, non-motile, living in both aerobic and anaerobic conditions. This pathogenic bacterium belonging to the order of Bacillales and to the family of Staphylococcaceae occupies human nasal carriage which provides a stagnant ground for this pathogen prior to its dissemination in other body areas, causing serious infections such as pneumonia, endocarditis, osteomyelitis.... It is also associated with skin and wound infections (Archer *et al.*, 2011). Being a common pathogen involved in hospital-acquired infections, *S. aureus* receives a considerable attention (Suresh *et al.*, 2019).

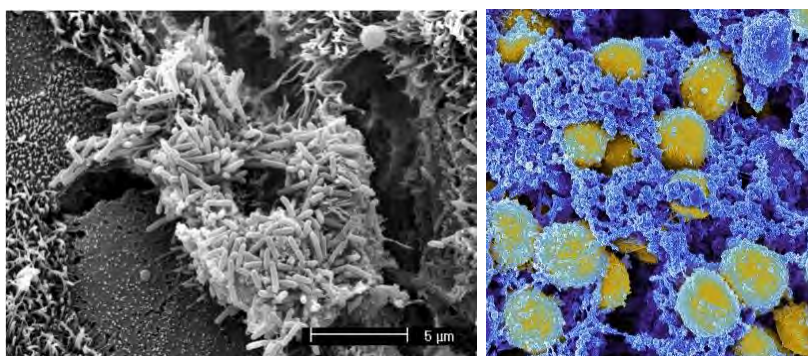


FIGURE 8 | *P. aeruginosa* biofilm developed on respiratory epithelial cells (Woodworth *et al.*, 2008). *S. aureus* in biofilm matrix (BoliOptics, 2020). (from left to right)

Due to the rapid emergence of multidrug resistant strains (MDR), these two pathogenic bacteria are classified by the Infectious Diseases Society of America (IDSA) as members of “ESKAPE pathogens” group which includes bacteria that are able to “escape” action of antibiotics (Pendleton *et al.*, 2013). Furthermore, *P. aeruginosa* and *S. aureus* are rated in the priority pathogens list defined by the World Health Organization (WHO) respectively as critical and high priority in the search for new therapeutic approaches, (WHO, 2017). Their advanced ability to form resilient biofilms offers a powerful armor for these pathogens resulting in life-threatening persistent and recurrent chronic infections.

III.1 BACTERIAL BIOFILMS

III.1.1 History of biofilm discovery

Going back to the works of the German scientist Robert Koch (1843 – 1910), father of modern microbiology, bacteria were considered as single-free microorganisms. This planktonic form has been used for the discovery of numerous antibiotics aiming to treat bacterial infections. However, the development of resistant bacterial strains led to a re-evaluation of bacterial lifestyle, and therefore many scientists affirmed that the majority of bacteria live in sessile form and are attached to a surface that gives them a kind of resistance or rather a loss of sensitivity to current antimicrobial treatments (Rabin *et al.*, 2015). Indeed, in the mid-20th century, some microbiologists suggested that microbes were most often detected in colonies form, characterized by a complex aspect which includes different microbial critters rather than a pure appearance. Furthermore, it has been proven that the attachment of these colonies to an appropriate surface was ensured by a common slimy substance (Cunningham *et al.*, 2010).

A direct relationship between these aggregated bacteria and diseases began to appear when *P. aeruginosa* aggregation was found in the lungs of cystic fibrosis patients (Hoiby *et al.*, 1977). Moreover, polysaccharide glycocalyx has been detected as an essential component of *Streptococcus mutans* cluster formed on teeth. After these discoveries, the term “Biofilm” was officially introduced in 1975 (Mack *et al.*, 1975).

III.1.2 Biofilm definition

Biofilm is defined as an aggregation of cells (here microorganisms) attached to a biotic or abiotic surfaces and enclosed in an extracellular polymeric matrix (EPS). It is also called “City of Microbes” owing to the huge diversity of microorganisms that inhabit this community, surrounded by the EPS matrix “House of Biofilm Cells” (Pan *et al.*, 2016). Zhang *et al.*, (2020) proposed that more than 90% of bacteria are competent to form biofilms but we may consider that all bacteria are able to form biofilm regarding their environment.

Besides surface-attached biofilms, these aggregates can also appear as mobile flocs (Flemming *et al.*, 2016). Biofilms are widely distributed in nature (river sediment biofilms, soil biofilms, plant roots and foliage biofilms...) as well as in industrial (biofouling layers) and medical (tissues, devices and implants biofilms) systems (Pan *et al.*, 2016).

In fact, biofilms commonly encountered in nature present a highly structured complex community that can contain millions of prokaryotic and even some eukaryotic cells in certain environmental biofilms (Stoodley *et al.*, 2002). On the other hand, in clinical biofilms, there is often a dominance of single-species aggregates even though they reside in multi-species infections (Burmolle *et al.*, 2010).

This complex structure is characterized by a high cell density ranging from 10^8 to 10^{11} cells g^{-1} wet weight, socially and physically interconnected, which differentiates biofilm lifestyle from that of free-living cells (Flemming *et al.*, 2016).

The proximity of cells within biofilm ensures an exchange of metabolites, genetic materials, signaling molecules, as well as defensive compounds. In fact, an intercellular communication based on the production and perception of signalling molecules named Quorum Sensing (QS) system is known as a key factor in the formation of a structured biofilm (Preda & Sandulescu, 2019).

Biofilms often present a three-dimensional structure crossed by channels and pores that ensure the transport of nutrients and oxygen, as well as the elimination of degradation products for the maintenance of the community (Ćirić *et al.*, 2019). Therefore, biofilm morphology depends on the constituent species, as well as on the growth (micro-environment) conditions (*Figure 9* for mono-bacterial biofilms) (Rabin *et al.*, 2015).

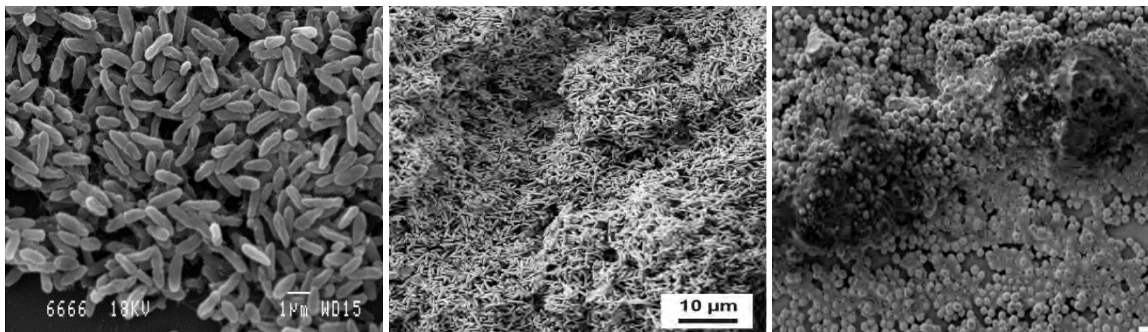


FIGURE 9 | Scanning electron microscopy (SEM) acquisition of bacterial biofilms. *Pseudomonas aeruginosa* cystic fibrosis isolates attaching to glass surfaces (Deligianni *et al.*, 2010). *Escherichia coli* biofilm on titanium oxide surface (Ludecke *et al.*, 2014). *Staphylococcus aureus* biofilm *in vitro* (Lee & Zhang, 2015; Lamret *et al.*, 2020) (from left to right).

Experts of biofilms noticed that this bacterial community is a dynamic entity that changes over time according to nutrient availability. Indeed, a depletion of nutrients can be perceived by the whole bacterial community within the biofilm through the intercellular cell-to-cell communication system (QS) ensuring bacterial migration. The colonization of other surfaces by these detached bacteria allows biofilm spreading (Ćirić *et al.*, 2019).

III.1.3 Biofilms: Bad or good?!

Biofilms have been known for long time as a detrimental structure responsible for significant problems in clinical, as well as in industrial fields. Indeed, according to the National Institutes of Health (NIH), bacterial biofilm is implicated in almost 65% of microbial diseases and in more than 80% of chronic infections (Olivares *et al.*, 2020). In addition to human infections (blood-stream, urinary tract infections...), biofilms are able to colonize all higher organisms such as plants and animals, leading to persistent invasions. The harm of biofilms is also presented by their ability to contaminate medical devices and implants, water systems as well as to spoil food (Hall-Stoodley *et al.*, 2004; Flemming *et al.*, 2016; Galie *et al.*, 2018). Besides its harmful impact on public health, biofilm formation leads to serious economic losses by damaging industrial equipment and contaminating various products. For all these reasons, biofilms are often considered as a socioeconomic issue costing billions of dollars annually (Gunn *et al.*, 2016).

Although most studies to date have focused on detrimental biofilms, different researches have highlighted the beneficial side of biofilm formation as their role in human, animal and vegetal ecosystems regarding healthy status (Louis *et al.*, 2007; Deng *et al.*, 2020) as well as the possibility of their applications in a wide array of fields, such as food (fermented and probiotic food...), agricultural (biofilm-based biofertilizers...), medical (probiotic and bacteriosin produced biofilms...) and environmental (energy, bioremediation, biogeochemical cycle...) fields (Turhan *et al.*, 2019).

After all, biofilms are considered as a double-edged sword that can present both beneficial and harmful effects in the same field. It all depends on the colonized surface and the species of microorganisms, pathogenic or not, living in these communities (Turhan *et al.*, 2019).

III.1.4 Biofilm life-cycle: from adhesion to dispersion

The transition from planktonic to sessile life and then the formation of structured biofilms involves five main steps: reversible attachment, irreversible attachment, proliferation, maturation, and dispersion (*Figure 10*) (Olivares *et al.*, 2020). Briefly, after bacterial adhesion to the surface, the cells multiply and proliferate in conjunction with extracellular polymeric matrix production in order to enable mature biofilm formation. Then, to ensure the biofilm life cycle, a dispersion step proceeds. Released bacteria can, therefore, colonize other sites initiating the formation of new biofilms (Rabin *et al.*, 2015).

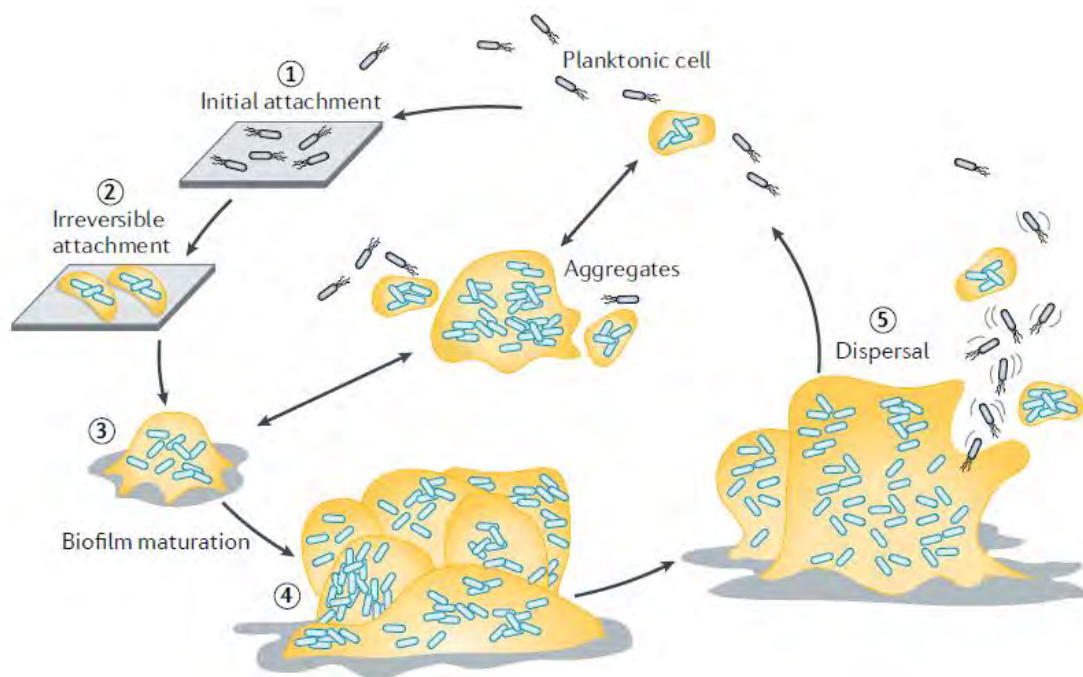


FIGURE 10 | Phases of bacterial biofilm formation (Rumbaugh & Sauer, 2020).

III.1.4.1 Reversible attachment

The capacity of bacteria to adhere to surfaces (including other cell surfaces) is critical for the establishment of biofilm. Initially, bacterial biofilm growth requires a favorable surface including what is known as the conditioning layer. This nutrient surface is composed of many organic and/or inorganic particles/structures ensuring bacterial colonization. Noted that bacterial adhesion is considered to be enhanced by rough and hydrophobic surfaces unlike smooth and hydrophilic surfaces (Olivares *et al.*, 2020). Bacteria detect these suitable substrates through different environmental signals such as oxygen, nutrient concentration, and pH variation and are then oriented by physical and biochemical (chemotaxis) forces involving bacterial appendages such as pili and flagella (Garrett *et al.*,

2008; Olivares *et al.*, 2020). In some description, approaching the surface is considered as the first step in biofilm formation.

As the name suggests, the initial reversible attachment involves weak and detachable bonds such as van der Waals, steric and electrostatic interactions known as DVLO forces. Briefly, DVLO theory describes the balance between attractive and repulsive forces among bacterial cells (considered as a particle) and substratum leading to the attachment and then the initiation of biofilm formation when attractive forces are greater than repulsive forces. However, repulsion of the negative charges on bacterial cells surface by negative charges of most environmental surfaces may led to bacterial repulsion, commonly occurring before conditioning of the surface (Xu *et al.*, 2021). It should be noted that such negative electrostatic interactions may be counterbalanced by the elements of microenvironment especially cationic ones (T. Wang *et al.*, 2019).

At a distance of less than 5 nm, physical contact between bacterial cells and substratum is conducted through more specific interactions involving surface receptors such as flagella, pili as well as their associated adhesins (Cunningham *et al.*, 2010). Once bacteria are initially attached by their appendages, they can quickly spin through the rotary movement of their fixed flagellum. Sometimes, bacteria in contact with the surface vibrate randomly following the Brownian movement provided by the surrounding fluid. On the other hand, some bacteria are capable to move through a flagella-independent movement called “twitching motility” which is ensured by extension and retraction of the pilus. This locomotion mode is also essential for microcolonies formation (Cunningham *et al.*, 2010). Interestingly, the leading role of bacterial appendages in the adhesion process is demonstrated by the inability of *P. aeruginosa* flagella-deficient mutants and type IV pili-deficient mutants to land onto surface and to form microcolonies, respectively (O'Toole & Kolter, 1998).

III.1.4.2 Irreversible attachment

After a few minutes of initial and close contact, irreversible attachment is held allowing the consolidation of bacteria – surface bonds. Besides the role of appendages, particularly of their associated adhesins in this stabilization step and their ability to stimulate chemical reactions (oxidation and hydration) in contact with the substratum, other interactions are involved (hydrogen bounds, ...) and above all the production of extracellular polymeric substances such as polysaccharides, proteins, eDNA plays a leading role in the irreversible attachment (Garrett *et al.*, 2008; Olivares *et al.*, 2020).

Indeed, it has been proven that permanent adhesion is time-dependent and induces the expression of genes encoding for matrix compounds like alginate, the major matrix component of *P. aeruginosa*. In this regard, (Davies *et al.*, 1993) have demonstrated that the quantity of alginate synthesized in biofilm population is significantly greater than in planktonic cells. In 1998, Lejeune and Tresse demonstrated respectively the involvement of specific appendages synthesis named curli and the under expression of OmpF in *E. coli* biofilms formation (Tresse *et al.*, 1997; Vidal *et al.*, 1998).

III.1.4.3 Proliferation and matrix production

Once attached, bacterial cells proliferate by binary division series leading to microcolonies, a process involving pilus-dependent motility as well as clonal growth of bacterial cells (Olivares *et al.*, 2020). It should be noted that at this stage, bacteria belonging to the same species or other species can be recruited to the biofilm from the surrounding environment. The organization of these bacteria within biofilm depends on their metabolic characteristics, for example, bacteria able to growth under anaerobic conditions are located in the deeper layers to avoid any contact with oxygen, while aerobic bacteria are situated in the superficial ones, including positive or negative interactions (Rabin *et al.*, 2015; Reigada *et al.*, 2021).

As soon as bacterial colonization and biofilm development are initiated, various biological processes occur, including deep modification in gene expression which leads to a phenotypic distinction between sessile cells and planktonic ones (Davies *et al.*, 1993). Indeed, given the restriction of movement within biofilm, the expression of genes encoding appendages is downregulated. In this context, Whiteley *et al.* have demonstrated through DNA microarrays analysis of *P. aeruginosa* biofilm that genes encoding for bacterial appendages synthesis are downregulated after biofilm formation (Whiteley *et al.*, 2001).

Moreover, the excretion of several synthesized products such as polysaccharides is ensured by some surface proteins (porins), such as Opr C and Opr E in *P. aeruginosa* (Garrett *et al.*, 2008). Subsequently, the resulting extracellular matrix provides mechanical cohesion between bacterial cells and thus defines the spatial configuration of the biofilm (Olivares *et al.*, 2020).

On the other hand, it has been proven that the formation of bacterial biofilm is directly dependent on a second messenger known as c-di-GMP (cyclic diguanosine-5'-monophosphate) whose concentration in the cytoplasm of bacterial cells is influenced by environmental signals (Pecastaings *et al.*, 2016; Olivares *et al.*, 2020).

At high concentrations, c-di-GMP promotes biofilm formation and structuration by stimulating extracellular matrix production and inhibiting bacterial motility within biofilm (Donne & Dewilde, 2015).

III.1.4.4 Maturation phase

Under optimal growth conditions, biofilm maturation step occurs, determined by a growth in thickness leading to the formation of a three-dimensional structure. Biofilm most often develops a “mushroom-like” shape enclosed in the extracellular matrix (*Figure 11*). The final structure is dependent on various parameters including the system hydrodynamics and biofilm age (Ghosh *et al.*, 2021) (Samrakandi, 1996).

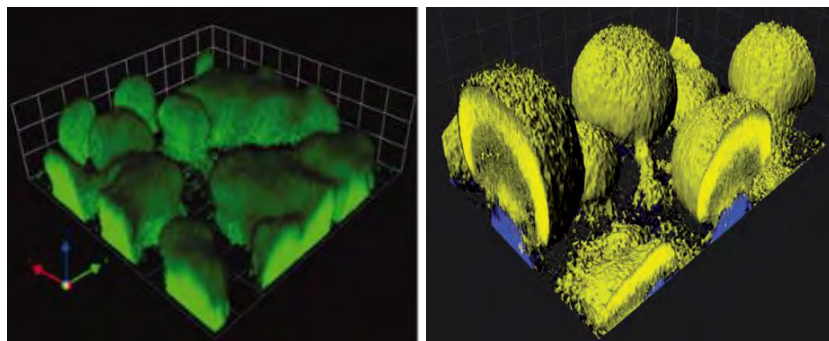


FIGURE 11 | Microscopic observations showing the mushroom structure of *P. aeruginosa* biofilm (Hickman *et al.*, 2005; Azeredo *et al.*, 2017).

Furthermore, gradients of oxygen and pH lead to a heterogeneous physicochemical environment within the biofilm and therefore to physiological heterogeneity. Consequently, microniches constituted of subpopulations that are genetically identical but physiologically different are generated (Olivares *et al.*, 2020). This physiological diversity guarantees biofilm sustainability by overcoming stressful conditions in comparison with a homogeneous population. Moreover, the presence of various bacterial species within the same biofilm (multispecies-biofilm) presents additional biological heterogeneity (Lebeaux & Ghigo, 2012).

III.1.4.5 Dispersion phase

After maturation, biofilm aging, lack of nutrients and intense competition can induce partial or total biofilm dispersion, with the release of bacterial cell aggregates and colonization of new sites, leading to the sustainability of a biofilm-related infection (Rabin *et al.*, 2015). Consequently, a new cycle of adhesion, proliferation, and maturation can happen again, which ensures the transmission of bacteria from their environmental reserves to the host,

the spread of bacterial infection within the same individual (biofilm metastasis) or the transportation of infection between hosts (Olivares *et al.*, 2020).

Initiation of biofilm detachment step depends either on mechanical factors such as abrasion or on chemical factors which are characterized by the secretion of degradative enzymes driven by QS system (Olivares *et al.*, 2020). In this regard, alginate lyase and N-acetyl-heparosanlyase are synthesized by *Pseudomonas aeruginosa* and *Escherichia coli* respectively, to trigger their biofilm dispersion (Garrett *et al.*, 2008).

Finally, the transition from the sessile form to the planktonic one involves phenotypic modifications, particularly concerning the motility. Indeed, it has been proven that the mobile phenotype in the released bacteria is restored by the up-regulation of genes encoding for flagella proteins (Garrett *et al.*, 2008).

The different factors involved in each phase of biofilm formation in the two pathogenic bacteria *P. aeruginosa* and *S. aureus* are listed in the table below (Table 8).

TABLE 8 | Overview of factors implicated in the establishment (chemotaxis) of *P. aeruginosa* and *S. aureus* biofilms and in their various formation phases. Based on (Schulze *et al.*, 2021).

Biofilm formation stage	<i>P. aeruginosa</i>	<i>S. aureus</i>
Approaching the surface / Adhesion	<ul style="list-style-type: none"> - Surface-appendages (flagella, type IV pili) - Cup fimbrial adhesins and lectins 	<ul style="list-style-type: none"> - Hydrophobic-surface - Electrostatic and <i>van der Waals</i> interactions - Teichoic acids - Adhesins
Proliferation and maturation	<ul style="list-style-type: none"> - Exopolysaccharide (alginate, Psl, Pel) - Extracellular DNA (eDNA) - Proteinaceous factors (Lectin A, Lectin B) - Rhamnolipids 	<ul style="list-style-type: none"> - Exopolysaccharide (Polysaccharide intercellular adhesin PIA) - Extracellular DNA (eDNA) - Proteinaceous factors (cell wall-anchored SasG) - Teichoic acids
Dispersion	<ul style="list-style-type: none"> - Alginate lyase - Rhamnolipids 	<ul style="list-style-type: none"> - Exoproteases (serine proteases SspA, SpIA-F and cysteine proteases SspB, ScpA)

III.1.5 The EPS matrix: a major biofilm component with essential functions

The extracellular polymeric matrix (EPS) is known as the major structural component of bacterial biofilms since it represents up to 90% of its organic matter. Moreover, the biofilm architecture, as well as its interaction with the external environmental world, are ensured by this structure (Flemming & Wingender, 2010; Flemming *et al.*, 2016; Olivares *et al.*, 2020).

The composition of this dynamic entity varies in time (age of the biofilms) and space (environmental conditions) (Samrakandi, 1996; Samrakandi *et al.*, 1997) and it also depends on the bacterial species (Campanac *et al.*, 2002; Pan *et al.*, 2016; Olivares *et al.*, 2020). Currently and especially in *P. aeruginosa* biofilms, the matrix forms a highly hydrated structure, made of 97% of water, which contains other functional and structural components such as polysaccharides, proteins, lipids, and eDNA released from bacterial cells (Figure 12) as well as non-organic compounds (minerals, ions,...) (Flemming & Wingender, 2010; Mauline *et al.*, 2016).

Its spatial configuration includes pores and channels between microcolonies ensuring the transport of oxygen and nutrients, thus inspiring the concept of a “rudimentary circulation system” within the biofilm (Wilking *et al.*, 2013; Flemming *et al.*, 2016).

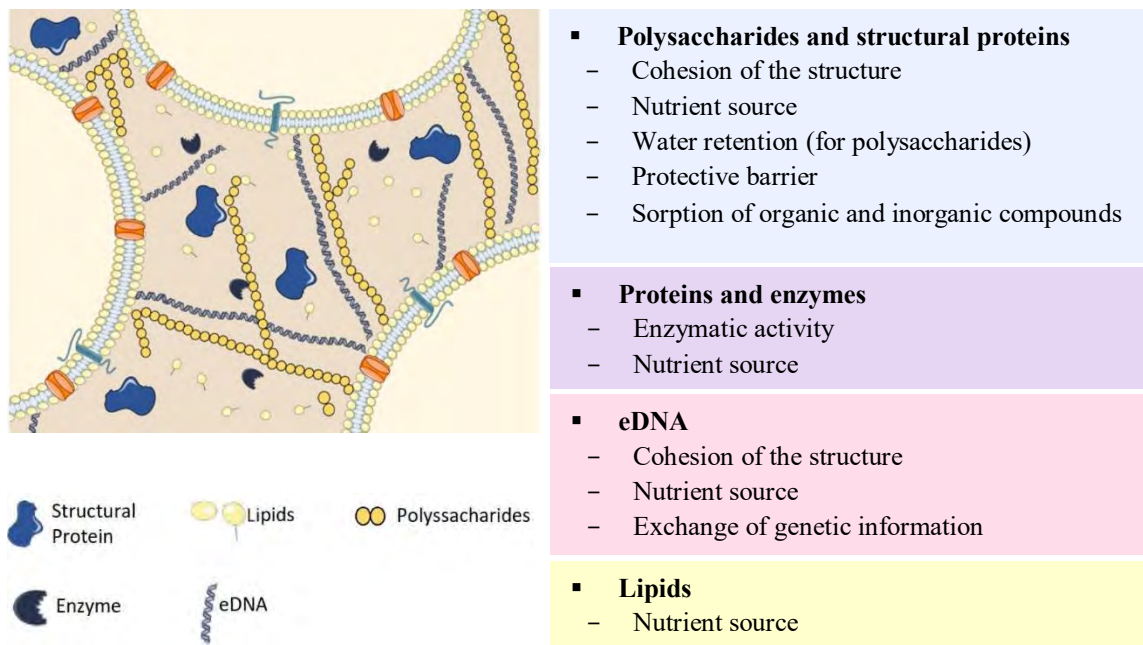


FIGURE 12 | The essential components of the biofilm extracellular matrix and their functions. Adapted from (Pinto *et al.*, 2020).

The importance of this structure in the maintenance and the persistence of bacterial biofilms is evidenced by its different functions, listed below:

- Tolerance to desiccation regularly faced by microorganisms due to the high proportion of hydrated polymer in the biofilm matrix serving as a hydrogel that retains water.

- Capture and digestion of organic and inorganic nutrient resources from the substratum or from the outer aqueous environment through the sorption properties of the sponge-like EPS biofilm matrix.
- Improvement of the intercellular signaling interaction which is strongly affected by the properties of biofilm matrix (Flemming *et al.*, 2016).
- Induction of the transition from reversible to irreversible adhesion of bacterial cells prior to the formation of a cohesive biofilm (Pan *et al.*, 2016).
- Diffusion barrier against antimicrobial agents: their activity can be inhibited by enzymatic degradation, by chelation or by neutralization/consumption by matrix components (Pinto *et al.*, 2020).

The following parts are dedicated to the main EPS components.

III.1.5.1 Matrix exopolysaccharides

Exopolysaccharides are high molecular weight polymers secreted by bacteria in the surrounding environment and composed of sugar residues. Glucose, galactose, mannose are the most abundant carbohydrates found in biofilm matrix, followed by galacturonic acid, arabinose, *N*-acetyl-glucosamine, fucose, xylose and rhamnose. These polymers have a considerable role in adhesion and act as scaffolds for other matrix components (Rabin *et al.*, 2015).

Interestingly, despite the fact that these polysaccharides are not specific to biofilms, their production is boosted under the stressful conditions associated with biofilm formation (Rabin *et al.*, 2015).

The composition and structure of these exopolysaccharides depend on the bacterial species present in the biofilm. Indeed, concerning *Staphylococcus* biofilms, the polysaccharide intercellular adhesin (PIA), also known as poly *N*-acetyl glucosamine, was characterized. This linear polymer is formed of β -1,6-linked glucosamine residues (Mack *et al.*, 1996).

On the other hand, in *P. aeruginosa* biofilm, three types of exopolysaccharides were well described (Moradali *et al.*, 2017):

1. Alginate (O-acetylated (1-4) linked D-mannuronic acid and variable proportions of L-guluronic acid). Its proportion varies regarding the mucoid characteristics of the strains (Marty *et al.*, 1998)
2. Psl polysaccharides (repeating pentasaccharide including D-mannose, D-glucose and L-rhamnose residues)

3. Pel polysaccharide (Partially acetylated (1-4) glycosidic linkages of N-acetylgalactosamine and N-acetylglucosamine)

III.1.5.2 Matrix extracellular proteins

Extracellular proteins are another major component of the matrix providing functional and structural benefits (Gunn *et al.*, 2016). Some proteins tend to bind to cell surfaces and to the exopolysaccharides, thus assisting in biofilm formation and stability (Rabin *et al.*, 2015).

The involvement of matrix extracellular proteins in biofilm formation and biofilm-related infections was demonstrated in different studies. Dueholm *et al.* proved that the overexpression of Fap amyloids, an insoluble fibrous protein, in *P. aeruginosa* enhances cell aggregation and therefore biofilm formation (Dueholm *et al.*, 2013). On the other hand, Cucarella *et al.* demonstrated the important role of a Bap protein (biofilm associated protein) in the initial adhesion of *S. aureus*, as well as in the formation of its biofilm (Cucarella *et al.*, 2001). The expression of this protein was also implicated in *S. aureus* pathogenicity and in the infection persistence in a murine catheter-induced infection model. Degradation enzymes were also detected in biofilm matrix. In fact, during starvation, these enzymes are able to provide the carbon and energy resources required for biofilm cells by the degradation of EPS matrix components, such as polysaccharides, other proteins, nucleic acids, and lipids. In addition, enzymatic functions are involved in the detachment and dispersion processes thus ensuring a new biofilm lifestyle (Rabin *et al.*, 2015).

III.1.5.3 Extracellular DNA

The extracellular DNA (eDNA) found in the biofilm matrix is not only derived from lysed cells, but can also be actively secreted by living bacteria through membranous vesicles fusion (Olivares *et al.*, 2020). As a result of their interaction with substrate receptors, eDNA plays an essential role in biofilm establishment by enhancing cells aggregation and adhesion to surfaces (Rabin *et al.*, 2015).

Furthermore, Whitchurch *et al.* hypothesized the early involvement of eDNA in the formation of *P. aeruginosa* biofilm since the addition of the DNase I enzyme prevented the formation of biofilms and was able to dissolve preformed ones (Whitchurch *et al.*, 2002).

III.1.6 Quorum sensing: microbial chatter orchestrating cells' behavior

III.1.6.1 Definition and discovery

Quorum sensing is an intercellular communication system that, depending on bacterial population density, is able to coordinate bacterial behavior via chemical signals regulating genes expression accordingly. The QS system involves the production, exchange and detection of signaling molecules, called autoinducers (AIs), which are constitutively synthesized and passively or actively excreted into the surrounding environment (Paul *et al.*, 2018). When a given number of cells (or cell contacts) is reached, along with an external accumulation of the signal molecules, bacteria leave their singular planktonic character and a shift in gene expression is detected leading to consider that the bacterial population may act collectively, as a group.

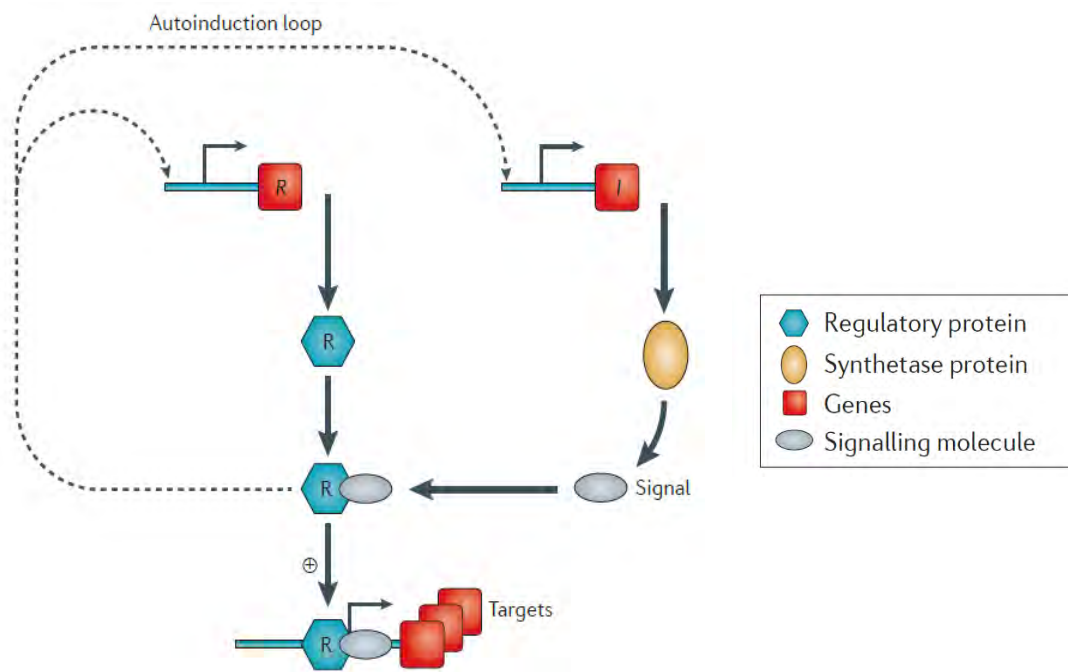
Interestingly, the QS communication system is not restricted to the one considered species but can occur cross species (Bachtiar & Bachtiar, 2020) and even kingdom barriers (Li *et al.*, 2019). Indeed, some signaling molecules are able to act on different bacterial species, as well as to affect the transcriptional programs of eukaryotic epithelial cells and host immune cells (Antonioli *et al.*, 2018; Song *et al.*, 2019; Medina-Rodriguez *et al.*, 2020)

QS system was discovered in 1970s in studying marine environment through an exceptional symbiotic association between the halophilic bacterium *Vibrio fischeri* and the Hawaiian bobtail squid *Euprymna scolopes*. Briefly, the high bacterial density of *V. fischeri* that colonizes the outer surface of the squid during the night was correlated with light production, providing a kind of squid camouflage against predators. Diffusible signal molecules and genetic cluster implicated in this phenomenon were then described in 1980s (Lami, 2019).

III.1.6.2 Quorum sensing circuit

Most of QS communication systems involve hierarchical auto-induction loops that consist of the synthesis, recognition and response to signal molecules (*Figure 13*). The enzymatic process of signal molecules production is generally catalyzed by a synthetase protein encoding by the gene *I* (inducer). While the bacterial density increased, along the AIs external concentration reach a critical threshold, a cell internalization (free diffusion or transport, *Table 9*) and a formation of complex between the signal molecule and the corresponding regulatory protein encoding by the gene *R* (receptor) occur.

The formed complex promotes (or reduces) the expression of target genes by acting as transcription factors. Also, as their name indicate, AIs are able to activate their own production (Papenfort & Bassler, 2016).



QS genes generally involved in:

- Biofilm formation and maintenance
- Antibiotic tolerance
- Extracellular components production
- Virulence factors production
- Surface motility
- Sporulation
- Conjugation
- Pigment production
- Regulatory gene (*R*)
- Synthetase gene (*I*)

FIGURE 13 | Simple scheme of the Quorum Sensing systems and implications. Adapted from (Bjarnsholt *et al.*, 2013; Moradali *et al.*, 2017).

A multitude of transcriptional programs are controlled by the QS systems (almost 15% of bacterial open reading frames), especially those related to biofilm formation and maintenance, to virulence factors production and to antibiotic tolerance via phenotypic modifications. QS communication system also regulates surface motility, conjugation, sporulation, as well as the production of extracellular components and pigments (Asad & Opal, 2008; Pena *et al.*, 2019; Zhao *et al.*, 2020).

III.1.6.3 *QS autoinducers in bacteria*

There is a wide structural diversity of signaling molecules and receptors that deeply differ between Gram-negative and Gram-positive bacteria (*Table 9*).

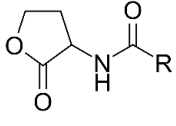
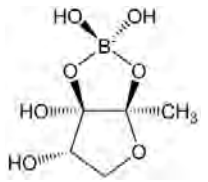
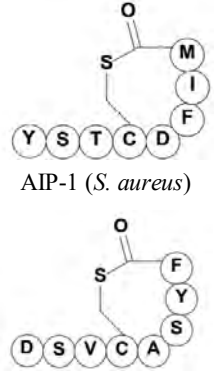
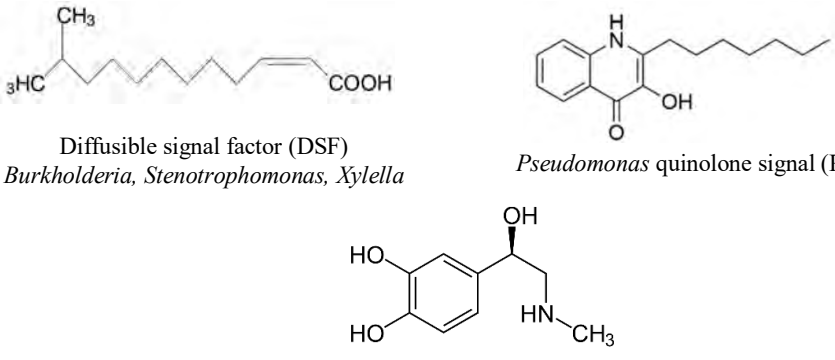
In fact, some AIs are produced exclusively by Gram-negative bacteria like *N*-acyl homoserine lactones (AHLs), that constitute their most common QS signals. Regarding Gram-positive bacteria, they possess a QS communication system based on short cyclic signaling peptides (AIPs). On the other hand, autoinducer type 2 (AI-2) is detected in both Gram-negative and Gram-positive cells, suggesting its implication in interspecies communication (Paul *et al.*, 2018). Furthermore, some signaling molecules are species-specific such as the unsaturated fatty acids (DFS) used by *Xanthomonas* spp., *Burkholderia* spp. and *Xylella* spp., the AI-3 (epinephrine) present in enterohemorrhagic bacteria and the *Pseudomonas* quinolone signal (PQS) (Mion *et al.*, 2019).

Interestingly, some bacteria are able to express the biosensor receptor without producing the corresponding signal molecule, as in the case of *E. coli* which does not produce AHLs but expresses its receptor (LuxR biosensor homologue SdiA). It is assumed that this feature allows *E. coli* to sense AHLs signaling molecules produced by other surrounding Gram-negative bacteria and to use this information to its own benefit (Ahmer, 2004; Asad & Opal, 2008). The non-species-specific role of AHLs was reported for a wide range of Gram-negative bacteria but also for inter-kingdoms relations (Patel *et al.*, 2013; Bez *et al.*, 2021).

III.1.6.4 Connection between QS and biofilm formation

As both QS and biofilm formation process focus on bacterial social aspect, they are known as two inextricably connected topics. In fact, QS network has been shown to play a critical role in all stages of biofilm formation, starting from attachment and surfaces colonization to biofilm dispersion (Parsek & Greenberg, 2005). Indeed, by controlling motility genes, QS is involved in the early adhesion phase, as well as in biofilm maturation and in its architecture via EPS synthesis. On the other hand, this system is able to regulate bacterial density in mature biofilm and to promote bacterial release when resources availability demands it (Asad & Opal, 2008). Overall, QS involvement in virulence factors expression has been many times described (Inat *et al.*, 2021; Luiz de Freitas *et al.*, 2021). However, due to the complexity and diversity of this communication system between bacterial species and also regarding its regulation, it is hard to generalize the QS regulatory mechanisms implicated in biofilm formation (Venturi, 2006; Zhou *et al.*, 2020).

TABLE 9 | Main QS autoinducers molecules in bacteria (Asad & Opal, 2008; Mion *et al.*, 2019).

Type	Signaling molecule	System operation	Features
Autoinducer type 1 (AI-1)	 <p><i>N</i>-acyl-homoserine lactone (AHL)</p>	<ul style="list-style-type: none"> - Synthesized by LuxI synthases - Diffused freely through cell membrane - Recognized by an intracellular receptors (LuxR) 	<ul style="list-style-type: none"> - Found in more than 200 different Gram-negative bacteria - Different structural variants of AHLs (length of the acyl chain, nature of the substitution at C3 position)
Autoinducer type 2 (AI-2)	 <p>Boron-containing AI-2 (<i>Vibrio</i>)</p> <p>Boron-free AI-2 (<i>Salmonella</i>)</p>	<ul style="list-style-type: none"> - Synthesized by LuxS protein - Two-component membrane receptor-cytoplasmic kinase complex 	<ul style="list-style-type: none"> - Found in Gram-negative and Gram-positive bacteria - Involved in interspecies communication
Autoinducer peptide (AIPs)	 <p>AIP-1 (<i>S. aureus</i>)</p> <p>AIP-1 (<i>S. epidermidis</i>)</p>	<ul style="list-style-type: none"> - Synthesized by synthetase proteins - Actively transported by a specialized transport system - Two-component sensor kinase-response regulator 	<ul style="list-style-type: none"> - Found in Gram-positive bacteria - Composed of 7 to 11 amino acids
Others	 <p>Diffusible signal factor (DSF) <i>Burkholderia</i>, <i>Stenotrophomonas</i>, <i>Xylella</i></p> <p><i>Pseudomonas</i> quinolone signal (PQS)</p> <p>AI-3 (Epinephrine) <i>Enterobacter</i>, <i>Escherichia</i>, <i>Legionella</i>, <i>Salmonella</i>, <i>Shigella</i></p>		

III.1.6.5 Quorum sensing network in *P. aeruginosa*

QS circuit in *P. aeruginosa* plays a critical role in surface/mucosa colonization and in the progression from acute to chronic infection. Indeed, the expression of various genes are regulated by this communication system, most of which are involved in virulence factors production, motility, biofilm maintenance and adaptation to stressful conditions (Moradali *et al.*, 2017). The reduced pathogenicity of *P. aeruginosa* strains deficient in QS systems and their increased sensitivity when under biofilm to antibiotics like tobramycin, indicate the high implication of this network in the establishment and resilience of biofilm-related infections (Bjarnsholt *et al.*, 2005; Feng *et al.*, 2016).

Four Quorum Sensing pathways have been identified in *P. aeruginosa* so far: the *las*, *rhl*, *pqs*, and *iqs* systems. They constitute dense and high interconnected circuits that regulate the expression of several functional elements (Figure 14) (Thi *et al.*, 2020).

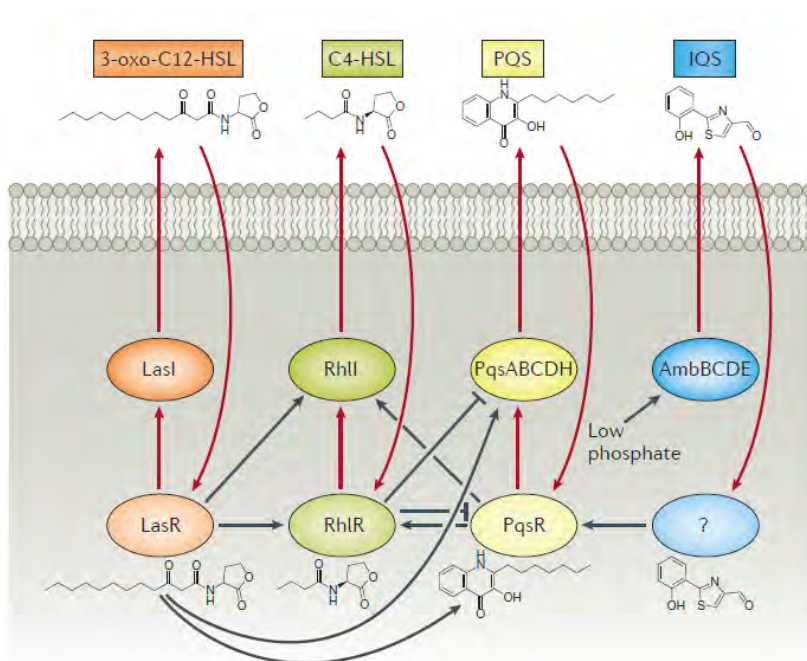


FIGURE 14 | The four interconnected QS pathways identified in *P. aeruginosa* (Papenfort & Bassler, 2016).

The *las* system (LasI/LasR), a AHL-mediated signaling pathway, occupies the top of this hierarchical cascade. The binding between the cytoplasmic receptor protein (LasR) and the corresponding signaling molecule (3-oxo-C12-HSL) promotes the expression of various downstream genes including *lasI*, which encodes its own synthase, as well as the regulatory genes of the other systems (*rhlI*, *rhlR*, *pqsABCDH*, *pqsR*), hence the auto-induction and the collective modulation (Thi *et al.*, 2020).

The *rhl* pathway (RhlI/RhlR) is also a AHL-dependent system that acts similarly to the *las* pathway, comprising C4-HSL as signaling molecule, and RhlR as receptor protein and RhlI as autoinducer synthase (Thi *et al.*, 2020).

On the other hand, the PQS-controlled quinolone system is a non-AHL mediated system recognized in *P. aeruginosa* which is based on signal molecules belonging to 2-alkyl-4-quinolone (AQs) class, especially the 2-heptyl-3-hydroxy-4-quinolone (PQS) whose synthesis depends on *pqsABCDH* operon. Besides the regulation of several functional genes expression, PqsR-PQS complex feeds back to activate *rhlI* and *rhlR* expression while the *pqs* system is inhibited by the *rhl* pathway. Furthermore, like AHL-based QS system, the *pqs* pathway is positively auto-regulated via its transcriptional regulator PqsR (Passos da Silva *et al.*, 2017). Finally, the *iqs* system was later identified as operating under phosphate-limiting conditions and carrying an aeruginaldehyde as signal molecule produced from the proteins encoded by *ambBCDE* genes. However, the receptor protein as well as IQS-regulated genes are still unknown (Thi *et al.*, 2020).

Different studies have documented the involvement of *P. aeruginosa* QS systems in biofilm formation and maintenance processes (Passos da Silva *et al.*, 2017).

TABLE 10 | Examples of QS-regulated factors that affect virulence and biofilm formation in *P. aeruginosa*. Based on (Lee & Zhang, 2015; Moradali *et al.*, 2017; Thi *et al.*, 2020).

QS-regulated product	QS-system involved	Benefits to <i>P. aeruginosa</i>
Rhamnolipids	<i>rhl</i>	<ul style="list-style-type: none"> - Maintain pores and channels between biofilm aggregates for the passage of nutrients - Role in swarming motility implicated in biofilm growth
Pyoverdine	<i>las</i>	<ul style="list-style-type: none"> - Sequester iron essential for biofilm development
Pyocyanin	<i>rhl</i> and <i>pqs</i>	<ul style="list-style-type: none"> - Induction of eDNA release - Increase in biofilm-environment interaction - Initiation of colonization and cellular aggregation
LasA elastase	<i>las</i>	<ul style="list-style-type: none"> - Enhancement of colonization
LasB elastase	<i>las</i> and <i>rhl</i>	<ul style="list-style-type: none"> - Crucial for tissue invasion
Alkaline protease	<i>las</i>	<ul style="list-style-type: none"> - Persistence of colonization
Exotoxin A	<i>las</i>	<ul style="list-style-type: none"> - Enhancement of colonization
Lectin A	<i>pqs</i>	<ul style="list-style-type: none"> - Important role in cell attachment, cell-cell interaction and biofilm growth
Hydrogen cyanide	<i>rhl</i> and <i>pqs</i>	<ul style="list-style-type: none"> - Enhancement of colonization

III.1.6.6 Quorum sensing network in *S. aureus*

The accessory gene regulator system *agr*, the most classical QS pathway in Gram-positive bacteria, has been described in *S. aureus* as a modulator of cell-density dependent virulence factors expression with a significant role in staphylococcal pathogenesis (Figure 15) (Painter *et al.*, 2014).

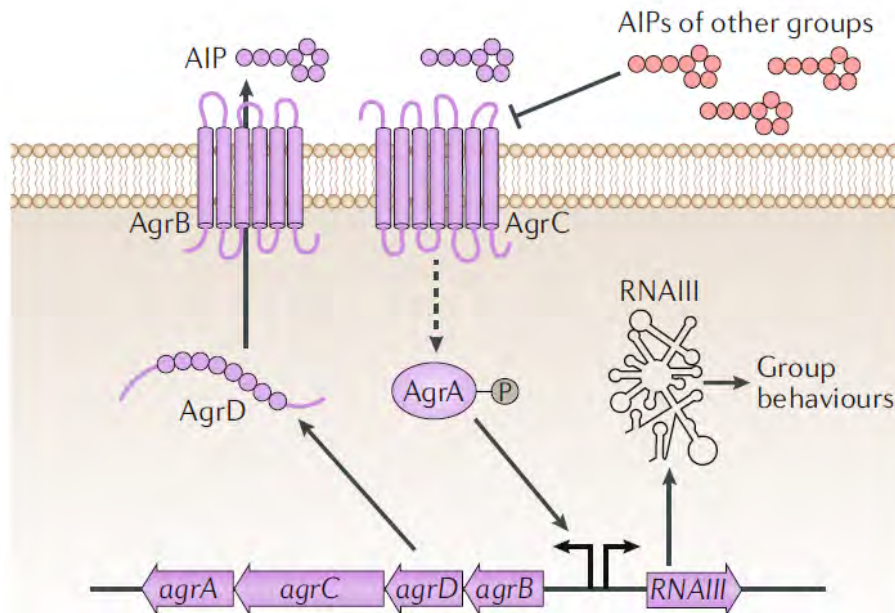


FIGURE 15 | Agr Quorum Sensing system in *S. aureus*. (Mukherjee & Bassler, 2019)

This cell-to-cell communication system is mediated by an auto-inducing peptide (AIP) constituted of thiolactone bond between a conserved cysteine and the C-terminal carboxyl group and whose precursor (AgrD) is encoded by *agrD* gene (Arciola *et al.*, 2012).

The export of this signaling molecule is conducted through a transmembrane endopeptidase (AgrB) that is also required for the post-translational modification of the AgrD pro-peptide and therefore the production of AIP as QS signal molecule.

At high concentration, AIP binds to AgrC, a histidine kinase receptor on the bacteria membrane, which in turn leads to the phosphorylation of the DNA-binding response regulator AgrA, thus presenting a classical two component signal transduction system (AgrC/AgrA). Once activated, AgrA binds to P2 and P3 chromosomal promoters to upregulate the transcription of the two divergent transcriptional units RNAlII and RNAlIII, respectively. In fact, RNAlII encodes the central QS network protein (*agrABCD* operon) allowing the auto-induction, while RNAlIII, the *agr* intracellular effector, regulates the expression of various virulence factors such as toxins (α -toxin, phenol-soluble modulins PSMs...) and degradative exoenzymes (proteases SspA, SspB...).

Interestingly, four *Agr* allelic variants that contribute to the production of different AIP (differ in amino acids residues) have been identified in *S. aureus*. Although AIP acts as QS activator in cells that produce it, it is able to inhibit QS system in other bacterial strains that produce different AIPs.

Regarding the role of this network in *S. aureus* biofilms, it has been shown that biofilm formation is negatively regulated by *agr* system given the significant increase in biofilm development in *S. aureus agr* mutants (Vuong *et al.*, 2000). However, the *agr* system is strongly involved in biofilm dispersion due to the positive regulation of several extracellular proteases expression conducted by AgrA response regulator (Boles & Horswill, 2008; Arciola *et al.*, 2012).

III.1.7 Biofilms: a resilient strength

Understanding the factors responsible for biofilms strength is crucial, especially in the search for treatments against those which are pathogenic and infectious.

Biofilm strength is the result of its ability to overcome harsh environmental conditions, tolerate high concentrations of antimicrobial agents, and escape from the host immune response in the case of clinical infection, which explains the fact that biofilm is the predominant and survival state of bacteria (Rabin *et al.*, 2015). Moreover, since it is tightly attached to a surface or tissues, biofilm can withstand the eradication factors such as water flow, blood-stream and shear forces.

Additionally, it has been shown that biofilm cells are about 100 to 1000 times more resistant than their planktonic form (Alasri *et al.*, 1992; Campanac *et al.*, 2002; Davies, 2003). In this context, Nickel *et al.*, (1985) have initially demonstrated that *P. aeruginosa* in floating form is significantly more sensitive to tobramycin antibiotic treatment than its biofilm state. Indeed, a significant proportion of adherent cells within the biofilm was found to be resistant to tobramycin treatment at a very high concentration (1.0 mg/mL). Furthermore, Luppens *et al.* (2002) have proved that *S. aureus* biofilms are respectively 50 and 600 times more resistant to benzalkonium chloride (BAC) and to the oxidizing agent sodium hypochlorite than suspension cells. In addition, we previously demonstrated the specific loss of sensitivity when bacteria are under biofilm regarding current disinfectants (Samrakandi *et al.*, 1994; Campanac *et al.*, 2002).

However, it has been proven that the sensitivity of bacteria to antimicrobial agents is restored after resuspension of biofilm cells, which shows the fact that this resilience characteristic is reversible, phenotypic, and non-heritable (Lebeaux & Ghigo, 2012).

Therefore, we talk about biofilm tolerance rather than resistance. Indeed, the resistance is defined as the ability of microorganisms to multiply in presence of antimicrobial agents due to different heritable or genetically acquired mechanisms such as the alteration of antibiotics target, modification of bacterial outer cell walls permeability, or destruction of the antimicrobial agents. By contrast, tolerant bacteria can survive in high concentrations of antimicrobial agents but with interrupted growth like sessile bacteria within the biofilm (Olivares *et al.*, 2020).

The resilience of biofilm is ensured by a combination of multiple mechanisms, summarized as follows and which can be inherent to the structural and functional characteristics of biofilm but also acquired by the transmission of genetic resistant material regarding cells connection (Figure 16, Table 11).

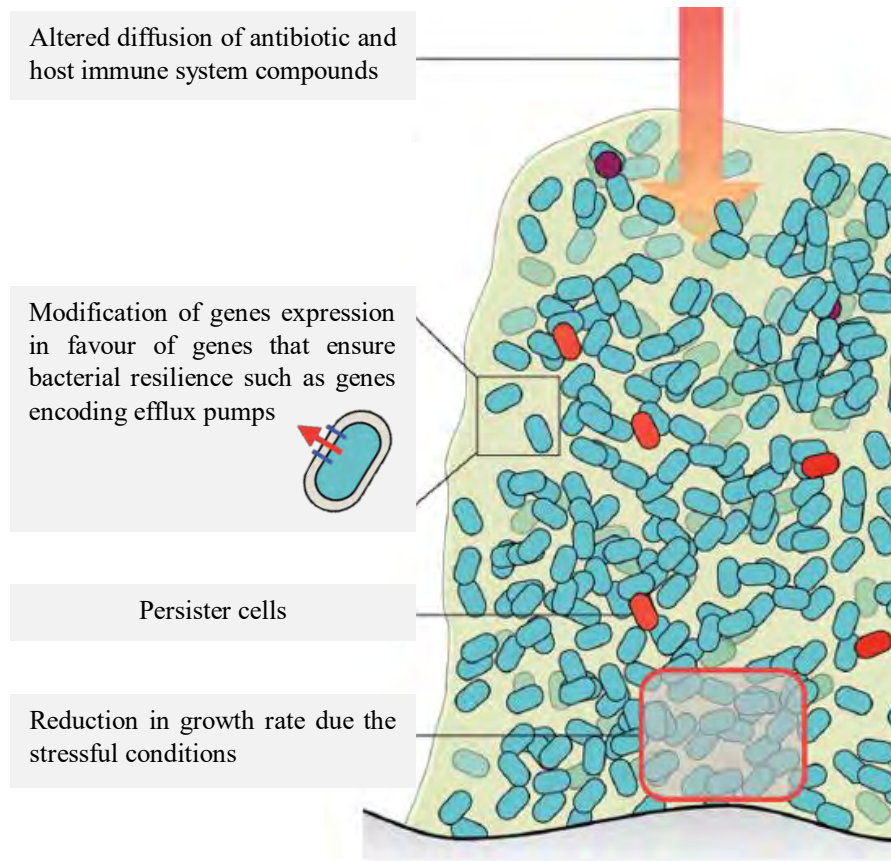


FIGURE 16 | Mechanisms of bacterial biofilms tolerance. Adapted from (Lebeaux & Ghigo, 2012).

TABLE 11 | Mechanisms of bacterial biofilms tolerance with their corresponding factors and characteristics. Adapted from (Preda & Sandulescu, 2019).

Biofilm tolerance mechanisms	Involved factors	Characteristics
Diffusion barrier	- EPS matrix components (polysaccharides, eDNA, enzymes...)	- Impairment of the penetration of antimicrobial agents and immune system components
Growth rate reduction	- Heterogeneity of nutrient and oxygen gradients within biofilm	- Alteration of the activity of antimicrobial agents that target cell division
Modification of genetic profile	- Stress response - Quorum sensing system	- Modification in the genetic profile in favor of increased tolerance and protection
Persister cells	- Stress response (nutrient deficit...) - Antibiotic exposure	- Involved in chronic infections due to their tolerance to antimicrobial agents
Horizontal gene transfer	- Spatial proximity of bacteria within biofilm	- Resistance genes transfer mainly by conjugation

III.1.7.1 Diffusion barrier

The complex architecture of biofilm mainly composed of extracellular polymeric matrix (EPS) presents an “innate” tolerance which provides mechanical barrier limiting antimicrobial agent penetration within the biofilm and therefore their effects on microorganisms (Olivares *et al.*, 2020).

The high viscosity and hydrophilicity of the extracellular polymeric matrix, as well as the electrostatic charge of some of these components (polysaccharides, eDNA...), ensure the trapping of different kinds of antibiotics which then prevent them from reaching their effective concentrations in the deep layers of biofilm to the cells (Rabin *et al.*, 2015; Olivares *et al.*, 2020). Consequently, due to this delayed invasion, bacteria located in the deeper layers can expand physiological adaptation (expression of porins,...) in comparison with surface cells which are more sensitive toward antibiotic treatment (Olivares *et al.*, 2020).

The involvement of EPS matrix in *P. aeruginosa* biofilm resistance towards antibiotics has been highlighted previously. In fact, Hentzer *et al.*, have demonstrated that *P. aeruginosa* biofilm developed by a strain that overproduces alginate, an essential component of *P. aeruginosa* matrix, exhibit an increased resistance to tobramycin in comparison with biofilm formed by wild-type strain (Hentzer *et al.*, 2001).

As for antimicrobial agents, the extracellular matrix forms a protective barrier against the penetration of host immune system compounds (cytokines, ...) in a manner that even immunocompetent individuals are unable to eradicate a biofilm infection (Olivares *et al.*, 2020). Indeed, the phagocytic cells of the innate immune system such as macrophages and neutrophils are generally activated by direct contact with bacterial surface and then they accumulate around the biofilm. In view of their difficult penetration through the extracellular matrix, they are slowed down which makes them more sensitive to the inactivation by bacterial enzymes. Consequently, the increased lysis of neutrophils leads to the release of harmful compounds and therefore to consecutive tissue damages (Watters *et al.*, 2016). Regarding the role of EPS matrix, tolerance to many disinfectants with oxidant properties is linked to the presence of reducing components like some proteins (Samrakandi *et al.*, 1997; Bridier *et al.*, 2011).

Nevertheless, this reduced diffusion cannot be considered as the radical tolerance mechanism of biofilm given the good penetration of some antibiotics (fluoroquinolone, ampicillin...) (Lebeaux & Ghigo, 2012). Thus, this protective barrier is strain and antibiotic dependent (Olivares *et al.*, 2020).

III.1.7.2 Reduction in growth rate

Gradients of oxygen and nutrients in mature biofilms lead to the formation of hypoxic, and stressful zones where bacteria are less metabolically active (Olivares *et al.*, 2020). Subsequently, some microorganisms, particularly those located in the deeper layers, tend to get back to the stationary phase by slowing down their growth and division rates (Rabin *et al.*, 2015).

Since the mode of action of the majority of antimicrobial agent targets dividing cells such as replication, transcription, and translation processes, the reduced growth rate of sessile cells is in part responsible for biofilm tolerance (Hoiby *et al.*, 2010).

In this context, it has been demonstrated that the bacteriolytic activity of β -lactams, antibiotics that act on dividing cells and inhibit the synthesis of bacterial cell wall, is diminished when they are used on *E. coli* biofilms (Rabin *et al.*, 2015). In addition, Bauer *et al.*, have reported the failure of some antibiotics such as fusidic acid (inhibitor of proteins synthesis) and moxifloxacin (inhibitor of DNA replication) to completely destroy *S. aureus* biofilms (Bauer *et al.*, 2013).

However, decreasing growth rate cannot explain biofilm tolerance to antibiotics that are considered bactericidal and effective also on stationary bacteria such as quinolones (Lebeaux & Ghigo, 2012).

III.1.7.3 Modification of genes expression: example of efflux pumps

The microarray analysis conducted by Wagner *et al.*, in order to investigate QS-regulated genes in *P. aeruginosa* highlighted the involvement of this key system in global gene expression by positive or negative regulation (Wagner *et al.*, 2003). In fact, some genes such as those implicated in the adaptation and protection as well as those encoding secreted factors (toxins, enzymes, alginates...) are upregulated by QS system. In addition, results demonstrated the involvement of this communication system in the positive regulation of three efflux pumps expression.

Indeed, these membrane transporters can pump out all kinds of intracellular toxins or xenobiotics, including antibiotics and therefore prevent them from reaching their targets (Rabin *et al.*, 2015).

Interestingly, it has been demonstrated that some efflux pumps genes are overexpressed in biofilm cells, which may contribute to their implication in biofilm tolerance (Rabin *et al.*, 2015). Indeed, Zhang & Mah (2008) have identified a novel efflux system in *P. aeruginosa* whose expression in sessile cells is higher than in planktonic cells. This efflux system enhances *P. aeruginosa* biofilm tolerance to tobramycin, gentamycin and ciprofloxacin given that the complete deletion of genes encoding this pump leads to the formation of sensitive biofilm (Zhang & Mah, 2008).

III.1.7.4 Persister cells

Another important mechanism which contributes to biofilm tolerance is the formation of a small bacterial subpopulation characterized by a growth rate of zero or extremely weak, called “persister cells” or dormant cells (Rabin *et al.*, 2015). Indeed, these metabolically inactive cells are not genetically resistant to antimicrobial agents but they display a transient phenotype giving them a tolerance to high concentrations of these agents, especially those targeting cell growth and division processes (Rabin *et al.*, 2015; Pang *et al.*, 2019). Furthermore, the downregulation of the expression of genes involved in motility and energy production has been shown in this type of cells (Olivares *et al.*, 2020).

Transition to the dormant state is induced by environmental stimuli and stresses, such as nutritional deficiency or antibiotic exposure (Harms *et al.*, 2016).

Moreover, it has been indicated that the QS communication system is implicated in this phenotypic switch. In fact, Moker *et al.* have demonstrated that the QS-regulatory signaling molecule 3-oxo-C12-HSL (*N*-(3-oxododecanoyl)-homoserine lactone) significantly increased the number of *P. aeruginosa* persister cells within the biofilm (Moker *et al.*, 2010).

Besides, it has been elucidated that the toxin-antitoxin (TA) system plays a leading role in the formation of persister cells of *P. aeruginosa*. Indeed, this system consists of a protein toxin that is able to block essential cellular mechanisms and of its antitoxin which can be a protein or a small non-coding-RNA whose function is to neutralize toxicity of the corresponding toxin (Pang *et al.*, 2019).

The imbalance of TA system in favor of toxin as a result of antitoxin degradation enhanced under stressful conditions is associated with dormant cell formation. In fact, MqsR/MqsA (toxin/antitoxin) of *E. coli* was the first TA system identified as being involved in persister cells formation. Kim & Wood have demonstrated that the deletion of *mqsRA* locus decreases persister cell formation whereas the overexpression of MqsR increased them (Kim & Wood, 2010).

In bacterial biofilm, persistent cells represent about 1% of the total population (Pang *et al.*, 2019). The fact that they are enclosed in the biofilm, they are protected from cellular and humoral response of host's immune system contrarily to planktonic ones. Their ability to survive in presence of antibiotics and to restore their metabolic activities after treatment interruption, prove the major role of this cell type in the persistence and recurrence of biofilm-related infection as well as the high prevalence of biofilm in chronic infections (Lebeaux & Ghigo, 2012).

III.1.7.5 Mutagenesis and horizontal gene transfer

It has been demonstrated that the starved biofilm environment as well as different environmental factors such as the presence of reactive oxygen species (ROS) stimulate random genetic mutation in sessile bacteria within biofilm leading to the acquisition of antibiotic resistance (Rodriguez-Rojas *et al.*, 2012).

Indeed, the high level of ROS found in chronic bacterial lung diseases such as in cystic fibrosis patients is mainly due to the vicious inflammatory response as well as to the reduction of antioxidant mechanisms. It has been shown that these reagents are involved in DNA damages and mutations leading to the progressive diversity of bacterial phenotypes within biofilm (Rodriguez-Rojas *et al.*, 2012).

On the other hand, the high bacterial density as well as their spatial proximity within a mature biofilm allow the propagation of antibiotic-resistant genes via horizontal transfer which is 1,000 more significant in bacterial community than between free cells (Olivares *et al.*, 2020). In this context, plasmid transfer by conjugation is facilitated between Gram negative bacteria but also Gram positive ones (Ghigo, 2001). The organization of *S. aureus* into a biofilm considerably increases mutation rate but also the horizontal transfer of plasmid-borne antibiotic resistance determinants by conjugation/mobilization (Savage *et al.*, 2013). This enhanced horizontal transfer has also been observed in *P. aeruginosa* biofilm (Tanner *et al.*, 2017).

III.1.8 Biofilm-related diseases

Biofilms pose a serious challenge for healthcare systems and public health due to their implication in the initiation and persistence of infections that are occasionally fatal. Indeed, in addition to its ability to colonize living tissues resulting in severe chronic infections (infections of lung, wounds, ear...), biofilms are frequently associated with medical devices, such as orthopedic implants and catheters... (Pinto *et al.*, 2020). In *Figure 17* and *Table 12* are represented the various bacterial species associated with the most common medical devices and chronic infections.

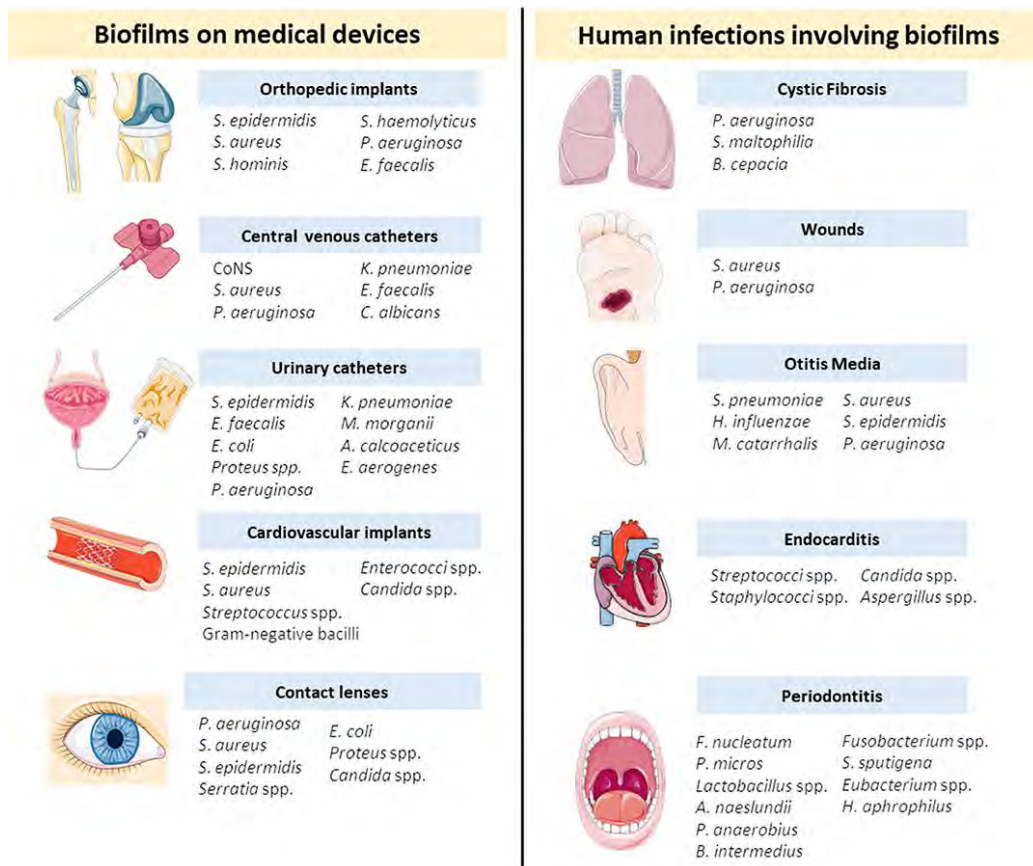


FIGURE 17 | Biofilms involved in medical devices and chronic diseases and the most common microorganisms for each device or disease (Pinto *et al.*, 2020).

III.1.8.1 Medical device-related biofilm and associated diseases

Although medical devices are designed to improve patient's health, they occasionally end up causing chronic pain and serious infections when they are invaded by bacterial biofilms. For example, it has been estimated that about 5% of orthopedic implants are infected. This infection is frequently due to opportunistic microorganisms such as *P. aeruginosa* and *S. aureus* originating from direct contamination of the device or from the wound (*Table 12*) (Stoica *et al.*, 2017).

TABLE 12 | Non-exhaustive list of human infections related to biofilms (Fux *et al.*, 2009).

Infection or disease	Common bacterial species involved
Dental caries	Acidogenic Gram-positive cocci (<i>Streptococcus</i> sp.)
Periodontitis	Gram-negative anaerobic oral bacteria
Otitis media	Nontypeable <i>Haemophilus influenzae</i>
Chronic tonsillitis	Various species
Cystic fibrosis pneumonia	<i>Pseudomonas aeruginosa</i>, <i>Burkholderia cepacia</i>
Endocarditis	Viridans group streptococci, staphylococci
Necrotizing fasciitis	Group A streptococci
Musculoskeletal infections	Gram-positive streptococci
Osteomyelitis	Various species
Biliary tract infection	Enteric bacteria
Infectious kidney stones	Gram-negative rods
Bacterial prostatitis	<i>Escherichia coli</i> and other Gram-negative bacteria
<u>Infections associated with foreign body material</u>	
Contact lens	<i>P. aeruginosa</i> , Gram-positive cocci
Sutures	Staphylococci
Ventilation-associated pneumonia	Gram-negative rods
Mechanical heart valves	Staphylococci
Vascular grafts	Gram-positive cocci
Arteriovenous shunts	Staphylococci
Endovascular catheter infections	Staphylococci
Peritoneal dialysis (CAPD) peritonitis	Various species
Urinary catheter infections	<i>E. coli</i> , Gram-negative rods
IUDs	<i>Actinomyces israelii</i> and others
Penile prostheses	Staphylococci
Orthopedic prosthesis	Staphylococci

Unfortunately, treatment of these infections is extremely difficult and usually requires the removal of the device which is not always convenient for the patient (Pinto *et al.*, 2020).

Mechanical cardiac valve can also be colonized by pathogenic biofilms. Despite the fact that these infections are less frequent, they are considered a worry owing to the high mortality rate that can attain 30% of implanted patients (Pinto *et al.*, 2020). In this case, the danger consists in the blockage of the artificial cardiac valve, as well as the diffusion of the infection via bloodstream as a result of the detachment of biofilm fragments (Rabin *et al.*, 2015).

III.1.8.2 Other biofilm-related diseases: example of cystic fibrosis

Cystic fibrosis (CF) is an autosomal recessive inherited genetic disorder associated with the secretion of a viscous layer of mucus on the respiratory epithelium. This disease is caused by one of more than 1,500 possible mutations in a membrane-bound chloride channel named cystic fibrosis transmembrane conductance regulator (CFTR), whose dysfunction leads to a warm, humid, stressful, and nutrient-rich environment suitable for bacterial colonization (Folkesson *et al.*, 2012).

Due to the high viscosity and dehydration of the mucus layer of CF patients, mucociliary clearance, a normal process in which the cilia of the epithelial cells in the upper respiratory tract remove particles and microbes trapped in the fluid mucus, is impaired, preventing the elimination of trapped microorganisms (Folkesson *et al.*, 2012). Unfortunately, CF patients also suffer from a dysregulation of the innate immune system leading to the evolution from colonization to lethal chronic infections (Doring & Gulbins, 2009).

Various bacterial species are associated with respiratory tract infection in CF patients with an age-dependent prevalence such as (Lipuma, 2010):

- *Staphylococcus aureus*
- *Pseudomonas aeruginosa*
- *Haemophilus influenzae*
- *Stenotrophomonas maltophilia*
- *Achromobacter xylosoxidans*
- *Burkholderia cepacia* complex
- *Burkholderia gladioli*
- *Ralstonia* species, *Cupriavidus* species, and *Pandoraea* species.

According to the Cystic Fibrosis Foundation report, *P. aeruginosa* and *S. aureus* are considered as the most commonly detected bacterial pathogens in CF patients (CFF, 2019). While the highest prevalence of *S. aureus* infection occurs in younger patients, *P. aeruginosa* colonization is most prominent in older adolescents and adults' patients (Figure 18).

The longevity of these infections is attributed to the formation of bacterial biofilms which are not only resilient to antibiotic treatments, but also serve as a reservoir for disease recurrence (Rabin *et al.*, 2015).

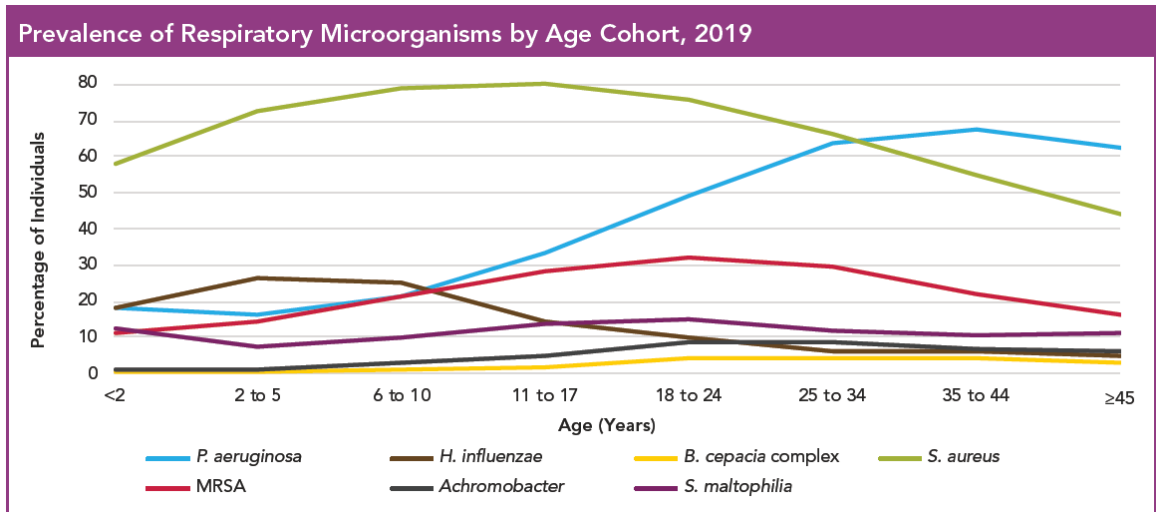


FIGURE 18 | Prevalence of microorganisms in CF patients according to their age (CFF, 2019).

III.2 CHALLENGE OF TREATING BIOFILM-ASSOCIATED INFECTIONS

III.2.1 How to handle with biofilms? Current therapeutic approaches and strategies

In view of the complexity of biofilm-associated infections, as well as the inherent resilience of this bacterial community, the search for effective treatments represents a veritable challenge. In fact, for accessible contaminated surfaces such as catheters, the conventional treatment consists in replacing the colonized material. However, for some implants, such as joint prostheses, this solution is considered the last option and antibiotherapy is initially set up. Moreover, the cost of these surgeries remains exorbitant (Ong *et al.*, 2018).

On the other hand, concerning biofilm-related infections of host tissue, the current therapies are still based on the use of conventional antimicrobial agents for extended periods with high concentrations and combinations. Unfortunately, these therapies are sometimes insufficient due to the failure in targeting more than one component of the heterogeneous biofilm microenvironment (Pinto *et al.*, 2020). In addition, such treatments often result in incomplete bacterial killing, allowing unaffected bacteria to ensure infection recurrence upon the withdrawal of the drug (Ong *et al.*, 2018). Moreover, besides the toxicity issues arising from the administration of high doses of antibiotics, too low concentrations may not only fail to destroy the biofilm, but also promotes biofilm formation/maintaining (Bjarnsholt *et al.*, 2013).

Over all, the new knowledge on bacterial behavior regarding colonization and infection underline the mismatch with current antibiotic targets.

Therefore, a great interest has been dedicated to the search for novel, multi-targeted or combinatorial therapies that can prevent or even eradicate biofilms during infections, hence the fundamental importance of understanding biofilm formation mechanisms.

In the following, four principle treatment approaches are emphasized, with (1) preventive, (2) weakening, (3) disruptive or (4) killing effect (*Figure 19*). It should be noted that the combination of these strategies will be most successful. Some examples with the corresponding advantages and disadvantages of each strategy combatting biofilm, especially regarding *P. aeruginosa* and *S. aureus*, are summarized in *Table 13*.

TABLE 13 | Non-exhaustive list of antibiofilm strategies with examples and disadvantages of each approach. Focus on *P. aeruginosa* and *S. aureus*.

Antibiofilm approaches	Applications/Targets	Examples	Effects	Disadvantages	References
Prevention of biofilm formation (more suitable for biomaterial devices)	Coating of medical devices with hydrophilic polymers	Copolymer derivatives of hyaluronic acid	Reduction of <i>S. aureus</i> adhesion on titanium surfaces	<ul style="list-style-type: none"> - Critical for partially implanted medical devices - Reduction of the anti-adhesive surface properties by the rapid formation of a host-derived glycoproteinaceous film - The release of the antimicrobial compound will eventually end 	(Palumbo <i>et al.</i> , 2015)
	Coating of medical devices with antimicrobial compounds	Coating of endotracheal tubes (ETT) with silver sulfadiazine (SSD)	Significant decrease in bacterial colonization		(Berra <i>et al.</i> , 2008)
	Sterilization of medical devices with ultraviolet light (UV)	UVC disinfection device	Disinfection of a catheter model contaminated for 3h by <i>P. aeruginosa</i> or <i>S. aureus</i> (<i>in vitro</i>)		<ul style="list-style-type: none"> - Only applicable for accessible medical devices - Time and species dependent
	Targeting bacterial surface components essential for their attachment (flagella, pili, eDNA, adhesins...)	<i>P. aeruginosa</i> flagella vaccine (containing flagella subtype antigens a0a1a2 and b)	Some prevention against the development of chronic infection in CF patients	Species specific	(Doring <i>et al.</i> , 2007)
		<i>P. aeruginosa</i> bivalent flagellin vaccine (serotypes a and b)	Protection of mice against fatal <i>P. aeruginosa</i> pneumonia		(Behrouz <i>et al.</i> , 2017)
Weakening of the biofilm	Interference with virulence factors	Immunization from β -lactamase of <i>P. aeruginosa</i>	<ul style="list-style-type: none"> - Development of antibodies against chromosomal β-lactamase - Improvement of lung functions 	<ul style="list-style-type: none"> - Species and strain specific - Effective only on early stages of infection - Targeting only a single virulence factor - High risk of antibodies to induce immunopathology (high inflammation due to an immune-complex-mediated reaction) 	(Ciofu <i>et al.</i> , 2002; Bjarnsholt <i>et al.</i> , 2013)
		Degradation of <i>P. aeruginosa</i> pyocyanin by pyocyanin demethylase	Inhibition of <i>P. aeruginosa</i> biofilm at different stages of development		(Costa <i>et al.</i> , 2017)
		Attenuation of Sortase A and Alpha-hemolysin virulence factors in <i>S. aureus</i> by Chalcone (Small natural compound)	Inhibition of <i>S. aureus</i> adhesion to fibronectin and biofilm formation		(Zhang <i>et al.</i> , 2017)

	Interference with QS communication system	Chitosan – derived from marine crab – Target <i>rhl</i> and <i>las</i> system in <i>P. aeruginosa</i>	- Inhibition in <i>P. aeruginosa</i> biofilm formation	Species and strain specific	(Rubini <i>et al.</i> , 2019)
		<u>HSL-analogues:</u> N-pyrimidylbutanamide (C11 compound) analogue of the N-butanoyl-L-homoserine lactone (C4-AHL)	- Inhibition of <i>P. aeruginosa</i> biofilm formation - Synergistic activity with conventional antibiotics		(Khalilzadeh <i>et al.</i> , 2010; Furiga <i>et al.</i> , 2016)
		Norlichexanthone – derived from <i>Penicillium algidum</i> fungi - Target <i>agr</i> system in <i>S. aureus</i>	- Inhibition of <i>S. aureus</i> biofilm formation		(Baldry <i>et al.</i> , 2016)
Interference with bacterial metabolism	Gallium Ga(NO ₃) ₃	- Inhibition of <i>P. aeruginosa</i> biofilm formation - Slowing down the growth of biofilm-forming cells - Inhibition of Fe uptake - Significant decrease in the biofilm in a chronic biofilm lung infection model	- Possible cytotoxicity - Deleterious impact on host's vital functions	(Kaneko <i>et al.</i> , 2007; Firoz <i>et al.</i> , 2021)	
Interference with c-di-GMP	Sulfathiazole	- Inhibition of <i>E. coli</i> biofilm formation - Decrease in c-di-GMP intracellular level	Narrow spectrum of activity	(Antoniani <i>et al.</i> , 2010; Lebeaux & Ghigo, 2012)	
Dispersion of the biofilm	Mechanical removal of biofilm	Biofilm removal by sonication or ultrasound	Periodontal diseases treatment	- Only applicable on accessible surfaces - Quenching effect of the host tissues (absorption of the ultrasound waves...) - Possible destruction of host tissues	(Bjarnsholt <i>et al.</i> , 2013)

TABLE 13 | Continued.

	Targeting EPS matrix	Alginate lyase	Synergistic activity with gentamycin to eradicate a mucoid <i>P. aeruginosa</i> biofilm	- Enzyme specificity - Species and strain specific - Possible initiation of an autoimmune response	(Alkawash <i>et al.</i> , 2006; Ong <i>et al.</i> , 2018)
		The aerosolized rhDNase (Pulmozyme®)	Decrease the burden of lung infections by <i>P. aeruginosa</i> and <i>S. aureus</i> in CF patients	Unable to eradicate chronic infections	(Frederiksen <i>et al.</i> , 2006; Bjamsholt <i>et al.</i> , 2013)
	Interference with c-di-GMP	Nitric oxide donor sodium nitroprusside	- Removal of <i>P. aeruginosa</i> biofilm - Increase in phosphodiesterase activity - Decrease in c-di-GMP intracellular level	- Possible cytotoxicity - Lack of specificity in targeting biofilm infections	(Barraud <i>et al.</i> , 2009; Koo <i>et al.</i> , 2017)
Killing the biofilm	Combination strategies	<u>Combination of antibiotics</u> Gentamycin + ciprofloxacin	- Eradication of 24h-preformed <i>P. aeruginosa</i> biofilm	-	(L. Wang <i>et al.</i> , 2019)
		<u>Combination of antibiotics with enzymes</u> - Meropenem + trypsin + DNase I - Amikacin + trypsin + DNase I	- Eradication of dual-species <i>P. aeruginosa</i> and <i>S. aureus</i> biofilm		(Fanaei Pirlar <i>et al.</i> , 2020)
		<u>Combination of antibiotics with QSI</u> - Cinnamaldehyde + tobramycin - Hamamelitannin + vancomycin	- Eradication of 24h-preformed <i>P. aeruginosa</i> biofilm - Eradication of 24h-preformed <i>S. aureus</i> biofilm		(Brackman <i>et al.</i> , 2011)
	Targeting biofilm by bacteriophages	Two <i>Pseudomonas</i> phages obtained from sewage treatment plant	Eradication of a preformed mucoid <i>P. aeruginosa</i> biofilm growing on the surface of CF bronchial epithelial cell line	- Species and strain specificity - Induction of adverse immune responses	(Alemayehu <i>et al.</i> , 2012; Bjamsholt <i>et al.</i> , 2013)

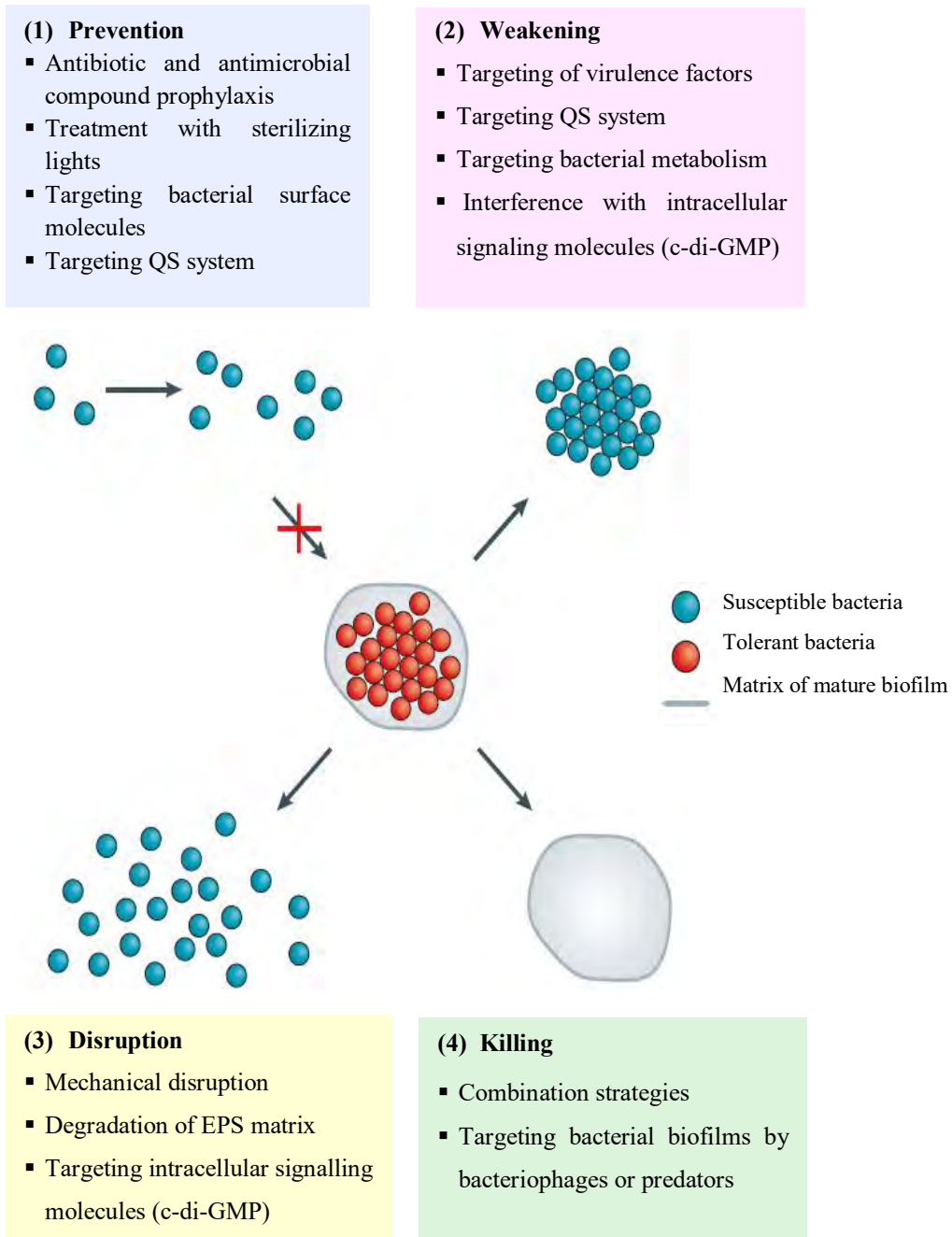


FIGURE 19 | Strategies for combating bacterial biofilms. Adapted from (Bjarnsholt *et al.*, 2013; Lee & Zhang, 2015).

III.2.1.1 Prevention of biofilm formation – Disruption of the initial phases

Inhibition of bacterial adhesion is considered the optimal weapon to avoid the establishment of biofilm-related infections. This strategy aims to maintain bacteria in planktonic form that are more susceptible to both antibiotics and host's defense system. However, since the initial stages of biofilm formation in a host organism lead to a very little inflammation, thus makes the detection of the initial bacteria very difficult and even impossible.

Indeed, the inflammation is only detectable after the establishment of the insensitive biofilm. For this reason, this strategy has to be considered in prophylactic setting, such as the prevention of implants and catheters contamination (Bjarnsholt *et al.*, 2013). Antibio prophylaxis may also be considered a way to control planktonic bacteria diffusion via bloodstream (i.e. before oral surgery for patients with cardiac risk) or at the surgical site (i.e. bone surgery) then preventing their adhesion.

On the one hand, for medical devices, the preventive global strategy towards bacterial adhesion consists in selecting less adherent materials and/or modifying the physicochemical properties of the surfaces by grafting/coating them with hydrophilic polymers, which hinders their interaction with hydrophobic bacterial surfaces (Ong *et al.*, 2018). On the other hand, covering biomaterials devices with antimicrobial agents or with antibiotics has shown effectiveness in delaying rather than totally preventing biofilm formation (Cho *et al.*, 2001). The major limitation of this approach which consists in coating medical devices to avoid biofilm formation lies in the rapid conditioning of these implants with host-derived glycoproteinaceous film (fibronectin, fibrinogen...) thus preventing the release of antibiotics and/or reducing the effectiveness of the anti-adhesion surfaces (Gristina, 1987). In addition, concerning partially implanted medical devices, the risk of being colonized is increased due to their exterior contact. It should be noted that no surface can be considered as “non-colonizable” (Costerton *et al.*, 1978).

Other preventive approaches, considered more species specific, have also been investigated such as targeting the components of bacterial surfaces that are involved in their initial attachment (flagella, pili, eDNA, polysaccharides...) by blocking the production pathways of these constituents or by employing specific neutralizing antibodies such as adhesin-binding antibody (Bjarnsholt *et al.*, 2013; Kisiela *et al.*, 2015).

III.2.1.2 Weakening of the biofilm by disarming bacteria

In situations where the preventive approach is not feasible nor efficient, another strategy consists in weakening and disarming bacteria within the biofilm by targeting the virulence factors and their biofilm-forming features can be applied. However, this approach is restricted by its specificity towards bacterial species and strains. Moreover, its effectiveness is only demonstrated on immature or developing biofilms, but not on mature ones and it never leads to the total eradication of the biofilm (Bjarnsholt *et al.*, 2013).

Several studies have focused on the search for compounds that weaken bacteria by acting on different targets. In the following, approaches that involve the interference with virulence factors, Quorum Sensing (QS) communication system, iron metabolism and sRNAs are discussed.

III.2.1.2.1 Interference with the expression/activity of virulence factors

In view of the essential involvement of virulence factors during the colonization, invasion and persistence of bacteria under biofilm in a susceptible host, anti-virulence therapy has emerged as a promising antimicrobial approach expected to be superior to conventional antibiotics (Totsika, 2017; Fleitas Martinez *et al.*, 2019) or at least to increase the antibiotherapy efficiency. The relevance of this strategy resides in the complexity of developing bacterial resistance towards the anti-virulence agents (Totsika, 2017).

However, this approach targets a single virulence factor which induces many disadvantages (Cegelski *et al.*, 2008):

- Virulence factors are species and sometimes strain specific
- Bacteria express various virulence factors and targeting only one generally induces a transient and low reduction in biofilm
- The transient effect observed refers also to bacterial adaptation with modification in expression or role of each virulence factor

III.2.1.2.2 Interference with QS communication system

As mentioned before, the intercellular communication system Quorum Sensing has a key role in biofilm formation, as well as in the regulation of multiple virulence factors expression. Therefore, a great interest has been attributed to the search for QS interference ways so as to disarm bacteria from their biofilm-forming proprieties and from their virulence (Remy *et al.*, 2018).

The interference with QS system can occur on different levels: on signal generation point, on signal molecules and on signal reception level. Indeed, QS inhibitor (QSI) molecules can target the signal generator by inhibiting signal molecules synthesis. Moreover, the complete degradation or inactivation of signal molecules by specific enzymes such as AHL-acylases and AHL-lactonases is another anti-QS mechanism of action. This strategy is known as Quorum Quenching. On the other hand, QS system can be blocked by antagonistic molecules that are able to interfere with signaling molecules for their binding to the corresponding receptor proteins (Husain *et al.*, 2019).

A wide variety of either naturally sourced (derived from plants, fungi, animals...) or chemically synthesized QSI molecules has been identified (Kalia, 2013).

Interestingly, the fact that QSI compounds have no direct effect on the bacterial life processes renders the emergence of bacterial resistance towards such drugs a minimized (to be under survey) phenomenon with effectiveness on antibiotic resistant bacteria (Hentzer & Givskov, 2003). In addition, this type of molecules has been found to enhance the susceptibility of bacteria to conventional antibiotics, as well as to the host's immune defense (Christensen *et al.*, 2012; Bjarnsholt *et al.*, 2013; Furiga *et al.*, 2016).

The disadvantages of this approach may be that (Krzyzek, 2019):

- QS systems and effectors are species specific. The inter-species and inter-kingdoms networks have been underlined in some papers (Lowery *et al.*, 2008; Wu & Luo, 2021) but the research on multi-species biofilm behavior regarding QS and QSI is just beginning.
- Bacteria present different QS systems interconnected and their adaptation capability has to be under survey when using QSI.

At last, the possible toxicity of QSI including impact on human/ animal ecosystems must be taken into consideration.

III.2.1.2.3 Interference with bacterial metabolism

Targeting bacterial metabolism is considered a classical target combatting pathogens but with renewed interest in the search for novel strategies to combat biofilm-related infections. For example, given the critical role of iron in biofilm formation in various pathogenic bacteria, antibiofilm approach that target bacterial iron metabolism has been explored (Banin *et al.*, 2005; Lin *et al.*, 2012).

The involvement of iron in the establishment of *P. aeruginosa* infection in cystic fibrosis patients has been earlier demonstrated. In fact, the mutation of CF airway cells (Δ F508-CFTR) enhances biofilm formation which is correlated with the increased availability of iron ensured by these mutated cells (Moreau-Marquis *et al.*, 2008).

Therefore, different attempts have been focused on the search for agents that can block bacterial iron-dependent pathways required for cell growth and biofilm formation. Indeed, the approach that consists in replacing iron by similar but metabolically inactive metals such as gallium has shown an interesting efficiency in reducing *P. aeruginosa* biofilm biomass in a chronic lung infection model (Kaneko *et al.*, 2007).

Also, the application of iron chelators in complement with metals (siderophore-gallium complex) or even with antibiotics (deferoxamine-tobramycin) have shown a significant antibiofilm activity against this opportunistic pathogen *in vitro* (Banin *et al.*, 2008; Moreau-Marquis *et al.*, 2009).

The major drawbacks of this approach are certainly the deleterious impact on host's vital functions as well as its potential cytotoxicity (Firoz *et al.*, 2021).

III.2.1.2.4 Interference with the second messenger c-di-GMP

c-di-GMP (cyclic diguanosine-5'-monophosphate) is a small diffusible molecule acting as a central second messenger which, depending on its intracellular concentration, controls the transition between the free bacterial life (planktonic) and the sessile one (biofilm) (Lebeaux & Ghigo, 2012). Generally, the high intracellular concentration of c-di-GMP induces biofilm formation while the decrease of its cytoplasmic level leads to biofilm dispersion. Synthesis and degradation of this signaling molecule are ensured by two enzymes with opposite activities and which are diguanylyl cyclase and phosphodiesterase, respectively (Bjarnsholt *et al.*, 2013). In this context, the application of c-di-GMP inhibitors can present a novel biofilm weakening strategy that aims to disrupt the genes network regulated by this signal molecule. Antoniani *et al.* have demonstrated the ability of sulfathiazole to inhibit *E. coli* biofilm formation by interfering with c-di-GMP metabolism (Antoniani *et al.*, 2010).

III.2.1.3 Dispersion of biofilms – Restoration of bacterial sensibility

As mentioned above, the tolerance to antimicrobial treatments of bacteria residing in a biofilm is a reversible phenotype that is reverted after their release. Therefore, a new approach that consists in inducing the disruption of this bacterial aggregation can reverse this physical tolerance, thus releasing planktonic cells that are more sensitive to conventional treatments. It should be noted that all biofilm dispersal approaches must be combined with antibiotic treatment in order to eliminate the released planktonic cells and avoid their spreading in the body (Bjarnsholt *et al.*, 2013).

Mechanical or surgical removal of the biofilm presents the most effective approach for treating biofilm-related infections but unfortunately this method is obviously only possible on accessible surfaces. The main example is the mechanical treatment applied for prevention and treatment of periodontal diseases (Apatzidou & Kinane, 2010).

Since the extracellular matrix has a definite role in biofilm stabilization and maintenance, targeting this structure by inhibiting its production or disrupting it can weaken the biofilm by rendering it susceptible to antibiotics and host defense system (Bjarnsholt *et al.*, 2013). In this context, Alkawash *et al.* demonstrated the ability of an alginate lyase combined with gentamycin to eradicate *P. aeruginosa* biofilms formed by a mucoid strain isolated from the lung of a CF patient (Alkawash *et al.*, 2006). The improvement of gentamycin activity in the presence of alginate lyase enzyme is probably due to the restoration of gentamycin bactericidal activity on susceptible cells released from the biofilm after degradation of the matrix alginate. However, this effect is restricted to the mucoid trait since the non-mucoid strain was not affected by alginate lyase treatment.

In addition, Frederiksen *et al.*, have demonstrated the efficacy of the aerosolized rhDNase (Pulmozyme®) in reducing the burden of lung infections in CF patients (Frederiksen *et al.*, 2006).

Regarding the various molecules implicated in EPS, the main part of these approaches may be limited by:

- Species and strain specific composition (as an example: alginate for *Pseudomonas* sp. biofilms).
- Adaptation of bacteria to microenvironment even regarding EPS composition (Samrakandi *et al.*, 1997).

On the other hand, since the dispersion of a mature bacterial biofilm is a normal process induced as a response to a starved condition, some studies have focused on identifying bacterial components responsible for this spreading. D. G. Davies & Marques have identified an unsaturated fatty acid *cis*-2-decenoic acid, a small messenger produced by *P. aeruginosa* and which is involved in biofilm dispersion (Davies & Marques, 2009). The addition of this compound was able to enhance the dispersion of biofilms formed by a range of Gram-positive (*S. aureus*, *B. subtilis*) and Gram-negative (*K. pneumoniae*, *E. coli*) bacteria. In addition, Allegrone *et al.*, have demonstrated the ability of natural (rhamnolipide produced by *P. aeruginosa*) and synthetic (Tween® 80 and Triton™ X-100) surfactants to significantly enhance *S. aureus* biofilm dispersion (Allegrone *et al.*, 2021).

Another strategy that aims to promote biofilm dispersion is the interference with the intracellular secondary messenger c-di-GMP. While the increase in the intracellular level of this molecule promotes biofilm formation, its reduction leads to biofilm dispersal (Koo *et al.*, 2017).

One well-characterized compound that is able to regulate c-di-GMP level leading to the removal of *P. aeruginosa* preformed biofilm is nitric oxide (NO). Indeed, upon the addition of NO donor sodium nitroprusside, an increase in phosphodiesterase activity (enzyme that govern c-di-GMP degradation) coupled to a decrease in the amount of intracellular c-di-GMP has been demonstrated (Barraud *et al.*, 2009).

This short review of anti-biofilm strategies underlines the difficulty to reach a rapid and deep efficiency with only one approach thus leading to evaluate the interest of combinations.

III.2.1.4 Killing of the biofilm – Combination strategies

It is clear that the combination of several strategies is the most effective approach that can potentially treat a biofilm-related infection. In fact, owing to the heterogeneous nature of this microbial community, it is essential to find a treatment that can target cells in their different metabolic states and environmental conditions, which is provided by the combinatory strategy (Grassi *et al.*, 2017; Belfield *et al.*, 2017). This approach relies on:

- Combination of two conventional antibiotics in an attempt to enhance their activity
- Combination of antibiotic with anti-matrix compounds such as matrix-degrading enzymes in order to facilitate the access of this antimicrobial agent to cells by impairing the protective barrier
- Combination of antibiotic with Quorum sensing inhibitors or anti-virulence compounds in an attempt to enhance biofilm eradication and mitigate the severity of infection

On the other hand, an innovative approach involving the application of bacteriophages has also been elucidated. Bacteriophages are viruses that replicate by infecting bacteria (lytic replication cycle) at the site of infection. In this context, Alemayehu *et al.* have demonstrated the ability of two phages to eradicate a preformed mucoid *P. aeruginosa* biofilm developed on a CF bronchial epithelial cell line (Alemayehu *et al.*, 2012). However, the specificity, as well as the risk of inducing adverse immune responses make the large application of bacteriophages limited.

III.2.2 Natural medicine: breakthrough in the search for antibiofilm agents

The administration of antibiotics is known as the conventional treatment for bacterial infections (Zhang *et al.*, 2020). However, in biofilm-associated infections, their effectiveness is reduced due the increased tolerance provided by this bacterial community towards these antimicrobial agents. Therefore, a great interest has been given to the search for novel treatments (Mishra *et al.*, 2020). In this context, natural medicine, which has been used since ancient times in healing and treatment of diseases, presents strong promises given the remarkable antibiofilm activity demonstrated for several natural products (single compounds and/or mixtures of natural products) (Yuan *et al.*, 2016; Zhang *et al.*, 2020; Mishra *et al.*, 2020; Furner-Pardoe *et al.*, 2020).

Interestingly, owing to their wide chemical diversity, natural products identified as anti-biofilm agents exhibit their action via various mechanisms. Some compounds showed a significant effect on the initial biofilm formation by inhibiting the early cell attachment. Other products were found to act on the extracellular matrix by reducing or interrupting its production, thus blocking biofilm development. Other natural molecules that attenuate biofilm formation by interfering with QS system were also described. Consequently, a reduction in QS-regulated virulence factors was demonstrated. On the other hand, the notable synergy demonstrated between some natural products and conventional antibiotics suggest their ability to restore the efficacy of these antimicrobial agents against bacterial cells under biofilm (Mishra *et al.*, 2020; Zhang *et al.*, 2020).

The advantage of natural medicine lies in the fact that, in general, natural products are expected to present fewer side effects than their synthetic counterparts (Mishra *et al.*, 2020). Nevertheless, the potential harmful effect of natural products should not be neglected and the realization of clinical trials is mandatory (Ćirić *et al.*, 2019). Furthermore, the unique chemical features and the structural complexity of some natural products, not easily obtained through chemical synthesis, allow a wide range of mechanisms compared to conventional antibacterial agents (Silva *et al.*, 2016).

Unfortunately, despite huge efforts and the encouraging preclinical *in vitro* and *in vivo* results, no natural antibiofilm product has been approved by the U.S Food and Drug Administration (FDA) so far (Lu *et al.*, 2019; Mishra *et al.*, 2020).

Besides the widely explored medicinal plants, various studies have highlighted the ability of compounds isolated from marine organisms (seaweeds, sponges...), as well as from microorganisms (fungi and bacteria) to present a valuable input in the search for new antibiofilm agents (Melander *et al.*, 2020).

Indeed, these organisms possess sophisticated defense mechanisms that involve the natural synthesis of secondary metabolites in order to overcome any kind of undesirable attacks (Paul *et al.*, 2018).

III.2.2.1 Plant derived compounds with antibiofilm activity

Medicinal plants have always played an important role in traditional medicine by exhibiting various benefits such as their ability to inhibit microorganisms' growth. In fact, their prominence was accentuated in the late 1990s, especially in the industrial countries, when the efficacy of antibiotics started to decrease as a result of their excessive and uncontrolled use. Interestingly, an endless number of extracts and compounds derived from plants have been documented for their broad spectrum of antimicrobial activity (Simoes *et al.*, 2009).

In the search for new antibiofilm agents, plant derived compounds have also occupied a preponderant place given their demonstrated capacity to inhibit biofilm formation and/or to eradicate a preformed one by showing different mechanisms of action (Guzzo *et al.*, 2020). A summary of some plant derived products that have exhibited antibiofilm activity, particularly against *P. aeruginosa* and *S. aureus* biofilms, and whose potential mechanism of action has been elucidated is presented below, with the identified chemical structures (Figure 20) (Table 14).

Baicalin (7-glucuronic acid, 5,6-dihydroxyflavone) isolated from *Scutellaria baicalensis* roots, is considered as one of the most medicinally active natural compounds given its broad spectrum of demonstrated biological activities. Interestingly, this natural flavonoid has shown significant antibiofilm activity against a range of bacterial species with a clear mode of action that relies mainly on the interference with their various QS systems (Ozma *et al.*, 2021). In this context, Luo *et al.*, have proven the ability of baicalin to inhibit *P. aeruginosa* biofilm formation by interfering with QS communication system, resulting in a downregulation of several QS-regulatory genes (*rhlI*, *rhlR*, *lasI*, *lasR*, *pqsR* and *pqsA*) expression (Luo *et al.*, 2017). Reduction in QS-regulated virulence factors such as LasA protease and LasB elastase has also been observed.

Moreover, it has been demonstrated that baicalin is able to suppress the expression of AI-2 QS signal molecule synthesis genes (*luxS* and *pfS*) in *E. coli* leading to a significant inhibition of biofilm formation (Peng *et al.*, 2019). This antibiofilm activity was associated with a downregulation of *fimA* (type I pili) and *csgA/csgB* (curli pili A) genes expression. On the other hand, the relevant effect of this molecule was not restricted to Gram-negative bacteria but a significant antibiofilm activity has also been detected for its aglycone (baicalein, 5,6,7-trihydroxyflavone) against the Gram-positive *S. aureus* by suppressing the expression of the QS-regulatory gene *agrA* (Chen *et al.*, 2016).

Moreover, given the prominent involvement of the EPS matrix in bacterial biofilm formation, it has presented the target of various plants derived compounds. In this context, Packiavathy *et al.* have demonstrated the capacity of curcumin extracted from *Curcuma longa* rhizomes to impair EPS production in *P. aeruginosa*, especially that of its major component, alginate (Packiavathy *et al.*, 2014). Similarly, the antibiofilm activity of cassipourol and β -sitosterol isolated from *Platostoma rotundifolium* against *P. aeruginosa* has been attributed to a reduction in EPS matrix production, paired with a downregulation of *pelA* gene expression, which is involved in the production of the extracellular cationic polysaccharide Pel (Rasamiravaka *et al.*, 2017).

This mechanism of action has also been observed in *S. aureus* where celasterol and emodin, two natural quinones, have been found to inhibit biofilm formation by restricting the production of extracellular proteins and carbohydrates (Woo *et al.*, 2017; Xiang *et al.*, 2017). Interestingly, Xiang *et al.* (2017) have shown that the antibiofilm activity of emodin is expressed during the adhesion and the proliferation phases of biofilm formation, which is correlated with its capacity to reduce the production of the polysaccharide intercellular adhesin (PIA), implicated in early stage of *S. aureus* biofilm formation (Arciola *et al.*, 2015) (Xiang *et al.*, 2017).

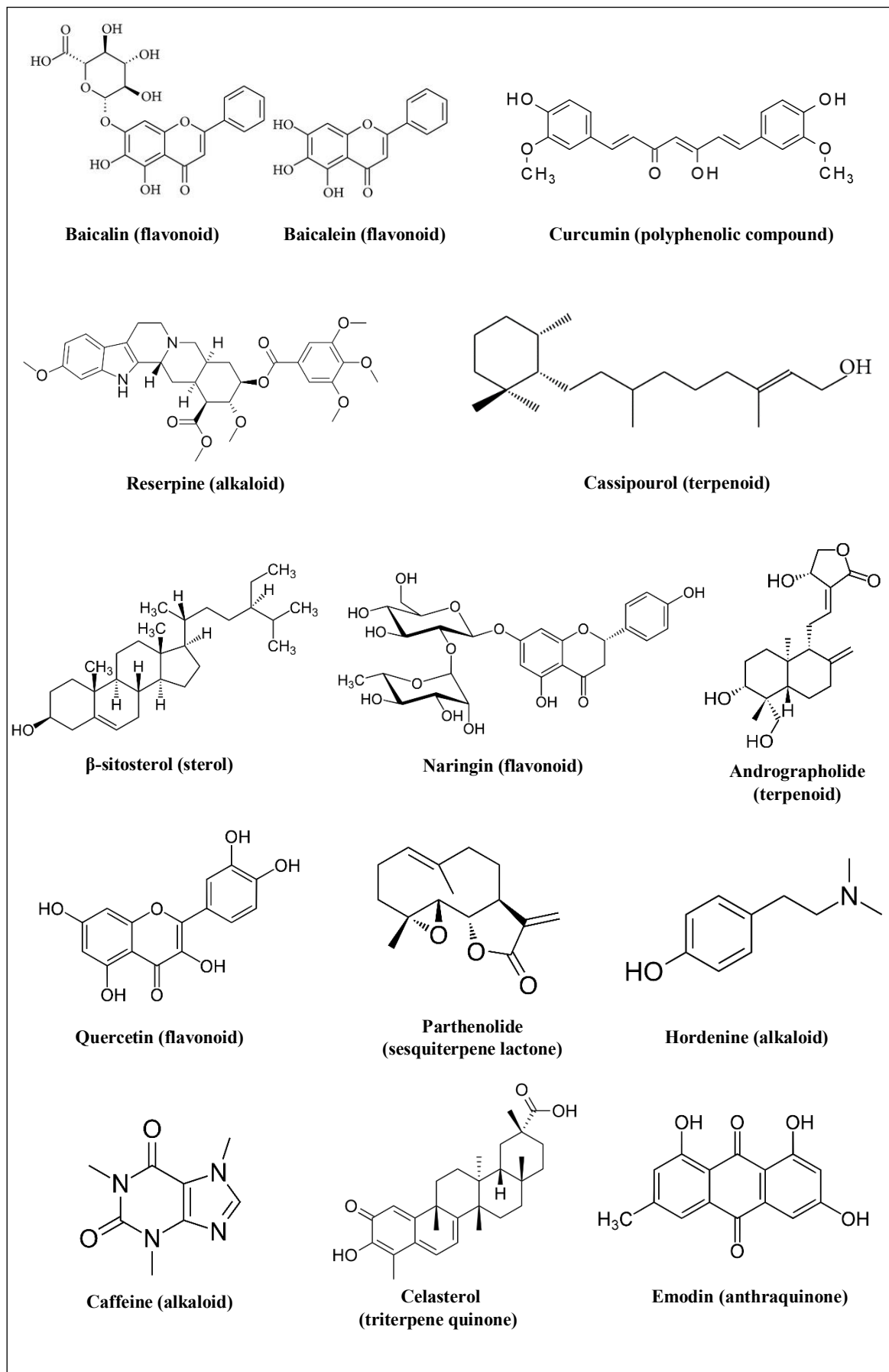


FIGURE 20 | Natural compounds isolated from plants that have presented an antibiofilm activity with elucidation of the potential mechanism of action.

TABLE 14 | Non-exhaustive summary of plants derived compounds with their demonstrated antibiofilm activity.

Plant species	Active extract/compound	Concentration	Target bacteria	Antibiofilm effect	Mechanism of action	References
<i>Scutellaria baicalensis</i>	Baicalin and its aglycone Baicalein	256 µg/mL	<i>P. aeruginosa</i> PAO1	<ul style="list-style-type: none"> - Inhibition of biofilm formation - Synergistic activity with antibiotics (tobramycin, levofloxacin and ceftazidime) 	<ul style="list-style-type: none"> - Downregulation of QS-regulatory genes expression (<i>lasI</i>, <i>lasR</i>, <i>rhlI</i>, <i>rhlR</i>, <i>pqsR</i>, and <i>pqsA</i>) - Reduction in 3-oxo-C12-HSL and C4-HSL production - Reduction in QS-regulated virulence factors production (LasA, LasB, pyocyanin and rhamnolipids) 	(Luo <i>et al.</i> , 2017)
		64 µg/mL	<i>S. aureus</i> (Clinical strains)	<ul style="list-style-type: none"> - Inhibition of biofilm formation - Synergistic activity with vancomycin 	<ul style="list-style-type: none"> - Downregulation of QS-regulatory gene (<i>agrA</i>) - Reduction in virulence factors production (α-hemolysin and enterotoxin A) 	(Chen <i>et al.</i> , 2016)
		50 µg/mL	<i>E. coli</i> (APEC-O78)	<ul style="list-style-type: none"> - Inhibition of biofilm formation 	<ul style="list-style-type: none"> - Downregulation of AI-2 synthesis genes expression (<i>luxS</i> and <i>pfs</i>) - Reduction in AI-2 autoinducer production - Downregulation of virulence factors genes (<i>fimB</i>, <i>csgA</i> and <i>csgB</i>). 	(Peng <i>et al.</i> , 2019)
		100 µM	<i>B. cenocepacia</i>	<ul style="list-style-type: none"> - Synergistic activity with tobramycin 	-	(Brackman <i>et al.</i> , 2011)
<i>Curcuma longa</i>	Curcumin	25 µg/mL	<i>P. aeruginosa</i> PAO1	<ul style="list-style-type: none"> - Inhibition of biofilm formation 	<ul style="list-style-type: none"> - Anti-QS activity using CV026 biosensor strain - Reduction in EPS production - Reduction in alginate and rhamnolipids production 	(Packiavathy <i>et al.</i> , 2014)
<i>Rauwolfia serpentina</i>	Reserpine	IC ₅₀ = 800 µg/mL EC ₅₀ = 300 µg/mL	<i>S. aureus</i> MTCC 96	<ul style="list-style-type: none"> - Inhibition of biofilm formation - Eradication of a preformed biofilm 	<ul style="list-style-type: none"> - Reduction in EPS matrix 	(Parai <i>et al.</i> , 2020)

TABLE 14 | Continued.

		400 µg/mL	<i>P. aeruginosa</i> PAO1	<ul style="list-style-type: none"> - Inhibition of biofilm formation - Eradication of a preformed biofilm 	<ul style="list-style-type: none"> - Downregulation of QS regulatory genes expression (<i>lasI</i>, <i>lasR</i>, <i>rhlI</i> and <i>rhlR</i>) - Reduction in EPS matrix - Reduction in QS-regulated virulence factors (protease, elastase, pyocyanin and rhamnolipids) 	(Parai <i>et al.</i> , 2018)
<i>Platostoma rotundifolium</i>	Cassipourol	200 µM / 100 µM	<i>P. aeruginosa</i> PAO1	<ul style="list-style-type: none"> - Inhibition of biofilm formation - Eradication of a preformed biofilm - Synergistic activity with tobramycin 	<ul style="list-style-type: none"> - Downregulation of QS-regulatory genes expression (<i>lasI</i>, <i>lasR</i>, <i>rhlI</i>, and <i>rhlR</i>) - Downregulation of QS-regulated virulence factors genes (<i>lasB</i> and <i>rhlB</i>) - Reduction in EPS and alginate production - Downregulation of <i>pelA</i> gene 	(Rasamiravaka <i>et al.</i> , 2017)
	β-sitosterol					
<i>Combretum albiflorum</i>	Naringin	410 µg/mL	<i>P. aeruginosa</i> MTCC 2488	<ul style="list-style-type: none"> - Eradication of a preformed biofilm - Synergistic activity with ciprofloxacin and tetracycline 	<ul style="list-style-type: none"> - Reduction in EPS matrix 	(Dey <i>et al.</i> , 2020)
<i>Andrographis paniculata</i>	Chloroform extract	1.25 mg/mL	<i>P. aeruginosa</i> PAO1	<ul style="list-style-type: none"> - Inhibition of biofilm formation 	<ul style="list-style-type: none"> - Downregulation of QS-regulatory genes expression (<i>lasI</i>, <i>lasR</i>, <i>rhlI</i> and <i>rhlR</i>) - Reduction in QS-regulated virulence factors (pyocyanin, elastase and rhamnolipids) 	(Banerjee, Moulick, <i>et al.</i> , 2017)
	Andrographolide	50 µg/mL	<i>S. aureus</i> MTCC 96	<ul style="list-style-type: none"> - Inhibition of biofilm formation 	-	(Banerjee, Parai, <i>et al.</i> , 2017)
		10 µg/mL	<i>E. coli</i> APEC-O78	<ul style="list-style-type: none"> - Inhibition of the initial adhesion 	<ul style="list-style-type: none"> - Decrease in AI-2 activity - Downregulation of adhesin genes expression (<i>fimC</i> and <i>papC</i>) 	(Guo <i>et al.</i> , 2014)
<i>Herba patriniae</i>	Water extract	1.6 mg/mL	<i>P. aeruginosa</i> PAO1	<ul style="list-style-type: none"> - Inhibition of biofilm formation 	<ul style="list-style-type: none"> - Downregulation of biofilm-associated genes expression (<i>algU</i>, <i>algA</i> and <i>pslM</i>) - Reduction in EPS production 	(Fu <i>et al.</i> , 2017)

TABLE 14 | Continued.

Ubiquitous in vegetables and fruits	Quercetin	16 µg/mL	<i>P. aeruginosa</i> PAO1	<ul style="list-style-type: none"> - Inhibition of biofilm formation - Inhibition of the initial adhesion 	<ul style="list-style-type: none"> - Downregulation of QS-regulatory genes expression (<i>lasI</i>, <i>lasR</i>, <i>rhlI</i> and <i>rhlR</i>) - Reduction in QS-regulated virulence factors (pyocyanin, protease and elastase) 	(Ouyang <i>et al.</i> , 2016)
<i>Tanacetum parthenium</i>	Parthenolide	1 mM	<i>P. aeruginosa</i> PAO1	<ul style="list-style-type: none"> - Inhibition of biofilm formation 	<ul style="list-style-type: none"> - Downregulation of QS-regulatory genes expression (<i>lasI</i>, <i>lasR</i>, <i>rhlI</i> and <i>rhlR</i>) - Reduction in QS-regulated virulence factors (pyocyanin and protease) 	(Kalia <i>et al.</i> , 2018)
Barley	Hordeinine	1 mg/mL	<i>P. aeruginosa</i> PAO1	<ul style="list-style-type: none"> - Inhibition of biofilm formation - Eradication of a preformed biofilm - Synergistic activity with antibiotics (netilmicin) 	<ul style="list-style-type: none"> - Downregulation of QS-regulatory genes expression (<i>lasI</i>, <i>lasR</i>, <i>rhlI</i> and <i>rhlR</i>) - Reduction in QS-regulated virulence factors (protease, elastase, pyocyanin, pyoverdine, rhamnolipids and alginate) 	(Zhou <i>et al.</i> , 2018)
Coffee bean	Caffeine	80 µg/mL	<i>P. aeruginosa</i> MTCC 424	<ul style="list-style-type: none"> - Inhibition of biofilm formation 	<ul style="list-style-type: none"> - Reduction in EPS production - Reduction in proteins production - Reduction in QS-regulated virulence factors (protease and pyocyanin) 	(Chakraborty <i>et al.</i> , 2020)
<i>Tripterygium</i> species	Celasterol	40 µmol/L	<i>S. aureus</i> MRSA	<ul style="list-style-type: none"> - Inhibition of biofilm formation - Eradication of a preformed biofilm 	<ul style="list-style-type: none"> - Reduction in EPS production (carbohydrates and proteins) 	(Woo <i>et al.</i> , 2017)
<i>Polygonum cuspidatum</i> and <i>Rheum palmatum</i>	Emodin	4 µg/mL	<i>S. aureus</i> CMCC 26003	<ul style="list-style-type: none"> - Inhibition of biofilm formation 	<ul style="list-style-type: none"> - Downregulation of biofilm-related genes expression (<i>agrA</i>, <i>icaA</i>, <i>cidA</i>, <i>dltB</i> and <i>SarA</i>) 	(Yan <i>et al.</i> , 2017)
Aloe		128 µg/mL	<i>S. aureus</i> ATCC 29213	<ul style="list-style-type: none"> - Inhibition of biofilm formation - Effect on initial adhesion and proliferation 	<ul style="list-style-type: none"> - Reduction in EPS production (proteins and PIA) 	(Xiang <i>et al.</i> , 2017)

III.2.2.2 Marine environment: a valuable source of antibiofilm molecules

The stressful conditions faced by organisms living in marine environment, especially those that are sessile, have led to the evolution of chemical defence systems in order to evade predation and prevent biofouling (Torres *et al.*, 2019). By definition, biofouling represents the undesirable development of micro (bacteria, protists...) and macroorganisms (invertebrates, algae...) on biotic or abiotic surfaces.

The uniqueness, as well as the huge structural and functional diversity of marine natural products, and therefore their wide spectrum of demonstrated biological activities, are surely related to the unique marine life conditions (Jimenez, 2018).

III.2.2.2.1 Seaweed derived compounds with antibiofilm activity

The ability of sessile seaweed to remain intact for long periods of time despite the high risk of biofouling encountered in the marine environment suggests their strong capacity to synthesize antifouling compounds. In this context, different studies have demonstrated the antifouling activity of extracts and/or of compounds, especially those derived from brown and red algae (Dahms & Dobretsov, 2017). However, despite the significant antibiofilm activity exhibited by some seaweed derived compounds against pathogenic bacteria, these marine organisms remain underexplored in novel antibiofilm agent's discovery scenario (Table 15).

Interestingly, the first natural molecule identified for its QS inhibitory activity and consequently biofilm formation inhibitory is the halogenated furanone isolated from the red alga *Delisea pulchra*. In fact, over 20 halogenated furanone compounds, differing in the structure and the substitution in their side chain, as well as in the number and nature of halogens, have been identified in this red alga, native to south eastern coast of Australia (de Nys *et al.*, 1993).

Initially, Manefield *et al.* have demonstrated the ability of a natural halogenated furanone to inhibit the AHL-based QS system by displacing AHL signal molecule from its LuxR receptor, which is explained by the structural similarity between this natural product and the AHLs signal molecules (Manefield *et al.*, 1999). Afterwards, the natural furanone has provided a source of inspiration to chemists who have focused on the synthesis of a wide variety of furanone analogues in order to elucidate the structure-activity relationships for future use in drug development (Lyons *et al.*, 2020).

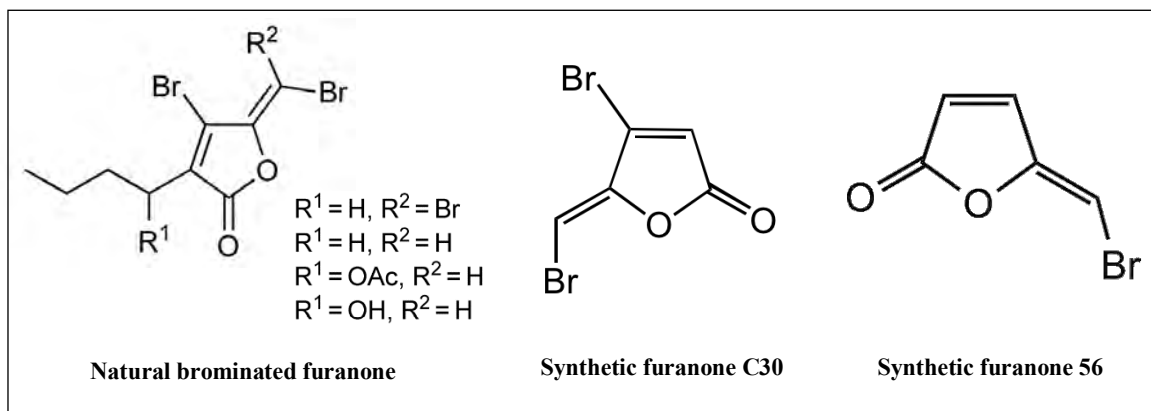


FIGURE 21 | Chemical structure of natural and synthetic halogenated furanones.

In this scenario, Manefield *et al.* have shown the ability of the synthetic furanone C30 to block AHLs-based QS system by accelerating the proteolytic degradation of LuxR receptor (Manefield *et al.*, 2002). Furthermore, a significant synergistic activity between C30 and tobramycin has also been observed against *P. aeruginosa* biofilm (Figure 21) (Hentzer *et al.*, 2003). On the other hand, another synthetic analogue named furanone 56 which lacks the side chain and contains a single bromine substitution, was able to enhance *P. aeruginosa* biofilm detachment and to reduce its virulence (Figure 21) (Hentzer *et al.*, 2002).

Interestingly, furanone's efficacy was not restricted on AHLs QS system but an antagonistic activity towards AI-2 QS system has also been demonstrated which indicates its wide range of activity as a non-specific intercellular signal antagonist (Ren *et al.*, 2001; Zang *et al.*, 2009).

Unfortunately, the clinical application of natural, as well as of synthetic halogenated furanones is hampered by their toxicity. In fact, the reactivity of these molecules, linked to the presence of halogen atoms in their structures makes them too toxic and therefore unsuitable for the treatment of bacterial infections (Husain *et al.*, 2019).

TABLE 15 | Non-exhaustive summary of seaweed derived compounds with a demonstrated antibiofilm activity.

Seaweed species	Active extract/compound	Concentration	Target bacteria	Antibiofilm effect	Mechanism of action	References
<i>Delisea pulchra</i> (Red alga)	Halogenated furanone (Natural)	50 μ M	<i>E. coli</i> recombinant strain (MT102)	–	– AHL antagonist (OHHL)	(Manefield <i>et al.</i> , 1999)
		5 and 10 μ g/mL	<i>E. coli</i> recombinant strain (JM109)	– Inhibition of biofilm formation at 60 μ g/mL	– Inhibition of AI-2 based QS system	(Ren <i>et al.</i> , 2001)
		100 μ M	<i>E. coli</i> recombinant strain (BL21)	–	– Inhibition of LuxS synthase protein	(Zang <i>et al.</i> , 2009)
	Halogenated furanone C30 (Synthetic)	10 μ M	<i>E. coli</i> recombinant strain (XL1-Blue)	–	– Promotes the degradation of the Lux R receptor	(Manefield <i>et al.</i> , 2002)
			<i>P. aeruginosa</i> PAO1	– Synergistic activity with tobramycin	– Reduction in QS-regulatory virulence factors production (elastase and pyoverdine)	(Hentzer <i>et al.</i> , 2003)
Halogenated furanone 56 (Synthetic)	5 μ g/mL	<i>P. aeruginosa</i> PAO1	– Stimulation of biofilm detachment	– AHL antagonist (OHHL) – Reduction in QS-regulatory virulence factor production (elastase)	(Hentzer <i>et al.</i> , 2002)	
<i>Asparagopsis taxiformis</i> (Red alga)	2-dodecanoyloxyethane- sulfonate (Expected active compound)	Identified in 100 mg/mL of methanolic extract	<i>Chromobacterium violaceum</i> biosensor strain (CV026)	–	– Anti-QS activity (C6-HSL)	(Jha <i>et al.</i> , 2013)
<i>Gracilaria fisheri</i> (Red alga)	α -resorcyclic acid	100 μ g/mL	<i>Vibrio harveyi</i> biosensor strain (BAA 1116)	– Inhibition of biofilm formation	– Inhibition of AI-2 based QS system	(Karnjana <i>et al.</i> , 2020)
	<i>N</i> -benzyl cinnamamide					
<i>Hizikia fusiforme</i> (Brown alga)	Phlorotannins	48 mg/mL	<i>P. aeruginosa</i> PAO1	– Inhibition of biofilm formation	– Reduction in pyocyanin production	(Tang <i>et al.</i> , 2020)

III.2.2.2.2 Other antibiofilm compounds derived from marine macro- and microorganisms

In addition to algae, the saline environment harbours a myriad of living organisms whose significant ability to produce unique bioactive molecules notably antibiofilm ones, have been demonstrated in various studies. The exploitation of marine microorganisms has received a great attention in this field in view of their huge diversity, their ability to produce active metabolites as well as the possibility of resource regeneration (Wang *et al.*, 2017; Goel *et al.*, 2021). In this context, marine sponges, which account for around 30% of bioactive marine molecules, present a valuable source of antibiofilm compounds, some of which are effective against pathogenic bacteria such as *P. aeruginosa* and *S. aureus* (Stowe *et al.*, 2011).

III.3 EXPERIMENTAL BIOFILM ASSAYS USED FOR BIOFILM STUDIES

Due to their rapid increase and their severity, biofilm-mediated infections are considered as an important concern that requires the search for novel and effective antibiofilm agents. However, the inerrant complexity and heterogeneity of biofilms make their exhaustive analysis by integrating all its components very challenging. In fact, different qualitative and quantitative methods have been applied, each of which targets a particular biofilm feature (matrix, living cells, both living and dead cells...). Therefore, the combination of various experimental approaches such as biochemical, genetic and physical ones is considered the most suitable to achieve a global biofilm analysis (Pantanella *et al.*, 2013). In the following, the most commonly methods used for the quantification and/or the visualization of biofilms are outlined, with their principles, their advantages, as well as their limitations (*Table 17*).

III.3.1 Counting method – CFU counts assay

One of the commonly used methods to quantify biofilm cells is the colony forming units (CFU) counts assay. Indeed, this method relies on the detachment of adhered cells from surface by scraping or sonication followed by their spreading on agar-plate culture prior to counting them. It should be noted that in comparison with two other assays (CV and resazurin tests), Allkja *et al.*, have highlighted the high responsiveness of this method in antimicrobial efficacy test (Allkja *et al.*, 2021).

However, one of the drawbacks of this method is the incomplete detachment of adhered cells (soft scraping) and/or the presence of aggregates, which can lead to an underestimation of biofilm cells number. Moreover, by CFU counts method, only the culturable cells are considered (no data on dormant and viable but non-culturable cells VBNC) (Pantanella *et al.*, 2013; Azeredo *et al.*, 2017).

III.3.2 Staining methods

III.3.2.1 Crystal violet assay (quantitative test)

Currently, one of the most widely used methods for bacterial biofilms quantification is the crystal violet (CV) staining method. This assay consists in labelling the biofilm biomass with CV.

The basic dye binds indifferently to the matrix polysaccharides, as well as to negatively charged bacteria. The stain fixed by biofilm biomass is extracted by an organic solvent, such as ethanol, methanol or acetic acid, prior to measuring the absorbance which is proportional to the total biomass (Pantanella *et al.*, 2013).

The main advantage of this method resides in the fact that it allows a high throughput screening which is crucial, especially in the evaluation of the antibiofilm activity of large libraries of compounds. However, since this assay quantifies the entire biofilm biomass including living cells, dead cells, and the EPS matrix, its combination with another approach that allows detachment and quantification of adhered cells such as the CFU count method is required (Pantanella *et al.*, 2013). Moreover, the sensitivity of this assay towards experimental conditions of biofilm growth, such as environmental factors and inoculum preparation method, reduces its reproducibility which makes the comparison of data between studies not feasible (Allkja *et al.*, 2020).

In addition, variation between bacterial species has also been recorded for this method making the standardization of a well-defined protocol unachievable (Stiefel *et al.*, 2016).

III.3.2.2 DMMB assay (quantitative test)

This colorimetric method is mostly used to quantify *S. aureus* biofilms. Briefly, the cationic dye DMMB (1,9-dimethyl methylene blue) is able to bind to the intercellular polysaccharide adhesin (PIA), a major component of *S. aureus* biofilm matrix (Pantanella *et al.*, 2013). The amount of fixed DMMB dye is determined by spectrophotometric measurement (OD) after addition of a decomplexation solution (Tote *et al.*, 2008).

The DMMB assay presents an economical, rapid and simple method. However, as the CV staining assay, it is unable to assess the number of living bacterial cells within the biofilm (Pantanella *et al.*, 2013).

The major limitations of biofilm quantification methods that are based on OD measurement lie in the detection limit, which is often high, as well as in the possible background signal. Moreover, when evaluating the antibiofilm activity of a product, its potential absorbance and/or its interaction with the dye can distort results (Stiefel *et al.*, 2016).

III.3.3 Microscopic observations

In order to decipher biofilm spatial structure and its associated functions, several imaging modalities have been used such as light microscopy, Scanning Electron Microscopy (SEM), and Confocal Laser Scanning Microscopy (CLSM).

The latter, which emerged in the early 90s, is considered the most versatile microscopic approach owing to the variety (Syto9, SYBR-Green, propidium iodide, fluorescent *in situ* hybridization FISH, Concanavalin A...) and specificity (microbial cells, matrix, specific microorganism...) of fluorescence probes that can be used (Azeredo *et al.*, 2017).

III.3.3.1 Fluorescent assay – focus on the most popular live/dead mixture

This method consists in differentiating biofilm's living cells from dead ones by using two distinct nucleic acid binding stains: Syto9, a green fluorescent dye, and propidium iodide (PI), a red fluorescent dye. Although both markers are able to bind DNA, Syto9 is characterized by its capacity to cross cell membranes of Gram-negative and Gram-positive bacteria and thus stains the intracellular DNA of living cells. However, PI only binds the DNA of damaged cells with compromised membranes (Pantanella *et al.*, 2013). Due to its higher affinity, PI replaces Syto9 when these two markers are exposed to the same extracellular DNA, leading to a red staining for dead cells and a green staining for living cells with sometimes intermediate color (Rosenberg *et al.*, 2019). After labelling, biofilm is visualized using a fluorescent optical microscopy.

Even if this method allows to estimate the proportion of living and dead cells in a biofilm, it is rather considered as a qualitative assay as the counting of total bacterial cells is not feasible (Pantanella *et al.*, 2013). Furthermore, since extracellular DNA is a major component of the biofilm matrix, an overestimation of dead cells can occur (Bjarnsholt *et al.*, 2013). Besides the staining of cells, various fluorescent labels which target different EPS matrix components are also used in the biofilms microscopic observations (Table 16).

TABLE 16 | Fluorescent dyes used to stain EPS matrix components.

Fluorescent dyes	Targeted matrix components	References
Concanavalin A	α -D-glucose and α -D-mannose	(Powell <i>et al.</i> , 2018)
TOTO-1	eDNA	(Powell <i>et al.</i> , 2018)
DAPI	eDNA	(Loza-Correa <i>et al.</i> , 2019)
SYPRO-Ruby	Proteins	(Powell <i>et al.</i> , 2018)
Nile red	Lipids	(Vega-Dominguez <i>et al.</i> , 2020)
Cascade blue	Glucan	(Rainey <i>et al.</i> , 2019)
Calcofluor white	Glycosidic bonds mainly β -(1,4) and β -(1,3)	(Soler-Arango <i>et al.</i> , 2019; Grecka <i>et al.</i> , 2020)
Wheat Germ Agglutinin	N-acetylglucosamine, or sialic acid residues	(Oniciuc <i>et al.</i> , 2016)

III.3.4 Metabolic methods

Biofilm quantification based on cells metabolic activity presents another strategy adopted in the study of microbial biofilms. The principle of this approach consists in the conversion through cellular metabolic activity of a specific substrate into a detectable product (by measurement of OD (XTT assay), fluorescence (resazurin assay), or of luminosity (ATP assay)) (Azeredo *et al.*, 2017).

III.3.4.1 Resazurin assay

Resazurin (7-hydroxy-3*H*-phenoxazin-3-one-10-oxide) is a non-fluorescent blue stain used to quantify living cells within biofilms, based on their metabolic activity. Indeed, living cells are able to reduce resazurin and irreversibly convert it into resorufin, a pink-fluorescent dye. The fluorescence measurement of the formed resorufin reflects the number of metabolically active living cells present within the biofilm (Pantanella *et al.*, 2013).

One of the major drawbacks of this method is its high sensitivity to metabolic rate of bacterial cells, which renders it dependent on their growth phase, as well as on biofilm age and thickness. Furthermore, the standardization of the experimental conditions is difficult as the time of resazurin reduction is species and strain specific (Van den Driessche *et al.*, 2014). In fact, Allkja *et al.*, have demonstrated the suitability of this method for *S. aureus* biofilm quantification. However, this assay has shown a poor responsiveness in treatment efficacy experiments which can be explained by an unknown interaction between resazurin and the tested compound (Allkja *et al.*, 2021).

III.3.4.2 The XTT assay

As for the resazurin test, the XTT (2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide salt) method relies on the ability of the respiratory metabolism of living cells to reduce the tetrazolium salt and to convert it to a water-soluble formazan. The absorbance measurement of the supernatant permits to determine the number of living cells within biofilm (Xu *et al.*, 2016).

Like other metabolic tests, heterogeneity and complexity of biofilm structure, resulting in a metabolic gradient, diminish the accuracy of XTT assay. On the other hand, the potential retention of XTT reduction or formazan release limits the relevance of this assay in mature biofilm studies (Pantanella *et al.*, 2013).

III.3.4.3 The ATP assay

This method relies on the quantification of viable bacteria by their mitochondrial adenosine triphosphate (ATP) which is directly proportional to their number. In fact, this assay is based on the reaction between luciferin and ATP, catalyzed by luciferase, leading to the formation of oxyluciferin with emission of a detectable luminescent signal (Herten *et al.*, 2017).

ATP assay is considered as a rapid, accurate, and sensitive method that can be used to quantify various bacterial species. However, the required materials (e.g. luciferase and luciferin) are relatively expensive, mainly for the analysis of numerous samples (Stiefel *et al.*, 2016; Herten *et al.*, 2017).

III.3.5 Molecular biology methods

III.3.5.1 Quantitative polymerase chain reaction qPCR

The application of qPCR allows quantification of bacteria contained in a biofilm through detection and quantification of specific gene sequences related to a bacterial species.

So, the major advantage of this technique in biofilm analysis is the ability to quantify different species within one sample (Azeredo *et al.*, 2017). However, this expensive method is not informative about the proportion of living cells in a biofilm since it does not differentiate between DNA of living and dead cells (Pantanella *et al.*, 2013).

On the other hand, RT-qPCR (Reverse Transcription quantitative PCR) method which is based on detection and quantification of mRNA with short half-life has been used in biofilms studies. In this context, Magalhaes *et al.*, have applied RNA-based qPCR in an attempt to investigate the interactions occurring in a dual-species biofilm composed by *P. aeruginosa* and *S. aureus* (Magalhaes *et al.*, 2019).

For both of these gene-based assays, the choice of the primer sequences is a crucial step in order to prevent the amplification of gene sequences not relevant for the objectives of the study (Pantanella *et al.*, 2013). In addition, the fact that these molecular biology methods are not performed directly on biofilm but on the resuspended adhered cells (potential modification of genes expression) present a drawback to these approaches.

TABLE 17 | The most common methods used in biofilms analysis.

Method	Assay	Principle of the assay	Advantages	Limits	References
Counting method	CFU counts method	Enumeration of living cells recovered from biofilm (scraping, sonication...) by agar-plate culture	<ul style="list-style-type: none"> - Simple - High responsiveness in treatment assays 	<ul style="list-style-type: none"> - Time-consuming - Detection of culturable cells only (dormant, viable but non-culturable cells are not quantified) - Underestimation due to the presence of aggregation - Underestimation due the lack of total recovery of biofilm cells (soft scraping...) 	(Azeredo <i>et al.</i> , 2017; Allkja <i>et al.</i> , 2021)
	Staining methods	Crystal violet (CV) assay (quantitative test)	Quantification of total biofilm biomass by CV dye	<ul style="list-style-type: none"> - High-Throughput screening - Economic - Rapid - Simple - Applied directly on biofilm 	<ul style="list-style-type: none"> - Low-reproducibility - Lack of sensitivity - Species specific hence the lack of a standardized protocol - No differentiation between live and dead cells - High detection limit - Possible interaction between dye and the tested product in treatment assays
	DMMB assay (quantitative test)	Quantification of <i>S. aureus</i> biofilm by binding to PIA of its matrix	<ul style="list-style-type: none"> - Rapid - Simple - Applied directly on biofilm 	<ul style="list-style-type: none"> - Low-reproducibility - Lack of sensitivity - Species specific hence the lack of a standardized protocol - No differentiation between live and dead cells - High detection limit - Possible interaction between dye and the tested product in treatment assays 	(Tote <i>et al.</i> , 2008; Pantanella <i>et al.</i> , 2013; Azeredo <i>et al.</i> , 2017; Allkja <i>et al.</i> , 2020)
	Fluorescent assay	<ul style="list-style-type: none"> - Differentiation between living and dead biofilm cells by a couple of fluorescent stains (Syto9/PI) - Detection of EPS components by specific fluorescent dyes 	<ul style="list-style-type: none"> - Visualisation of biofilm morphology and estimation of living and dead cells proportion - Visualisation of biofilm spatial structure 	<ul style="list-style-type: none"> - Qualitative assay - Overestimation of dead cells due to eDNA - Relatively expensive - Possible interference between fluorescence probes and biofilm proprieties 	(Pantanella <i>et al.</i> , 2013; Bjarnsholt <i>et al.</i> , 2013; Azeredo <i>et al.</i> , 2017)
Metabolic methods	Resazurin assay	Quantification of metabolically active living cells by resazurin dye	<ul style="list-style-type: none"> - High-throughput screening - Versatile (can be applied on various microorganisms) - Applied directly on biofilm 	<ul style="list-style-type: none"> - High sensitivity to metabolic rate - Low detection limit - Possible interaction between dye and the tested compound in treatment assays - Species and strain specific hence the lack of a standardized protocol - Not suitable for mature biofilm studies 	(Pantanella <i>et al.</i> , 2013; Azeredo <i>et al.</i> , 2017)
	XTT assay	Quantification of metabolically active living cells by XTT tetrazolium salt	<ul style="list-style-type: none"> - Applied directly on biofilm 	<ul style="list-style-type: none"> - High sensitivity to metabolic rate - Low detection limit - Possible interaction between dye and the tested compound in treatment assays - Species and strain specific hence the lack of a standardized protocol - Not suitable for mature biofilm studies 	(Pantanella <i>et al.</i> , 2013; Azeredo <i>et al.</i> , 2017)

TABLE 17 | Continued.

	ATP assay	Quantification of metabolically active living cells by their ATP content	<ul style="list-style-type: none"> - Rapid - Accurate - Sensitive - Applied directly on biofilm 	<ul style="list-style-type: none"> - Relatively expensive 	(Stiefel <i>et al.</i> , 2016; Herten <i>et al.</i> , 2017)
Molecular biology methods	qPCR (quantitative polymerase chain reaction)	Determination of total number of cells based on the amplification of a targeted gene by PCR	<ul style="list-style-type: none"> - Detection and quantification of a specific microorganism in a biofilm - Quantification of various species within one sample 	<ul style="list-style-type: none"> - Quantification of both living and dead cells - Overestimation due to the presence of eDNA - High-cost - Laborious method - Crucial and judicious choice of primers sequence - Performed on resuspended adhered cells and not directly on biofilm 	(Guilbaud <i>et al.</i> , 2005; Pantanella <i>et al.</i> , 2013; Azeredo <i>et al.</i> , 2017)
	RT-qPCR (reverse transcription quantitative PCR)	Detection and quantification of mRNA	Genes expression studies in an attempt to elucidate mechanisms of action and interactions	<ul style="list-style-type: none"> - High cost - Laborious method - Crucial and judicious choice of primers sequence - Performed on resuspended adhered cells and not directly on biofilm 	(Xie <i>et al.</i> , 2011; Pantanella <i>et al.</i> , 2013)

MAIN OBJECTIVES

The previous literature review highlights the richness of seaweed in bioactive molecules that can be valorized in different fields. Indeed, although the exploration of algae for their potential insecticidal and mostly for their antibiofilm activity remains limited, some extracts and/or compounds derived from seaweed have been documented for their effectiveness in these two areas. In this context, we focus in this study on the exploration of three algae collected from Lebanon in terms of their potential insecticidal and antibiofilm activity against the fruit fly *D. melanogaster* and two pathogenic bacteria (*P. aeruginosa* and *S. aureus*), respectively.

- Regarding the evaluation of the insecticidal activity, the effect of the green alga *U. lactuca* on the fruit fly *D. melanogaster* was assessed using different methods, each of which presents a specific mode of exposure.
- Concerning the evaluation of the antibiofilm activity, the potential ability of extracts derived from three algae (green *U. lactuca*, brown *S. scoparium*, and red *P. capillacea*) to impair both *P. aeruginosa* and *S. aureus* biofilm was assessed. Different methods have been used in an attempt to decipher the antibiofilm mechanism of action exhibited by the active extracts.

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Green seaweed: potential alternative to chemical insecticide

ARTICLE I – Published:

Rima, M., Chbani, A., Roques, C., & El Garah, F. (2021). Comparative study of the insecticidal activity of a high green plant (*Spinacia oleracea*) and a chlorophytae algae (*Ulva lactuca*) extracts against *Drosophila melanogaster* fruit fly. *Ann Pharm Fr*, 79(1), 36-43. doi: 10.1016/j.pharma.2020.08.005

▪ CHAPTER PREVIEW

In this chapter, the evaluation of the insecticidal activity of extracts derived from the green alga *Ulva lactuca* against the fruit fly *Drosophila melanogaster* is presented. In addition to the agricultural damage caused by this pest on sweet fruits during their production and also with large economic impact, this fruit fly is considered the best model for the study of insecticidal activity, which justifies its use in our study. Green extraction solvents such as ethanol and acetone were favored. In parallel to the organic and aqueous extracts, the potential insecticidal activity of pigments purified from this green alga was also evaluated. In this context, the green plant *Spinacia oleracea*, a valuable source of chlorophyll, was used as a control. The insecticidal and the repellent activities of extracts were assessed following different complementary methods.

The work presented in this chapter was done in the Applied Biotechnology Laboratory (LBA3B-ER032) – AZM Center for Research in Biotechnology and its applications – Lebanese University – Tripoli – Lebanon and resulted in a published article (Rima *et al.*, 2021).

In the first part of this chapter, materials and methods used to complete this work are detailed. The publication preceded by a brief summary of the main results is then integrated. Supplementary unpublished data are finally presented.

I. MATERIALS & METHODS

I.1. MATERIALS

I.1.1 Organic solvents

The organic solvents used are listed below:

- Ethanol 96% (Sigma-Aldrich)
- Acetone > 99.5% (Sigma-Aldrich)
- Methanol 99.8% (Sigma-Aldrich)
- Diethyl ether > 99.0% (Sigma-Aldrich)
- Petroleum ether (Sigma-Aldrich)

I.1.2 Chemical compounds

The chemical compounds used are mentioned below:

- Sodium carbonate (Sigma-Aldrich)
- Sodium sulfate (Sigma-Aldrich)
- Potassium hydroxide (Sigma-Aldrich)

I.1.3 Algal material

The green alga *Ulva lactuca* used in this study was manually collected from the North Lebanese coast of the Mediterranean, particularly from El Mina - Tripoli in July 2017. In order to remove undesirable impurities such as adhered sand particles and epiphytes which can contaminate the samples, a rigorous washing with the surrounding seawater was applied to the collected seaweed. Then, alga was immediately transported to the laboratory of AZM center of research in biotechnology and its applications where a second wash with distilled water was carried out. Alga samples were air-dried at room temperature in the dark for several weeks and weighed continuously until complete drying (Al Monla *et al.*, 2020). Dried algae were then stored in sealed bags at room temperature in the dark until use.

I.1.4 Plant material

Spinach leaves (*Spinacia oleracea*) used in this study were purchased from a Tripoli – Lebanon local market in July 2017. The same washing, drying, and storage procedures as for the green alga were applied to this plant.

Oranges (*Citrus sinensis*) were used for the insecticide bioassays. They were purchased from a Tripoli – Lebanon local market, washed with distilled water to remove impurities, and manipulated on the same day.

I.1.5 Biological material

Fruit flies (*Drosophila melanogaster*) were taken from an infected orange containing larvae at different stages of growth picked from a field in Tripoli – Lebanon in February 2018 (Figure 22). This infected orange was placed in a plastic jar covered with muslin. After two weeks, adults fruit flies emerged corresponding to the first generation of *drosophila* reared under laboratory conditions.

Flies were reared in new plastic jars covered with muslin cloth allowing the air to pass through. Non-contaminated pieces of oranges were offered as a food source, place of reproduction, and egg-laying.



FIGURE 22 | Orange infected by *D. melanogaster* larvae. (A) external view, (B) internal view, (C) rearing jar.

I.2 METHODS

I.2.1 Preparation of crude extracts

The dried and milled samples of the green alga and spinach leaves were extracted separately by maceration in distilled water, ethanol, and acetone with a ratio of 1 g : 4 mL (Saritha *et al.*, 2013). Extraction was carried out at room temperature and under continuous orbital shaking. After 24 hours, crude extracts were recovered by filtration using a whatman filter paper and stored at 4°C until use.

I.2.2 Quantification of pigments content in acetic and ethanolic extracts

The amount of chlorophyll pigments (Chlorophyll a and Chlorophyll b) in ethanolic (OD_{665nm} and OD_{649nm}) and acetic extracts (OD_{662nm} and OD_{645nm}) as well as of carotenoids (OD_{470nm}) was calculated by measuring the absorbance (Evolution 60 UV-visible spectrophotometer, Thermo Scientific) at the corresponding wavelengths and by applying the appropriate formula (Lichtenthaler & Wellburn, 1983). It should be noted that the quantity of pigments was calculated to investigate the potential correlation between their amount in extract and the insecticidal activity.

I.2.3 Separation of chlorophyll and carotenoid pigments by the differential solubility method

In order to evaluate their potential insecticidal and repellent activity towards the fruit fly *D. melanogaster*, green pigments (Chl a and Chl b) and carotenoids (carotene and xanthophyll) were extracted from the green alga (*U. lactuca*) and spinach leaves (*S. oleracea*) following the differential solubility method previously described by (Prat, 2007) (Figure 23).

Extraction of pigments with acetone and preparation of ethereal solution

10.0 g of plant and algal materials were ground in a porcelain mortar with 60.0 mL of acetone. Small pinches of sodium carbonate and sodium sulfate were added to neutralize the acidity and to reduce the moisture, respectively. Extract was then filtered through a Whatman filter paper and transferred to a separating funnel.

75.0 mL of petroleum ether were mixed with the acetic extracts. As acetone and petroleum ether are two miscible solvents, 25.0 mL of distilled water were added in order to dissolve the acetone and separate the two phases.

After delicate shaking and decanting, two phases were obtained: an upper phase (to be kept) containing pigments in petroleum ether and a lower one (to be discarded) containing acetone, water, and debris.

It is recommended to rinse the pigment phase several times with distilled water in order to obtain a well purified ethereal solution.

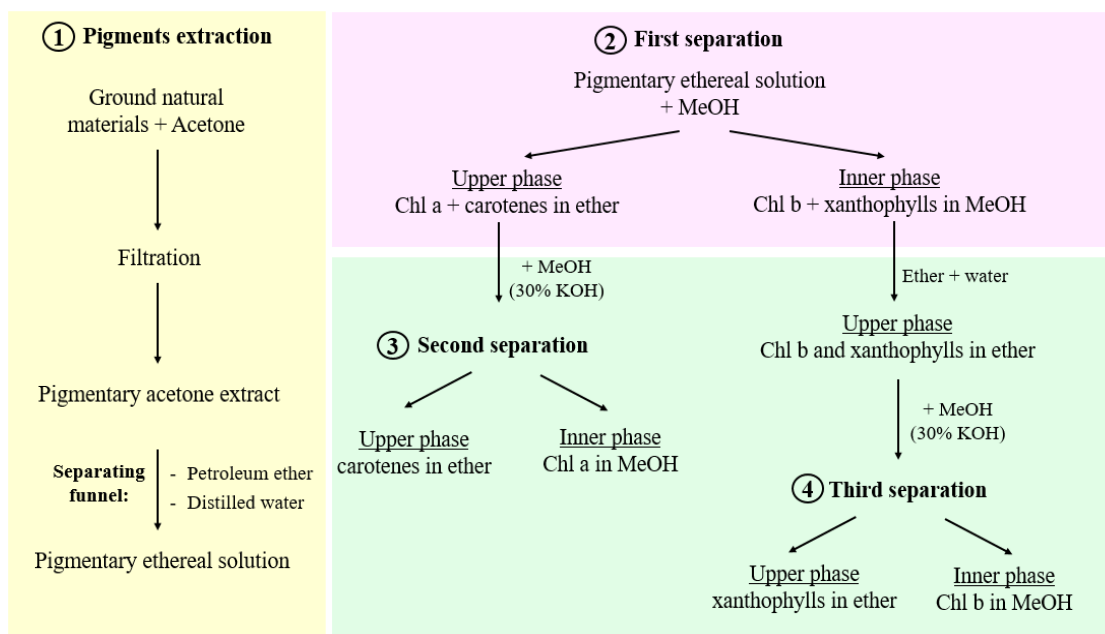


FIGURE 23 | Separation of pigments from the green alga (*U. lactuca*) and spinach leaves (*S. oleracea*) by the differential solubility method.

First separation

The first separation consists of adding 25.0 mL of methanol to 25.0 mL of the ethereal solution. After agitation and decantation, two phases were separated: an upper one containing Chl a and carotenes dissolved in petroleum ether and a lower one containing Chl b and xanthophyll in methanol (reserved for the third separation).

Second separation

To the ethereal solution (Chl a + carotenes), 25.0 mL of a freshly prepared solution of methyl alcohol with 30% of potash (facilitates the separation) were added. After mixing and decanting, two phases were formed: an upper one containing carotenes in ether and a lower one containing Chl a in methanol.

Third separation

To the methanolic fraction enclosing Chl b and xanthophyll, 50.0 mL of diethyl ether were added. 25.0 mL of distilled water were then added leading to the formation of two phases: an upper one containing pigments in diethyl ether and a lower one containing methanol and water (to be discarded).

Finally, 25.0 mL of the methyl alcohol (30% of potash) were added to the ether phase leading to the separation of Chl b and xanthophyll (xanthophyll dissolved in diethyl ether in the upper phase and Chl b solubilized in methanol in the lower phase).

The absorbance spectrum between 400 and 700 nm of each purified pigment was plotted to confirm the proper separation (Suppl. III.1).

1.2.4 Insecticidal activity bioassays

The potential insecticidal activity of aqueous, ethanolic, and acetonetic extracts derived from the green alga (*U. lactuca*) and spinach leaves (*S. oleracea*) against the fruit fly (*D. melanogaster*) was evaluated following various complementary methods. Interestingly, each test exhibits a specific mode of exposure. Effect of purified pigments (Chl a, Chl b, carotene, and xanthophyll) was also tested. Extraction solvents were used as negative control and each test was performed in triplicate. Insecticidal activity of extracts was determined using Sun-Shepard formula (non-uniform population) (1), which corrects the efficacy by nullifying solvent effect (Püntener, 1981).

$$(1) \quad \frac{\text{mortality \% in treated plot} + \text{change \% in control plot population}}{100 + \text{change \% in control plot population}} \times 100$$

- With change % in control plot population =

$$\frac{\text{Population in control after treatment} - \text{population in control before treatment}}{\text{Population in control before treatment}} \times 100$$

1.2.4.1 Test 1: Spraying oranges

In this first test, natural conditions were imitated on a laboratory scale by using the protocol previously described by (Chaieb *et al.*, 2010) with some modifications (*Figure 24, A*). Oranges were sprayed with 1.0 mL of extract or not and after drying, each one was placed in an individual plastic jar. Then, 15 ± 5 adult flies were distributed into each jar. The number of dead flies in each jar was recorded after 24, 48, and 72h. It should be noted that in this assay the tested extract can penetrate into the insect by simple contact and/or by ingestion.

1.2.4.2 Test 2: Ingestion toxicity

In this second test, the potential ability of extracts to kill flies by ingestion was assessed following the protocol developed by (Aboussaid *et al.*, 2010) with some modifications (*Figure 24, B*).

So, a piece of orange impregnated with 1.0 mL of extract was placed in a plastic jar in presence of 15 ± 5 fruit flies. Mortality was determined after 24, 48, and 72h of exposure.

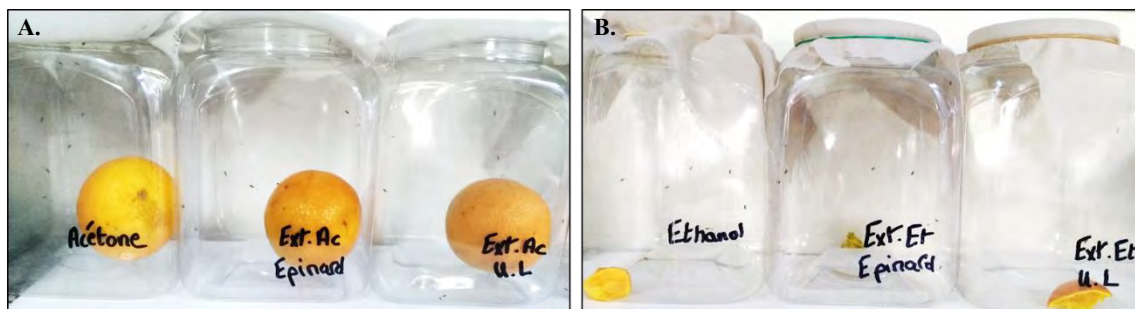


FIGURE 24 | Insecticidal activity bioassays. (A) Spraying oranges, (B) Ingestion toxicity.

1.2.4.3 Test 3: Repellent activity

Based on the method « choice bioassay » described by (Renkema *et al.*, 2017), the potential capacity of extract to repel fruit flies was evaluated (Figure 25). Briefly, a small tube containing a piece of orange impregnated with 1.0 mL of extract was placed in a plastic jar. In a parallel jar, a tube carrying a piece of orange impregnated with 1.0 mL of the corresponding extraction solvent was placed. The two jars were connected with transparent perforated paper to allow the passage of air. To facilitate their handling, fruit flies were anesthetized by holding them at -4°C for 5 - 7min.

The anesthetized flies (15 ± 5) were then placed in the jar containing the extract and their location was determined after 2, 6, and 24h. The repellent percentage was calculated using the following formula (2).

$$(2) \quad \frac{\text{Total number of flies} - \text{number of flies remaining in the jar containing extract}}{\text{Total number of flies}} \times 100$$



FIGURE 25 | Repellent activity bioassay.

1.2.4.4 Statistical analysis

Results are expressed as means \pm SEM of three independent experiments using SPSS 22.0 software (SPSS, IBM Corporation, Armonk, NY, USA)

II. ARTICLE SUMMARY – FOCUS ON THE MAIN RESULTS

The aim of this study is to evaluate the insecticidal activity of aqueous, acetic and ethanolic extracts derived from the green alga *U. lactuca* as well as those obtained from the spinach *S. oleracea* against the fruit fly *D. melanogaster* in an effort to find natural, efficient and ecofriendly alternatives to the currently used toxic pesticides. It should be noted that the choice of extraction solvents was based on health and safety reasons as well as on the ability of acetone and ethanol to extract pigments of interest. The insecticidal effect of pigments (chlorophylls and carotenoids) purified from these two natural sources was also assessed. Three complementary *in vivo* assays, each one with a specific mode of exposure, were used: application by spraying oranges (effect by contact and/or ingestion), toxicity by ingestion and repellent activity.

Interestingly, results showed a correlation between the quantity of chlorophyllian pigments in acetic and ethanolic extracts and their insecticidal activity determined by the spraying oranges method (*Table 18*).

TABLE 18 | Summary table of the demonstrated insecticidal activity of extracts and green pigments derived from the green alga *U. lactuca* and from spinach *S. oleracea*. ND: not determined. NA: not active.

Nature of extract		Pigment content (Total chlorophyll)	Insecticidal activity		
			Spraying oranges	Ingestion toxicity	Repellent activity
Aqueous	<i>S. oleracea</i>	ND	++	NA	> 80%
	<i>U. lactuca</i>	ND	NA	NA	< 80%
Acetic	<i>S. oleracea</i>	High	++	+	ND
	<i>U. lactuca</i>	Moderate	++	++	ND
Ethanolic	<i>S. oleracea</i>	High	++	NA	< 80%
	<i>U. lactuca</i>	Weak	NA	NA	< 80%
Purified pigments (Chlorophyll a and b)			++	NA	< 30%
Mortality % > 80%		++			
Mortality % < 80%		+			
Mortality % < 30%		NA			

In fact, the two acetic extracts as well as the ethanolic extract originated from spinach exhibited an interesting insecticidal activity leading to ≈ 96 and 82% of flies' mortality, respectively. On the other hand, the considerable insecticidal effect of the purified green pigments (Chl a and Chl b) recorded only by the spraying oranges method suggests a potential transcutaneous mode of action of these green pigments.

Moreover, the strong insecticidal effect observed for the acetonic extract originated from the green alga *U. lactuca* whether sprayed on oranges or mixed with flies' nutrient source suggests its richness in other unique bioactive compounds.

The obtained results summarized in the table below highlighted the potential ability of green pigments as well as of extracts originated from the green alga *U. lactuca* to be exploited as an effective natural alternative to synthetic insecticide. These encouraging findings require further experiments in order to analyze the chemical composition of the extracts and thus identify novel bioactive molecules.



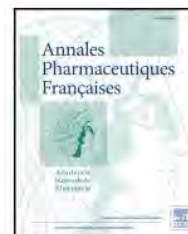
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ORIGINAL ARTICLE

Comparative study of the insecticidal activity of a high green plant (*Spinacia oleracea*) and a chlorophytae algae (*Ulva lactuca*) extracts against *Drosophila melanogaster* fruit fly



Étude comparative de l'activité insecticide des extraits issus d'une plante verte (Spinacia oleracea) et d'une algue verte chlorophyte (Ulva lactuca) contre la mouche de fruit Drosophila melanogaster

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HIGHLIGHTS

- This study highlights the ability of natural extracts of *Spinacia oleracea* and *Ulva lactuca* to present an alternative to chemical insecticide.
- Acetone extracts, rich in green pigments, presented the best insecticidal activities.
- The purified green pigments showed an interesting insecticidal activity by the spraying method.
- The algal acetonic extract presented a cumulative activity by both the spraying and ingestion methods.
- The aqueous extract of spinach displayed an interesting repellent activity against *D. melanogaster*.

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KEYWORDS

Ulva lactuca;
Spinacia oleracea;
 Pigments;
 Extracts;
 Insecticidal activity;
Drosophila melanogaster

Summary

Objectives. – Currently, the global interests tend to take advantage of the plant world as a renewable source of a natural and effective molecule, to find an eco-friendly, cost-effective, and less toxic alternative to the current synthetic pesticide. In this context, the present research was carried out in an attempt to study the insecticidal activity of extracts and pigments derived from the green plant *Spinacia oleracea* and the green alga *Ulva lactuca* against the fruit fly *Drosophila melanogaster* as an alternative to chemical insecticide.

Methods. – The toxicity of the aqueous, acetic and ethanolic extracts as well as of the purified pigments (Chlorophylls and carotenoids) was determined by complementary in vivo tests (application by spraying oranges, toxicity by ingestion and repellent activity). Interestingly, each one of these methods corresponds to a specific mode of exposure.

Results. – Results showed that acetone extracts, which are rich in green pigments, present the best insecticidal activities. On the other hand, the purified chlorophyllian pigments exhibited an interesting activity only by spraying method. Regarding the repellent activity, the aqueous extract of spinach displayed higher effectiveness.

Conclusion. – Our study suggests the potential of tested plant and algal extracts, as well as of chlorophyllian pigments, to provide a safer alternative way to the use of synthetic pesticides.

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MOTS CLÉS

Ulva lactuca ;
Spinacia oleracea ;
 Pigments ;
 Extraits ;
 Activité insecticide ;
Drosophila melanogaster

Résumé

Objectifs. – Actuellement, l'intérêt mondial tend à profiter du monde végétal comme étant une source renouvelable des molécules bioactives afin de chercher des alternatives écologiques, moins toxiques et rentables aux pesticides synthétiques. Dans ce contexte, cette étude est menée dans le but d'évaluer l'activité insecticide des extraits, ainsi que des pigments issus de l'épinard (*Spinacia oleracea*) ainsi que d'une algue verte (*Ulva lactuca*) contre la mouche de fruit *Drosophila melanogaster*.

Méthodes. – L'activité insecticide des extraits aqueux, acétoniques et éthanologiques, ainsi que des pigments purifiés (chlorophylles et caroténoïdes), a été déterminée par différents tests complémentaires (pulvérisation des oranges, toxicité par ingestion et activité répulsive). À noter que chacun de ces tests présente un mode d'exposition spécifique.

Résultats. – Les résultats ont montré que les extraits acétoniques riche en pigments verts présentent la meilleure activité insecticide. D'autre part, les pigments chlorophylliens purifiés de l'épinard ainsi que de l'algue verte ont montré une activité intéressante uniquement par la méthode de la pulvérisation. Concernant l'activité répulsive, l'extrait aqueux issu de l'épinard a été le plus efficace.

Conclusion. – Cette étude suggère la capacité des extraits évalués ainsi que des pigments chlorophylliens à fournir une alternative aux pesticides chimiques nocives.

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Introduction

Despite the ability of synthetic pesticides to eradicate pests, their harmful effects on human health and the environment have generated worldwide interest in the development of alternative strategies [1]. Moreover, their massive use has contributed to the selection of resistant varieties of pests [2]. In this context, the search for natural, less toxic, cost-effective, and eco-friendly biopesticides has become a global challenge. Since terrestrial plants, as well as marine algae, are rich in bioactive substances that are naturally synthesised as a defence method against different types of infections; they have received

attention as an effective alternative to chemical pesticides [1,3].

Spinach (*Spinacia oleracea*) is a leafy green vegetable, native to south-western Asia. This plant is rich in vitamins, minerals, and in antioxidant agents [4]. In addition to its value in the medical field, an application in the agricultural field is possible. An earlier study demonstrated the capacity of spinach ethanol extract to inhibit the growth of *Aspergillus niger*, a fungus responsible for the black mold of fruits [5].

The seaweed *Ulva lactuca* or "sea lettuce" is a green macroalga, with a cosmopolitan distribution. Vitamins, minerals, proteins, lipids, and polyunsaturated fatty acids have

been found in this alga [6]. Interestingly, several studies have demonstrated the potential of this seaweed to present an alternative to chemical pesticides. Abbassy et al. proved an antifungal activity of this alga against some phytopathogenic fungi [7]. Also, they demonstrated an insecticidal activity against different stages of development of the cotton worm *Spodoptera littoralis*, a pest of legumes [7].

Among the bioactive molecules produced by terrestrial and marine plants, chlorophyllian pigments have found great interest. Indeed, these green pigments have a broad spectrum of biological activities (antioxidant, anti-inflammatory, anticancer activity.) [8,9]. Despite the scarcity of studies looking for the application of these pigments in the agriculture field, a potential antifungal activity of an organic extract rich in chlorophyll from the green alga *Ulva linza* against the phytopathogenic fungus *Penicillium digitatum* responsible for citrus green mold has been demonstrated [10].

Therefore, the purpose of the present study is to evaluate the insecticidal activity against the fruit fly *Drosophila melanogaster* of extracts and pigments (chlorophylls and carotenoids), from spinach leaves (*Spinacia oleracea*) and a seaweed (*Ulva lactuca*) collected from Lebanon. These two potential sources of bio-pesticides have been selected as they are abundant in Lebanon and inexpensive, which make their valorisation possible even on a large scale. Moreover, they are rich in chlorophylls of interest.

In addition to the agricultural damage caused by *D. melanogaster* on sweet fruits and the economic impact on their storage [1], this fruit fly is considered the best model for the study of insecticidal activity. Indeed, it is easy to handle, requires a small breeding space and its life cycle is short enough to allow a large number of repetitions for a more accurate study [11]. The correlation between pigment contents and insecticidal activity was evaluated. *Spinacia oleracea* and *Ulva lactuca* have been selected, as they are abundant in Lebanon and rich in chlorophylls of interest.

Materials/methods

Plant material

To test their insecticidal activity, the green alga *Ulva lactuca* and the spinach leaves of *Spinacia oleracea* were used as algal and vegetal material, respectively. The green alga was freshly and manually harvested from a coastal area of the Mediterranean, El Mina–Tripoli–Lebanon in July 2017. In order to eliminate the impurities that contaminate the seaweed samples (adhered sand particles, epiphytes, parasitic micro-organisms), rigorous washing with seawater and then with distilled water was applied to the algae. They were transported in moistened plastic bags to the laboratory of the AZM research centre at the Lebanese University, Tripoli, Lebanon. The algae samples were air-dried at room temperature and in the dark for several weeks, weighed continuously until complete drying. The same washing and drying procedures were applied to spinach leaves of *Spinacia oleracea*, purchased from a Tripoli local market in July 2017. Before extraction, the dried plant and algal materials were milled into fine particles in order to facilitate extraction.

Table 1 The formula used for calculating the content of pigments (in $\mu\text{g/mL}$ plant extract) (Lichtenthaler & Wellburn, 1985).

Les formules utilisées dans le calcul du teneur des pigments (en $\mu\text{g/mL}$ d'extrait) (Lichtenthaler & Wellburn, 1985).

Solvent	Corresponding formulas
Acetone (100%)	Chlorophyll a: $11.75 A_{662} - 2.35 A_{645}$ Chlorophyll b: $18.61 A_{645} - 3.96 A_{662}$ Carotenoids: $\frac{1000A_{470} - 2.27 \text{ Chl a} - 81.4 \text{ Chl b}}{227}$
Ethanol (96%)	Chlorophyll a: $13.95 A_{665} - 6.88 A_{649}$ Chlorophyll b: $24.96 A_{649} - 7.32 A_{665}$ Carotenoids: $\frac{1000A_{470} - 2.05 \text{ Chl a} - 114.8 \text{ Chl b}}{245}$
Methanol	Chlorophyll a: $15.65 A_{666} - 7.34 A_{653}$ Chlorophyll b: $27.05 A_{653} - 11.21 A_{666}$

To calculate in mg/g DW (dry weight), we multiplied by the volume of the extract in mL and then we divided by the dry mass of the sample in gram. A = Absorbance at a specific wavelength. Chl a: Chlorophyll a; Chl b: Chlorophyll b.

Preparation of crude plant extracts

Dry samples of *Ulva Lactuca* alga and *Spinacia oleracea* leaves were macerated separately in distilled water, ethanol (96%, Sigma–Aldrich), and acetone (>99.5%, Sigma–Aldrich), with a ratio of 1/4 (m/v) at room temperature with regular shaking [3]. After 24 hours, the extracts were filtered off with a Whatman filter paper and stored at 4°C until use.

Quantification of pigments content

In order to study the correlation between the quantity of chlorophylls and carotenoids pigments and the insecticidal activity, the content of pigments in acetonic and ethanolic extracts of the green alga and spinach was calculated. Note that pigments' content was only quantified in these two extracts and not in aqueous extract, which is unsuitable for pigments extraction. Absorbance was measured using an Evolution 60 UV-Visible spectrophotometer (Thermo Scientific). The formula used to calculate the level of pigments in each organic solvent is presented in Table 1 [12].

Purification of chlorophyll and carotenoid pigments

In order to separate the green pigments (Chl a and Chl b) and the carotenoid pigments (carotene and xanthophyll) from *Spinacia oleracea* leaves and the *Ulva lactuca* alga, the method based on the differential solubility in

organic solvents was used [13]. At the end of the purification, chlorophylls a and b were recovered separately in methanol (99.8%, Sigma–Aldrich). Their concentration of these green pigments in methanol was calculated using the corresponding formula (Table 1). On the other hand, carotene and xanthophyll were solubilised in diethyl ether (>99.0% Sigma–Aldrich). Noted that methanol and ether were used as negative control in the evaluation of the insecticidal activity of chlorophyll and carotenoid pigments, respectively.

Insect culture

Drosophila melanogaster fruit flies were obtained from infected orange containing larvae at different stages of development, brought from a field in Tripoli, Lebanon in February 2018. The damaged oranges were cut into pieces and deposited in a plastic jar. After two weeks, adult flies emerged. They correspond to the first generation of flies reared under laboratory conditions. Adult fruit flies were reared in plastic jars covered with a muslin cloth to allow the passage of air, with an orange cut into two pieces as a source of food, place of reproduction, and laying.

Insecticidal activity bioassay

The insecticidal activity against *D. melanogaster* adults was determined from various methods, each one having a specific mode of exposure.

We evaluated the activity of the aqueous, acetonetic and

ethanolic extracts, as well as the purified pigments (Chlorophyll a, Chlorophyll b, Carotene, Xanthophyll) isolated from *Spinacia oleracea* leaves and the green alga *Ulva Lactuca*. Extraction solvents were used as negative control. Each test was repeated three times. The corrected efficacy of the extracts that nullifies any solvent activity (negative control) was calculated by the following Sun–Shepard formula (non-uniform population) [14].

$$\frac{\text{Mortality \% in treated plot} + \text{Change \% in control plot population} * 100}{100 + \text{Change \% in control plot population}}$$

With Change % in control plot population

$$= \frac{\text{Population in control plot after treatment} - \text{Population in control plot before treatment} * 100}{\text{Population in control plot before treatment}}$$

Spraying oranges

In order to mimic the natural conditions in which oranges are infected with the fruit fly, the method described by Chaieb et al. [15] was used with some modifications. Oranges were washed superficially with distilled water and then each one was sprayed with 1 mL of extract. After drying, each orange was placed in a plastic jar with *D. melanogaster* adults (15 ± 5 flies). The mortality was recorded after 24 h, 48 h,

and 72 h. It should be noted that this method has two different modes of action. Indeed, the extract can penetrate into the insect by simple contact or by ingestion.

Ingestion toxicity

The ability of an extract to kill the fruit flies by ingestion was determined through the method described by Aboussaid et al. [16], with some modifications. A small piece of orange impregnated with 1.0 mL of extract was placed in a plastic jar with *D. melanogaster* adults (15 ± 5 flies). The mortality of flies was recorded after 24, 48, and 72 h.

Repellent activity

In order to study the repulsive activity of extracts against fruit flies, the method entitled "Choice Bioassays" was used with some modifications [17]. Briefly, a tube containing a piece of orange impregnated with 1.0 mL of extract was placed firstly in a plastic jar. On the other hand, and at a distance of 18 cm, a tube containing a piece of orange impregnated with the extraction solvent is placed in another jar (negative control). The two pots were connected by transparent paper with holes for breathing. To stop their movement and, therefore, facilitate their handling, adult *Drosophila* were anaesthetised by placing them in the refrigerator at -4°C for 5–7 minutes. The anaesthetised flies (15 ± 5 flies) were then placed in the jar containing the extract and their location was recorded after 2 h, 6 h, and 24 h. Repellent percentage was calculated by the following formula.

The repulsive activity of extract (%):

$$\frac{\text{Total number of flies} - \text{Number of flies remaining in the jar containing extract} * 100}{\text{Total number of flies}}$$

Statistical analysis

After calculating percentage mortality, all values are expressed as mean ± SEM of three parallel measurements using the SPSS statistical software (Version 22).

Results and discussion

Preparation of crude plant extracts

Aqueous, ethanolic, and acetonetic extracts of spinach and seaweed were prepared to evaluate their potential insecticidal activity. It should be noted that the solvents were chosen

Table 2 The amount of pigments presents in the acetonic and the ethanolic extracts of spinach (*Spinacia oleracea*) and green alga (*Ulva Lactuca*), in mg/g DW (Dry weight). All values are expressed as mean \pm SEM of three parallel measurements.

La quantité des pigments présente dans les extraits acétoniques et éthanoliques de l'épinard (*Spinacia oleracea*) et de l'algue verte (*Ulva Lactuca*), en mg / g PS (poids sec). Les valeurs sont exprimées en moyenne \pm SEM de trois mesures.

Nature and origin of the extract		Chl t	Chl a	Chl b	Carotenoids
Acetone	<i>S. oleracea</i>	0.68 \pm 0.13	0.52 \pm 0.00	0.16 \pm 0.13	0.16 \pm 0.10
	<i>U. lactuca</i>	0.13 \pm 0.02	0.07 \pm 0.01	0.05 \pm 0.01	0.006 \pm 0.001
Ethanol	<i>S. oleracea</i>	0.55 \pm 0.12	0.43 \pm 0.11	0.12 \pm 0.01	0.10 \pm 0.01
	<i>U. lactuca</i>	0.07 \pm 0.01	0.039 \pm 0.001	0.034 \pm 0.010	0.12 \pm 0.001

Chl a: Chlorophyll a; Chl b: Chlorophyll b; Chl t: Total Chlorophyll (Chl a + Chl b).

for health and safety reasons [18] as well as for the ability of ethanol and acetone solvents to extract the pigments [19].

Estimation of pigment contents

In order to study the correlation between pigment contents and insecticidal activity, the quantity of pigments present in acetone and ethanol extracts of spinach and alga was calculated from spectrophotometry measurements (Table 2).

The results show that the level of chlorophyllian pigments (Chl a and Chl b) and of carotenoids is significantly greater in spinach than in alga, in both acetonic and ethanolic extracts. These findings confirm that spinach is an essential source of pigments [20]. We notice also that acetone allows for better extraction of pigments than ethanol. This is in agreement with the study of Su et al., which revealed that the amount of chlorophylls extracted from aquatic plants by 90% acetone is greater than that extracted by 95% ethanol [21].

Insecticidal activity bioassay

Spraying oranges

The crude extracts of spinach and alga and the purified pigments were used in this test. The mortality rate of *D. melanogaster* was measured after 24, 48, and 72 h of exposure (Fig. 1).

Regarding aqueous extracts, only the spinach's shows an effect on flies resulting in 95.3 \pm 3.3% of mortality after 72 hours (Fig. 1A). The activity of this extract may be due to its richness in phenolic compounds, which have shown significant insecticidal activity in several studies, although their mechanism of action has not been elucidated yet [22,23]. Furthermore, ethanolic extracts of spinach and green alga show an insecticidal activity causing 82.5 \pm 5.5% and 26.0 \pm 7.4% of mortality respectively, after 72 hours. The most interesting results were obtained with both acetonic extracts as they led to the death of almost all the flies after 48 h (Fig. 1A).

On the other hand, concerning the effect of the purified pigments, only the chlorophyllian pigments show an insecticidal activity (Fig. 1B) [no effect was recorded for carotenoids pigments (data not shown)]. The mortality rate of flies, in the presence of oranges sprayed with Chl a, derived from spinach and alga is respectively 95.6 \pm 2.8% and 94.11 \pm 3.56%, after 72 hours. Moreover, the high activity of Chl b appears after 72 hours, reaching 91.6 \pm 5.6% for spinach fraction and 93.2 \pm 5.1% for alga one.

Considering our results on the amount of total chlorophylls in the most effective extracts (ethanolic extract of spinach and both acetonic extracts) (Table 2), one can attribute their activity to the presence of the green pigments. According to our knowledge, this is the first study demonstrating the insecticidal activity of green pigments. Surprisingly, the acetonic extract of *Ulva lactuca* shows a high insecticidal activity while a moderate level of total chlorophylls compared to the ethanolic extract of *Spinacia oleracea*.

Ingestion toxicity

In Fig. 2 are presented the mortality rate of flies in the presence of a food source impregnated with the crude extracts after 24, 48 h and 72 h of exposure.

Interesting ingestion toxicity was observed for the acetonic extracts of plant and alga origin after only 24 hours, 46.2 \pm 4.3% and 85.1 \pm 5.2% of mortality, respectively. This percentage remains constant over time. Regarding aqueous and ethanolic extracts, low or no toxicity was observed against the *D. melanogaster* fruit fly. Note that all purified pigments had no effect in this assay (data not shown).

The difference of results with the previous test can be explained by the delivery method of the extract. Since green pigments have shown an effect only by spraying oranges, this can be explained by the fact that they have a transcutaneous insecticidal mode of action. Indeed, the passage of an insect on a surface treated with an insecticide allows the penetration of this agent into the insect and then acts by targeting

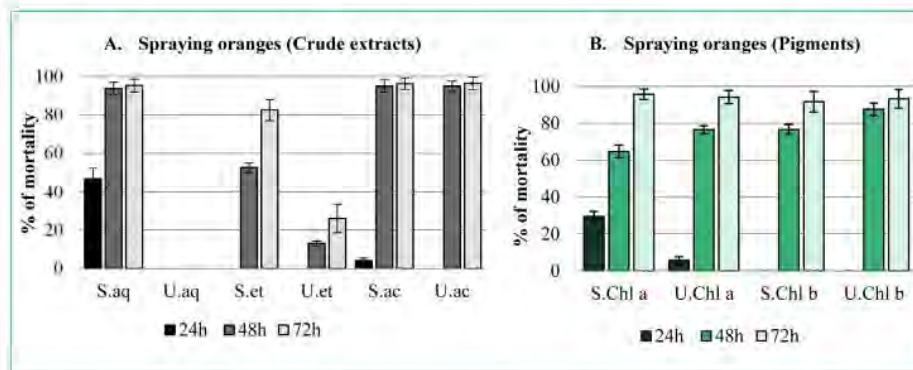


Figure 1. Histogram representing the variation of the mortality rate of flies with respect to the crude extracts' nature (A) and the pigments (B) sprayed on the oranges, originated from spinach (*Spinacia oleracea*) and green algae (*Ulva Lactuca*) after 24 h, 48 h and 72 h of exposure. All values are expressed as mean \pm SEM of three parallel measurements. The percentage of mortality in negative control (acetone alone and methanol alone) was $10 \pm 5\%$. No mortality was recorded when the negative control was water or ethanol. S.aq: Spinach aqueous extract, U.aq: Alga aqueous extract, S.et: Spinach ethanolic extract, U.et: Alga ethanolic extract, S.ac: Spinach acetonic extract, U.ac: Alga acetonic extract, S.Chl a (0.14 mg/mL) and S. Chl b (0.15 mg/mL): Chlorophyll a and Chlorophyll b purified from Spinach, U.Chl a (0.02 mg/mL) and U. Chl b (0.12 mg/mL): Chlorophyll a and Chlorophyll b purified from alga.

Histogramme représentant la variation du taux de mortalité des mouches en fonction de la nature des extraits bruts (A) et des pigments (B) pulvérisée sur les oranges, issus de l'épinard (*Spinacia oleracea*) et de l'algue verte (*Ulva lactuca*) après 24 h, 48 h et 72 h d'exposition. Les valeurs sont exprimées en moyenne \pm SEM de trois mesures parallèles. Le pourcentage de mortalité dans le contrôle négatif (acétone et méthanol) était $10 \pm 5\%$. Aucune mortalité n'a été enregistrée quand le contrôle négatif était l'eau ou l'éthanol. S.aq : extrait aqueux de l'épinard, U.aq : extrait aqueux de l'algue verte, S.et : extrait éthanolic de l'épinard, U.et : extrait éthanolic de l'algue verte, S.ac : extrait acétonique de l'épinard, U.ac : extrait acétonique de l'algue. S.Chl a (0.14 mg/mL) and S. Chl b (0.15 mg/mL): Chlorophyll a et Chlorophyll b purifiés de l'épinard, U.Chl a (0.02 mg/mL) and U. Chl b (0.12 mg/mL): Chlorophyll a et Chlorophyll b purifiés de l'algue verte.

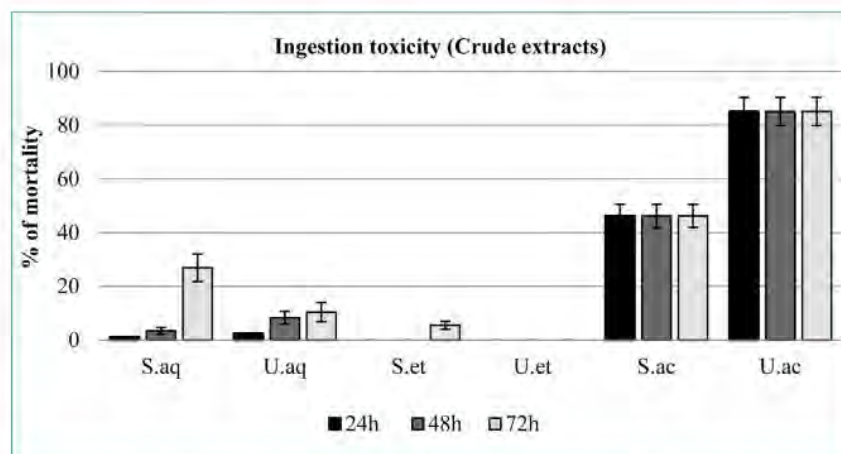


Figure 2. Histogram representing the variation of the mortality percentage of the flies with respect to the nature of the extract mixed with the food, after 24 h, 48 h and 72 h of exposure. All values are expressed as mean \pm SEM of three parallel measurements. The percentage of mortality in acetone alone was $10 \pm 5\%$. No mortality was recorded when the negative control was water or ethanol. S.aq: Spinach aqueous extract, U.aq: Alga aqueous extract, S.et: Spinach ethanolic extract, U.et: Alga ethanolic extract, S.ac: Spinach acetonic extract, U.ac: Alga acetonic extract.

Histogramme représentant la variation du taux de mortalité des mouches en fonction de la nature des extraits bruts (A) et des pigments (B) mélangés avec un morceau d'orange après 24 h, 48 h et 72 h d'exposition. Les valeurs sont exprimées en moyenne \pm SEM de trois mesures parallèles. Le pourcentage de mortalité avec l'acétone était $10 \pm 5\%$. Aucune mortalité n'a été enregistrée quand le contrôle négatif était l'eau ou l'éthanol. S.aq : extrait aqueux de l'épinard, U.aq : extrait aqueux de l'algue verte, S.et : extrait éthanolic de l'épinard, U.et : extrait éthanolic de l'algue verte, S.ac : extrait acétonique de l'épinard, U.ac : extrait acétonique de l'algue. Les pigments purifiés n'ont pas enregistré une activité insecticide par cette méthode d'évaluation.

its specific site of action [24]. Moreover, the mode of action by contact of the chlorophyllian pigments is confirmed by the presence of a correlation between its quantity in the extracts and its insecticidal activity only by the method of spraying oranges.

On the other hand, the difference in results between these two assays may have a chemical context. Indeed, a possible reactivity between the active compounds of extract and the natural acids (ascorbic and citric acid) present in the inner part of orange in the ingestion test could explain

Table 3 Repulsive activity of the aqueous, ethanolic and acetonetic extracts derived from the spinach (*Spinacia oleracea*) and the algae (*Ulva lactuca*) after 2 h, 6 h, and 24 h of exposure.

Activité répulsive des extraits aqueux, éthanoliques et acétoniques des extraits issus de l'épinard (*Spinacia oleracea*) et de l'algue verte (*Ulva lactuca*) après 2 h, 6 h et 24 h d'exposition.

Nature and origin of the extract		Repellence (mean ± SEM)			Mean repellency (%)
		2 h	6 h	24 h	
Aqueous	<i>Spinacia oleracea</i>	76.7 ± 4.7	86.7 ± 9.4	100.0 ± 0.0	87.8 ± 1.6
	<i>Ulva lactuca</i>	35.6 ± 7.9	43.5 ± 5.0	59.4 ± 0.8	46.2 ± 4.0
Ethanolic	<i>Spinacia oleracea</i>	32.3 ± 1.5	45.8 ± 5.9	60.4 ± 2.9	46.2 ± 2.5
	<i>Ulva lactuca</i>	32.4 ± 7.2	36.9 ± 0.8	63.1 ± 0.8	44.1 ± 2.4
Acetonetic	<i>Spinacia oleracea</i>	51.2 ± 1.7	58.6 ± 2.0	ND	—
	<i>Ulva lactuca</i>	46.2 ± 1.1	52.1 ± 9.4	ND	—

The repellence is expressed in percentage. ND: Note determined as the flies are anesthetised by the accumulation of acetone vapours in the jars. All values are expressed as mean ± SEM of two parallel measurements.

the loss of activity of the chlorophyllian pigments, as well as of the spinach aqueous extract, in comparison with the spraying test.

However, the high insecticidal activity of the acetonetic extract of *Ulva lactuca* in both methods can be explained by the fact that algae contain unique bioactive compounds given the stressful marine environment [3,25]. These bioactive molecules may present toxicity by contact as well as by ingestion (cumulative activity observed in the spraying method). Furthermore, the ingestion toxicity of acetonetic extract of spinach suggests the presence of active compounds other than green pigments and which presents toxicity by feeding (cumulative activity observed in the spraying test). The insecticidal activity of both acetonetic extracts is probably due to the presence of different active chemical compounds [23,26]. Indeed, secondary metabolites (phenolic compounds, quinones, terpenoids, alkaloids, etc.) have been reported to block metabolic pathways by interfering with vital enzymes or to act on the nervous system leading to permanent nerve stimulation and insect death [22,27].

Repellent activity

Concerning the repulsive activity of extracts, the aqueous extract of spinach presented the best ability to repel *D. melanogaster* fruit fly until 24 h with a mean repellence of 87.78 ± 1.57%. (Table 3). The repellent activity of plants is due to the presence of certain chemicals that can irritate the olfactory sense of insects such as alkaloids, phenolics, terpenoids and flavonoids [28,29]. In this sense, the richness of the aqueous extract of spinach in phenolic compounds, flavonoids, and terpenoids, can explain its important repulsive activity [23,30]. Moreover, we should note that a recent study has demonstrated the ability of certain natural terpenoids to disturb the sense of *D. melanogaster* fruit fly thus exhibiting an antifeedant activity [31].

No interesting repellent activity was recorded for the purified pigments (data not shown).

Overall, our results reveal the capacity of natural plant-based insecticide to replace synthetic pesticides. Although natural products are not immune to insect pest resistance, it has been shown that plant extracts that contain

several bioactive compounds are more complex than synthetic pesticides, which delays the acquisition of pest resistance. Indeed, natural extracts are mixtures of secondary metabolites that have different insecticidal modes of action, which makes the process of insect resistance more complicated [22].

Conclusion

In the present study, we assessed the benefit of using natural extracts of a Lebanese plant and alga as insecticidal and repulsive agents against *Drosophila* fruit flies. Several extracts showed good activity, according to the delivery methods. These encouraging results allow us to consider further experiments to elucidate the phytochemical composition of the most active extracts and the identification of the bioactive molecules. Overall, this work constitutes a first interesting step in the development of Lebanese *Spinacia oleracea* and *Ulva lactuca* extracts as natural, efficient, and affordable alternatives to currently used synthetic pesticides.

Acknowledgments

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Disclosure of interest

The authors declare that they have no competing interest.

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III. SUPPLEMENTARY UNPUBLISHED DATA

III.1 Absorption spectra of purified pigments

In order to ensure that pigments (Chl a, Chl b, carotene, and xanthophyll) were properly separated by the differential solubility method, their absorption spectrum between 400 nm and 700 nm were plotted.

Each pigment is characterized by a particular absorbance profile. In fact, the spectra of Chl a and b are recognized by two well-spaced peaks: one in blue at 425 nm and 458 nm, respectively, and the other in red at 660 nm for Chl a and 645 nm for Chl b. Regarding the absorption spectrum of carotenoids, it is characterized by three peaks for xanthophyll and two peaks for carotenes, positioned between 400 and 500 nm (Lichtenthaler, 1987) (Bhagavathy *et al.*, 2011).

Each pigment, whether purified from spinach (*Figure 26*) or from the green alga (*Figure 27*) presented its characteristic spectrum. The richness of spinach in pigments is highlighted by their optical density which is higher than that of pigments derived from the green alga.

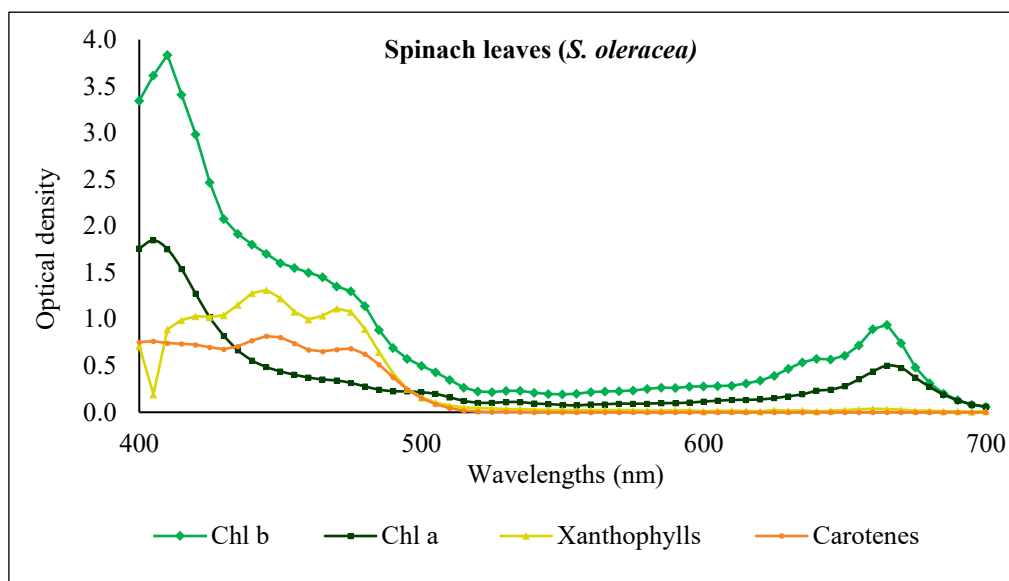


FIGURE 26 | UV-Vis spectrum of pigments purified from spinach leaves (*S. oleracea*) shows λ_{\max} 425 and 660 nm for Chl a, λ_{\max} 458 and 645 nm for Chl b. The characteristic spectrum of carotenoids is also shown.

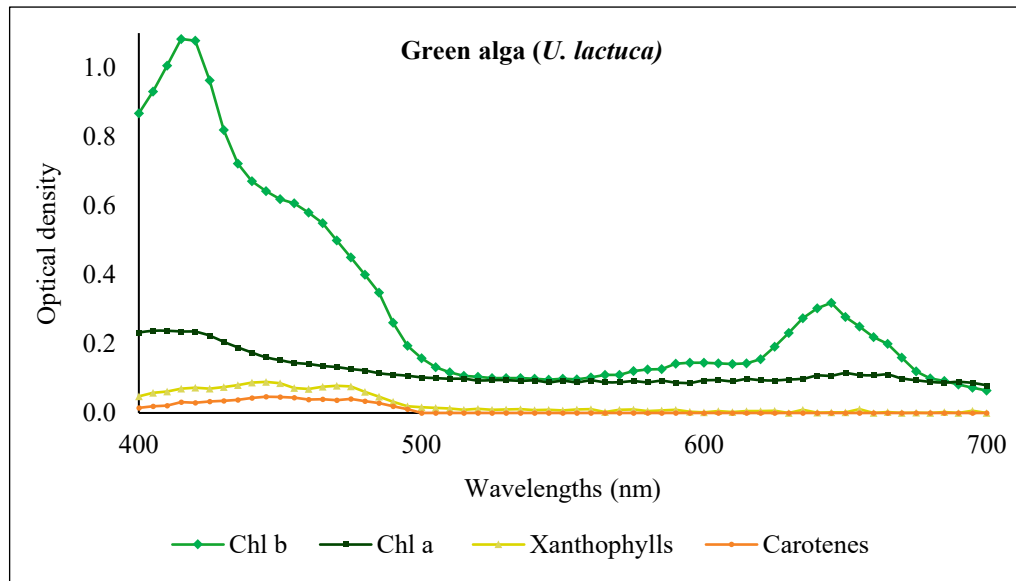


FIGURE 27 | UV-Vis spectrum of pigments purified from the green alga (*U. lactuca*) shows λ_{\max} 425 and 660 nm for Chl a, λ_{\max} 458 and 645 nm for Chl b. The characteristic spectrum of carotenoids is also shown.

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Seaweed extracts: a promising source of antibiofilm agents against pathogenic bacteria

“Particularly against P. aeruginosa & S. aureus”

▪ CHAPTER PREVIEW

In this chapter, the evaluation of the potential antibiofilm activity of the different extracts derived from the three tested algae (*U. lactuca*, *S. scoparium* and *P. capillacea*) against *P. aeruginosa* and *S. aureus* pathogenic bacteria is presented.

In the first part, the materials and methods followed are detailed. Then, the second and the third part of this chapter are devoted to the presentation and discussion of the results obtained following the evaluation of the antibiofilm effect against *P. aeruginosa* and *S. aureus*, respectively.

Part I:

Materials & Methods

▪ PREVIEW

After the listing of materials used in this part of the study, the method used for the preparation of algal extracts is described. Then, the protocols followed in the evaluation of the antibiofilm activity (biofilm inhibition and reduction, biofilm microscopic analysis, synergistic activity with conventional antibiotics) of algal extracts against *P. aeruginosa* gram-negative bacterium are detailed. In addition, the principle of the biosensor-based assay carried out in order to assess the potential capacity of extracts to inhibit AHL-based QS systems is also outlined. It should be noted that the biosensor strains used in this assay were obtained from the Laboratory of Microbial Biodiversity and Biotechnology (USR3579 – LBBM) – Banyuls-sur-Mer, France and we would like to thank Dr. Raphaël LAMI and his team for welcoming me in his laboratory to train me to conduct this test.

Similarly, the methods used in the assessment of the antibiofilm activity of extracts against *S. aureus* are presented. On the other hand, the contact angle measurement method which allows the determination of the hydrophobicity of bacteria previously treated with extracts is described.

I. MATERIALS & METHODS

I.1 MATERIALS

I.1.1 Laboratory materials and devices

Microplates used for evaluating the antibacterial and antibiofilm activities of extracts are 96-well microtiter plates (Falcon, TC-treated, polystyrene) and 24-well plates (Falcon, TC-treated, polystyrene), respectively. Biofilms visualized with epifluorescence microscope are prepared in 6-well plates (Falcon, TC-treated, polystyrene).

Extracts solutions were sterilized by filtration using a syringe filter (Cellulose Acetate Syringe Filter, 0.45 μm , GE Healthcare Whatman).

Cellulose acetate membrane filter (0.45 μm , Sigma-Aldrich) was used for preparation of bacterial layer in the contact angle measurement assay.

List of employed devices is presented below:

- Ultrasonic bath (VWR ultrasonic cleaning bath, 45 KHz)
- Orbital shaker (MaxQ 4000, Thermo Fisher Scientific)
- Microplate spectrophotometer (MultiskanTM GO, Thermo Fisher Scientific)
- Epifluorescence microscope: Zeiss – Axiotech microscope using a 20 X / 0.50 (Zeiss, EC Plan-Neofluar) objective and equipped with an HXP 120 C light source
- Fluorometer plate reader (BMG Labtech)
- Digidrop contact angle meter (GBX Scientific Instruments, Romans-sur-Isère, France)

I.1.2 Organic solvents

The solvents used in the algae extraction are listed below:

- Cyclohexane 99.5% (Sigma-Aldrich, France)
- Dichloromethane 100% (VWR, France)
- Ethyl acetate 99.9% (VWR, France)
- Methanol 99.8% (Sigma-Aldrich, France)

On the other hand, ethanol 96% (Sigma-Aldrich, France) was used to extract the crystal violet fixed by the bacterial biomass in the evaluation of the antibiofilm activity.

I.1.3 Chemical products

All the chemicals products employed in this study are categorized below:

- Crystal violet (Sigma-Aldrich, France)
- Antibiotics:
 - Tobramycin (Sigma-Aldrich, France)
 - Colistin (Colistin sodium methanesulfonate, Sigma-Aldrich, France)
 - Tetracycline (Sigma-Aldrich, France)
 - Gentamycin solution 10 mg/mL (Sigma-Aldrich, France)
- Fluorescent markers:
 - Syto9 (5 mM, Invitrogen™, ThermoFisher Scientific)
 - Propidium iodide (1 mg/mL, Invitrogen™, ThermoFisher Scientific)
 - Concanavalin A (Tetramethylrhodamine conjugate, ThermoFisher Scientific)
 - SYPRO Ruby stain (Invitrogen™, FilmTracer™, SYPRO™ Ruby biofilm matrix stain)
- Chemical compounds used in biosensor-based assay:
 - N-Hexanoyl-L-homoserine lactone (C₆-HSL) (Sigma-Aldrich, France)
 - N-(3-Oxodecanoyl)-L-homoserine lactone (Oxo-C₁₀-HSL) (Sigma-Aldrich, France)
 - The synthetic compound C11 (analogue of the N-butanoyl-L-homoserine lactone (C₄-AHL) (Khalilzadeh *et al.*, 2010)
 - Cinnamaldehyde (Sigma-Aldrich, France)
 - BacTiter-Glo™ Microbial Cell Viability Assay (PROMEGA, France)

I.1.4 Algal materials

The three species of algae (green alga *Ulva lactuca*, brown alga *Stypocaulon scoparium* and red alga *Pterocladia capillacea*) used in this study were manually collected from the North Lebanese coast of the Mediterranean, particularly from El Mina - Tripoli in September 2019. In order to remove undesirable impurities such as adhered sand particles and epiphytes which can contaminate the samples, a rigorous washing with seawater was applied to the collected algae. Then, they were immediately transported to the laboratory of AZM center of research in biotechnology and its applications where a second wash with

distilled water was carried out. Algae samples were air-dried at room temperature in the dark for several weeks and weighed continuously until complete drying.

The dried samples were ground into fine powder and were then transported in sealed bags to the Laboratoire de Génie Chimique of Toulouse, France, where the extraction were carried out.

I.1.5 Bacterial strains and culture media

Bacterial strains involved in this study are *Pseudomonas aeruginosa* PAO1 (CIP 104116) and *Staphylococcus aureus* (CIP 4.83) which are obtained from the collection of Pasteur Institute (Paris, France) and are stored at -80°C in a protective solution (3.0 g/L of beef extract powder, 5.0 g/L of Tryptone Pancreatic digest of casein, 150.0 g/L of glycerol, pH of 6.9 ± 0.2 at $20 \pm 2^{\circ}\text{C}$). It should be noted that the bacterial inoculum used in each assay was taken from a second overnight subculture on Trypticase soy agar (TSA) that was incubated under aerobic conditions at 37°C . The culture media used are mentioned below:

- **Trypticase soy agar (TSA)** (BioMérieux, Crapone, France) used for bacterial subculture and CFU counts.
- **Mueller-Hinton broth (MHB)** (Oxoid microbiology products, Basingstoke, UK) used to determine the minimal inhibitory concentration (MIC) of extracts.
- **Modified biofilm broth (MBB)** used in *P. aeruginosa* biofilm culture and thus for the evaluation of the antibiofilm activity of extracts against this bacterium. The MBB 10X medium is composed of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.005 g/L), Na_2HPO_4 (12.5 g/L), KH_2PO_4 (5.0 g/L), $(\text{NH}_4)_2\text{SO}_4$ (1.0 g/L), glucose (0.5 g/L) and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2 g/L) (Khalilzadeh *et al.*, 2010). All these compounds were purchased from Sigma-Aldrich, France.
- **Biofilm broth (BB)** used in *S. aureus* biofilm culture and thus for the evaluation of the antibiofilm activity of extracts against this bacterium. The BB 10X medium is composed of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.005 g/L), Na_2HPO_4 (12.5 g/L), KH_2PO_4 (5.0 g/L), $(\text{NH}_4)_2\text{SO}_4$ (1.0 g/L), lactose (0.25 g/L), yeast extract (1.0 g/L), vitamin assay casamino acids (1.0 g/L) and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2 g/L) (Campanac *et al.*, 2002). Except for yeast extract (Bacto™, ThermoFisher scientific) and vitamin assay casamino acids (Difco™, ThermoFisher scientific) all these compounds were purchased from Sigma-Aldrich, France.

All media were prepared by dissolution of ingredients in distilled water and sterilized just after preparation (121°C, 15 min).

The biosensor bacterial strains used in the evaluation of the anti-QS activity of extracts are *Escherichia coli* MT102 (pJBA132) and *Pseudomonas putida* F117 (pRK-C12) which detect short-chain AHLs (≤ 8 carbons in the acyl side chain) and long chain AHLs (> 8 carbons in the acyl side chain), respectively. These reporter strains were obtained from the Laboratory of Microbial Biodiversity and Biotechnology (USR3579 – LBBM) – Banyuls-sur-Mer, France and were stored at -80°C. The culture media used for these strains are:

- **LB broth (Lennox)** (Sigma-Aldrich, France) supplemented with **agar** (Sigma-Aldrich, France) used for bacterial subculture.
- **LB broth (Lennox)** (Sigma-Aldrich, France) used for liquid bacterial culture as well as in the biosensor-based assay

Media are prepared as indicated before. It should be noted that both LB broth and LB agar are supplemented with the corresponding antibiotic, tetracycline for MT102 (final concentration 25.0 $\mu\text{g/mL}$) and gentamycin for F117 (final concentration 20.0 $\mu\text{g/mL}$) in order to maintain the selection pressure.

I.2 METHODS

I.2.1 Preparation of algal extracts

Prior to extraction, the dry samples of the three algae species (green alga *U. lactuca*, brown alga *S. scoparium* and red alga *P. capillacea*) collected and dried in the Laboratory of Applied Biotechnology – Lebanese University – Tripoli – Lebanon, were separately ground into fine particles in order to facilitate the penetration of extraction solvents. Seaweeds extracts were prepared by successive extraction in selective solvents with increasing polarity (cyclohexane, dichloromethane, ethyl acetate and methanol) so as to extract the majority of algae constituents (Kohoude *et al.*, 2017). The extraction was carried out in the Laboratoire de Génie Chimique – Toulouse – France.

So, 100.0 g of the dried samples of each alga were macerated successively in 1 L of each solvent for 2h, at room temperature and under magnetic agitation (*Figure 28*). Crude extracts were then recovered after filtration using the Büchner funnel. The extraction with the same solvent was repeated when the filtrate has a dark color, indicating that the algal matrix may still be rich in compounds soluble in this solvent (extraction guided by progressive discoloration). In this case, filtrates obtained from the same solvent were combined. Finally, dry extracts were obtained following solvent evaporation using a rotary evaporator under vacuum at 40°C. The extraction yield was calculated using the following formula (1) where W2 is the weight of the extract residue after solvent evaporation and W1 is the weight of the algal matrix initially used in the extraction (100.0 g).

$$(1) \quad \text{Extraction yield (\%)} = (W2 / W1) \times 100$$

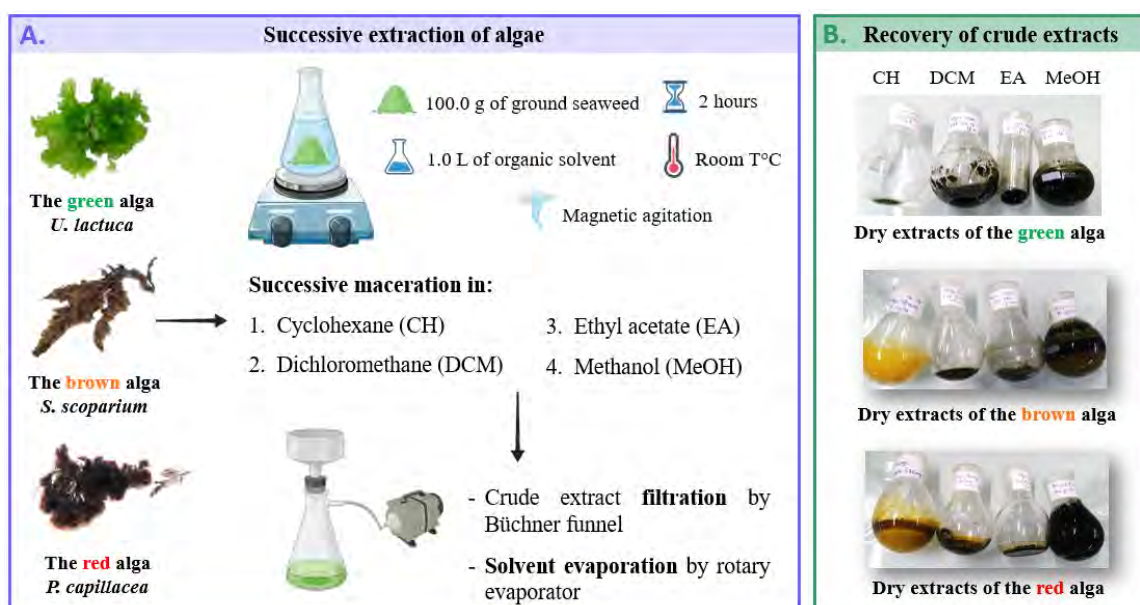


FIGURE 28 | Protocol used for the preparation of seaweed extracts. CH, DCM, EA, and MeOH are cyclohexane, dichloromethane, ethyl acetate, and methanol, respectively.

Stock solutions of extracts were prepared in sterile distilled water (SDW) at a concentration of 100.0 µg/mL using an ultrasonic bath for almost 6 hours in order to improve their solubility. They were then sterilized by filtration through a syringe filter (0.45µm) prior to the evaluation of their antibacterial, antibiofilm and anti-QS activities.

I.2.2 Assessment of the potential antibiofilm activity of seaweed extracts against the pathogenic bacteria *P. aeruginosa*

I.2.2.1 Evaluation of the antibacterial activity of extracts – MIC determination

The MIC of all extracts against *P. aeruginosa* PAO1 (CIP 104116) was determined using the broth microdilution method, according to the guidelines of CA-SFM/EUCAST 2020. Indeed, the determination of the MIC of extracts is essential in order to use a sub-MIC concentration when evaluating their antibiofilm activity and thus to exclude a potential “classical” antibacterial effect especially on planktonic growth. In view of the lack of growth of *P. aeruginosa* planktonic cells in MBB, this assay was performed in MHB as indicated in CA-SFM/EUCAST 2020.

Briefly, 100.0 μL of algal extract stock solution (100.0 $\mu\text{g}/\text{mL}$) were introduced into the wells of the first column of a sterile 96-well microtiter plate and subjected to 2-fold serial dilutions with 100.0 μL of Mueller-Hinton broth (MHB) to achieve final concentrations ranging from 50.0 to 0.098 $\mu\text{g}/\text{mL}$. The bacterial suspension used in this assay was prepared extemporaneously in SDW and adjusted to an optical density of 0.150 at 640 nm, corresponding to a concentration of 10^8 CFU/mL. This suspension was then subjected to a 2-fold dilution in SDW prior to the inoculation of the microtiter-plate using a manual multipoint inoculator (1.0 μL) in order to obtain a final concentration of 5×10^5 CFU/mL. Note that wells in the last column were used as sterility controls (SDW + MHB). The previous column was dedicated to growth control (SDW + MHB + inoculum). After incubation at 37°C for 24h, the MIC, defined as the lowest concentration of the tested extract which can prevent the visible bacterial growth (clear well), was determined. Assays were performed in duplicate.

I.2.2.2 Evaluation of the antibiofilm activity of extracts – Extract added at t_0

I.2.2.2.1 Formation of a treated biofilm

Biofilms were developed in 24-well plates. The bacterial suspension prepared in MBB (2X) was initially adjusted to 10^8 CFU/mL followed by a ten-fold serial dilution up to 10^{-6} with the same medium. 1.0 mL of the 10^{-6} dilution (equivalent to 10^2 CFU/mL) was introduced in each well. In order to test their effect on the biofilm, 1.0 mL of the algal extract (100.0 $\mu\text{g}/\text{mL}$) was added to each well, corresponding to a final concentration of 50.0 $\mu\text{g}/\text{mL}$. Wells containing 1.0 mL of SDW + 1.0 mL of un-inoculated MBB (2X) or 1.0 mL SDW + 1.0 mL inoculated MBB (2X), were considered as sterility and biofilm growth controls,

respectively. The plate was then incubated for 24h at 37°C. All assays were performed in triplicate.

1.2.2.2.2 Screening of algal extracts for their effect on PAO1 biofilm formation and growth – Crystal violet staining method

The first screening of extracts for their potential effect on PAO1 biofilm formation and development was carried out using the crystal violet (CV) staining method (Figure 29). In fact, CV is a dye which marks the negatively charged surface molecules as well as polysaccharides, the major fraction of *P. aeruginosa* biofilm matrix. So, the objective of this method is to quantify the total biofilm biomass (adhered cells + matrix) (Peeters *et al.*, 2008).

The growth of biofilms treated with algae extracts was realized as described above (I.2.2.2.1). The protocol followed is illustrated in (Figure 29). After overnight incubation, biofilms were washed twice with 2.0 mL of SDW to remove non-adherent planktonic cells. The plate was then air-dried for 1h. To stain the adhered biomass, 2.0 mL of an aqueous CV solution (1%) was added to the wells and consecutively incubated for 15 min at room temperature. In order to remove the excess stain, wells were rinsed twice with 2.0 mL of SDW followed by drying for 30 min before quantification. 1.0 mL of ethanol was finally added to extract bound stain and the inhibition percentage (IP_{CV}) was calculated according to the following formula (2).

$$(2) \quad IP_{CV}(\%) = \frac{OD_{570nm} \text{ of biofilm growth control} - OD_{570nm} \text{ of tested extract}}{OD_{570nm} \text{ of biofilm growth control}} \times 100$$

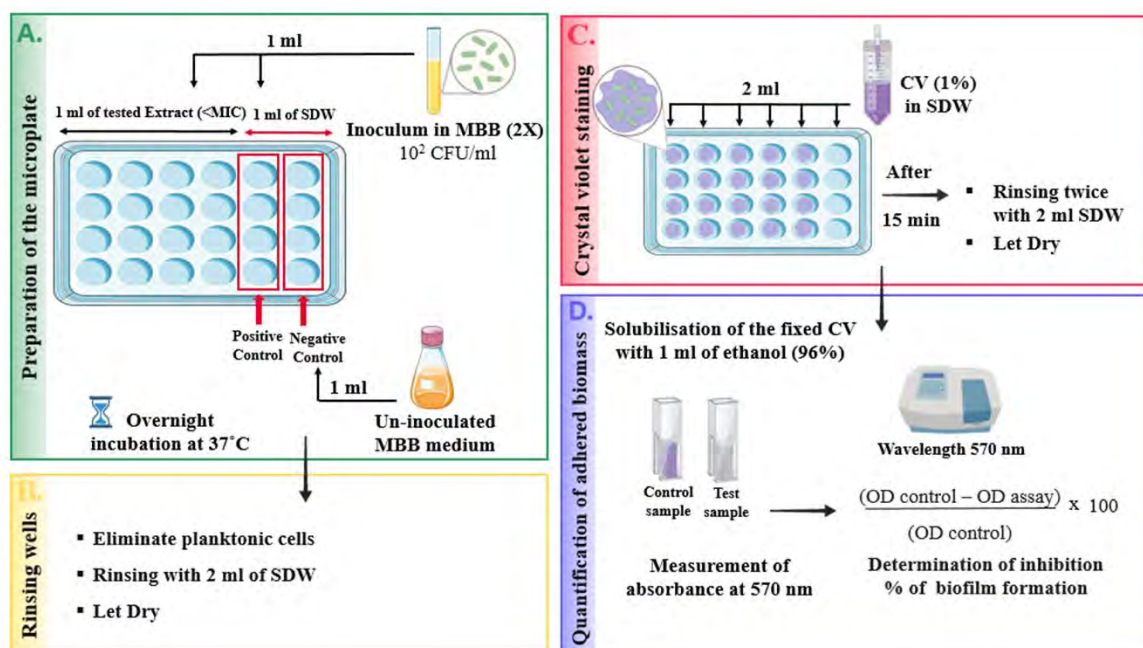


FIGURE 29 | Crystal violet staining method used for the evaluation of extract's antibiofilm activity.

1.2.2.2.3 Effect of the potentially active extracts on the number of adhered bacteria – CFU counts method

After screening of extracts for their effect on the total biofilm biomass by CV staining method, the algal extracts showing an inhibition percentage higher than 50% ($IP_{CV} > 50\%$) were selected to assess their effect on the number of adhered cells by the CFU counts method. In this assay, the protocol developed by (Campanac *et al.*, 2002) and (Khalilzadeh *et al.*, 2010) was followed with some modifications.

Treated biofilms were developed as described above (1.2.2.2.1) and the detailed protocol is described in (Figure 30).

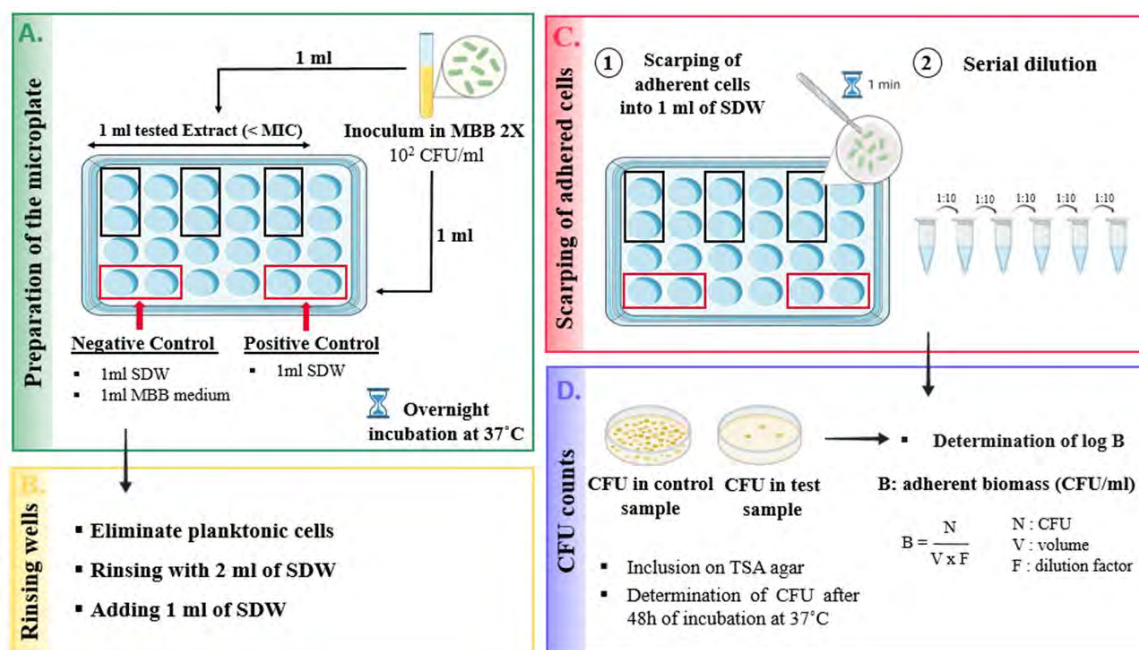


FIGURE 30 | CFU counts method used for the evaluation of extract's antibiofilm activity.

After 24h of incubation, planktonic cells were discarded by rinsing twice with 2.0 mL of SDW. Adhered cells were then recovered by scarping for 1 min with a sterile spatula into 1.0 mL of SDW. These recuperated cells were diluted by ten-fold serial dilution (from 10^1 to 10^{-6}) and 900.0 μL of each dilution was inoculated by inclusion in TSA plates. After 48h of incubation at 37°C , the numbers of CFU were counted by considering only those between 15 and 300 CFU. The adhered biomass was then calculated and subjected to logarithmic transformation by the following formula (3). The logarithmic reduction as well as the IP_{CFU} with respect to the corresponding untreated control were also calculated using the formulas below (4)(5).

- (3) $\text{Log of adhered biomass (log CFU/mL)} = \log \frac{\text{number of colonies (CFU)}}{\text{Dilution factor} \times \text{inoculated volume}}$
- (4) $\text{Log CFU/mL reduction} = \log \text{CFU/mL}_{\text{for control}} - \log \text{CFU/mL}_{\text{for treated biofilm}}$
- (5) $\text{IP}_{\text{CFU}} (\%) = \frac{\text{Adhered cells}_{\text{Control}} (\text{CFU/mL}) - \text{Adhered cells}_{\text{Sample}} (\text{CFU/mL})}{\text{Adhered cells}_{\text{Control}} (\text{CFU/mL})} \times 100$

1.2.2.2.4 Phenotypic observations of biofilms by epifluorescence microscopy

The potential effect of extracts, added at t_0 and after 24h of incubation in MBB on PAO1 biofilm morphology as well as on bacterial cell organization was examined by epifluorescence microscopy. For this analysis, PAO1 biofilms were grown as described above but in a 6-well microplate and with a total volume of 6.0 mL (Figure 31). 3.0 mL of PAO1 bacterial suspension prepared in MBB (2X) (10^2 CFU/mL) was added to 3.0 mL of tested extract. Alga extract was replaced by 3.0 mL of SDW in the control well.

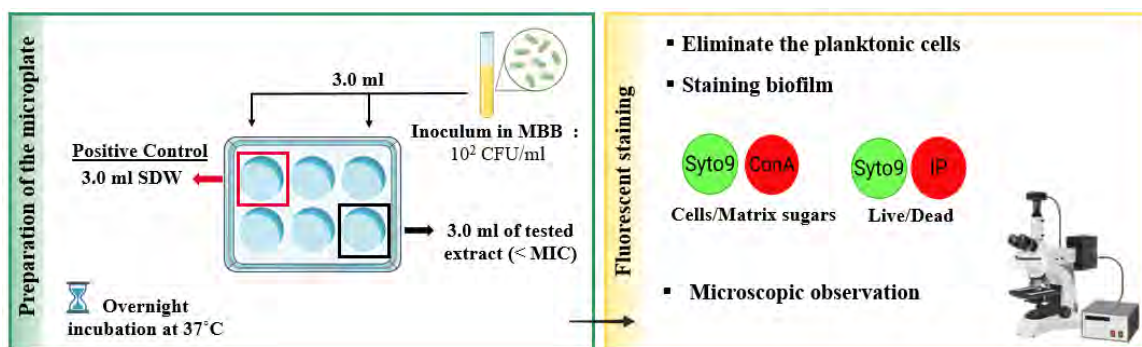


FIGURE 31 | Effect of extract on biofilm morphology by epifluorescence microscopic analysis.

After 24 hours of incubation at 37°C , wells content was carefully discarded. In order to examine the potential effect of extracts on biofilm matrix, 1.0 mL of Concanavalin A (ConA) prepared at a concentration of $100.0 \mu\text{g/mL}$ in 0.1 M of sodium bicarbonate, was added after withdrawing wells content. ConA is a lectin that exhibits an affinity for certain osidic residues, in particular for α -mannopyranosyl and α -glucopyranosyl residues. It is important to note that Strathmann *et al.*, have proved that ConA may also bind to alginate, an essential component of *P. aeruginosa* biofilm matrix (Strathmann *et al.*, 2002). Its conjugation to tetramethylrhodamine allows the emission of orange-red visible fluorescence upon the excitation with a green light. After 20 min of incubation in dark at room temperature, wells were delicately rinsed twice with 1.0 mL of SDW. Just before proceeding to the microscopic observations, 6.0 mL of SDW, as well as $1.0 \mu\text{l}$ of Syto9 (bacteria staining), were added.

Furthermore, in order to differentiate between living and damaged cells, biofilms were stained with 1.0 μL of Syto9 (5 mM) and 1.0 μL of Propidium iodide (1 mg/mL) respectively. So, after 24 hours of incubation, wells content was removed and substituted with 6.0 mL of SDW in which the two markers were added. In fact, Syto9 is a cell-permeable nucleic acid stain which is able to bind intracellular nucleic acid whatever the cell status (viable, damaged or dead).

However, PI can only stain nucleic acid of dead cells with a damaged membrane due to its inability to cross intact bacterial membranes. So, during co-staining with these two fluorescent markers, living cells are labelled with syto9 and turn green after excitation with blue-light, while damaged/dead bacteria are stained with PI and appear red when excited with green-light. Indeed, a yellow to red fluorescent signal appears when both stains are exposed to the same nucleic acid of a dead cell given the affinity of PI to bind DNA which is higher than that of syto9 stain (Rosenberg *et al.*, 2019).

Microscopic observations were made with Zeiss – AxioTech microscope using a 20 X / 0.50 (Zeiss, EC Plan-Neofluar) objective and equipped with an HXP 120 C light source. Images were acquired with a digital camera (Zeiss AxioCam ICm 1) and then the set of photos was processed with ZEN software.

1.2.2.3 Effect of selected algal extracts on PAO1 24h-old biofilms – CV staining method

The potential ability of the selected extracts ($\text{IP}_{\text{CV}} > 50\%$) to eradicate a 24h-preformed biofilm was evaluated using CV staining method. In fact, 1.0 mL of algal extract stock solution (100.0 $\mu\text{g}/\text{mL}$) was added with 1.0 mL of MBB (2X) into wells of a 24-well plate in which a 24h-preformed biofilm was developed as previously described (I.2.2.2.1). After 24h of further incubation at 37°C, wells were rinsed twice with 2.0 mL of SDW and biofilms were stained with 2.0 mL of an aqueous CV solution (1%) for 15 min. Wells were then rinsed with 2.0 mL of SDW and allowed to dry for 30 min at room temperature. After dissolution of the CV fixed by the remaining biomass of the treated biofilm with 1.0 of ethanol, eradication percentage (EP_{CV}) was determined by the following formula (6). Assay was performed in triplicate.

$$(6) \quad \text{EP}_{\text{CV}}(\%) = \frac{\text{OD}_{570\text{nm}} \text{ of untreated control} - \text{OD}_{570\text{nm}} \text{ of tested extract}}{\text{OD}_{570\text{nm}} \text{ of untreated control}} \times 100$$

1.2.2.4 Effect of the most active extract on PAO1 24h-old biofilms – CFU counts method

The most active extract that has exhibited an eradication percentage (EP_{CV}) greater than 80% by CV staining method was also evaluated by the CFU counts method. In this case, both the planktonic cells released from biofilm as well as the remaining adhered cells were quantified to evaluate the potential effect of extracts in promoting the dispersion and detachment of biofilm cells, respectively.

Also here, 24h-old biofilms were treated with 1.0 mL of the active extract (100.0 µg/mL), which is EA extract originated from the green alga *U. lactuca*. 1.0 mL of MBB (2X) was also added to the wells. After 24h of incubation at 37°C and before rinsing the wells twice with 2.0 mL of SDW, 1.0 mL of the supernatant was withdrawn to quantify biofilm-released cells. Adhered cells were then recuperated by scraping for 1 min in 1.0 mL of SDW. Finally, planktonic and adhered cells were submitted to ten-fold serial dilution followed by inoculation in TSA (900.0 µL) for CFU quantification. The number of CFU counted after 48h of incubation at 37°C was subjected to logarithmic transformation based on the following formula (7). Assay was performed in triplicate.

$$(7) \quad \text{Log CFU/mL} = \log \frac{\text{number of colonies (CFU)}}{\text{Dilution factor} \times \text{inoculated volume}}$$

1.2.2.5 Control of extracts effect on PAO1 planktonic growth

1.2.2.5.1 Assessment of the impact of CH and EA extracts on planktonic growth – CFU counts method

In order to exclude the potential bactericidal effect of the two active extracts (CH and EA extracts derived from the green alga *U. lactuca*) at the tested concentration (50.0 µg/mL) as well as to ensure that EA extract has no planktonic-growth promoting effect, their impact on PAO1 planktonic cells was assessed. The protocol developed by Feuillolay *et al.*, was used in this assay (Feuillolay *et al.*, 2016).

Briefly, 5.0 mL of PAO1 bacterial suspension (10⁵ or 10² CFU/mL) prepared in MBB (2X) medium and supplemented with 5.0 mL of sterile distilled water were incubated for 24 hours at 37°C. Water was replaced by 5.0 mL of extracts (CH or EA extracts) in the sample tubes. The potential bactericidal activity of extracts was determined on both suspension (10⁵ and 10² CFU/mL). Tubes were maintained under agitation (100 rpm) in an orbital shaker (MaxQ 4000, Thermo Fisher Scientific, Waltham, USA).

The number of planktonic cells was monitored after 24 hours of incubation at 37°C by CFU counts. Prior to their quantification, samples were homogenized then 1.0 mL was taken and serially diluted (10^{-1} to 10^{-6}). 900 μ l of each dilution were inoculated by inclusion in TSA agar plates and overnight incubated at 37°C for cell quantification. Assays were performed in duplicate. Results were expressed as ratio (log CFU/mL for sample / log CFU/mL for untreated control). The protocol followed is illustrated in the figure below (Figure 32).

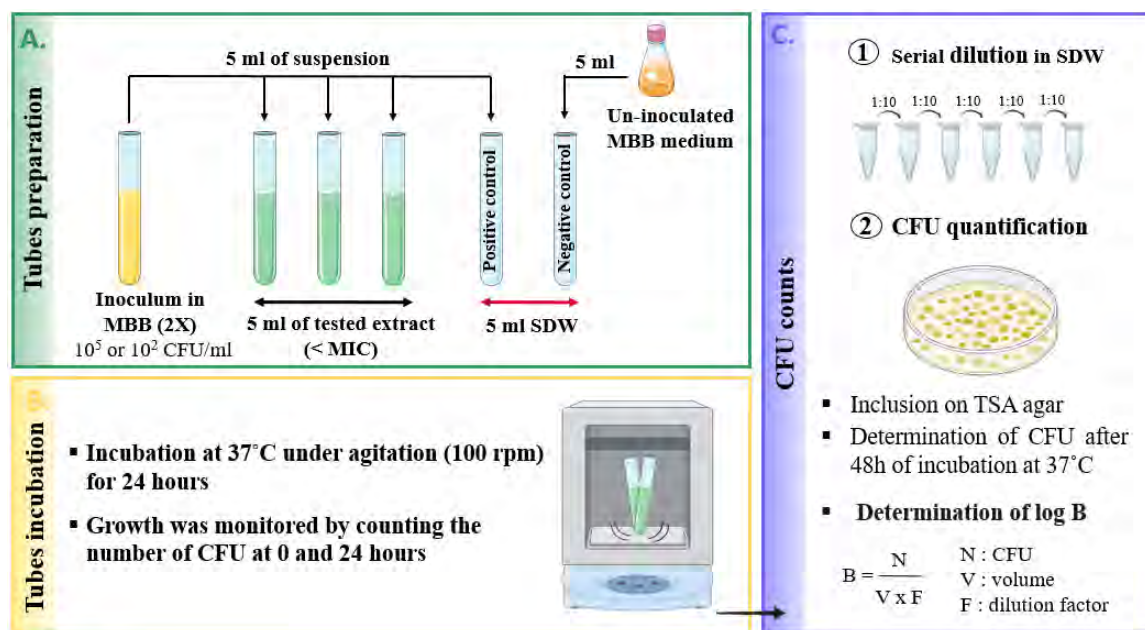


FIGURE 32 | Control of extracts effect on planktonic growth by CFU counts method.

1.2.2.5.2 Assessment of the impact of culture media and EA extract on PAO1 planktonic growth kinetics – OD measurement

In order to justify the use of MBB in the antibiofilm assays as a low-nutritive medium that allow the growth of bacteria in adhered form rather than in planktonic form, a comparison of PAO1 planktonic growth in MBB versus that in rich medium MHB was carried out. PAO1 growth kinetics curves in MHB, MBB (2X), as well as in presence of EA extract (to ensure the absence of planktonic-growth promoting effect) were performed. Briefly, 100.0 μ L of tested media (MHB and MBB) was introduced respectively into the wells of a sterile 96-well microtiter plate supplemented with 100.0 μ L of SDW. In order to evaluate the potential effect of EA extract on the PAO1 growth curve, 100.0 μ L of EA extract stock solution (100.0 μ g/mL) were added to 100.0 μ L of MBB (2X).

The bacterial suspension used in this assay was prepared in SDW and adjusted to an OD_{640nm} of 0.150 corresponding to a concentration of 10^8 CFU/mL followed by dilution (1:10) to achieve a concentration of 10^7 CFU/mL.

Then, the microtiter-plate was inoculated using a manual multipoint inoculator. Note that wells in the last column were used as sterility controls (100.0 μ L of SDW + 100.0 μ L of tested media).

The plate was incubated at 37°C for 24h in a microplate spectrophotometer under continuous agitation. The optical density measurement was carried out at 640 nm every one hour. The measured values were plotted as a function of time. Assay was performed in duplicate.

1.2.2.6 Evaluation of the synergistic antibiofilm activity of EA extract in combination with tobramycin and colistin antibiotics

The potential synergistic antibiofilm effect between the active EA extract originated from the green alga *U. lactuca* and two conventional antibiotics active on *P. aeruginosa* planktonic cells (tobramycin and colistin) was evaluated following the protocol developed by (Furiga *et al.*, 2016) with some modifications. The protocol used is summarized in the table below (*Table 19*).

TABLE 19 | Protocol used for the evaluation of the synergistic activity between EA extract and two conventional antibiotics. The final concentrations of EA extract, tobramycin and colistin, alone or in combination, are 50.0, 2 and 16 μ g/mL, respectively.

	Control	EA extract control	Tobramycin control	Colistin control	EA extract / tobramycin	EA extract / colistin
t₀	↓ + 1.0 mL SDW	↓ + 1.0 mL of extract	↓ + 1.0 mL SDW	↓ + 1.0 mL SDW	↓ + 1.0 mL of extract	↓ + 1.0 mL of extract
t_{24h}	Rinsing of the wells and addition of 1.0 mL of fresh medium					
	1.0 mL SDW	1.0 mL of extract	1.0 mL of tobramycin	1.0 mL of colistin	1.0 mL of the combination	1.0 mL of the combination
t_{48h}	Scarping time and quantification					

“↓” is inoculation time point

Biofilm formation was performed as described above (I.2.2.2.1). Briefly, at t₀, 1.0 mL of bacterial suspension (10² CFU/mL) prepared in MBB (2X) was added to the wells of a 24-well microplate supplemented either with 1.0 mL of SDW (control, tobramycin and colistin controls) or with 1.0 mL of a solution of 100.0 μ g/mL of EA extract (EA extract control and combination assays; final concentration 50.0 μ g/mL).

After 24 hours of incubation at 37°C, wells were rinsed and 1.0 mL of fresh medium was added, supplemented either by 1.0 mL of SDW (control), 1.0 mL of tobramycin alone (tobramycin control; final concentration 2.0 µg/mL), 1.0 mL of colistin alone (colistin control; colistin sodium methanesulfonate; final concentration 16.0 µg/mL), 1.0 mL of EA extract (EA extract control; final concentration 50.0 µg/mL) or a solution of EA extract in mixture with tobramycin in one hand, or with colistin in the other hand for the combination assays. The final concentrations of EA extract, tobramycin and colistin were 50.0 µg/mL, 2.0 µg/mL and 16.0 µg/mL, respectively. The concentrations of tobramycin and colistin were chosen based on their average level reached *in vivo* and which have partial impact on *P. aeruginosa* biofilms in order to detect a potential synergistic activity.

For all conditions, the number of adherent cells was quantified after 48 hours of incubation by the CFU counts method and subjected to logarithmic transformation by the formula above (3). Log reduction was then calculated according to the formula (4). Assay was performed in triplicate.

1.2.2.7 Evaluation of the potential ability of extracts to inhibit AHL-based QS systems – Biosensor-based assay

In view of the crucial role of QS communication system in the control of various bacterial behaviors, notably those related to biofilm formation and maintenance as well as to virulence factors production, all algal extracts were screened for their potential capacity to inhibit AHL-based QS systems.

To do that, AHL biosensor assay was carried out following the protocol described by (Blanchet *et al.*, 2017) and illustrated in the figure below (*Figure 33*).

E. coli MT102 (pJBA132) and *P. putida* (pKR-C12) were used for the detection of short-chain AHLs (≤ 8 carbons in the acyl side chain) and long chain AHLs (> 8 carbons in the acyl side chain), respectively. In fact, *E. coli* MT102 biosensor strain harbors the plasmid pJBA132 which is based on *Vibrio fischeri* QS components (*luxR* gene). However, *P. putida* F117 biosensor strain (AHL-negative derivative of the rhizosphere *P. putida* IsoF) carries pKR-C12 plasmid which codes for the component of *P. aeruginosa* QS system (*lasR* gene). Both plasmids include *gfp* gene encoding the green fluorescent protein (GFP) as reporter system (Steindler & Venturi, 2007).

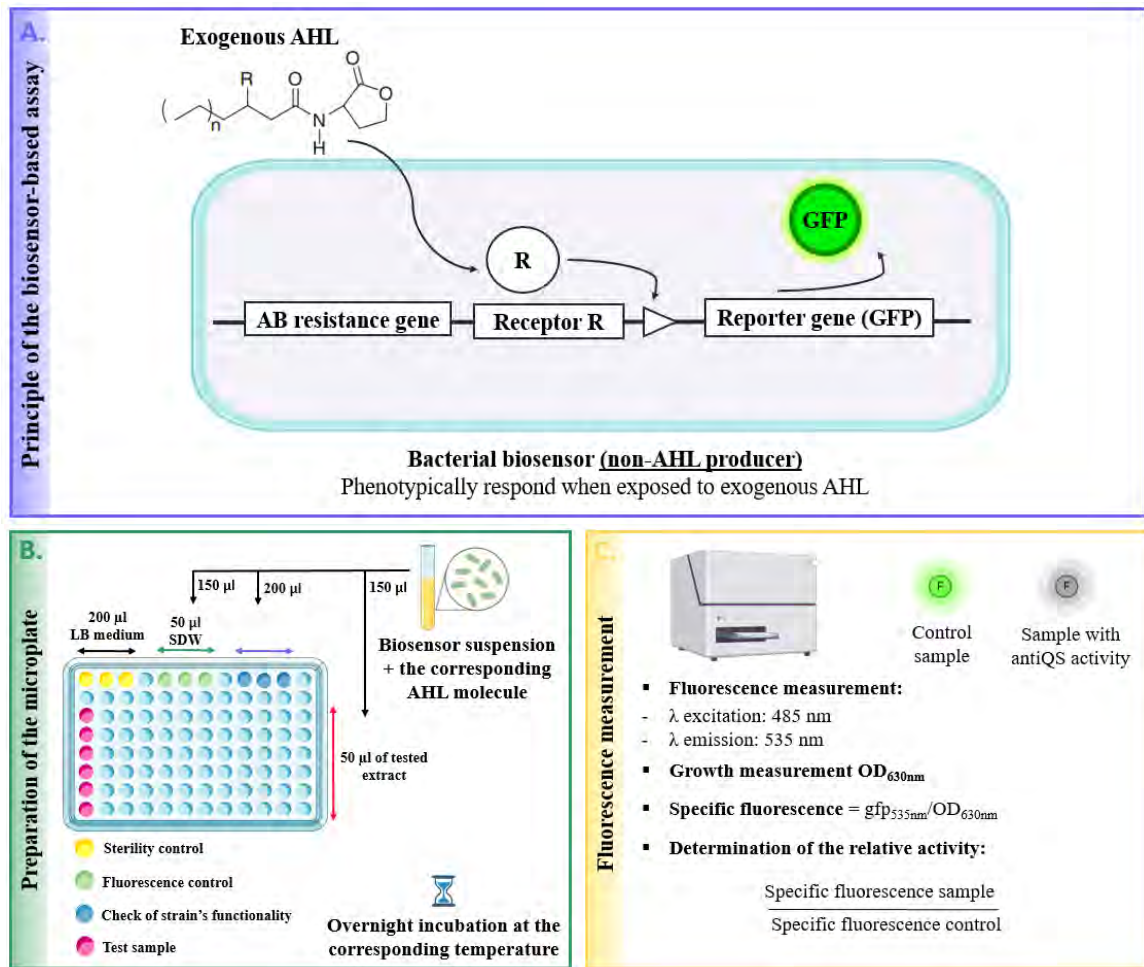


FIGURE 33 | Biosensor-based assay used to evaluate the potential ability of extract to inhibit QS system.

The first subculture of these biosensor strains was done from aliquots (stored at -80°C) on LB agar supplemented with the corresponding antibiotic (tetracycline at a final concentration of $25\ \mu\text{g}/\text{mL}$ and gentamycin at a final concentration of $20\ \mu\text{g}/\text{mL}$ for M102 and F117, respectively) and incubated for 24h at the appropriate temperature (37°C and 30°C for MT102 and F117, respectively). Then, the preparation of the second subculture was carried out in LB broth also supplemented with the corresponding antibiotic (5.0 mL of LB broth + $12.5\ \mu\text{L}$ of tetracycline ($25\ \mu\text{g}/\text{mL}$) for MT102 and 5.0 mL of LB broth + $10\ \mu\text{L}$ gentamycin ($20\ \mu\text{g}/\text{mL}$) for F117). These cultures were incubated overnight with continuous agitation.

After checking the OD_{630nm} of biosensor strain overnight cultures, which must be between 0.6 and 0.8, an inoculation in fresh LB broth supplemented with the corresponding antibiotic and AHL molecule was realized (dilution 1:50). So, $500.0\ \mu\text{L}$ of bacterial suspension were diluted in 25.0 mL of fresh LB broth.

62.5 μL of tetracycline (25 $\mu\text{g}/\text{mL}$) and 25.0 μL of C₆-HSL (final concentration 1 μM) were added to MT102 bacterial suspension. On the other hand, 50.0 μL of gentamycin (20 $\mu\text{g}/\text{mL}$) and 25.0 μL of oxo-C₁₀-HSL (final concentration 1 μM) were mixed into F117 bacterial suspension.

These fresh biosensor cultures were distributed into the wells of a 96-well microplate (150.0 $\mu\text{L}/\text{well}$) supplied with 50.0 μL of tested seaweed extract (final concentration 50.0 $\mu\text{g}/\text{mL}$). 50.0 μL of the synthetic product C11 (final concentration 50 μM), designed to be a structural analogue of the N-butanoyl-L-homoserine lactone (C4-AHL) as we previously demonstrated (Khalilzadeh *et al.*, 2010), was employed as positive control in *E. coli* MT102 assay. On the other hand, cinnamaldehyde (1:2000) was used as positive control in *P. putida* F117 assay (10.0 μL of cinnamaldehyde + 40.0 μL of SDW).

Wells containing 200.0 μL of LB broth or 150.0 μL of biosensor bacterial suspension supplemented with 50.0 μL of SDW were used as sterility (SC) and fluorescence (FC) controls, respectively. In order to confirm the proper functioning of the biosensor strains, 200.0 μL of bacterial suspension were added to the wells. Noted that the AHL-dependent fluorescence was verified by measuring the fluorescence of 200.0 μL of the biosensor suspension devoid of AHL molecule.

After overnight incubation at the corresponding temperature, fluorescence (λ excitation: 485 nm, λ emission: 535 nm) was measured by a fluorometer. OD_{630nm} was also determined using a microplate spectrophotometer in order to verify bacterial growth. After calculation of the specific fluorescence ($\text{gfp}_{535 \text{ nm}} / \text{OD}_{630 \text{ nm}}$), the relative activity of samples was determined following the formula below (8). Assay was performed in triplicate.

$$(8) \quad \text{Relative activity} = \frac{\text{Specific fluorescence of sample}}{\text{Specific fluorescence of control (FC)}}$$

In order to exclude the possible cytotoxic effect of extracts on the biosensor strains, viable cells were quantified by measuring the adenosine triphosphate (ATP). To do that, 50.0 μL of the cell viability assay kit were added to 50.0 μL of the biosensor culture. Luminescence was then recorded after brief agitation. Emitted luminosity is proportional to the amount of ATP, which directly reflects the number of living cells.

1.2.2.8 Statistical analysis

All values are expressed as mean \pm SD for three independent experiments. Student t-test was used to calculate the significance of the differences between the mean effects of the extract and those for the associated untreated control in the CFU counts method (log CFU/mL) after checking equality of variances with Levene's test (P -value < 0.05). Student t-test was also used to analyze results obtained by CV staining method. The significance of differences was determined between the mean OD_{570 nm} of CV fixed by the treated biofilm and that of the related un-treated biofilm. Regarding biosensor-based assay, the significance of differences was calculated between the mean specific fluorescence in sample and that of the related control, also using student t-test. Statistically significant values were defined as a P -value (* < 0.05 , ** < 0.01 or *** < 0.001). SPSS 22.0 software (SPSS, IBM Corporation, Armonk, NY, USA) was used in the statistical analysis.

I.2.3 Assessment of the potential antibiofilm activity of seaweed extracts against the pathogenic bacteria *S. aureus*

I.2.3.1 Evaluation of the antibacterial activity of extracts – MIC determination

Prior to the assessment of their potential effect on biofilm, the evaluation of antibacterial activity of extracts on *S. aureus* is essential to ensure the use of a sub-MIC concentration in antibiofilm activity assays. For this purpose, the minimal inhibitory concentration (MIC) of all extracts against *S. aureus* was determined using the broth microdilution method, according to the guidelines of CA-SFM/EUCAST 2020 and as described above for *P. aeruginosa*.

I.2.3.2 Evaluation of the antibiofilm activity of extracts

First, the ability of extracts derived from the three tested algae to inhibit *S. aureus* biofilm formation and growth was determined. Extracts that exhibited a significant activity were selected in order to assess their potential capacity to reduce a 24h-preformed biofilm. Furthermore, to investigate the biofilm growth phase targeted by the active extracts, a third experiment was conducted by adding extract to a *S. aureus* biofilm at various development stages (t_{2h} , t_{4h} , t_{6h} , and t_{24h}). It should be noted that in all assays, the quantification of *S. aureus* biofilm was realized by CFU counts method. In fact, the application of CV staining method is not suitable here due to the limited quantity of biomass produced by *S. aureus* biofilm (below the detection limit) under the culture conditions adopted in this study. Assays were performed in triplicate.

*I.2.3.2.1 Effect of extracts (added at t_0) on *S. aureus* biofilm formation and growth*

The influence of extracts on the number of adhered cells was evaluated following the colony counts method previously described by (Khalilzadeh *et al.*, 2010) with some modifications.

The bacterial suspension used in this assay was prepared in the low-nutritive medium BB (2X) and was adjusted to 10^8 CFU/mL ($OD_{640nm} = 0.150$) followed by ten-fold serial dilution up to 10^{-6} with the same medium. Then, 1.0 mL of the 10^{-6} dilution (equivalent to 10^2 CFU/mL) was introduced into the wells of a 24-well plate. 1.0 mL of algal extract (100.0 μ g/mL) was added at t_0 , corresponding to a final concentration of 50.0 μ g/mL. Algal extract was replaced by 1.0 mL of SDW in biofilm growth control. Wells containing 1.0 mL of SDW + 1.0 mL of un-inoculated BB (2X) medium was considered as negative control.

After overnight incubation at 37°C, wells content was discarded followed by rinsing (x 2) with 2.0 mL of SDW in order to remove unattached planktonic cells. Adhered cells were then recovered by scraping for 1 min with a sterile spatula into 1.0 mL of SDW followed by a ten-fold serial dilution (from 10⁻¹ to 10⁻⁶). 900.0 µL of each dilution was then inoculated by inclusion in TSA plates.

After 48h of incubation at 37°C, the numbers of CFU were determined by considering only plates with 15 to 300 CFU. The adhered biomass was then calculated and subjected to logarithmic transformation by the following formula (9). The logarithmic reduction with respect to the corresponding untreated control was calculated using the formula below (10).

$$(9) \quad \text{Log of adhered biomass (log CFU/mL)} = \log \frac{\text{number of colonies (CFU)}}{\text{Dilution factor x inoculated volume}}$$

$$(10) \quad \text{Log CFU/mL reduction} = \log \text{CFU/mL}_{\text{for control}} - \log \text{CFU/mL}_{\text{for treated biofilm}}$$

1.2.3.2.2 Phenotypic observations of biofilms by epifluorescence microscopy

The biofilm formed in presence of the most active extracts (***, *P*-value < 0.001; **, *P*-value < 0.01) was visualized using an epifluorescence microscopy. For this analysis, *S. aureus* biofilms were grown as described above (1.2.3.2.1) but in a 6-well microplate and with a total volume of 6.0 mL (3.0 mL of *S. aureus* bacterial suspension prepared in BB 2X (10² CFU/mL) + 3.0 mL of tested extract or 3.0 mL of SDW for the untreated control). After 24 hours of incubation at 37°C and in order to evaluate the potential effect of extracts on *S. aureus* biofilm matrix, 1.0 mL of SYPRO Ruby stain was added after discarding wells content. This stain binds to most classes of proteins including glycoproteins, lipoproteins, phosphoproteins and fibrillar proteins. After 30 min of incubation in dark at room temperature, wells were carefully washed twice with 1.0 mL of SDW. 6.0 mL of SDW was then added supplemented with 1.0 µL of Syto9 stain for cells observation.

Microscopic observations were made with Zeiss – AxioTech microscope using a 20 X / 0.50 (Zeiss, EC Plan-Neofluar) objective and equipped with an HXP 120 C light source. Images were acquired with a digital camera (Zeiss AxioCam ICm 1) and then the set of photos was processed with ZEN software.

I.2.3.2.3 Determination of biofilm development stage targeted by the selected extracts

Extracts that showed the most significant activity (in comparison with the untreated control) (***, P -value < 0.001; **, P -value < 0.01) on *S. aureus* biofilm formation and growth were selected. With the aim of specifying biofilm development phase targeted by these active extracts, *S. aureus* biofilm was treated at different stages of growth (t_{2h} , t_{4h} , t_{6h} , and t_{24h}) as outlined in the table below (Table 20).

TABLE 20 | Protocol used for the addition of extract at different time points.

Stage of biofilm formation	Time point of extract addition				
	0	2h	4h	6h	24h
0	↓+	↓	↓	↓	↓
2h		+			
4h			+		
6h				+	
24h		Scarping time			+
48h		–			Scarping time

“↓” is inoculation time point and “+” is extract addition time point.

Briefly, at t_0 , 1.0 mL of bacterial suspension (10^2 CFU/mL) prepared in BB (2X) was introduced into the wells of a 24-well plate either with 1.0 mL of SDW (untreated control and later treated biofilm) or with 1.0 mL of extract (final concentration 50.0 μ g/mL). 1.0 mL of un-inoculated BB (2X) + 1.0 mL of SDW were introduced into sterility control wells. Plate was then incubated at 37°C.

At different time point (t_{2h} , t_{4h} , t_{6h} , and t_{24h}), the formed biofilm (in BB without extract) was washed twice with 2.0 mL of SDW and 1.0 mL of extract (final concentration 50.0 μ g/mL) was added supplemented with 1.0 mL of un-inoculated BB (2X). Extract was replaced by 1.0 mL of SDW in the corresponding control wells.

Plate was incubated at 37°C and adhered cells were recovered by scarping after 24h or 48h of incubation. After quantification and logarithmic transformation of the number of adhered cells using the formula above (9), the efficiency of each extract at every biofilm development stage was determined after calculation (formula 10) of the logarithmic reduction with respect to the corresponding untreated control.

1.2.3.3 Evaluation of the potential effect of selected extracts on bacterial hydrophobicity by measuring the contact angle

In view of the reported correlation between bacterial hydrophobicity and their adhesiveness, the potential effect of the selected extracts on *S. aureus* hydrophobicity was assessed so as trying to explain their significant effect demonstrated only on the early stage of biofilm formation. For this purpose, the method that consists in measuring the contact angle of a water drop on a bacterial layer was carried out. In fact, the contact angle presents an indirect and proportional measure of the hydrophobicity (a higher contact angle indicates a greater surface hydrophobicity) (Figure 34) (Braga & Reggio, 1995).

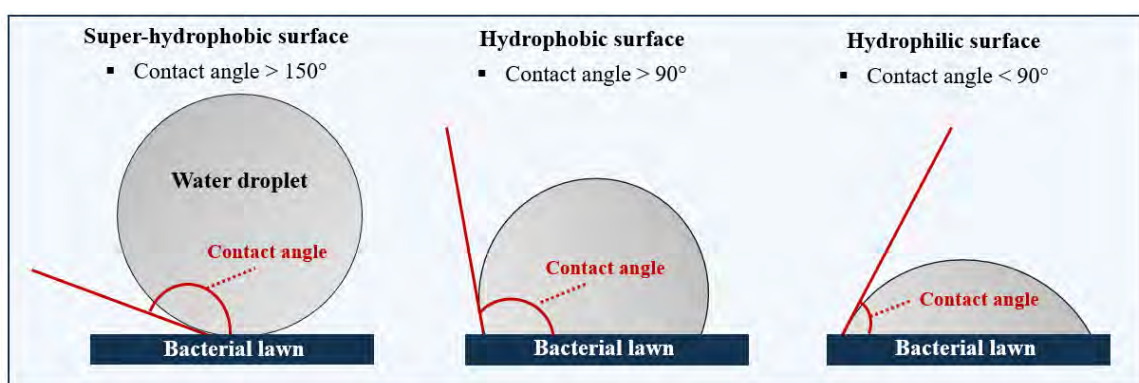


FIGURE 34 | The correlation between the contact angle and bacterial lawn hydrophobicity.

The protocol described by (Elabed *et al.*, 2017) was applied with some modifications. Briefly, 5.0 mL of *S. aureus* suspension prepared in BB (2X) ($OD_{640nm} = 0.3$) was added to 5.0 mL of extract (final concentration 50.0 $\mu\text{g/mL}$). Extract was replaced by 5.0 mL of SDW in the control tube.

After 2 hours of incubation at 37°C and in order to remove extracts and SDW, bacteria were recovered by vacuum filtration on a sterile cellulose acetate membrane filter (0.45 μm) that was left to dehydrate for almost 30 min at room temperature.

The contact angle between a water drop (1-2 μL) and the bacteria lawns was then measured under ambient conditions using a Digidrop contact angle meter (GBX Scientific Instruments). The measurements were computed automatically by Windrop++ software. It should be noted that the measurement should be done within 3-4 s after depositing the drop in order to avoid its penetration in the bacterial layer. Contact angle was determined at 5 random points per bacterial film. Results are expressed as mean contact angle \pm the corresponding standard deviation.

1.2.3.4 Statistical analysis

All values are expressed as mean \pm SD for three independent experiments. The student t-test was used to calculate the significance of the differences between the mean effects of the extract and those for the associated untreated control after checking equality of variances with Levene's test (P -value < 0.05). Statistically significant values were defined as a P -value (* < 0.05 , ** < 0.01 or *** < 0.001). SPSS 22.0 software (SPSS, IBM Corporation, Armonk, NY, USA) was used.

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Part II:

Evaluation of the antibiofilm activity of seaweed extracts against *P. aeruginosa*

ARTICLE II – Published:

Rima, M., Trognon, J., Latapie, L., Chbani, A., Roques, C., & El Garah, F. (2022) Seaweed extracts: A promising source of antibiofilm agents with distinct mechanisms of action against *Pseudomonas aeruginosa*. *Mar. Drugs*, 20(2), 92. doi: //doi.org/10.3390/md20020092

▪ PREVIEW

In this part, the potential ability of extracts derived from the three Lebanese seaweed (green alga *U. lactuca*, brown alga *S. scoparium*, and red alga *P. capillacea*) to exhibit an antibiofilm activity against the pathogenic bacterium *P. aeruginosa* was investigated. The potential antibiofilm mechanisms of action of the most active extracts was elaborated. In addition, the most active extract was tested in combination with two antibiotics which are generally used to combat *P. aeruginosa* biofilm infections, in order to detect a possible synergistic antibiofilm activity.

The work presented in this part was done in the Laboratoire de Génie Chimique (LGC – UMR5503) – Toulouse – France and resulted in a published article (Rima *et al.*, 2022).

After a brief summary of the article with a highlight on the main results, the publication is integrated followed by the supplementary materials.

On the other hand, a screening of extracts for their potential capacity to inhibit AHL-based QS system is presented.

II.1 ARTICLE SUMMARY – FOCUS ON THE MAIN RESULTS

Screening of extracts derived from the three algae evaluated in this study for their capacity to inhibit PAO1 biofilm formation and growth (extract added at t_0) permitted the selection of CH and EA extracts obtained from the green alga *U. lactuca* as the most promising. It should be noted that the antibiofilm activity was assessed following two complementary methods: by crystal violet staining, which allows the quantification of total biofilm biomass (cells + matrix), and by quantification of adhered bacteria (CFU counts method). Interestingly, a consistency between these two methods was demonstrated for CH extract ($IP_{CV} = 69.4 \pm 13.6\%$; $IP_{CFU} = 67.2 \pm 17.2\%$). However, the significant antibiofilm effect of EA extract was only observed by CV staining method ($IP_{CV} = 84.0 \pm 9.6\%$) which suggests the involvement of two distinct antibiofilm mechanisms of action for these extracts.

The epifluorescence microscopic analysis of biofilm developed in presence of these two active extracts supports this hypothesis. In fact, CH extract has led to the formation of an unstructured biofilm formed by separated bacterial aggregates with an associated matrix. On the other hand, a dispersed biofilm with a diffused matrix was developed in presence of EA extract.

The potential effect of EA extract on the production and/or the degradation of PAO1 biofilm matrix was also evidenced by its ability to significantly reduce 24h-preformed biofilm biomass ($EP_{CV} = 85.5 \pm 7.4\%$) as well as to promote the release of biofilm cells.

In view of the significant involvement of EPS matrix in biofilm tolerance towards antimicrobial agents, the possible synergistic antibiofilm activity between EA extract, a potential matrix disruptor, and two conventional antibiotics (tobramycin and colistin), was evaluated. Interestingly, EA extract significantly improved the antibiofilm activity of tobramycin against EA extract-pretreated biofilm.

These encouraging findings summarized in the table below (*Table 21*) emphasize the relevance of the green alga *U. lactuca* as promising source of antibiofilm molecules with different modes of action and that can be used alone or in combination with antibiotics in the treatment of biofilm-related infections.

TABLE 21 | Summary table of the demonstrated activity of the two selected active extracts (CH and EA extracts) derived from the green alga *U. lactuca*. CV and CFU are crystal violet staining and CFU counts methods, respectively. CH and EA are cyclohexane and ethyl acetate extracts, respectively. IP: Inhibition percentage. ND: not determined.

Test conditions		CH extract	EA extract
Extract added at t ₀	CV	++	+++
	CFU	++	+
	Different mechanisms of action		
	Microscopic analysis	- Unstructured biofilm - Defined matrix	- Unstructured biofilm - Undefined and spread matrix
Extract added at t _{24h}	CV	+	+++
	CFU	ND	- No effect on adhered cells - Increase in planktonic cells
Synergistic activity with antibiotics		ND	- Synergistic activity with tobramycin

+++ IP > 80%

++ 60% < IP < 80%

+ IP < 60%

II.2 ADDITIONAL EXPERIMENTS

II.2.1 Screening of extracts for their ability to inhibit AHL-based QS system – Biosensor-based assay

In an attempt to understand the mechanism of action implicated in the antibiofilm activity of extracts, the potential capacity of all extracts to inhibit QS communication system which plays a key role in biofilm formation and maintenance as well as in virulence factors production was evaluated following biosensor-based assay. The most common AHL-dependent QS system, only found in Gram-negative bacteria, was targeted in this assessment. To do that, the two biosensor strains *E. coli* MT102 (pJBA132) and *P. putida* F117 (pKR-C12) which respectively detect exogenous short-chain AHLs (< 8 carbons in the acyl side chain) and long chain AHLs (> 8 carbons in the acyl side chain) were used.

Regarding the ability of extracts to hinder the detection and the response to the short-chain AHLs (C₆-HSL) by *E. coli* MT102 biosensor strain, the extracts derived from the green alga *U. lactuca* displayed the most promising activity (Figure 35). In fact, CH, EA and MeOH originated from this alga were able to significantly (***, *P*-value < 0.001) reduce the fluorescence emitted by MT102 biosensor strain following the addition of the exogenous C₆-HSL. Furthermore, a significant activity (**, *P*-value < 0.01) was also recorded for DCM extract derived from this green alga as well as for the four extracts originated from the brown alga.

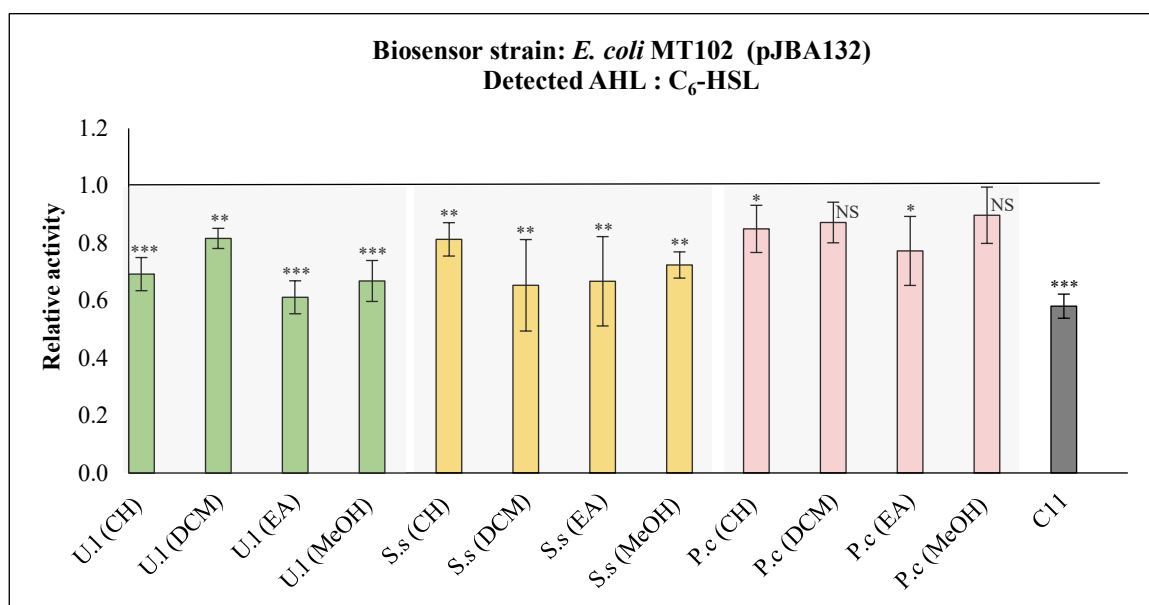


FIGURE 35 | Anti-QS activity of seaweed extracts (50 µg/mL) using *E. coli* MT102 biosensor strain. Results are expressed as means \pm SD of the relative activity calculated by dividing the specific fluorescence (gfp_{535nm}/OD_{630nm}) of sample by that of the control. Statistically significant differences (***, *P*-value < 0.001; **, *P*-value < 0.01; *, *P*-value < 0.05) between the specific fluorescence of sample and that in the appropriate control are indicated. NS: not significant.

On the other hand, concerning the effect of extracts on QS system of *P. putida* F117 biosensor strain regulated by the long chain AHLs (oxo-C₁₀-HSL), the green alga also exhibited an interesting activity (Figure 36). In fact, a significant (***, *P*-value < 0.001) decrease in the emitted fluorescence was detected in presence of CH, DCM and EA extracts. Moreover, a significant (***, *P*-value < 0.001) anti-QS activity was also recorded for DCM and EA extracts derived from the brown alga *S. scoparium*.

It should be noted that extracts originated from the red alga *P. capillacea* failed to show a noticeable anti-QS effect towards the two biosensor strains used in this assay.

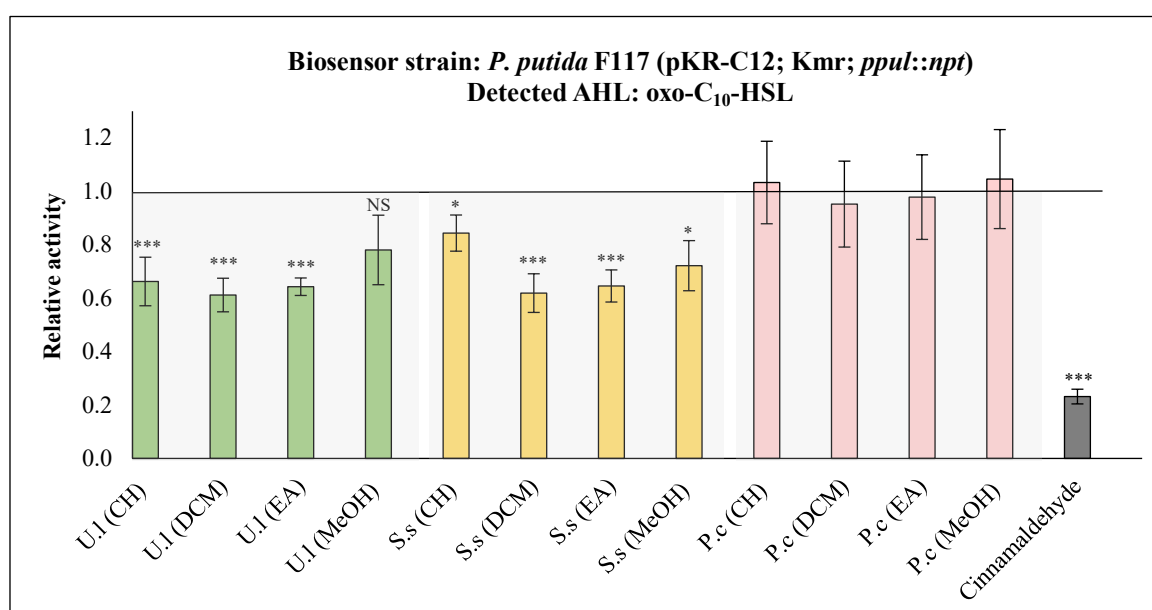


FIGURE 36 | Anti-QS activity of seaweed extracts (50 µg/mL) using *P. putida* F117 biosensor strain. Results are expressed as means ± SD of the relative activity calculated by dividing the specific fluorescence (gfp535nm/OD630nm) of sample by that of the control. Statistically significant differences (***, *P*-value < 0.001; **, *P*-value < 0.01; *, *P*-value < 0.05) between the specific fluorescence of sample and that in the appropriate control are indicated. NS: not significant.

The anti-QS activity exhibited by some extracts such as MeOH extracts derived from both the green and the brown alga that did not show an antibiofilm activity against *P. aeruginosa* makes us reconsider the involvement of this complex system in the antibiofilm activity of the active extracts. In this context, additional experiments are required in order to accurately decipher the implicated mechanism of action.

Article

Seaweed Extracts: A Promising Source of Antibiofilm Agents with Distinct Mechanisms of Action against *Pseudomonas aeruginosa*

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Abstract: The organization of bacteria in biofilms is one of the adaptive resistance mechanisms providing increased protection against conventional treatments. Thus, the search for new antibiofilm agents for medical purposes, especially of natural origin, is currently the object of much attention. The objective of the study presented here was to explore the potential of extracts derived from three seaweeds: the green *Ulva lactuca*, the brown *Stypocaulon scoparium*, and the red *Pterocladia capillacea*, in terms of their antibiofilm activity against *P. aeruginosa*. After preparation of extracts by successive maceration in various solvents, their antibiofilm activity was evaluated on biofilm formation and on mature biofilms. Their inhibition and eradication abilities were determined using two complementary methods: crystal violet staining and quantification of adherent bacteria. The effect of active extracts on biofilm morphology was also investigated by epifluorescence microscopy. Results revealed a promising antibiofilm activity of two extracts (cyclohexane and ethyl acetate) derived from the green alga by exhibiting a distinct mechanism of action, which was supported by microscopic analyses. The ethyl acetate extract was further explored for its interaction with tobramycin and colistin. Interestingly, this extract showed a promising synergistic effect with tobramycin. First analyses of the chemical composition of extracts by GC-MS allowed for the identification of several molecules. Their implication in the interesting antibiofilm activity is discussed. These findings suggest the ability of the green alga *U. lactuca* to offer a promising source of bioactive candidates that could have both a preventive and a curative effect in the treatment of biofilms.

Keywords: seaweed extracts; *Ulva lactuca*; anti-biofilm; *Pseudomonas aeruginosa*; synergistic activity; biofilm-matrix



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1. Introduction

Although the discovery of antibiotics has revolutionized modern medicine and has saved the lives of millions of patients, their massive use has contributed to a selection pressure on bacteria leading to the rapid emergence of multidrug-resistant strains (MDR) [1]. Unfortunately, the World Health Organization (WHO) has warned of a “post-antibiotic” world in which a supposedly life-saving drug will lose its effectiveness [2].

Pseudomonas aeruginosa, an opportunistic human pathogen often associated with chronic and nosocomial infections, is one of the three bacteria (*Acinetobacter baumannii* and Enterobacteriaceae) classified by the WHO as a critical priority in the search for new therapeutic strategies, due to its phenotypic and genotypic resistance towards most conventional antibiotics [3]. This ubiquitous Gram-negative bacterium is characterized by its

versatile metabolic capacity, which allows it to adapt and colonize, as biofilms, different biotic and abiotic surfaces [4].

Biofilms are defined as organized populations of microorganisms adhering to each other and to a surface, enclosed in a matrix consisting of highly hydrated extracellular polymeric substances (EPS), essentially composed of exopolysaccharides, proteins, nucleic acids, and minerals [5]. This matrix, also known as the “House of Biofilm Cells”, represents up to 90% of total biofilm biomass and its value is reflected in its structural, as well as in its functional benefits to the biofilm [6]. In addition to its essential role in maintaining the architecture, stability, and growth of the biofilm, EPS ensures an “innate” tolerance by forming a mechanical barrier against the penetration of antimicrobial agents and host immune system components [7,8]. At the same time, transfer limitation participates in drastic modifications of the cellular physiology. According to the National Institutes of Health (NIH), bacterial biofilms are implicated in 65% of microbial diseases and 80% of chronic infections [9].

P. aeruginosa biofilm presents the hallmark of long-term infection persistence and progression from colonization to infection that can lead to death, particularly in immunocompromised subjects and in cystic fibrosis (CF) patients [4]. In addition to its intrinsic and acquired resistance, the extraordinary ability of this bacterium to form biofilm accentuates its strength by providing a protective barrier against host defenses, as well as against anti-*Pseudomonas* antibiotics [10].

For all these reasons, the search for approaches to effectively prevent or treat biofilm-associated infections is currently the focus of great interest. However, despite the protective effect bestowed on bacterial cells in the biofilm state, an important feature to be considered is the total reversibility of the specific resistance when biofilms are disrupted, leading the phenomenon to be considered as a transitory loss of susceptibility rather than true resistance [8,9]. This definitely encourages the search for new strategies to inhibit biofilm formation and disrupt existing biofilms.

In this context, natural compounds can be a boon for the discovery of novel bioactive agents, including biofilm inhibitors [11]. In particular, the capacity of marine organisms to overcome stressful environmental conditions and their ability to protect themselves from bacterial invasion suggest their great richness in bioactive compounds [12]. Macroalgae, which are traditionally used for both nutritional and medicinal purposes, offer a valuable source of bioactive molecules with a wide spectrum of biological activities (anti-inflammatory, antitumor, antiviral, antimicrobial, neuroprotective, etc.), proved both *in vitro* and *in vivo* [13,14]. The availability of algal resources and the diversity of their chemical composition within green (Chlorophyta), red (Rhodophyta), and brown (Phaeophyta) algae, point to their huge potential for industrial applications [15,16].

Interestingly, a halogenated furanone isolated from the red alga *Delisea pulchra*, endemic to the south-eastern coast of Australia, was the first molecule identified as having an inhibitory activity on the bacterial communication system known as quorum sensing (QS), a mechanism essential to biofilm formation [17]. In particular, this natural molecule has been demonstrated to interfere with the *N*-acylhomoserine lactone (AHL) based quorum sensing regulatory systems of several Gram-negative bacteria [1] and several studies have proved the interest of using natural products as sources of QS inhibitors [18,19].

The present study aims to explore the potential of three seaweed species as sources of antibiofilm agents against *P. aeruginosa*. The green (*Ulva lactuca* “Sea lettuce”), the brown (*Stypocaulon scoparium* “Sea broom”), and the red (*Pterocladia capillacea*) algae were chosen for their wide range of demonstrated bioactivities [12,14,20]. The originality of this study lies in the fact that algae are scarcely explored for their potential antibiofilm activity [21–24]. After preparation of different extracts, their antibiofilm activity was evaluated using two complementary assays: the crystal violet staining method and the quantification of adhered living cells by the colony-forming unit (CFU) counting method. Both effects on the initial adhesion and biofilm progression and on 24-h-old biofilms were evaluated. Fluorescence microscopy observation was combined in order to confirm our results and to

demonstrate a potential modification of the biofilm morphology. Finally, the potential synergistic antibiofilm activity between the most active extract and tobramycin and colistin, two antibiotics which are generally used to combat *P. aeruginosa* lung infections, was analyzed [25].

2. Results

2.1. Extraction Yields of Different Seaweed Extracts

Seaweed extracts were prepared by successive maceration in different solvents with increasing polarity, with cyclohexane as the least polar solvent used (P' : 0.2) and methanol the most polar one (P' : 5.1). As expected, the yields of seaweed extracts were affected by the polarity of the extraction solvent used (Table 1). In fact, for the three algae evaluated in this study, the highest extraction yield was recorded for the methanolic extracts, resulting in 12.1, 1.4, and 7.3% (w/w) for green, brown, and red seaweed, respectively. Moreover, the number of extraction repetitions required with methanol to achieve a complete extraction demonstrates the richness of these algae in polar compounds in comparison with their content in non-polar ones.

Table 1. Characteristics of extracts according to the extraction solvents.

Seaweed Species		CH P': 0.2	DCM P': 3.1	EA P': 4.4	MeOH P': 5.1
Green alga <i>U. lactuca</i>	N° of repetitions	×1	×2	×2	×4
	Color	Pale yellow	Dark green	Dark green	Dark green
	Yield (w/w %)	0.2	0.3	0.1	12.1
Brown alga <i>S. scoparium</i>	N° of repetitions	×2	×3	×3	×3
	Color	Dark yellow	Dark green	Dark green	Green
	Yield (w/w %)	0.2	0.2	0.5	1.4
Red alga <i>P. capillacea</i>	N° of repetitions	×2	×3	×3	×4
	Color	Dark yellow	Dark green	Dark green	Dark green
	Yield (w/w %)	0.4	0.8	0.9	7.3

P': Polarity index. CH: cyclohexane, DCM: dichloromethane, EA: ethyl acetate, MeOH: methanol.

2.2. Assessment of the Inhibitory Effect of Extract on BIOFILM formation—Extracts Added at t_0

2.2.1. Screening of Algal Extracts for Their Inhibitory Effect on PAO1 BIOFILM Formation and Growth—Crystal Violet (CV) Staining Method

The initial screening was carried out by the crystal violet staining method, which allowed the entire biomass of the biofilm to be quantified. Note that all antibiofilm assays were conducted in the minimum modified biofilm broth (MBB), which promotes the formation of the biofilm, rather than planktonic growth, by creating stressful conditions [26]. This was confirmed by comparing the PAO1 growth curve in this medium with growth in the rich MHB medium (Supplementary Materials Figure S2). In order to evaluate their effect on the first stage of bacterial biofilm formation (from adhesion to proliferation under adherent status), algal extracts (50.0 $\mu\text{g}/\text{mL}$) were first added at t_0 . Their ability to reduce the biofilm biomass is compared to the control and expressed as inhibition percentages (IP_{CV}) in Figure 1.

Concerning CH extracts, only the one derived from the green alga was able to significantly reduce PAO1 biofilm biomass ($\text{IP}_{\text{CV}} = 69.4 \pm 13.6\%$) (***, p -value < 0.001). On the other hand, DCM extracts obtained from both green and brown algae exhibited considerable antibiofilm activity leading to biomass reductions of $52.9 \pm 9.2\%$ (**, p -value < 0.01) and $75.2 \pm 15.4\%$ (***, p -value < 0.001), respectively. Regarding EA extracts, results showed that the one derived from the green alga had the best ability to reduce PAO1 biofilm biomass ($\text{IP}_{\text{CV}} = 84.0 \pm 9.6\%$) (***, p -value < 0.001). EA extract obtained from the brown alga also presented a notable activity ($\text{IP}_{\text{CV}} = 64.8 \pm 9.2\%$) (***, p -value < 0.001). Note that no significant activity was recorded for any MeOH extracts or red alga *P. capillacea* extracts.

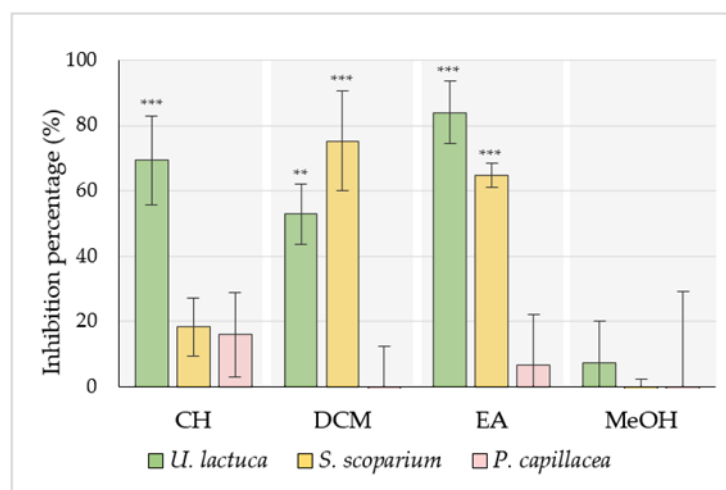


Figure 1. Effect of different algal extracts (50.0 $\mu\text{g/mL}$) on PAO1 biofilm formation, assessed using the CV staining method. Extracts were added at t_0 to evaluate their effect on biofilm formation and growth. Results are expressed as the inhibition percentage ($\text{IP}_{\text{CV}} \%$) mean \pm SD, from three independent experiments. CH, DCM, EA, and MeOH are cyclohexane, dichloromethane, ethyl acetate, and methanol extracts, respectively. Statistically significant difference (**, p -value < 0.01 , ***, p -value < 0.001) between the extract and the related untreated control is indicated.

2.2.2. Effect of Selected Active Extracts on the Number of Adhered Bacteria—CFU Counts Method

Following the screening by the CV method, extracts with an IP_{CV} higher than 50% were selected for evaluation by the CFU counting method of their effect on adhered cells. Results obtained by the CFU counting method and by the CV staining method (already displayed in Figure 1) are presented in Table 2 in order to compare them and thus search for a potential correlation. Results showed that the CH extract of the green alga *U. lactuca* was the only one to show a significant inhibitory activity (**, p -value < 0.01), leading to 5.9 ± 0.1 log CFU/mL versus 6.4 ± 0.2 log CFU/mL in the related untreated control (0.5 ± 0.1 log reduction). While the activity of the EA extract derived from the green alga was only demonstrated by the CV staining method, a consistency between the two methods ($\text{IP}_{\text{CV}} = 69.4 \pm 13.6\%$; $\text{IP}_{\text{CFU}} = 67.2 \pm 17.2\%$) was observed for the CH extract. This can be explained by two different modes of antibiofilm action for these extracts.

In order to confirm this finding, the PAO1 biofilms treated with these two active extracts were analyzed by epifluorescence microscopy.

Table 2. Comparison of the antibiofilm activity of selected algal extracts (50.0 $\mu\text{g/mL}$) using the crystal violet staining method and the numeration of adherent bacteria (CFU counts) method.

Seaweed Species	Nature of the Extract	CV Method		CFU Method
		$\text{IP}_{\text{CV}} \%$	$\text{IP}_{\text{CFU}} \%$	Log Reduction in Relation to Untreated Control
Green alga (<i>U. lactuca</i>)	CH	69.4 ± 13.6	67.2 ± 17.2	0.5 ± 0.1 **
	DCM	52.9 ± 9.2	NA	0
	EA	84.0 ± 9.6	44.3 ± 16.5	0.2 ± 0.2 NS
Brown alga (<i>S. scoparium</i>)	DCM	75.2 ± 15.4	28.1 ± 24.1	0.1 ± 0.1 NS
	EA	64.8 ± 3.6	NA	0

Extracts were added at t_0 . Results are expressed as means of inhibition percentage (IP_{CV} and IP_{CFU}) \pm SD and log reduction in comparison with the related untreated control (log reduction (log CFU/mL) \pm SD) for the CFU counts method, from three independent experiments. Statistically significant difference (**, p -value < 0.01) between the extract and the related untreated control is indicated. NS: not significant, NA: not active ($\text{IP} < 10\%$).

2.2.3. Phenotypic Observations of Biofilms by Epifluorescence Microscopy

For the CH and EA extracts originating from the green alga, *U. lactuca*, the effect on the biofilm structure and composition was examined by epifluorescence microscopy, by labeling (i) cells and matrix sugars and (ii) live/damaged cells (Figure 2). The phenotype of the biofilm was displayed after 24 h of incubation in MBB medium, with or without extract.

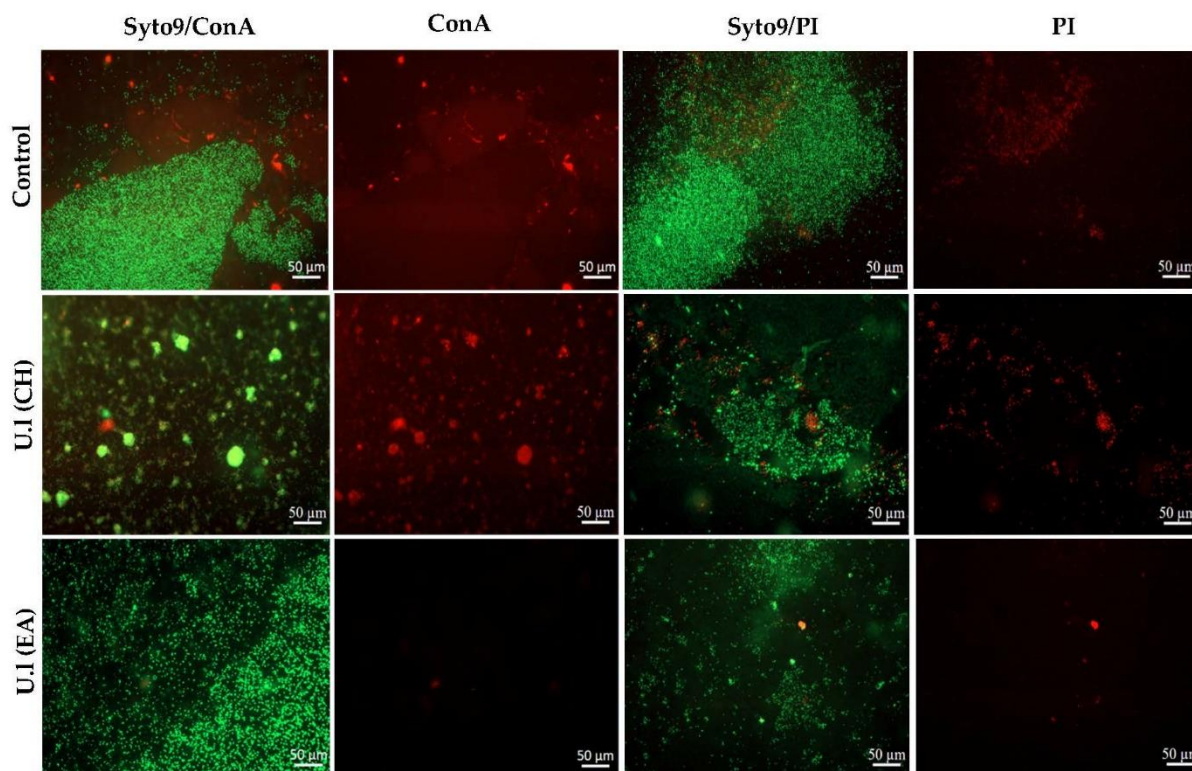


Figure 2. Epifluorescence microscopy images of PAO1 biofilms incubated in MBB medium at 37 °C for 24 h without extract (control) or with one of the two active extracts (cyclohexane or ethyl acetate extract) of the green alga *U. lactuca* at 50.0 µg/mL. Extracts were added at t_0 . Biofilms were stained with Syto9 for cells (green-fluorescent), with concanavalin A for the matrix sugars (red-fluorescent), and with Syto9 and propidium iodide (PI) to differentiate live and damaged cells, respectively. U.I (CH) and U.I (EA) are cyclohexane and ethyl acetate extract, respectively, derived from the green alga *U. lactuca*. (Magnification: $\times 20$).

Compared to the typical control biofilm consisting of bacterial cells surrounded by a well-distributed matrix, biofilms grown in the presence of extracts showed dissimilar structures. A decrease in cell number was confirmed when the CH extract was added at t_0 , with characteristic separated bacterial aggregates encased in an associated matrix (conA staining). Damaged cells or eDNA are also more likely to be in the form of aggregates than in isolation. On the other hand, a potential effect on the matrix was demonstrated in the biofilm treated with the EA extract, leading to scattered adherent cells lacking matrix (conA staining). Furthermore, the differentiation between living and damaged cells by Syto9/PI revealed the prevalence of living cells.

2.3. Effect of Selected Algal Extracts on PAO1 24 h-Old Biofilm—Extracts Added at 24 h

The extracts selected after the first screening ($IP_{CV} > 50\%$) were subjected to an evaluation of their ability to eradicate a 24-h-old biofilm. For this purpose, extracts were added at t_{24h} , followed by overnight incubation. The biomass remaining adhered after treatment of PAO1 biofilm with extracts or not was first quantified by the CV staining method (Figure 3). Results are expressed as eradication percentages.

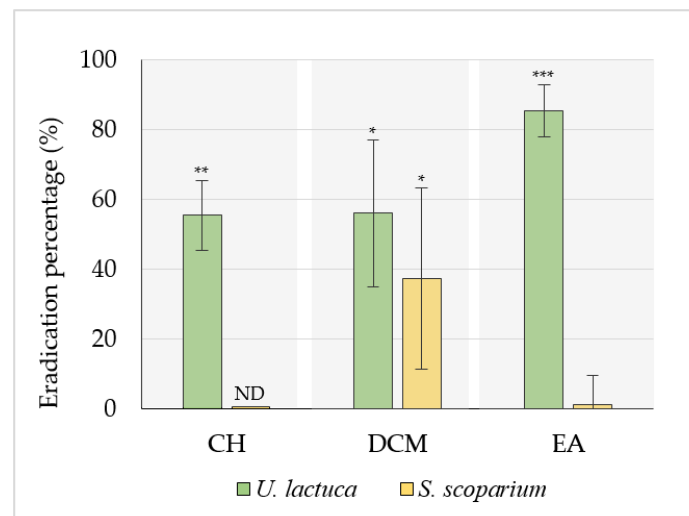


Figure 3. Effect of selected algal extracts (50.0 µg/mL) on PAO1 24 h-old biofilm assessed using the CV staining method. Extracts were added at t_{24h} to evaluate their effect on 24 h-old biofilms. Results are expressed as the eradication percentage mean \pm SD from three independent experiments. Statistically significant difference (*, p -value < 0.05, **, p -value < 0.01, ***, p -value < 0.001) between the extract and the related untreated control is indicated. ND: not determined.

Results revealed that the EA extract of the green seaweed exhibited the best eradication activity ($EP_{CV} = 85.6 \pm 7.4\%$). In addition, CH and DCM extract also obtained from *U. lactuca* displayed a moderate activity on PAO1 24 h-old biofilm, leading to $55.5 \pm 10.0\%$ and $56.1 \pm 21.0\%$ of eradication, respectively.

On the other hand, the effect of the most active extract (EA extract with $EP_{CV} > 80\%$) was evaluated using the CFU counting assay, with quantification of both adhered and detached (planktonic) cells (Figure 4). While no notable effect was observed on adhered cell counts, a significant increase in the number of detached cells was measured (**, p -value < 0.01) in the presence of EA extract (8.0 ± 0.3 log CFU/mL) compared to the control (7.1 ± 0.5 log CFU/mL). In order to exclude a possible growth promoter effect of the EA extract, its effect on planktonic growth was examined by plotting the growth curve. Results validated the absence of any significant effect of the EA extract on planktonic growth (Supplementary Materials Figure S2).

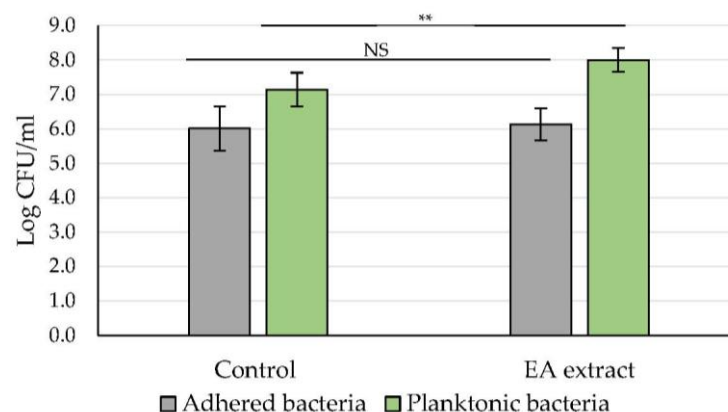


Figure 4. Effect of the *U. lactuca* EA extract (50.0 µg/mL) on PAO1 24 h-formed biofilm using the CFU counting assay. Both adherent and planktonic bacteria were quantified (CFU counts) after 24 h incubation in the MBB medium. Results are expressed as mean (log CFU/mL) \pm SD from three independent experiments. Statistically significant difference (**, p -value < 0.01) between extract and control is indicated. EA: ethyl acetate extract. NS: not significant.

2.4. Evaluation of the Synergistic Antibiofilm Activity of EA Extract in Combination with Tobramycin or Colistin

Since the CV staining method showed EA extract originating from the green alga *U. lactuca* to be the most effective in reducing the formation of the biofilm, as well as in eradicating previously formed biofilms, the potential synergy of the extract with two conventional antibiotics was evaluated following the CFU counting method. The choice of this evaluation method was based on the demonstrated responsiveness of CFU counting method in treatment efficacy testing in comparison with the CV staining method [27]. For comparison, the antibiofilm activity of tobramycin and colistin alone was evaluated on 24 h-old untreated biofilms, while the effect of the antibiotic/EA extract combination was determined on 24 h-old biofilms, previously exposed to the EA extract for 24 h (Figure 5). Results confirmed that EA extract had no significant effect on adherent CFU counts, while tobramycin and colistin were able to induce a 3- or 2-log significant reduction, respectively. The potential synergy was expressed by comparing the logarithmic reduction in biofilm treated with each antibiotic alone with that in biofilm treated with the corresponding EA extract/antibiotic combination. Results showed that the logarithmic reduction relative to the corresponding untreated control was statistically higher after treatment with the tobramycin/EA extract combination (4.9 ± 1.2 CFU/mL of log reduction) than that obtained with tobramycin treatment alone (3.3 ± 1.5 CFU/mL of log reduction). In contrast, no significant synergy was observed between the EA extract and colistin.

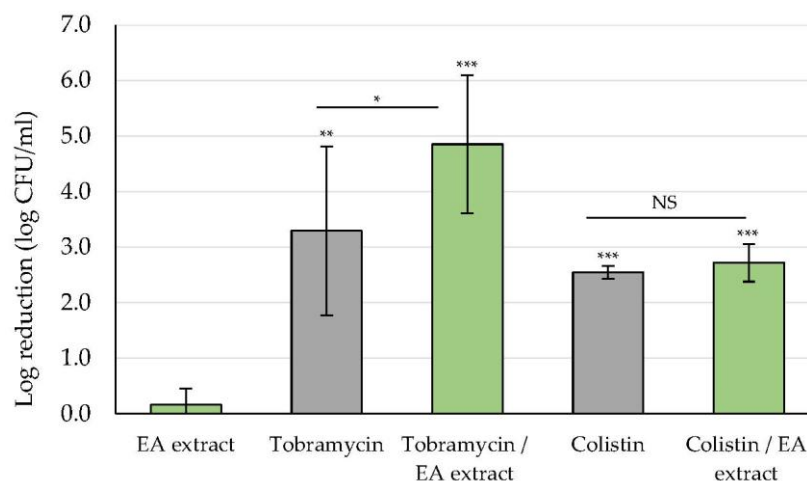


Figure 5. Synergistic effect of the *U. lactuca* EA extract (50.0 $\mu\text{g}/\text{mL}$) and tobramycin (2 $\mu\text{g}/\text{mL}$) and colistin (16 $\mu\text{g}/\text{mL}$) on PAO1 biofilms using the CFU counting assay method. The EA extract was added at t_0 . The EA extract/antibiotic combination was added after 24 h of incubation at 37 °C as antibiotics alone. Results are expressed as means of log reduction in comparison with the related untreated control (log reduction (log CFU/mL) \pm SD) from three independent experiments. Statistically significant differences (**, p -value < 0.01, ***, p -value < 0.001) between the log CFU/mL number remaining after treatment with the EA extract/antibiotic combination or with the antibiotics alone and that in the appropriate untreated control are indicated. Statistically significant difference (*, p -value < 0.05) between the log CFU/mL number remaining after treatment with the EA extract/antibiotic combination vs. antibiotic alone. NS: not significant.

2.5. Analysis of the Chemical Composition of Extracts by GC–MS

In an attempt to identify molecule(s) responsible for the demonstrated antibiofilm activity of the two selected active extracts, an analysis of the chemical composition of extracts was carried out by GC–MS (Table 3, Supplementary Materials Figure S5). Among the identified molecules, we found three phenolic compounds: 2,4-di-tert-butylphenol, 2,6-bis(1,1-dimethylethyl)-4-(1-methyl-1-phenylethyl)-phenol, and 2,4-Bis(dimethyl benzyl)-6-t-butylphenol. However, these compounds were also detected in inactive extracts.

Table 3. Compounds identified in the extracts derived from the green alga *U. lactuca*, the brown alga *S. scoparium*, and the red alga *P. capillacea*. MF: Molecular formula. MW: Molecular weight. RT: Retention time.

Identified Molecules	MF	MW (g/mol)	RT (min)														
			<i>U. lactuca</i>				<i>S. scoparium</i>				<i>P. capillacea</i>						
			CH	DCM	EA	MeOH	CH	DCM	EA	MeOH	CH	DCM	EA	MeOH			
2,4-Dithiapentane	C ₃ H ₈ S ₂	108								7.37							
2,4-Di-tert-butylphenol/2,5-bis(1,1-dimethylethyl)-phenol	C ₁₄ H ₂₂ O	206		18.81	18.96	18.55				18.76	19.36	18.45		19.04	19.24	18.54	
Heptadecane	C ₁₇ H ₃₆	240				20.33	19.72	20.24				20.04	19.88	20.5	20.68		
3,5-bis(1,1-dimethylethyl)-4-hydroxy-methyl ester benzenepropanoic acid	C ₁₈ H ₂₈ O ₃	292				23.5		24.5									
2,6-bis(1,1-dimethylethyl)-4-(1-methyl-1-phenylethyl)-phenol	C ₂₃ H ₃₂ O	324	25.66	26.08	26.2				26.03	26.59			25.67	26.29	26.41		
2,4-Bis(dimethylbenzyl)-6-t-butylphenol	C ₂₈ H ₃₄ O	386	32.49	33.48	33.7		32.22	33.37	34.77				32.52	34.03	34.27		
1-ethynyl-4-methyl benzene	C ₉ H ₈	116		9.67										9.93			
6,10,14-trimethyl-2-pentadecanone	C ₁₈ H ₃₆ O	268		22.59													
Hexadecanoic acid methyl ester	C ₁₇ H ₃₄ O ₂	270	23.08	23.48									23.09		23.8		
Decane	C ₁₀ H ₂₂	142	7.37														
Nonanal	C ₉ H ₁₈ O	142	9.39														
Isopropyl myristate	C ₁₇ H ₃₄ O ₂	270	21.83														
Tetratriacontane	C ₃₄ H ₇₀	478	26.29														
Hexadecanoic acid ethyl ester	C ₁₈ H ₃₆ O ₂	284							24.61						24.46		
2,4-bis(1-methyl-1-phenylethyl) phenol	C ₂₄ H ₂₆ O	330	33.12		34.5				35.58						35.03		
1-ethoxy-2-propanol	C ₅ H ₁₂ O ₂	104													5.79		
4-hydroxy-4-methyl-2-pentanone	C ₆ H ₁₂ O ₂	116													6.99		
1-Ethoxypropane-2-yl-acetate	C ₇ H ₁₄ O ₃	146													7.68		
4-(2,6,6-trimethyl-2-cyclohexen-1-yl)-3-buten-2-one	C ₁₃ H ₂₀ O	192													18.12		
5,6,7,7a-tetrahydro-4,4,7a-trimethyl-2(4H)-benzofuranone	C ₁₁ H ₁₆ O ₂	180													20.93		
Methyl tetradecanoate	C ₁₅ H ₃₀ O ₂	242													21.52		

3. Discussion

At this critical time when pathogens' development of multiple resistance pathways has enabled them to outgrow our ability to effectively control them, we find ourselves facing a serious public health problem, since most conventional antimicrobial agents are no longer functional. In this context, the marine world, a habitat of immense biodiversity, offers a source of inspiration in the search for natural alternatives with novel mechanisms to prevent and/or treat life-threatening diseases [16]. Despite the richness of seawater in bacteria (≈ 1 million cells/mL of seawater), such as *Pseudomonas* species, and the correspondingly high risk of colonization by a bacterial biofilm, many marine organisms, particularly sessile ones such as algae, successfully control this bacterial threat, which suggests their innate ability to synthesize metabolites to protect themselves [28]. Several studies are emerging, bringing evidence of the significant antimicrobial, antibiofilm, and antifouling activities of extracts and compounds derived from green, brown, and red macroalgae [12,20].

In the present study, the extracts of three macroalgae were explored for their potential antibiofilm activity against the "superbug" *P. aeruginosa*. Great interest has been focused on the search for synthetic and natural alternatives to conventional antibiotics to overcome the strong ability of this pathogen to form deleterious biofilms that override antibiotherapy [29]. In this context, the present study focuses on the screening of various seaweed extracts (mixtures of compounds) for their possible antibiofilm activity. Different approaches are combined in an attempt to determine their potential mechanisms of action and select the most promising extracts for further studies.

To explore their potential antibacterial and antibiofilm activities, different extracts were prepared from the three seaweeds examined in this study, using solvents of increasing polarity. As expected, the extraction yield depends on the polarity of the solvent used. Results showed that the dry matter of the three tested algae was richer in polar compounds than in nonpolar ones, since the highest yield of the crude extract was obtained with methanol (Table 1). These results are not unexpected, given that macroalgae are characterized by a high carbohydrate content (that can reach approximately 76% of their dry weight) versus a low lipid content [30]. Besides, this is in accordance with recent studies that have demonstrated the richness of the red alga *P. capillacea* and the green alga *U. lactuca* in polar compounds [31,32].

The first screening of extracts (50.0 $\mu\text{g}/\text{mL}$) for their ability to inhibit the formation and the development of PAO1 biofilms was performed using the CV staining method. Although this method provides a good estimate of the total biofilm biomass by marking EPS, especially the polysaccharides, it is not informative on the viability and the number of adhered cells. This makes it necessary to combine the CV assay with the more accurate CFU counting method [33]. Furthermore, Allkja et al., 2021 proved that the CFU counting assay is more responsive in treatment experiments than CV staining, due to potential interaction between the treatment and the dye [27]. Results obtained by adding extracts at t_0 revealed that those derived from the green alga *U. lactuca*, particularly CH and EA extracts, are the most promising in reducing bacterial adherent biomass, in comparison to the two other algae tested here (Table 2). It should be noted that the potential bactericidal effect of these two selected extracts at the tested concentration (50.0 $\mu\text{g}/\text{mL}$) was checked in order to confirm that the observed effect is definitely related to an antibiofilm activity. No bactericidal effect (neither on 10^2 nor on 10^5 CFU/mL) was recorded for these two extracts (Supplementary Materials Table S3).

By evaluating different types of extract, the value of green alga *U. lactuca* has been highlighted by various studies revealing its richness in bioactive compounds suitable for pharmaceutical (antioxidant, anti-proliferative, etc.) cosmetic, nutritional, and energy applications [34–36].

To the best of our knowledge, the only publication that has evaluated the antibiofilm activity of this green alga against *P. aeruginosa* by the CV method demonstrated the ability of a MeOH extract, prepared by a single maceration in methanol, to reduce total biomass [37]. This difference with our results can be attributed to many parameters, such as the extraction

method, the bacterial strain and the biofilm formation conditions. Whereas rich media are commonly used for the evaluation of antibiofilm activity, in this study, PAO1 biofilms were grown in a low-nutritive medium, and using a low inoculum concentration, in order to promote biofilm formation through the growth of adherent cells. Moreover, our extracts were tested at a rather low concentration (50.0 µg/mL) to avoid solubility issues. On the other hand, due to the high variability of protocols and conditions in biofilm experiments (environmental factors, inoculum preparation, etc.) and quantification, especially those based on spectrophotometry, such as the CV staining method; data comparison between studies is very complicated [38].

Regarding the CH extract derived from the green alga *U. lactuca*, results of CV ($IP_{CV} = 69.4 \pm 13.6\%$) and CFU ($IP_{CFU} = 67.2 \pm 17.2\%$) assays were consistent, which implies a significant effect on biofilm biomass formation and growth, as well as on the number of adhered cells (Table 2). An alteration in the morphology of the cell aggregates that formed was also revealed by microscopic analysis (Figure 2). On the other hand, when tested on a 24 h-old biofilm, the ability of CH extract to reduce biofilm biomass was moderate, which suggests an effect restricted to the early stages of biofilm formation (Figure 3). Such a mechanism of action has been observed with a synthetic compound, N-(2-pyrimidyl)butanamide (C11 compound), designed to be a structural analogue of the N-butanoyl-L-homoserine lactone (C4-AHL) [26]. AHLs are signal molecules involved in the quorum sensing (QS) cell-to-cell communication system, a key factor in virulence and in biofilm formation. This “chat circuitry” requires the production, detection, and response to signal molecules leading to the synchronization of bacterial group behavior. In *P. aeruginosa*, three major QS systems are well described: *rhl* and *las* systems based on signal molecules belonging to acyl-homoserine lactones (C₄-HSL and C₁₂-HSL) and the *pqs* system regulated by 2-alkyl-4-quinolone (AQs) molecules [39,40]. Interestingly, C11 is able to prevent *P. aeruginosa* biofilm formation and proliferation only when added during the initial stages, with a dose-dependent effect, and with demonstrated antagonistic effect of the C4-AHL [26,41].

Concerning the effect of the EA extract, also derived from the green alga *U. lactuca*, on PAO1 biofilm formation and growth, a significant activity was recorded only with the CV staining method ($IP_{CV} = 84.0 \pm 9.6\%$) (Table 2). Since the main objective of this assay is to quantify the total biofilm biomass, including EPS, these results can be explained by a potential action on the production and/or degradation of the biofilm matrix. This hypothesis is supported by the epifluorescence microscopic analysis, which proved that addition of the EA extract leads to the formation of a biofilm characterized by an undefined spread matrix (Figure 2). Interestingly, this extract also showed considerable efficiency in reducing a 24 h-old biofilm by the CV method ($EP_{CV} = 85.5 \pm 7.4\%$) (Figure 3). This finding allowed us to select the EA extract and use the CFU method to explore the effect of the extract on the number of remaining adhered cells, as well as on the number of planktonic cells released. Results showed a significant increase in planktonic cells, while no effect on the adhered cell counts was observed (Figure 4), which can be attributed to a matrix modification that promotes the release of biofilm cells. This mechanism of action targeting biofilm structure and morphology has been described for usnic acid, a secondary lichen metabolite [42]. *P. aeruginosa* biofilm grown on a usnic acid-loaded polymer formed an altered structure consisting of microcolonies separated by interstitial void areas. Furthermore, Powell et al., 2018 have demonstrated the ability of alginate oligosaccharides derived from the brown alga *Laminaria hyperborea* to decrease *P. aeruginosa* biofilm biomass by disrupting its EPS network [43]. The function of the EPS is not limited to providing a protective barrier against exogenous factors, it also ensures nutrition, hydration, and intercellular interaction within the biofilm. In this scenario, and given its major role in the formation, development, and maintenance of biofilms, the EPS matrix has become a potential target in the search for novel anti-biofilm strategies such as the use of alginate lyase, DNase, or mucolytic agents, which aim to impair the complex structure of biofilms and consequently eradicate them or reduce their high resistance to antimicrobial treatments [44].

On the other hand, several studies have focused on the search for a therapy that combines an antimicrobial agent with an innovative adjuvant, especially one that can disassemble the biofilm matrix. This can be considered as a good therapy that aims to minimize the long-term administration of high doses of antibiotics [44]. The lack of biofilm sensitivity towards antibiotics is a well-known, ubiquitous phenomenon caused by a combination of factors. Generally, a biofilm's complexity and heterogeneity can hinder the efficiency of antibiotics by many mechanisms: (1) the restricted penetration ensured by the EPS matrix components interacting with antibiotics, (2) the physiological tolerance associated with the formation of a subpopulation within the biofilm, characterized by a slower cell metabolism, leading to the inactivity of antibiotics that target fundamental cellular processes (replication, protein or cell wall synthesis, etc.), (3) tolerance based on specific genes whose expression is strictly associated with biofilm formation [45].

Thus, the possible synergistic activity between the active EA extract, which acts by potentially affecting the PAO1 matrix structure, and tobramycin or colistin antibiotics, commonly used in the treatment of *P. aeruginosa* infections, was evaluated. Tobramycin is a polycationic aminoglycoside antibiotic with hydrophilic properties. Its antibacterial mechanism of action is based on its ability to bind to ribosomal subunits, resulting in suppression of mRNA translation and subsequently the inhibition of protein synthesis [46]. Colistin is a polypeptide antibiotic belonging to the polymyxin family, with amphiphilic and cationic properties. Its binding to LPSs and phospholipids of the outer membrane of Gram-negative bacteria leads to the disruption of the cell membrane with leakage of intracellular contents and, finally, to cell death [47].

In the present study, EA extract/antibiotic combinations were evaluated on 24 h-old biofilms exposed to the EA extract. It should be noted that the tested antibiotic concentrations were selected in a previous study based on the level reached in the serum (for tobramycin) or sputum (for colistin) 1 h after administration of a single dose, which corresponded to 8 µg/mL for tobramycin and 32 µg/mL for colistin [41]. However, since these concentrations led to a strong biofilm reduction *in vitro* (data not shown), they were lowered to 2 and 16 µg/mL for tobramycin and colistin, respectively, in order to detect a potential synergistic effect. Results showed a significant increase in the antibiofilm activity of tobramycin against EA-extract-pretreated biofilm (Figure 5). In contrast, no synergistic activity was recorded with colistin. This can be explained by the difference in the mechanisms involved in biofilm-associated tolerance to aminoglycoside antibiotics (tobramycin) and antimicrobial peptide (colistin) and/or by a possible denaturing effect of EA extract on colistin.

For aminoglycoside antibiotics, which act at the intracellular level by targeting bacterial protein synthesis, various studies have highlighted the major role played by negatively charged EPS matrix in limiting the diffusion of such polycationic compounds through the biofilm, thus blocking their effects. For instance, alginate, a polyanionic exopolysaccharide and a component of *P. aeruginosa* biofilm matrix, has been shown to have a crucial function in protecting the biofilm from polycationic aminoglycosides, such as tobramycin, through ionic interactions [48].

In the present study, the EA extract has been proven to significantly reduce the total biomass, potentially by altering the EPS matrix structure and architecture of *P. aeruginosa* biofilms. Thus, the synergistic effect observed with tobramycin may be explained by the partial restoration of the susceptibility of PAO1 EA extract-pretreated biofilms. The absence of total recovery of biofilm sensitivity to tobramycin may be linked to other factors related to the biofilm state itself, such as the involvement of efflux pumps (e.g., MexAB-OprM) or the modification of cellular targets [49]. Interestingly, a synergistic effect with tobramycin has also been demonstrated with the C4-HSL analogue (C11) mentioned above, and also the halogenated furanone, known as a substance antagonistic to the bacterial QS communication system [41,50], since the efficacy of tobramycin on furanone-treated *P. aeruginosa* biofilms is exerted on both the surface cells and those present in the deepest layers, while the antibiotic had a limited effect on untreated biofilms.

On the other hand, the absence of synergy between the EA extract and colistin can be explained by its lower retention by the EPS matrix, in comparison with the polycationic tobramycin. Furthermore, evidence has been provided that biofilm tolerance to antimicrobial peptides is correlated with eDNA-mediated activation of *pmr/arn* operon, encoding the LPS modification enzyme [45].

To progress towards the identification of the bioactive compounds present in the two selected extracts, an analysis of the chemical composition of all extracts was performed by GC–MS (Table 3). Various molecules have been identified, some of which have already been described for their biological activity, such as the 2,4-di-tert-butylphenol [51]. In fact, Viszwapriya et al., 2016 have demonstrated the ability of this phenolic compound to inhibit *Streptococcus pyogenes* biofilm formation along with a reduction in EPS matrix production [52]. Moreover, a synergistic antibiofilm activity of this phenol with gentamycin has been reported against *Serratia marcescens* [53]. However, since most of the identified compounds were also detected in the inactive extracts, such as the extracts derived from the red alga, their specific implication in the demonstrated antibiofilm activity of the two active extracts has to be confirmed by further purification and analyses to identify and quantify the active molecule and/or the effective mixture.

Finally, as the QS communication system is a key factor in bacterial biofilm formation, the two active extracts discovered in this study may potentially act on this complex system and/or on other factors regulated by QS, such as the production of rhamnolipids. This biosurfactant, controlled by the *rhl* QS system, is involved in the different stages of biofilm formation, particularly in the mediation of cell dispersion [54]. Thus, the present results encourage towards elucidating the potential direct and/or indirect anti-QS activity of these extracts.

4. Materials and Methods

4.1. Collection of Algal Materials

Seaweed samples belonging to three different groups (green alga *Ulva lactuca*, brown alga *Stypocaulon scoparium*, and red alga *Pterocladia capillacea*) were manually collected in the Mediterranean Sea, from the northern Lebanese coast, particularly from El Mina in Tripoli in September 2019 (Supplementary Materials Figure S1). After collection, the fresh macroalgae were rinsed with seawater to remove impurities such as particles of adhered sand or epiphytes. The samples were immediately transported to the laboratory of applied biotechnology, AZM research center, Lebanese university, Tripoli, Lebanon, where they were rigorously washed with distilled water. Then, seaweed samples were air-dried in a dark place at room temperature (20–27 °C) for several weeks and weighed continuously until they were completely dry. The dried samples were ground into a fine powder in order to facilitate extractions, and were then transported in sealed bags to the Laboratoire de Génie Chimique of Toulouse, France, where the extractions were carried out.

4.2. Organic Solvents, Chemicals and Antibiotics

The solvents used in this study were cyclohexane 99.5% (Sigma–Aldrich, St. Quentin Fallavier, France), dichloromethane 100% (VWR, Rosny-sous-Bois, France), ethyl acetate 99.9% (VWR, Rosny-sous-Bois, France), methanol 99.8% (Sigma–Aldrich, St. Quentin Fallavier, France) and ethanol 96% (Sigma–Aldrich, St. Quentin Fallavier, France). Unless otherwise mentioned, all chemicals, including dyes and antibiotics, were purchased from Sigma–Aldrich, St. Quentin Fallavier, France.

4.3. Bacterial Strain and Culture Media

The bacterial strain used in this study was *Pseudomonas aeruginosa* PAO1 (CIP 104116), purchased from the collection of the Pasteur Institute (Paris, France) and preserved at –80 °C. The inoculum used in each experiment came from a second subculture on Trypticase soy agar (BioMérieux, Craponne, France) that was incubated under aerobic conditions at 37 °C for 24 h. A low-nutritive medium, named minimum biofilm broth (MBB) was used

for the biofilm formation and the evaluation of the antibiofilm activity of extracts, in order to create stressful conditions and subsequently promote biofilm formation and growth of adherent cells rather than planktonic growth. The MBB 10X medium is composed of FeSO_4 , $7 \text{ H}_2\text{O}$ (0.005 g/L), Na_2HPO_4 (12.5 g/L), KH_2PO_4 (5.0 g/L), $(\text{NH}_4)_2\text{SO}_4$ (1.0 g/L), glucose (0.5 g/L) and MgSO_4 , $7 \text{ H}_2\text{O}$ (0.2 g/L) [26].

4.4. Preparation of Seaweed Extracts

In order to extract a maximum of seaweed constituents, a successive extraction method using selective solvents with increasing polarity (cyclohexane, dichloromethane, ethyl acetate, and methanol) was adopted [55]. One hundred grams of the dried samples of each alga were macerated successively in 1 L of each solvent for 2 h under magnetic agitation. Crude extracts were recovered after filtration using a Büchner funnel followed by solvent evaporation using a rotary evaporator under vacuum at 40 °C. Note that maceration with the same solvent was repeated until discoloration of the filtrate. In this case, the different extracts obtained from the same solvent were combined.

The extraction yield was then calculated using the following formula (1), where W_2 is the weight of the extract residue after solvent evaporation and W_1 is the weight of the algal matrix initially used in the extraction (100.0 g).

$$\text{Extraction yield (\%)} = \left(\frac{W_2}{W_1} \right) \times 100 \quad (1)$$

To evaluate their bioactivity, extract solutions were prepared by dissolving the extracts in sterile distilled water (SDW) at 100.0 $\mu\text{g}/\text{mL}$, using an ultrasonic bath (VWR ultrasonic cleaning bath, 45 kHz) for 1 to 6 h until complete dissolution. Extract solutions were then sterilized by filtration through a syringe filter (Cellulose Acetate Syringe Filter, 0.45 μm , GE Healthcare Whatman).

4.5. Assessment of the Inhibitory Effect of Extract on Biofilm Formation—Extract Added at t_0

4.5.1. Formation of PAO1 Biofilms

Biofilms were developed in 24-well plates (Falcon, TC-treated, polystyrene). The bacterial suspension prepared in MBB (2X) was initially adjusted to 10^8 CFU/mL followed by a serial dilution to 10^{-6} with the same medium. One milliliter of the 10^{-6} dilution (equivalent to 10^2 CFU/mL) was introduced into each well. In order to test its effect on the biofilm, 1.0 mL of the algal extract (100.0 $\mu\text{g}/\text{mL}$) (sub-MIC Supplementary Materials S4) was added to each well, corresponding to a final concentration of 50.0 $\mu\text{g}/\text{mL}$. Wells containing 1.0 mL of SDW + 1.0 mL of un-inoculated MBB or 1.0 mL SDW + 1.0 mL inoculated MBB, were considered as sterility and biofilm growth controls, respectively. The plate was then incubated for 24 h at 37 °C. All assays were performed in triplicate.

4.5.2. Screening of Algal Extracts for Their Effect on PAO1 Biofilm Formation and Growth—Crystal Violet Staining Method

The objective of this method was to quantify the total biomass of the biofilm (adhered cells + matrix) by crystal violet (CV) staining and consequently to evaluate the effect of the extract on the formation and proliferation of the biofilm [33]. The protocol adopted by Genovese et al., 2021 was followed with some modifications [56]. After overnight incubation, biofilms were washed twice with 2.0 mL of SDW to remove non-adherent planktonic cells. The plate was then air-dried for 1 h. To stain the adhered biomass, 2.0 mL of an aqueous 1% CV solution was added to the wells and consecutively incubated for 15 min at room temperature. In order to remove the excess stain, wells were rinsed twice with 2.0 mL of SDW followed by drying for 30 min before quantification. One milliliter of

ethanol was finally added to extract bound stain and the inhibition percentage (IP_{CV}) was calculated according to the following Formula (2):

$$IP_{CV}(\%) = \frac{OD_{570 \text{ nm}} \text{ of biofilm growth control} - OD_{570 \text{ nm}} \text{ of tested extract}}{OD_{570 \text{ nm}} \text{ of biofilm growth control}} \times 100 \quad (2)$$

The absence of any interference between the extracts and CV staining was checked using blank wells (1.0 mL of extract + 1.0 mL cell-free MBB).

4.5.3. Effect of the Potentially Active Extracts on the Number of Adhered Bacteria—CFU Counts Method

In this assay, the protocol developed by [8,26] was used with some modifications. After 24 h of incubation, wells were rinsed twice with 2.0 mL of SDW, and then the attached cells were scraped (for 1 min) with a sterile spatula into 1 mL of SDW. The recovered suspension was diluted by serial dilution (from 10^{-1} to 10^{-6}) and 900 μ L of each dilution was inoculated by inclusion in TSA agar plates. After 48 h of incubation at 37 °C, the numbers of CFU were counted by considering only plates with 15 to 300 CFU. The adhered biomass was then calculated and subjected to logarithmic transformation by Formula (3). The logarithmic reduction and the IP_{CFU} with respect to the corresponding untreated control were also calculated using Formulas (4) and (5).

$$\log \text{ of adhered biomass (log CFU/mL)} = \log \frac{\text{number of colonies (CFU)}}{\text{Dilution factor} \times \text{inoculated volume}} \quad (3)$$

$$\log \text{ CFU/mL reduction} = \log \text{ CFU/mL for control} - \log \text{ CFU/mL for treated biofilm} \quad (4)$$

$$IP_{CFU} (\%) = \frac{\text{Adhered cells}_{\text{Control}}(\text{CFU/mL}) - \text{Adhered cells}_{\text{Sample}}(\text{CFU/mL})}{\text{Adhered cells}_{\text{Control}}(\text{CFU/mL})} \times 100 \quad (5)$$

4.5.4. Phenotypic Observations by Epifluorescence Microscopy

The potential effect of extracts, added at t_0 , on PAO1 formed biofilm morphology and on bacterial cell organization was examined by epifluorescence microscopy (EM). For this analysis, *P. aeruginosa* biofilms were grown as described above but in a 6-well microplate (Falcon, TC-treated, polystyrene) and with a total volume of 6.0 mL (3.0 mL of PAO1 bacterial suspension prepared in MBB 2X (10^2 CFU/mL) + 3.0 mL of tested extract or 3.0 mL of SDW for the control).

After 24 h of incubation at 37 °C, well content was carefully discarded and replaced by 6.0 mL of SDW. Live and damaged cells were differentiated by staining with 1.0 μ L of Syto9 (5 mM, Invitrogen™, ThermoFisher Scientific, Illkirch, France) and 1.0 μ L of propidium iodide (1 mg/mL, Invitrogen™, ThermoFisher Scientific), respectively.

Moreover, to examine the potential effect of extracts on the biofilm matrix, 1.0 mL of concanavalin A (ConA, tetramethylrhodamine conjugate, ThermoFisher Scientific) prepared at a concentration of 100.0 μ g/mL in 0.1 M of sodium bicarbonate, was added to the well after its contents had been withdrawn. ConA is a lectin that exhibits an affinity for certain osidic residues, in particular for α -mannopyranosyl and α -glucopyranosyl residues. It is important to note that Strathman et al. [57] have proven that ConA may also bind to alginate, a component of *P. aeruginosa* biofilm matrix. Its conjugation to tetramethylrhodamine leads to the emission of orange-red visible fluorescence upon excitation with a green light. After 20 min of incubation in the dark at room temperature, wells were delicately rinsed twice with 1.0 mL of SDW. Just before proceeding to the microscopic observations, 6.0 mL of SDW, together with 1.0 μ L of Syto9, were added. Microscopic observations were made with Zeiss—AxioTech microscope using a 20 X/0.50 (Zeiss, EC Plan-Neofluar) objective and equipped with an HXP 120 C light source. Images were acquired with a digital camera (Zeiss AxioCam ICm 1) and the set of photos was processed with ZEN software.

4.6. Effect of Selected Algal Extracts on PAO1 24 h-Old Biofilms—Extract Added at t_{24h}

Extracts for which the CV staining method revealed an effect on the biofilm formation (i.e., $IP_{CV} > 50\%$) were subjected to an experiment to evaluate their potential impact on a 24-h-old biofilm. In this assay, 1.0 mL of algal extract solution (100.0 $\mu\text{g}/\text{mL}$) was added with 1.0 mL of MBB into wells of a 24-well plate in which a 24-h-old biofilm was developed as previously described. The plate was then re-incubated at 37 °C for 24 h. After incubation, wells were rinsed twice with 2.0 mL of SDW before the remaining biomass was quantified by the CV staining method.

The eradication percentage was calculated using the following Formula (6):

$$EP_{CV}(\%) = \frac{OD_{570\text{ nm}} \text{ of untreated control} - OD_{570\text{ nm}} \text{ of tested extract}}{OD_{570\text{ nm}} \text{ of untreated control}} \times 100 \quad (6)$$

The extract exhibiting an eradication percentage (EP_{CV}) greater than 80% was also evaluated by the CFU counts method. In this case, both the adhered and the detached (planktonic) cells were quantified. To do this, before the wells were rinsed and scraped, 1.0 mL of the supernatant was withdrawn and submitted to serial dilution followed by inoculation in TSA agar for CFU quantification of planktonic cells. The adherent cells were quantified as described above. The number of CFU counted after 48 h of incubation at 37 °C was subjected to logarithmic transformation based on the above Formula (3).

4.7. Evaluation of the Synergistic Antibiofilm Activity of the Active Extract in Combination with Tobramycin or Colistin on 24 h-Old Treated Biofilms

The potential synergistic antibiofilm effect of the *U. lactuca* ethyl acetate (EA) active extract with tobramycin and colistin was evaluated on 24 h-old biofilms, previously treated with the EA extract or not, following the protocol developed by Furiga et al., 2016 with some modifications. Since the objective here was to detect a potential synergistic effect, the tested concentrations of antibiotics had to be lower than the concentration that would be fully effective in eradicating PAO1 biofilm, hence the choice of 2 and 16 $\mu\text{g}/\text{mL}$ for tobramycin and colistin, respectively [41].

First, 1.0 mL of bacterial suspension (10^2 CFU/mL) prepared in MBB (2X) was added into each well of a 24-well microplate, supplemented either with 1.0 mL of SDW (control, tobramycin, and colistin control) or with 1.0 mL of a solution of 100.0 $\mu\text{g}/\text{mL}$ of EA extract (EA extract control and combination assays; final concentration 50.0 $\mu\text{g}/\text{mL}$). After 24 h of incubation at 37 °C, the supernatant was removed, and replaced by 1.0 mL of SDW (control) or 1.0 mL of tobramycin alone (tobramycin control; final concentration 2.0 $\mu\text{g}/\text{mL}$) or 1.0 mL of colistin alone (colistin control; colistin sodium methanesulfonate; final concentration 16.0 $\mu\text{g}/\text{mL}$) or 1.0 mL of EA extract (EA extract control; final concentration 50.0 $\mu\text{g}/\text{mL}$) or a solution of EA extract mixed with either tobramycin or colistin for the combination assays. The final concentrations of EA extract, tobramycin, and colistin were 50.0 $\mu\text{g}/\text{mL}$, 2.0 $\mu\text{g}/\text{mL}$ and 16.0 $\mu\text{g}/\text{mL}$, respectively. MBB medium was then added to all wells (1.0 mL/well). For all conditions, the number of adherent cells after 48 h of incubation was quantified by the CFU counts method, as described above. Log reduction was then calculated using Formulas (3) and (4).

4.8. Analysis of the Chemical Composition of Extracts by GC–MS

The chemical composition of all extracts was analyzed first by GC–MS; extracts were prepared at a concentration of 2.5 mg/mL in the corresponding extraction solvent (cyclohexane, dichloromethane, ethyl acetate, or methanol). Analyses were performed using GC–MS system (TRACETM 1310—ThermoFisher Scientific) equipped with a Rtx-502.2 fused silica capillary column (30 m in length, 0.25 mm in diameter, 1.4 μm in film thickness). The column oven temperature was programmed as follows: initial temperature was 50 °C (for 2 min) then gradually increased to 150 °C (for 5 min) at a rate of 20 °C/min, and finally increased to 290 °C at a rate of 10 °C/min and maintained for 10 min. Ionization of the sample components was performed in electron impact mode (EI, 70 eV) with 220 °C as

ion temperature. The injector and detector temperatures were 250 and 220 °C, respectively. Hydrogen was used as carrier gas at a flow rate of 1.0 mL/min. The injection volume of the prepared extract solution (2.5 mg/mL) was 5.0 µL. The total running time of the GC–MS system was 36 min. Finally, molecules were identified using Xcalibur software.

4.9. Statistical Analysis

All values were expressed as mean ± SD for three independent experiments. The student t-test was used to calculate the significance of the differences between the mean effects of the extract and those for the associated untreated control in the CFU counts method after checking equality of variances with Levene's test (p -value < 0.05). Statistically significant values were defined as a p -value (* <0.05, ** <0.01 or *** <0.001). SPSS 22.0 software (SPSS, IBM Corporation, Armonk, NY, USA) was used in the statistical analysis.

5. Conclusions

In the present study, the screening of extracts derived from three algae for their antibiofilm activity against the pathogenic bacterium *P. aeruginosa* allowed two *U. lactuca* extracts (CH and EA extracts) to be selected as the most promising for valorization in this field. CH extract appears to impair microcolony growth, resulting in a significant reduction in the number of adherent cells, while an effect on the production and the degradation of the biofilm matrix has been suggested as a potential mode of action of EA extract. In light of these encouraging results, further experiments are envisaged to analyze the chemical composition of the two active extracts and isolate active components as pure molecules. The evaluation of the antibiofilm effect of these extracts on other pathogenic bacteria would identify a broad spectrum of activities. Overall, this study raises the possibility of extracting bioactive compounds from the green alga, *U. lactuca*, which can potentially be used alone or in combination with antibiotics in the treatment of biofilm-related infections.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/md20020092/s1>, Figure S1: Map showing the area where seaweed samples were collected, Figure S2: Planktonic growth kinetics of PAO1 in MHB, MBB and in presence of EA, Table S3: Evaluation of the potential bactericidal activity of CH and EA extracts on PAO1. Figure S5: Chromatograms of extracts derived from the green alga *U. lactuca*.

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Seaweed extracts: A promising source of antibiofilm agents with distinct mechanisms of action against *Pseudomonas aeruginosa*

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S1: Collection of algal materials

Seaweed samples belonging to three different groups (green alga *Ulva lactuca*, brown alga *Stypocaulon scoparium*, and red alga *Pterocladia capillacea*) were manually collected in the Mediterranean Sea, from the northern Lebanese coast, particularly from El Mina in Tripoli in September 2019 (Figure S1).

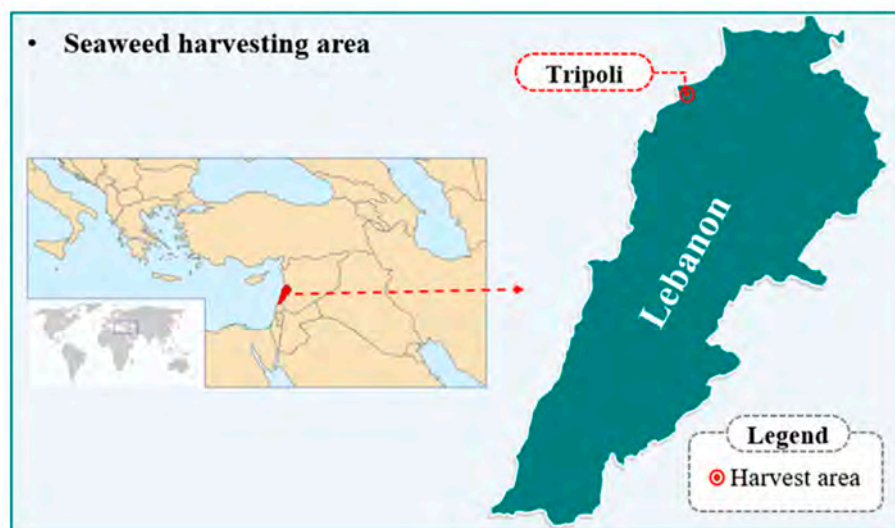


Figure S1. Map showing the area where seaweed samples were collected.

S2: PAO1 planktonic growth kinetics in the rich medium MHB, in the low-nutritive medium MBB and in presence of EA-Extract – optical density measurement

PAO1 growth kinetics curves in MHB, MBB, and in MBB in the presence of EA extract were also performed. Briefly, 100 µl of tested media (MHB and MBB 2X) were introduced into the wells of a sterile 96-well microtiter plate (Falcon, TC-treated, polystyrene) supplemented with 100 µl of sterile distilled water. In order to evaluate the potential effect of EA extract on the PAO1 growth curve, 100 µl of EA extract stock solution (100.0 µg/ml) were added to 100 µl of MBB. The bacterial suspension used in this assay was prepared in sterile distilled water and adjusted to an optical density of 0.150 at 640 nm, corresponding to a concentration of 10⁸ CFU/ml followed by dilution (1:10) to achieve a concentration of 10⁷ CFU/ml. Then, the microtiter-plate was inoculated using a manual multipoint inoculator. Note that wells in the last column were used as sterility controls (100 µl of sterile distilled

water + 100 µl of tested media). The plate was incubated at 37°C for 24h in a microplate spectrophotometer (Multiskan™ GO, Thermo Fisher Scientific, Waltham, USA) under continuous agitation. The optical density measurement was carried out at 640 nm every one hour. The measured values were plotted as a function of time. Results are expressed as means \pm SD (OD_{640nm}) of two independent assays (Figure S2).

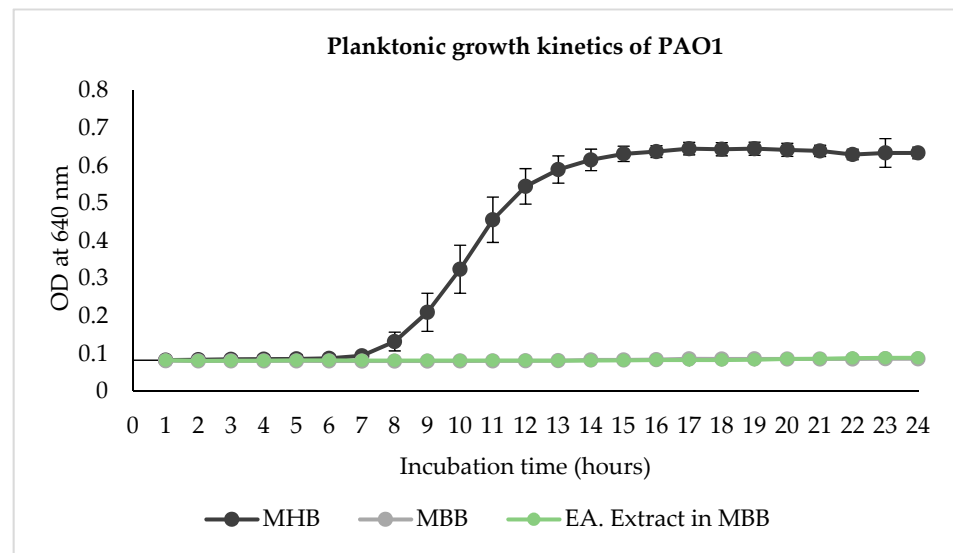


Figure S2. Planktonic growth kinetics of PAO1 in MHB, MBB and in presence of EA. Extract (50.0 µg/ml) originated from the green alga *U. lactuca*. Results are expressed as means \pm SD of the optical density measured at 640 nm of two independent experiments. EA is ethyl acetate extract. MHB and MBB are Mueller-Hinton broth and modified biofilm broth, respectively.

S3: Checking the potential bactericidal activity of CH and EA extracts derived from the green alga *U. lactuca* – CFU counts method

In order to exclude the potential bactericidal effect of the two active extracts (CH and EA extracts derived from the green alga *U. lactuca*) at the tested concentration (50.0 µg/ml), their effect on PAO1 planktonic cells was assessed. The protocol developed by Feuillolay et al., 2016 was used in this assay. Briefly, 5.0 ml of PAO1 bacterial suspension (10^5 or 10^2 CFU/ml) prepared in MBB (2X) medium and supplemented with 5.0 ml of sterile distilled water were incubated for 24 hours at 37°C. Water was replaced by 5.0 ml of extracts (CH or EA extracts) in the sample tubes. The potential bactericidal activity of extracts was determined on both suspension (10^5 and 10^2 CFU/ml). Tubes were maintained under agitation (100 rpm) in an orbital shaker (MaxQ 4000, Thermo Fisher Scientific, Waltham, USA). The number of planktonic cells was monitored after 24 hours of incubation at 37°C by CFU counts. Prior to their quantification, samples were homogenized then 1.0 ml was taken and serially diluted (10^{-1} to 10^{-6}). 900 µl of each dilution were inoculated by inclusion in TSA agar plates and overnight incubated at 37°C for cell quantification. Assays were performed in duplicate. Results expressed as ratio (log CFU/ml for sample / log CFU/ml for control) are presented in Table S3.

Table S3. Evaluation of the potential bactericidal activity of CH and EA extracts (50.0 µg/ml) derived from the green alga *U. lactuca* on PAO1 (10⁵ CFU/ml or 10² CFU/ml). The number of planktonic cells was measured after 24 hours of incubation at 37°C under agitation. Results are expressed as means of ratio (log CFU/ml for sample/ log CFU/ml for control) ± SD from two independent experiments. CH and EA are cyclohexane and ethyl acetate extracts, respectively.

Initial bacterial suspension	CH extract	EA extract
10 ⁵ CFU/ml	1.03 ± 0.01	1.01 ± 0.01
10 ² CFU/ml	1.00 ± 0.02	1.00 ± 0.01

Feuillolay, C., Pecastaings, S., Le Gac, C., Fiorini-Puybaret, C., Luc, J., Joulia, P., & Roques, C. (2016). A *Myrtus communis* extract enriched in myrtucummulones and ursolic acid reduces resistance of *Propionibacterium acnes* biofilms to antibiotics used in *acne vulgaris*. *Phytomedicine*, 23, 307-315.

S4: Effect of extracts on PAO1 planktonic growth – MIC determination

The antibacterial activity was evaluated in order to determine the appropriate concentration of the extracts to be used in the antibiofilm activity assays (sub-MIC) in a way that they did not present classical bacteriostatic/bactericidal effects since we were looking for an effect on the biofilm formation. The MIC of each extract against *P. aeruginosa* was determined using the broth microdilution method, according to the guidelines of CA-SFM/EUCAST 2020. Briefly, 100.0 µl samples of algal extract solution (100.0 µg/ml) were introduced into the wells of the first column of a sterile 96-well microtiter plate (Falcon, TC-treated, polystyrene) and subjected to 2-fold serial dilutions with Mueller-Hinton broth (MHB) (100 µl/well) to achieve final concentrations ranging from 50.0 to 0.098 µg/ml. The bacterial suspension used in this assay was prepared in SDW and adjusted to an optical density of 0.150 at 640 nm, corresponding to a concentration of about 10⁸ CFU/ml. This suspension was then subjected to a 2-fold dilution in SDW prior to the inoculation of the microtiter-plate using a manual multipoint inoculator (1.0 µl), in order to obtain a final concentration of 5 × 10⁵ CFU/ml. Note that wells in the last column were used as sterility controls (SDW + MHB). The previous column was dedicated to growth control (SDW + MHB + inoculum). After incubation at 37°C for 24 h, the MIC, defined as the lowest concentration of the tested extract that could prevent visible bacterial growth, was determined. Assays were performed in duplicate. According to the results, no antibacterial activity was demonstrated at the highest concentration tested (50.0 µg/ml) for any of the extracts. Therefore, the concentration adopted for the antibiofilm activity assays was 50.0 µg/ml for all extracts.

S5: Chromatograms of extracts derived from the green alga *U. lactuca* – GC/MS

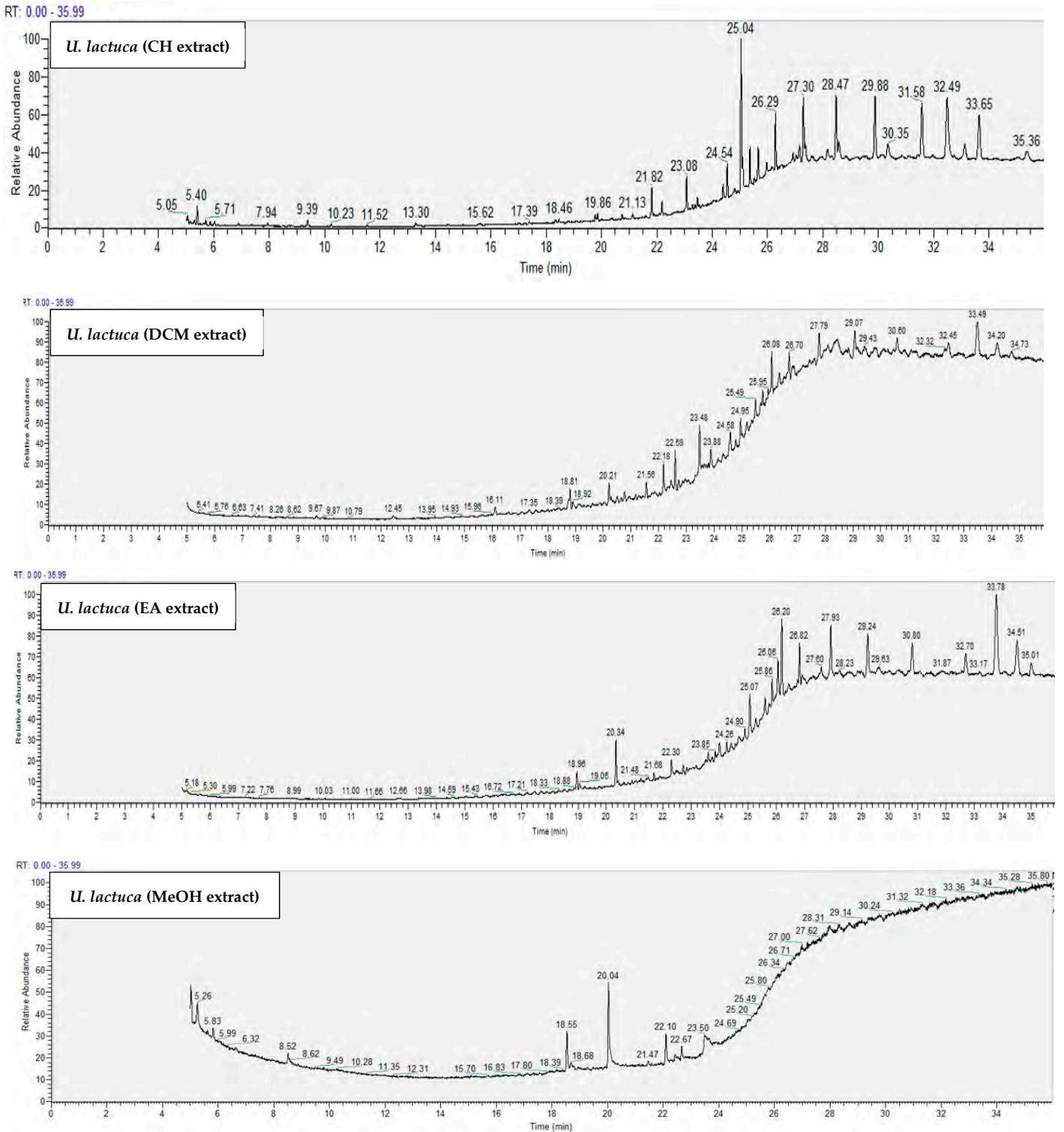


Figure S5. Chromatograms of extracts derived from the green alga *U. lactuca*.

Part III:

Evaluation of the antibiofilm activity of seaweed extracts against *S. aureus*

ARTICLE III – To submit:

Rima, M., Chbani, A., Roques, C., & El Garah, F. Seaweed extracts as an effective gateway in the search for novel antibiofilm agents against *Staphylococcus aureus*.

▪ PREVIEW

In this part, the potential ability of extracts derived from the three Lebanese seaweed (green alga *U. lactuca*, brown alga *S. scoparium*, and red alga *P. capillacea*) to exhibit an antibiofilm activity against the bacterium *S. aureus* was investigated.

The work presented in this part was done in the Laboratoire de Génie Chimique (LGC – UMR5503) – Toulouse – France and resulted in an article for submission.

After a brief summary of the article with a highlight on the main results, the manuscript is integrated followed by the supplementary materials.

III.1 ARTICLE SUMMARY – FOCUS ON THE MAIN RESULTS

The potential ability of various extracts derived from the three algae tested in this study (green alga *U. lactuca*, brown alga *S. scoparium*, and red alga *P. capillacea*) to present a good candidate in the search for novel antibiofilm agents against *S. aureus* bacterium was assessed. First, the potential effect of extracts on biofilm formation and growth (extract added at t_0) was evaluated using CFU counts method. It should be noted that the application of CV staining method, widely used in the quantification of biofilm biomass, is not suitable in our case due to the limited quantity of matrix produced by *S. aureus* (below the detection limit) in our culture conditions. This first experiment allowed us to select four extracts (CH and DCM extracts derived from the green alga *U. lactuca* and CH and EA extracts derived from the brown alga *S. scoparium*) showing the most promising antibiofilm activity (***, P -value < 0.001; **, P -value < 0.01). The microscopic analysis supports their ability to reduce the number of adhered cells and even some extracts such as (DCM and CH extracts derived from the green and the brown alga, respectively) have shown an effect on matrix proteins.

Then, with the aim of specifying biofilm development stage targeted by these selected active extracts, *S. aureus* biofilm was treated at different stages of growth (t_0 , t_{2h} , t_{4h} , t_{6h} , t_{24h}). Results showed a progressive reduction in the effectiveness of extracts by delaying their addition which suggests their potential effect on the initial adhesion and proliferation stages.

In an attempt to decipher the possible mechanism of action exhibited by these extracts, their potential effect on the hydrophobicity of *S. aureus* cells was evaluated in view of the strong involvement of its hydrophobic properties in its adhesion. Interestingly, results showed the ability of the two extracts (CH and DCM extracts) derived from the green alga *U. lactuca* to significantly reduce the hydrophobicity of *S. aureus* which can explain their demonstrated effect on the early stages of biofilm formation (up to 6h).

These encouraging results summarized in the table below (Table 22) emphasize algae as a promising source of antibiofilm agents against *S. aureus*. However, additional experiments are required in order to go further in the elucidation of the mechanism of action exhibited by the selected active extracts.

TABLE 22 | Summary table of the demonstrated antibiofilm activity of the four selected active extracts against *S. aureus*. U.l and S.s are *U. lactuca* green alga and *S. scoparium* brown alga, respectively. CH, DCM, and EA are cyclohexane, dichloromethane, and ethyl acetate, respectively.

Experiments		U.l (CH)	U.l (DCM)	S.s (CH)	S.s (EA)
Extract added at t_0		++	+	+	+
Microscopic analysis	Cells	Reduction in the number of adhered cells			
	Matrix proteins	-	Decrease in matrix proteins	Decrease in matrix proteins	-
Extract added at different times point		Gradual reduction in the effect (up to <u>6h</u>)	Gradual reduction in the effect (up to <u>6h</u>)	Rapid reduction in the effect	Rapid reduction in the effect
Effect on cells hydrophobicity		+	++	-	-

++, significant effect with P -value < 0.001

+, significant effect with P -value < 0.01

-, no effect

III.2 ARTICLE TO BE SUBMITTED

Seaweed extracts as an effective gateway in the search for novel antibiofilm agents against *Staphylococcus aureus*

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ABSTRACT

Background: Currently, the treatment of biofilm-associated infections has become a major challenge in biomedical and clinical fields due to the failure of conventional treatments in controlling this highly complex and tolerant structure. Therefore, the search for novel antibiofilm agents with increased efficacy and few side effects as those provided by natural products, presents an urgent need.

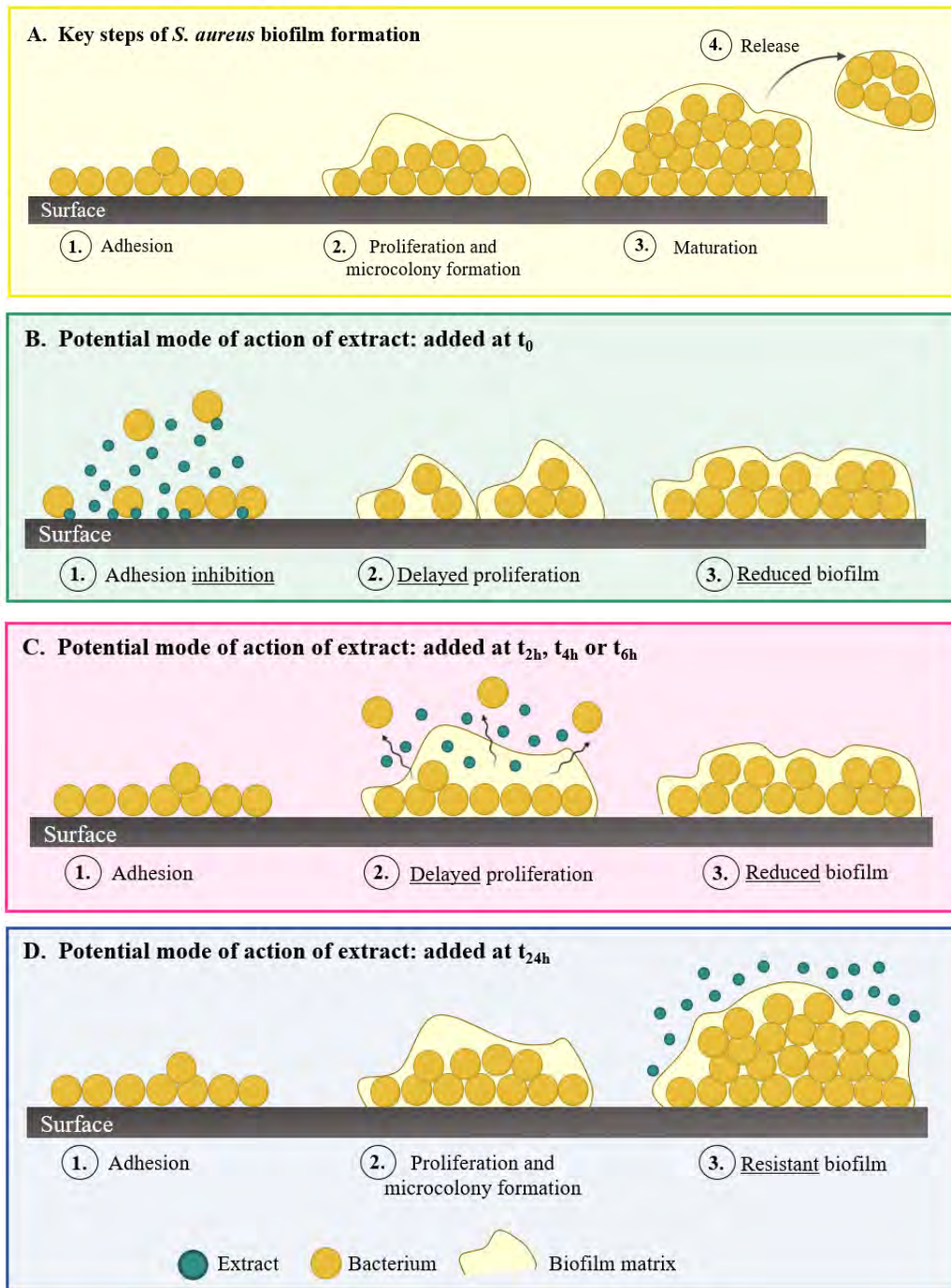
Purpose: The aim of this study is to explore extracts derived from three algae (green *Ulva lactuca*, brown *Stypocaulon scoparium*, red *Pterocladia capillacea*) for their potential antibiofilm activity against *Staphylococcus aureus*, bacterium responsible for several acute and chronic infections by colonizing tissue and artificial surfaces.

Methods: Seaweed extracts were prepared by successive maceration in four solvents with increasing polarity (cyclohexane, dichloromethane, ethyl acetate, and methanol). The ability of the different extracts to inhibit *S. aureus* biofilm formation was assessed using colony-forming unit (CFU) counts method. Epifluorescence microscopic analysis of biofilm formed in presence of the potentially active extracts was also carried out. Effects of active extracts on growth cycle of biofilm formation (extract added at various times of biofilm development) as well as on *S. aureus* surface hydrophobicity were evaluated.

Results: The obtained results revealed the ability of four extracts (CH and DCM extracts derived from the green alga and CH and EA extracts originated from the brown one) to significantly (***, P -value < 0.001; **, P -value < 0.01) inhibit *S. aureus* biofilm formation. These findings were supported by microscopic analyses. The gradual reduction in the number of adherent bacteria when the selected extracts were added at various times (t_0 , t_{2h} , t_{4h} , t_{6h} , and t_{24h}) reveals their potential effect on the initial adhesion and proliferation stages of *S. aureus* biofilm development. Concerning DCM extract derived from the green alga, its demonstrated ability to significantly (***, P -value < 0.001) reduce *S. aureus* surface hydrophobicity may account to its effect on the early stages of biofilm formation.

Conclusion: These findings present new insight into the exploration of seaweed as a valuable source of antibiofilm agents with preventive effect by inhibiting and/or delaying biofilm formation.

Keywords: *Staphylococcus aureus*, anti-biofilm, seaweed extracts, hydrophobicity, anti-adhesion.



Graphical abstract

1. INTRODUCTION

Although the huge marine biodiversity is far from being completely explored, previous studies have evidenced the richness of the marine world in organisms producing a library of bioactive secondary metabolites that arise from millions of years of natural selection and evolution (Kiuru *et al.*, 2014; Jimenez, 2018). Seaweed, benthic marine macroalgae widely distributed on rocky shores as well as at various sea depth, are part of sea's treasure trove that have been used for centuries as sea vegetables, fertilizers and medicines (Leandro *et al.*, 2019). In fact, algae are well known for their richness in unique bioactive compounds synthesized from the simple resources found in the marine environment as a natural response and a self-preservation way of facing the stressful environmental conditions (Shannon & Abu-Ghannam, 2016; Leandro *et al.*, 2019).

In addition to the abiotic challenges (salinity, temperature changes, UV radiation exposure...) encountered in seawater, algae are also exposed to biotic threats represented by a considerable risk of being infected by undesirable microorganisms such as bacteria (Leandro *et al.*, 2019). In this context, different studies have proven the wide spectrum of antibacterial activity of algal metabolites demonstrated against several Gram-negative and Gram-positive pathogenic bacteria which provides a promising gateway in the search for novel drugs (Bhowmick *et al.*, 2020).

It is obvious that the rapid emergence of multidrug resistant bacteria poses a global threat for human health which calls for intensive efforts in order to overcome the problem of antibiotic failure. Besides the well-known genetic mechanisms involved in the bacterial resistance phenomenon as well as the horizontal transfer of antibiotic-resistance genes, bacteria also exhibit an adaptive strategy that consists in the formation of a strongly structured cells assembly named "biofilm", embedded in a self-produced extracellular matrix and adhered to a biotic or abiotic surfaces. Due to the collective recalcitrance of this bacterial association towards antibiotics as well as its ability to evade the host immune defenses, treatment of biofilms related infections is increasingly challenging (Uruen *et al.*, 2020).

The Gram-positive “superbug” *Staphylococcus aureus* is one of the common pathogenic bacteria well-known as a biofilm producer. Classified by the Infectious Diseases Society of America (IDSA) as member of “ESKAPE pathogens” group and defined by the World Health Organization (WHO) as a high priority in the search for novel therapeutic strategies, *S. aureus* receives a considerable attention (Pendleton *et al.*, 2013; WHO, 2017). This opportunistic bacterium is one of the principle human pathogens that is widely associated with hospital acquired infections and responsible for several biofilms related infections worldwide (Tong *et al.*, 2015). Besides its ability to colonize living tissues leading to severe infections such as osteomyelitis, endocarditis, and respiratory infections, *S. aureus* readily forms a resilient biofilm on catheters and implanted medical devices surfaces (Archer *et al.*, 2011).

Typically, bacterial biofilm formation occurs in three main steps initiated by cell adhesion to a surface followed by bacterial aggregates proliferation leading to the establishment of a multi-layered structure of biofilm. Then, to ensure the biofilm life cycle, a dispersion step proceeds (Rumbaugh & Sauer, 2020).

In *S. aureus*, the initial attachment to surface is mainly mediated by hydrophobic and electrostatic interactions followed by the production of the extracellular matrix (polysaccharides, teichoic acids, extracellular DNA, proteins...) which is highly involved in mature biofilm resilience by providing a diffusion barrier against antimicrobial agents (Suresh *et al.*, 2019).

The current treatments of *S. aureus* biofilm related infections are based on the ablation of the infected foreign bodies when it's possible, otherwise, the administration of conventional antimicrobial agents at high concentration and for an extended period is often used (Suresh *et al.*, 2019). Thus, the exploration of new approaches to prevent and/or to treat *S. aureus* biofilm presents an area of active research. In this context, natural medicine, which has been used for centuries in healing and treatment of diseases, presents strong promises given the remarkable antibiofilm activity demonstrated for several natural products (Mishra *et al.*, 2020; Guzzo *et al.*, 2020) .

Therefore, the aim of this study is to evaluate the potential capacity of extracts derived from three algae (the green alga *U. lactuca*, the brown alga *S. scoparium* and the red alga *P. capillacea*) collected from the Lebanese coast to control *S. aureus* biofilm. The potential antibiofilm effect of the different prepared extracts was first assessed on biofilm formation and development using colony-forming unit (CFU) counting method. An epifluorescence microscopic examination of the biofilm formed in the presence of the most active extracts was also carried out. Then, the biofilm development phase targeted by the selected active extracts was determined by adding extract at different stages of *S. aureus* biofilm growth. Furthermore, the potential influence of the selected extracts on *S. aureus* surface hydrophobicity known to be correlated to its adhesiveness was evaluated by contact angle measurement method.

2. MATERIALS AND METHODS

2.1 Collection of algal materials

The three algae chosen to be evaluated in this study are the green alga *Ulva lactuca*, the brown alga *Stypocaulon scoparium*, and the red alga *Pterocladia capillacea*. Seaweed samples were manually collected from the North Lebanese coast of the Mediterranean (El Mina – Tripoli – Lebanon) in September 2019. Algae were rinsed directly with seawater followed by a distilled water wash in laboratory to remove associated impurities such as epiphytes and adhered sand particles. Seaweed samples were then air-dried in dark at room temperature (20 - 27°C) for several weeks and weighted continuously until complete drying. Dried algae were powdered in fine-milled form prior to extraction. They were then transported in sealed bags to Laboratoire de Génie Chimique of Toulouse – France where the extractions were done.

2.2 Organic solvents and chemicals

Solvents used for preparation of seaweed extracts are cyclohexane 99.5% (Sigma-Aldrich, France), dichloromethane 100% (VWR, France), ethyl acetate 99.9% (VWR, France), and methanol 99.8% (Sigma-Aldrich, France). The dyes employed in the microscopic analysis were purchased from ThermoFisher Scientific – France.

2.3 Bacterial strain and culture media

The bacterial strain used in this study is *Staphylococcus aureus* (CIP 4.83), purchased from the collection of Pasteur Institute (Paris, France) and preserved at - 80°C. Before each experiment, two successive overnight subcultures were realized on Trypticase soy agar TSA (BioMérieux, Craponne, France) and incubated under aerobic conditions at 37°C. Mueller-Hinton broth MHB (Oxoid microbiology products, Basingstoke, UK) was used as culture medium for the evaluation of the antibacterial activity of extracts (CA-SFM/EUCAST 2020). On the other hand, the antibiofilm activity assays were conducted in the previously selected low-nutritive medium named biofilm broth (BB) in order to create stressful conditions and subsequently promote biofilm formation and adherent cells growth rather than planktonic growth (Campanac *et al.*, 2002).

The BB 10X is composed of FeSO₄, 7H₂O (0.005 g/L), Na₂HPO₄ (12.5 g/L), KH₂PO₄ (5.0 g/L), (NH₄)₂ SO₄ (1.0 g/L), lactose (0.25 g/L), yeast extract (1.0 g/L), vitamin assay casamino acids (1.0 g/L) and MgSO₄, 7H₂O (0.2 g/L) (Campanac *et al.*, 2002). Except for yeast extract (Bacto™, ThermoFisher scientific) and vitamin assay casamino acids (Difco™, ThermoFisher scientific), all these compounds were purchased from Sigma-Aldrich, France.

2.4 Preparation of seaweed extracts

In order to extract the maximum seaweed's constituents, a successive extraction method using selective solvents with increasing polarity (cyclohexane, dichloromethane, ethyl acetate, and methanol) has been adopted (Kohoude *et al.*, 2017). Thus, 100.0 g of the dried samples of each alga were macerated successively in 1L of each solvent for 2 hours under magnetic agitation. Crude extracts were recovered after filtration using the Büchner funnel followed by solvent evaporation using a rotary evaporator under vacuum at 40°C. Note that maceration with the same solvent was repeated until the progressive discoloration of the filtrate. In this case, the different extracts obtained from the same solvent were combined. The extraction yield was then calculated using the following formula (1), where W2 is the weight of the extract residue after solvent evaporation and W1 is the weight of the algal matrix initially used for the extraction (100.0 g).

$$(1) \quad \text{Extraction yield (\%)} = (W2 / W1) \times 100$$

To evaluate their bioactivity, stock solutions were prepared by dissolving the extracts in sterile distilled water (SDW) at 100.0 µg/mL, using an ultrasonic bath (VWR ultrasonic cleaning bath, 45 KHz) for almost 6 hours to promote the solubility. Stock solutions were then sterilized by filtration through a syringe filter (Cellulose Acetate Syringe Filter, 0.45 µm, GE Healthcare Whatman).

2.5 Effect of extracts on *S. aureus* planktonic growth – MIC determination

Prior to the assessment of their potential effect on biofilm, the evaluation of antibacterial activity of extracts on *S. aureus* is essential for ensuring the use of a sub-MIC concentration in antibiofilm activity assays. For this purpose, the minimal inhibitory concentration (MIC) of all extracts against *S. aureus* was determined using the broth microdilution method, according to the guidelines of CA-SFM/EUCAST 2020.

Briefly, 100.0 μL of algal extract stock solution (100.0 $\mu\text{g}/\text{mL}$) were introduced into the wells of the first column of a sterile 96-well microtiter plate (Falcon, TC-treated, polystyrene) and subjected to 2-fold serial dilutions with Mueller-Hinton broth (MHB) (100.0 $\mu\text{L}/\text{well}$) to achieve final concentrations ranging from 50.0 to 0.098 $\mu\text{g}/\text{mL}$.

The bacterial suspension used in this assay was prepared in SDW and adjusted to an optical density of 0.150 at 640 nm, corresponding to a concentration of 10^8 CFU/mL. This suspension was then subjected to a 2-fold dilution in SDW prior to the inoculation of the microtiter-plate using a manual multipoint inoculator (1.0 μL). Note that wells in the last column were used as sterility controls (SDW + MHB). The previous column was dedicated to growth control (SDW + MHB + inoculum). After incubation at 37°C for 24h, the MIC, defined as the lowest concentration of the tested extract which can prevent the visible bacterial growth was determined. Assays were performed in duplicate.

2.6 Evaluation of the antibiofilm activity of extracts

First, the ability of extracts derived from the three algae to inhibit *S. aureus* biofilm formation and growth (extract added at t_0) was determined. The biofilms formed in the presence of the potentially active extracts were visualized by epifluorescence microscopy. Then, the most active extracts were selected in order to investigate the targeted biofilm growth phase by adding extract to a *S. aureus* biofilm at various development stages (t_{2h} , t_{4h} , t_{6h} , and t_{24h}). It should be noted that in all assays, the quantification of *S. aureus* biofilm was performed by counting the adhered cells recovered by scraping. Assays were performed in triplicate.

2.6.1 Effect of extracts on *S. aureus* biofilm formation and growth (extract added at t_0)

The influence of extracts on the number of adhered cells was evaluated following the CFU counts method previously described by (Khalilzadeh *et al.*, 2010) with some modifications. The bacterial suspension used in this assay was prepared in the low-nutritive medium BB (2X) and was adjusted to 10^8 CFU/mL ($\text{OD}_{640\text{nm}} = 0.150$) followed by ten-fold serial dilution up to 10^{-6} with the same medium. Then, 1.0 mL of the 10^{-6} dilution (equivalent to 10^2 CFU/mL) was introduced into the wells of a 24-well plates (Falcon, TC-treated, polystyrene). 1.0 mL of algal extract (100.0 $\mu\text{g}/\text{mL}$) was added at t_0 , corresponding to a final concentration of 50.0 $\mu\text{g}/\text{mL}$. Algal extract was replaced by 1.0 mL of SDW in biofilm

growth control. Wells containing 1.0 mL of SDW + 1.0 mL of un-inoculated BB (2X) medium was considered as sterility control. After overnight incubation at 37°C, wells' content was discarded followed by rinsing (x 2) with 2.0 mL of SDW in order to remove unattached planktonic cells. Adhered cells were then recovered by scraping for 1 min with a sterile spatula into 1.0 mL of SDW followed by a ten-fold serial dilution (from 10⁻¹ to 10⁻⁶).

900.0 µL of each dilution was then inoculated by inclusion in TSA agar plates. After 48h of incubation at 37°C, the numbers of CFU were determined by considering only plates with 15 to 300 CFU. The adhered biomass was then calculated and subjected to logarithmic transformation by the following formula (2). The logarithmic reduction with respect to the corresponding untreated control was calculated using the formula below (3).

$$(2) \quad \text{Log of adhered biomass (log CFU/mL)} = \log \frac{\text{number of colonies (CFU)}}{\text{Dilution factor} \times \text{inoculated volume}}$$

$$(3) \quad \text{Log CFU/mL reduction} = \log \text{CFU/mL}_{\text{for control}} - \log \text{CFU/mL}_{\text{for treated biofilm}}$$

2.6.2 Epifluorescence microscopic analysis of treated biofilms (extract added at t₀)

Biofilms formed in presence of the potentially active extracts were visualized by epifluorescence microscopy. For this analysis, *S. aureus* biofilms were grown as described above but in a 6-well microplate (Falcon, TC-treated, polystyrene) and with a total volume of 6.0 mL (3.0 mL of *S. aureus* bacterial suspension prepared in BB 2X (10² CFU/mL) + 3.0 mL of tested extract or 3.0 mL of SDW for the biofilm growth control).

After 24 hours of incubation at 37°C and in order to evaluate the potential effect of extracts on *S. aureus* biofilm matrix, 1.0 mL of SYPRO Ruby stain (Invitrogen™, FilmTracer™, SYPRO™ Ruby biofilm matrix stain) was added after discarding wells content. This stain binds to most classes of proteins including glycoproteins, lipoproteins, phosphoproteins and fibrillar proteins. After 30 min of incubation in dark at room temperature, wells were carefully washed twice with 1.0 mL of SDW. 6.0 mL of SDW was then added supplemented with 1.0 µL of Syto9 stain for cells visualization.

Microscopic observations were made with Zeiss – AxioTech microscope using a 20 X / 0.50 (Zeiss, EC Plan-Neofluar) objective and equipped with an HXP 120 C light source. Images were acquired with a digital camera (Zeiss AxioCam ICm 1) and then the set of photos was processed with ZEN software.

2.6.3 Determination of biofilm development stage targeted by the selected extracts

Extracts that showed the most significant activity (***, P -value < 0.001; **, P -value < 0.01) (in comparison to the biofilm growth control) on *S. aureus* biofilm formation and growth were selected. With the aim of specifying biofilm development phase targeted by these active extracts, *S. aureus* biofilm was treated at different stages of growth as outlined in Table 1.

Table 1: Protocol for the addition of extract at different time points.

Stage of biofilm formation	Time point of extract addition				
	0	2h	4h	6h	24h
0	↓+	↓	↓	↓	↓
2h		+			
4h			+		
6h				+	
24h		Scarping time			+
48h			–		Scarping time

“↓” is inoculation time point and “+” is extract addition time point.

Briefly, at t_0 , 1.0 mL of bacterial suspension (10^2 CFU/mL) prepared in BB (2X) was introduced into the wells of a 24-well plate either with 1.0 mL of SDW (control and later treated biofilms) or with 1.0 mL of extract (final concentration 50.0 μ g/mL). 1.0 mL of un-inoculated BB (2X) + 1.0 mL of SDW were introduced into sterility control wells. Plate was then incubated at 37°C.

At different time point (t_{2h} , t_{4h} , t_{6h} , t_{24h}), the formed biofilm was washed twice with 2.0 mL of SDW and 1.0 mL of extract (final concentration 50.0 μ g/mL) was added supplemented with 1.0 mL of un-inoculated BB (2X). Extract was replaced by 1.0 mL of SDW in the corresponding control wells.

Plate was incubated at 37°C and adhered cells were recovered by scraping after 24h or 48h of incubation. After quantification and logarithmic transformation of the number of adhered cells, the logarithmic reduction with respect to the corresponding untreated control was calculated using the formula above (3).

2.7 Effect of the selected extracts on *S. aureus* hydrophobicity – contact angle measurement method

In order to evaluate the effect of the selected extracts on *S. aureus* hydrophobicity, the sessile drop technique which consists in measuring the contact angle of a water drop on a bacterial layer was carried out. The protocol described by (Elabed *et al.*, 2017) was adopted with some modifications. Briefly, 5.0 mL of *S. aureus* suspension prepared in BB (2X) ($OD_{640nm} = 0.3$) was added to 5.0 mL of extract (final concentration 50.0 µg/mL). Extract was replaced by 5.0 mL of SDW in the control tube. After 2 hours of incubation at 37°C and in order to remove extracts, bacteria were recovered by vacuum filtration on a sterile cellulose acetate membrane filter (0.45 µm, Sigma-Aldrich, France) that was dehydrated for almost 30 min at room temperature prior to the measurement of the contact angle.

The contact angle between a water drop (1-2µL) and the bacterial lawns was then measured under ambient conditions using a Digidrop contact angle meter (GBX Scientific Instruments, Romans-sur-Isère, France). The measurements were computed automatically by Windrop++ software. It should be noted that the measurement should be done within 3-4 s after depositing the drop in order to avoid its penetration in the bacterial layer. Contact angle was determined at 5 random points per bacterial film. Results are expressed as mean contact angle \pm the corresponding standard deviation.

2.8 Statistical analysis

All values are expressed as mean \pm SD for three independent experiments. The student t-test was used to calculate the significance of the differences between the mean effects of the extract and those for the associated untreated control after checking equality of variances with Levene's test (P -value < 0.05). Statistically significant values were defined as a P -value (* < 0.05 , ** < 0.01 or *** < 0.001). SPSS 22.0 software (SPSS, IBM Corporation, Armonk, NY, USA) was used.

3. RESULTS

3.1 Extraction yields of different seaweed extracts

Seaweed extracts were prepared by maceration in different solvents with increasing polarity. As expected, the yields of seaweed extracts were affected by the polarity of the extraction solvent used (Table 2). In fact, for the three algae evaluated in this study, the highest extraction yield was recorded for the methanolic extracts resulting in 12.1, 1.4, and 7.3% for green, brown, and red seaweed, respectively. Moreover, the number of extraction repetitions required with methanol to achieve a complete extraction demonstrates the richness of these algae in polar compounds in comparison with their content in non-polar ones.

Table 2: Characteristics of extracts according to the extraction solvents

Seaweed species		CH P': 0.2	DCM P': 3.1	EA P': 4.4	MeOH P': 5.1
Green alga <i>U. lactuca</i>	Nb of repetitions	x 1	x 2	x 2	x 4
	Color	Pale yellow	Dark green	Dark green	Dark green
	Yield (%)	0.2	0.3	0.1	<u>12.1</u>
Brown alga <i>S. scoparium</i>	Nb of repetitions	x 2	x 3	x 3	x 3
	Color	Dark yellow	Dark green	Dark green	Green
	Yield (%)	0.2	0.2	0.5	<u>1.4</u>
Red alga <i>P. capillacea</i>	Nb of repetitions	x 2	x 3	x 3	x 4
	Color	Dark yellow	Dark green	Dark green	Dark green
	Yield (%)	0.4	0.8	0.9	<u>7.3</u>

P': Polarity index

CH: cyclohexane, DCM: dichloromethane, EA: ethyl acetate, MeOH: methanol.

3.2 Effect of extracts on planktonic growth – MIC determination

The antibacterial activity was evaluated in order to determine the appropriate concentration of the extracts (sub-MIC) to be used in the antibiofilm activity assays providing that they do not present classical antibacterial effects. Indeed, for all extracts, no antibacterial activity was demonstrated at the highest concentration tested (50.0 µg/mL). Therefore, the concentration adopted in the antibiofilm activity assays was 50.0 µg/mL.

3.3 Evaluation of the antibiofilm activity of extracts

3.3.1 Effect of extracts on *S. aureus* biofilm formation and growth (extract added at t_0)

The influence of extracts derived from the three tested seaweed on *S. aureus* biofilm formation and proliferation was evaluated by adding the extract at t_0 followed by adhered

biomass quantification after 24h of incubation (Figure 1).

Results showed that the antibiofilm activity exhibited by extracts originated from the green alga *U. lactuca* was the most promising. By comparing with the associated untreated control, a significant reduction (***, P -value < 0.001) of adhered cells number was recorded in the biofilm treated with CH extract derived from this green seaweed leading to 2.9 ± 0.7 log CFU/mL versus 5.3 ± 0.4 log CFU/mL in the corresponding untreated control (log reduction of 2.2 ± 0.7 log CFU/mL). A significant decrease in biofilm was also observed with DCM (**, P -value < 0.01) and EA (*, P -value < 0.05) extracts treatments leading to 1.8 ± 0.5 and 1.1 ± 0.4 of log reduction (log CFU/mL), respectively.

Concerning extracts derived from the brown alga *S. scoparium*, both CH and EA extracts showed a significant effect (**, P -value < 0.01) by reducing 1.4 ± 0.0 and 1.3 ± 0.2 log CFU/mL of the adhered biomass, respectively. However, EA extract was the only extract derived from the red alga *P. capillacea* to reveal a significant (*, P -value < 0.05) effect (log reduction of 1.0 ± 0.7 log CFU/mL).

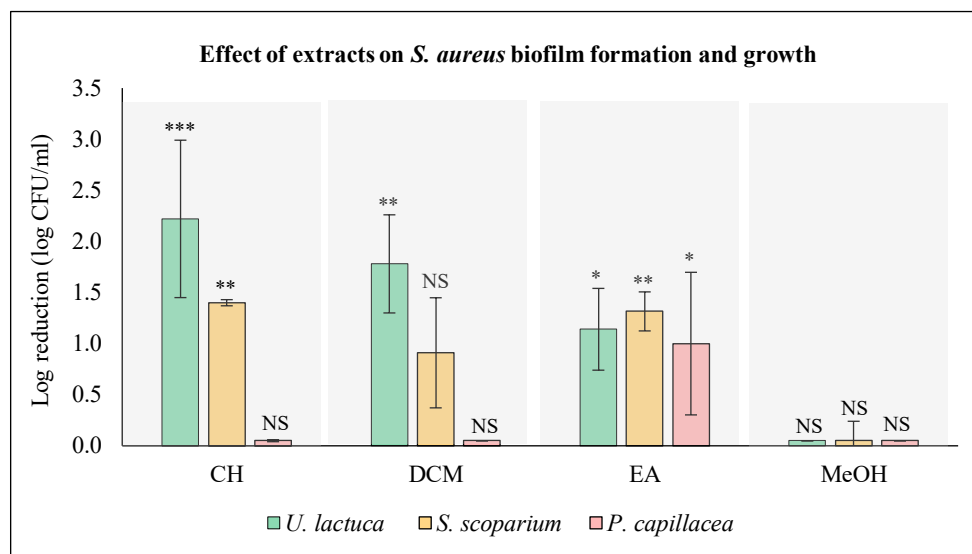


Figure 1: Effect of extracts (50.0 µg/mL) derived from the green alga *U. lactuca*, the brown alga *S. scoparium*, and the red alga *P. capillacea* on *S. aureus* biofilm formation and growth in BB medium. Extracts were added at t_0 . Results are expressed as means of log reduction in comparison with the related untreated control (log reduction (log CFU/mL) \pm SD) from three independent experiments. Statistically significant differences (***, P -value < 0.001, **, P -value < 0.01, *, P -value < 0.05) between log CFU/mL number in the extract treated biofilm and that in the appropriate untreated control are indicated. NS: not significant.

On the other hand, biofilms formed in presence of the most active extracts (***, P -value < 0.001 and **, P -value < 0.01) were visualized by epifluorescence microscopy (Figure 2). The captured images confirmed the impact of extracts on the number of adhered cells since the treated biofilms density was reduced compared to the control. In addition, a potential effect on the proteins matrix was recorded for DCM and CH extracts derived from the green and the brown alga, respectively.

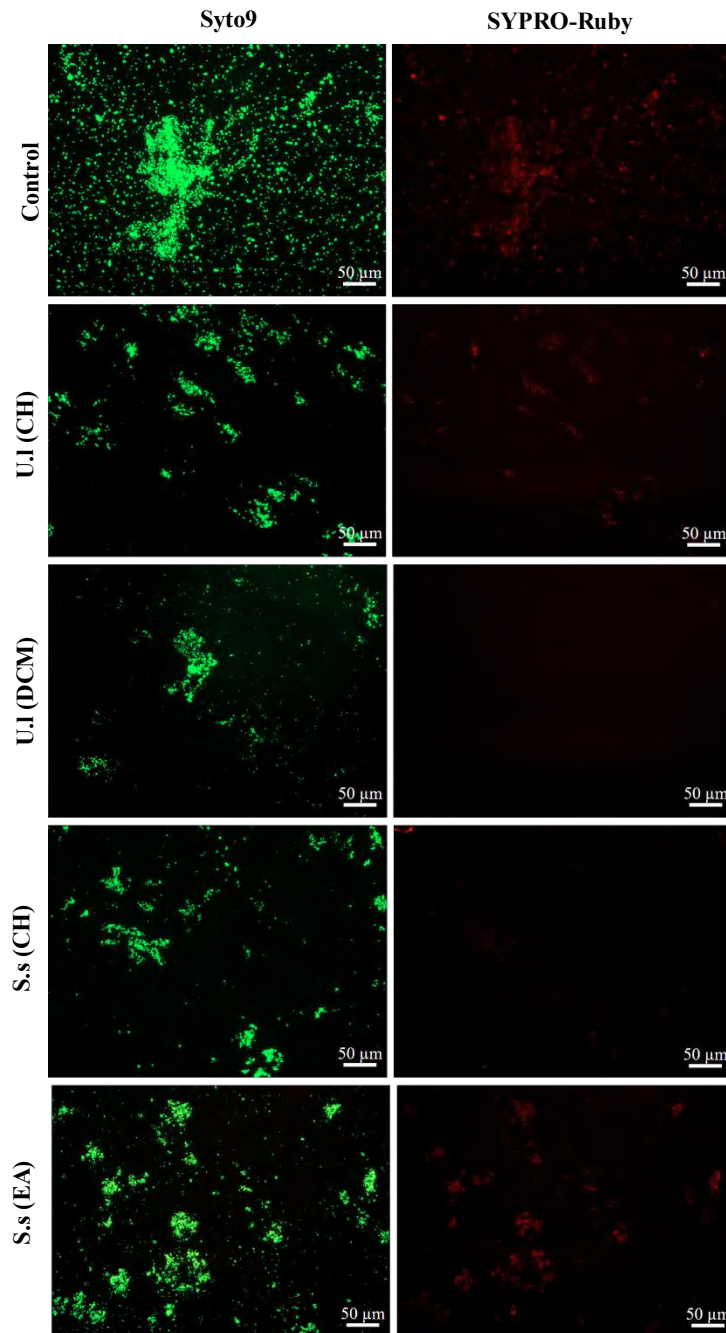


Figure 2: Epi-fluorescence microscopy images of *S. aureus* biofilms incubated in BB medium at 37°C for 24h without extract (control) or with the selected extracts (cyclohexane and dichloromethane extracts of the green alga *U. lactuca* and cyclohexane and ethyl acetate extracts derived from the brown alga *S. scoparium* at 50.0 µg/mL. Biofilms were stained with Syto9 for cells (green-fluorescent) and with SYPRO-Ruby for matrix proteins (red-fluorescent). U.l and S.s are *U. lactuca* and *S. scoparium* algae, respectively. (Magnification x 20).

3.3.2 Determination of biofilm development stage targeted by the selected extracts

With the aim of detecting biofilm formation stage affected by the selected extracts, *S. aureus* biofilm was treated at different time points followed by a quantification of the adhered cells number after overnight incubation. As results showed, the efficacy of the selected extracts decreased by retarding its addition with a total loss of this efficacy on 24h-preformed biofilm which suggests an influence on the early stages of biofilm formation (Figure 3). However, the significance of the antibiofilm activity (**, P -value < 0.001) of DCM extract derived from the green alga was maintained both when added at t_0 leading to 1.6 ± 0.2 log CFU/mL of log reduction (3.9 ± 0.6 log CFU/mL versus 5.5 ± 0.8 log CFU/mL in the related untreated control) and even when added on a 6h-preformed biofilm with a log reduction of 1.0 ± 0.3 log CFU/mL (4.9 ± 0.5 log CFU/mL versus 5.9 ± 0.6 log CFU/mL in the associated untreated control) (Figure 3B). It should be noted that results showed no relevant effect of all these selected extracts when added on *S. aureus* 24h-preformed biofilm.

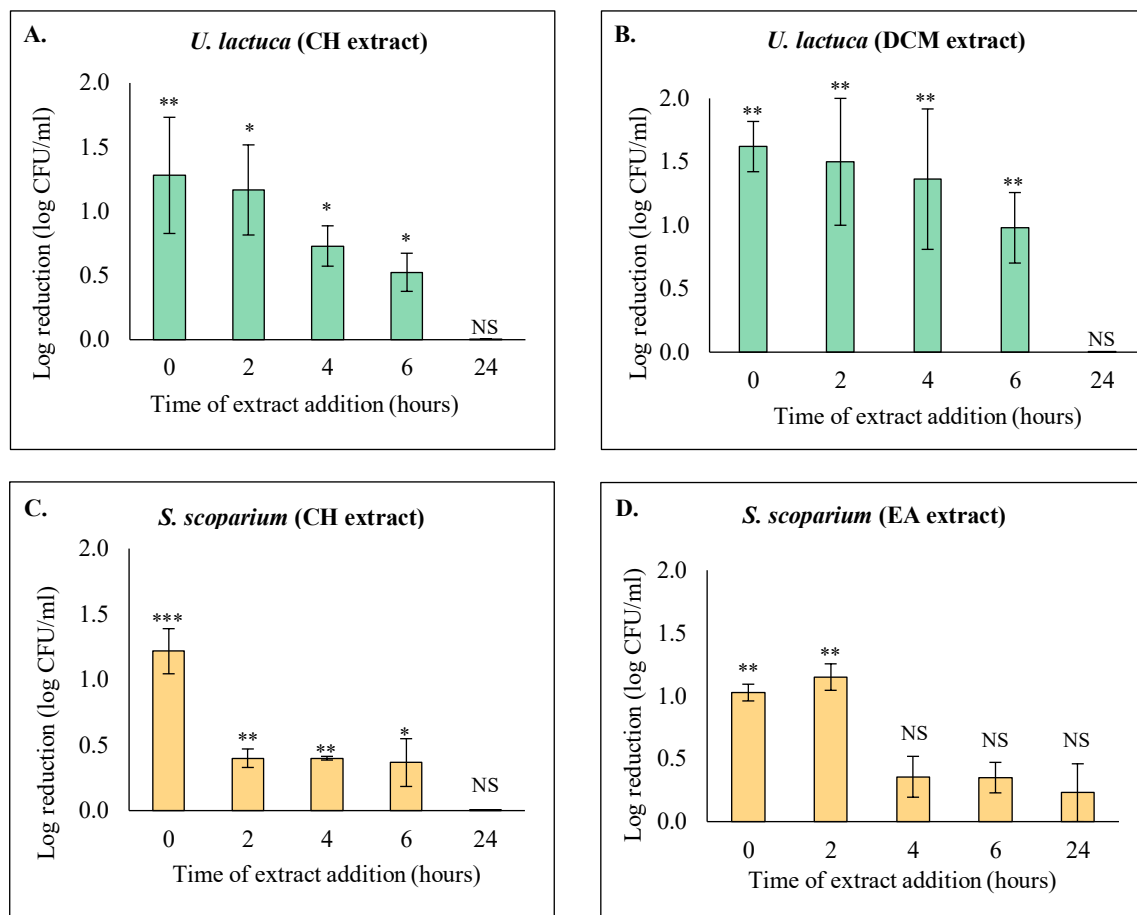



Figure 3: Effect of selected algal extracts (50.0 μ g/mL) on *S. aureus* biofilm in BB medium. Extracts were added at different time point (t_0 , t_{2h} , t_{4h} and t_{6h}). Results are expressed as means of the adhered cells number (log CFU/mL) \pm SD from three independent experiments. Statistically significant differences (***, P -value < 0.001, **, P -value < 0.01, *, P -value < 0.05) between log CFU/mL number with extract treated biofilm and that in the appropriate untreated control are indicated. NS: not significant.

3.4 Effect of the selected extracts on *S. aureus* hydrophobicity – contact angle measurement method

The potential impact of the selected extracts on *S. aureus* surface hydrophobicity was evaluated by measuring the contact angle of a drop of water deposited on a layer of previously treated bacteria (Table 3). Results showed that the DCM extract derived from the green alga *U. lactuca* was the most potent in reducing bacterial surface hydrophobicity (***, P -value < 0.001) ($\Theta^\circ = 57.9 \pm 8.1^\circ$ versus $94.2 \pm 3.8^\circ$ for the untreated control). A significant effect (**, P -value < 0.01) of CH extract derived from the same alga was also recorded ($\Theta^\circ = 85.6 \pm 0.9^\circ$).

Table 3: Effect of the selected extracts (50.0 $\mu\text{g/mL}$) on *S. aureus* surface hydrophobicity assessed by measuring the contact angle Θ° . Results are expressed as mean of Θ° determined at 5 random points per bacterial film ($\Theta^\circ \pm \text{SD}$). Statistically significant differences (***, P -value < 0.001, **, P -value < 0.01) between the extract treated bacterial layer and the untreated control one are indicated. NS: not significant.

Sample	Contact angle Θ°	Water droplet deposited on the bacterial layers
Control	$94.2 \pm 3.8^\circ$	
U.1 (CH)	$85.6 \pm 0.9^{\circ**}$	
U.1 (DCM)	<u>$57.9 \pm 8.1^{\circ***}$</u>	
S.s (CH)	$94.1 \pm 4.1^{\circ\text{NS}}$	
S.s (EA)	$90.8 \pm 6.3^{\circ\text{NS}}$	

4. DISCUSSION

Biofilm formation presents one of the strategies adopted by bacteria in order to overcome treatment with antimicrobial agents as well as to escape from host immune defenses (Uruen *et al.*, 2020). Indeed, besides the protection provided by the extracellular matrix against the penetration of antimicrobial agents, the heterogeneity within the biofilm represented by nutrient and oxygen gradients, lead to the formation of cells with different metabolic states, which promotes the resilience of this bacterial community (Campanac *et al.*, 2002; Preda & Sandulescu, 2019). Therefore, a great interest has been dedicated to the search for novel antibiofilm agents in an attempt to prevent and/or treat biofilm-related infections (Bjarnsholt *et al.*, 2013; Koo *et al.*, 2017). In this context, natural products are considered as a strong promises given their remarkable antibiofilm activity demonstrated against various pathogenic bacteria by exhibiting different mechanisms of action (Mishra *et al.*, 2020; Zhang *et al.*, 2020).

Interestingly, various studies have highlighted the ability of compounds isolated from marine organisms such as seaweed and sponges to present a valuable input in the search for new antibiofilm agents (Stowe *et al.*, 2011; Dahms & Dobretsov, 2017; Melander *et al.*, 2020). Indeed, these organisms living in the stressful conditions of the marine environment, possess sophisticated defense mechanisms that involve the natural synthesis of secondary metabolites in order to overcome any kind of undesirable attacks (predation, biofouling...) (Shannon & Abu-Ghannam, 2016).

So, the aim of this study is to explore the potential ability of extracts derived from three algae (the green *U. lactuca*, the brown *S. scoparium*, and the red *P. capillacea* seaweed) to control the biofilm formed by *S. aureus*, a common pathogen involved in hospital-acquired infections (Suresh *et al.*, 2019). Concerning the green alga *U. lactuca*, various studies have highlighted the significant bioactivity (antimicrobial, cytotoxic, antioxidant, insecticidal activities...) of its extracts (acetonic, methanolic, aqueous...) (Saeed *et al.*, 2019; Anjali *et al.*, 2019; Rima *et al.*, 2021). On the other hand, although the number of studies that have evaluated the potential bioactivity of the brown alga *S. scoparium* is limited, some biological proprieties such as antioxidant, antibacterial, anti-inflammatory as well as cytotoxic activities have been demonstrated for its extracts (Campos *et al.*, 2018; Guner *et al.*, 2019). Regarding the red alga *P. capillacea*, Ismail *et al.*, and Shobier *et al.*, have

respectively demonstrated the ability of its extracts to exhibit antioxidant, antidiabetic, and antifungal activities (Shobier *et al.*, 2016; Ismail *et al.*, 2020).

After preparation of the different extracts by successive maceration in four solvents (cyclohexane, dichloromethane, ethyl acetate, and methanol) with increasing polarity, their antibacterial activity against *S. aureus* was evaluated. As the objective of this study is to search for an antibiofilm effect rather than a classical antibacterial activity, this potential impact of extracts on *S. aureus* planktonic growth was investigated in order to ensure that the tested concentration in the antibiofilm assays is sub-MIC and therefore the obtained results will be restricted to an effect on the biofilm. Results showed that for all extracts, no visible antibacterial effect was recorded at the highest tested concentration (50.0 µg/mL).

This is in accordance with two previous studies conducted by Pushparaj *et al.*, and De Alencar *et al.*, which indicated the absence of an inhibitory effect on *S. aureus* bacterial growth of extracts derived from the green alga *U. lactuca* (acetonic, ethyl acetate, methanolic...extracts) and the red one *P. capillacea* (hexane and ethanolic extracts), respectively (Pushparaj *et al.*, 2014; De Alencar *et al.*, 2016). On the other hand, Dulger *et al.*, demonstrated the capacity of methanolic extract obtained from the brown alga *S. scoparium* to inhibit the growth of *S. aureus* but at much higher concentration (Dulger *et al.*, 2009). It should be noted that the selection of 50.0 µg/mL as the maximum concentration tested is intended to avoid solubility issues that may lead to the deposition of precipitates on the bottom of wells and thereby distorts results and this for both antibacterial and antibiofilm assays.

The evaluation of the potential ability of extracts to inhibit *S. aureus* biofilm formation and growth was first assessed by adding extract at t_0 . Results showed that CH and DCM extracts derived from the green alga *U. lactuca* as well as CH and EA extracts obtained from the brown alga *S. scoparium*, are the most promising in exhibiting a significant (***, P -value < 0.001; **, P -value < 0.01) antibiofilm activity (Figure 1). The epifluorescence microscopic analysis of *S. aureus* biofilm formed in presence of these four potential active extracts support their demonstrated ability to reduce the number of adhered cells associated for some extracts (DCM and CH extracts derived from the green alga *U. lactuca* and the brown alga *S. scoparium*, respectively) with a decrease in matrix proteins (Figure 2).

According to our knowledge, the brown alga *S. scoparium* has never been explored for their potential antibiofilm activity. Regarding the green alga *U. lactuca*, the study conducted by Yuvaraj & Arul, is the only one to evaluate the antibiofilm activity of this alga against *S. aureus* (Yuvaraj & Arul, 2014). In fact, its methanolic extract, prepared by a single maceration, was able to significantly reduce *S. aureus* biofilm biomass using the crystal violet (CV) staining method, commonly used in the quantification of total biofilm biomass by marking both adherent cells and matrix (Pantanella *et al.*, 2013). It should be noted that this method of biofilm quantification has been widely used in the exploration of natural products such as gallic acid and ellagic acid rhamnoside for their antibiofilm activity against *S. aureus* (Fontaine *et al.*, 2017; Liu *et al.*, 2017). However, the application of CV staining method is not suitable in our case due to the limited quantity of matrix produced by *S. aureus* (below the detection limit) in our culture conditions, especially in the low-nutritive medium used (Suppl. S1). Furthermore, Allkja *et al.*, proved that in comparison with CV staining method, the CFU counting assay followed in our study is more responsive in treatment experiments, making it the most suitable method to use in treatment efficacy testing due to the possible interference between the treatment and the dye (Allkja *et al.*, 2021).

In order to gain insight into their potential mechanism of action, the selected extracts were added at different times point (t_0 , t_{2h} , t_{4h} , t_{6h} and t_{24h}) during the development of *S. aureus* biofilm. Results showed a gradual biofilm reduction when extracts were added at t_0 , t_{2h} , t_{4h} , and t_{6h} (Figure 3). However, regarding the number of remaining cells after extract treatment, the 24h-old biofilm was completely resistant to the extracts which suggests their potential effect on the initial adhesion and proliferation stages. In this context, Xiang *et al.*, have demonstrated the ability of aloe-emodin, natural product derived from *Rheum officinale* plant, to interfere with the early stages of biofilm formation by progressively reducing *S. aureus* biofilm biomass (Xiang *et al.*, 2017). In fact, this antibiofilm activity restricted to the early stages of biofilm formation was explained by a reduction in matrix components production such as proteins and polysaccharide intercellular adhesin (PIA) that are involved in *S. aureus* attachment (Foster *et al.*, 2014; Arciola *et al.*, 2015).

Moreover, this mode of action has been previously reported for some antibiotics such as vancomycin and moxifloxacin whose efficacy has been observed only on *S. aureus* young biofilm (6h-old biofilm) and not on mature one (24-hour-old biofilm) (Bauer *et al.*, 2013). On the other hand, it is recognized that the hydrophobic proprieties of bacterial surfaces are strongly involved in the adhesion to biotic and abiotic surfaces, especially to medical devices made of hydrophobic materials such as silicone and stainless steel (Krasowska & Sigler, 2014). In *S. aureus*, the attachment to abiotic surfaces is often mediated by ionic and hydrophobic interactions through surface-anchored proteins such as Bap (biofilm associated protein) and autolysin, as well as by wall teichoic acid and lipoteichoic acid (Heilmann, 2011). Indeed, the prevalence of hydrophobic patches compared to hydrophilic ones on the surface of *S. aureus* was demonstrated in the study conducted by Forson *et al.*, in which the adhesion was favored on the hydrophobic surface (Forson *et al.*, 2020). In addition, Kouidhi *et al.*, have highlighted a correlation between the surface hydrophobicity of various *S. aureus* strains associated with dental caries and their adhesiveness on polystyrene plates (Kouidhi *et al.*, 2010). In this context, the potential effect of the selected extracts on *S. aureus* hydrophobicity was assessed in an attempt to elucidate their potential mechanism of action. To do that, the sessile drop technique which consists in measuring the contact angle of a water drop on a bacterial surface was adopted. Basically, the contact angle presents an indirect and proportional measure of the hydrophobicity as a higher contact angle indicates a greater surface hydrophobicity (Braga & Reggio, 1995).

The obtained results have revealed the high hydrophobicity of *S. aureus* cells ($\Theta = 94.2 \pm 3.8^\circ$) (Table 3). Interestingly, a significant reduction in the hydrophobicity of *S. aureus* cells treated either with CH (**, P -value < 0.01) or DCM (***, P -value < 0.001) extracts derived from the green alga was shown. Combined with the demonstrated ability of these two extracts to gradually reduce *S. aureus* biofilm when added at the early stages of formation (up to 6h) (Figure 3), their potential mechanism of action may be based on the inhibition of the initial adhesion (when added at t_0) by decreasing surface hydrophobicity and/or delay biofilm proliferation by altering cells/plate surface interactions. This mechanism of action has already been described for the brodimoprim, an antibacterial agent whose ability to reduce the adhesiveness of *S. aureus* to human epithelial buccal cells has been correlated with a decrease in bacterial surface hydrophobicity (Braga & Reggio,

1995). In addition, Allegrone *et al.*, have demonstrated the capacity of natural rhamnolipids and Triton™ – X100 (synthetic surfactant) to significantly reduce *S. aureus* surface hydrophobicity, as well as to inhibit its adhesion to a surfactant-precoated silicone surface (Allegrone *et al.*, 2021).

5. CONCLUSION

In the present study, the exploration of various extracts derived from three algae for their potential ability to present an antibiofilm activity against *S. aureus* permitted the selection of four extracts (CH and DCM extracts obtained from the green seaweed and CH and EA extracts derived from the brown one) as the most promising. Their significant antibiofilm effect was restricted to the early stages of biofilm formation. Regarding the potential antibiofilm mechanism of action exhibited by CH and DCM extracts originated from the green alga, a decrease in *S. aureus* surface hydrophobicity may explain in part their ability to hinder bacterial adhesion and/or to delay biofilm proliferation. In light of these encouraging results, further experiments are envisaged in an attempt to decipher the possible mechanism of action of the selected active extracts, particularly through molecular analysis. Furthermore, it will be interesting to analyze the chemical composition of the active extracts in an effort to isolate highly active molecules. Overall, the findings of this study pave the way for possible future applications of seaweed in the prevention of biofilms formation.

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CONFLICT OF INTERESTS

The authors declare they have no conflict of interest.

SUPPLEMENTARY MATERIALS

S1: Quantification of *S. aureus* biofilm by crystal violet staining method

In order to screen extracts derived from the three algae for their ability to inhibit *S. aureus* biofilm formation and growth, the crystal violet staining method, commonly used for the quantification of bacterial biofilms biomass (adhered cells + matrix), was first adopted. Briefly, after overnight incubation of the treated biofilms, they were washed twice with 2.0 mL of SDW to remove non-adherent planktonic cells. The plate was then air-dried for 1h. To stain the adhered biomass, 2.0 mL of an aqueous 1% CV solution was added to the wells and consecutively incubated for 15 min at room temperature. In order to remove the excess stain, wells were rinsed twice with 2.0 mL of SDW followed by drying for 30 min before quantification. 1.0 mL of ethanol was finally added to extract bound stain prior to the absorbance measurement (OD_{570nm}).

Results showed that the evaluation of the antibiofilm activity of extracts against *S. aureus* by this method is not feasible in our culture conditions due to the low quantity of biofilm biomass detected in the control wells ($OD_{570nm} = 0.06 \pm 0.03$). For this reason, CFU counts method was the only assay used in this study.

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GENERAL CONCLUSION

The increased worldwide interest in the exploration and cultivation of seaweed is owed to their richness in a wide variety of heterogeneous bioactive compounds that can exhibit multidimensional functionalities (Nakhate & van der Meer, 2021). In fact, algae are recognized by their high ability to produce different metabolites involving complex metabolic pathways largely distinct from those of terrestrial organisms. While some of these compounds are essential for their own growth, several molecules synthesized by seaweed are implicated in their ability to overcome the extreme marine environmental conditions (salinity, pollutants, temperature...) as well as to escape the biotic threats (predators, microbial infections...) faced in seawater (Leandro *et al.*, 2019; Bhowmick *et al.*, 2020). Interestingly, the benefits of seaweed are not restricted to one field but extend to cover different areas such as pharmaceutical, cosmetics, food industries, and even the agricultural field (Leandro *et al.*, 2019).

In this context, the present study investigates the possible valorization of three algae (green alga *U. lactuca*, brown alga *S. scoparium*, and red alga *P. capillacea*) collected from Tripoli-Lebanon in two distinct domains: agricultural and pharmaceutical fields.

I. Agricultural field – Evaluation of the insecticidal activity of extracts and pigments derived from the green alga *U. lactuca* against the fruit fly *D. melanogaster*

In order to evaluate the potential ability of the green alga *U. lactuca* to present a good alternative to synthetic pesticides, the insecticidal activity of extracts as well as of pigments (chlorophylls and carotenoids) derived from this seaweed was assessed against the fruit fly *D. melanogaster* using different complementary methods (application by spraying oranges, ingestion toxicity, and repellent activity), each of which providing a specific mode of exposure (Rima *et al.*, 2021). Interestingly, a significant insecticidal activity of chlorophylls (purified or present in extracts), potentially based on a transcutaneous insecticidal mode of action was demonstrated. To the best of our knowledge, this is the first study that highlight an insecticidal activity of these green pigments. On the other hand, the efficiency of the acetonic extract derived from the green alga *U. lactuca* proved by both spraying oranges and ingestion toxicity assays, reveals the richness of this alga in various bioactive compounds with several mechanisms of action.

Although for the moment no algae-based biopesticides are commercially available, various studies have underlined the interesting insecticidal activity exhibited by extracts and/or compounds derived from seaweed against different phytopathogenic insects (Zemolin *et al.*, 2014; González-Castro *et al.*, 2019; Elbrense & Gheda, 2021). Regarding the green alga *U. lactuca*, some studies have demonstrated its considerable larvicidal and/or insecticidal activity against certain insects such as *Aedes aegypti*, *Culex pipiens*, and *Spodoptera littoralis* without elucidating the implicated mechanism of action (Abbassy *et al.*, 2014; Ishwarya *et al.*, 2018). Indeed, the insecticidal activity of natural products can be attributed to a possible effect on insect nervous system such as the inhibition of acetylcholinesterase and/or the blockage of GABA-gated chloride channel resulting in a lack of neuromuscular coordination and in nervous hyper-excitation, respectively. On the other hand, a potential impact on vital mitochondrial activity may also occur (Singh Rattan, 2010).

1.1 Perspectives

Overall, the findings obtained in this part of the study emphasize the ability of the green alga *U. lactuca* to be a good candidate in the search for natural, eco-friendly, efficient, and affordable alternatives to currently used synthetic pesticides. In light of the encouraging results observed, further experiments are to be considered:

- In an attempt to identify the bioactive molecules responsible for the considerable insecticidal activity of the acetic extract derived from the green alga *U. lactuca*, the analysis of its chemical composition needs to be performed (GC-MS and LC-MS analysis) in order to define if specific molecules are involved in the activity. This approach may also involve the elucidation of mechanism of action by enzyme assay such as the acetylcholinesterase biochemical assay as well as by tests at cellular and genomic levels (Hematpoor *et al.*, 2017; Ruttanaphan *et al.*, 2020).
- The evaluation of the ovicidal and larvicidal activity of this active extract, its potential effect on *D. melanogaster* reproduction as well as its potential insecticidal activity against other pests will also be of relevance in order to extend the action spectrum of this alga.
- In the same way, the limit of the extract spectrum has to be defined to ensure its potential large use without deleterious environmental impact, especially on pollinating insects.

- It is essential to verify the absence of cytotoxic effect of this extract on human prior to moving towards applications and trials in the field.
- The risk of selecting resistant flies (or other susceptible insects) has also to be explored.
- It will be also interesting to assess the possible synergistic activity between the active extract and conventional insecticides in an attempt to reduce their consumption and therefore their adverse effects.

Regarding biopesticides currently on the market, the development of algal extracts or molecules originated from alga as insecticides needs a deep evaluation (field trials, development of a potential resistance, toxicological proprieties, synergistic activity...) which can be compared to those of *Bacillus thuringiensis*, the most used and studied bioinsecticide in the world (Tetreau *et al.*, 2013; ANSE, 2015).

At last, field tests are needed to validate the conditions of use and the real impact on environment, the wildlife and humans.

Finally, this part of the study conducted in the Applied Biotechnology Laboratory (LBA3B-ER032) – Lebanese University – Tripoli – Lebanon provides a very interesting development perspectives, including on the economic level.

II. Pharmaceutical field – Evaluation of the antibiofilm activity of extracts derived from three algae (green alga *U. lactuca*, brown alga *S. scoparium*, and red alga *P. capillacea*) against *P. aeruginosa* and *S. aureus*

In the second part of this study, various extracts derived from the three tested algae were evaluated for their potential ability to inhibit biofilm formation and/or to eradicate a preformed one. Indeed, this exploration is based on the fact that the first molecule identified as having an inhibitory activity on QS system and therefore on biofilm formation is the halogenated furanone isolated from the red alga *Delisea pulchra* (Manefield *et al.*, 1999; Ren *et al.*, 2001). In addition, seaweeds are recognized for their capacity to synthesize various metabolites in order to withstand the high risk of being colonized by bacterial biofilms (Dahms & Dobretsov, 2017). Therefore, in this study we have focused on the search for novel antibiofilm agents against two critical pathogenic bacteria: The Gram-negative *P. aeruginosa* and the Gram-positive *S. aureus*. It should be noted that biofilms culture was conducted in low-nutritive media which were MBB (minimum biofilm broth) and BB (biofilm broth) for *P. aeruginosa* and *S. aureus*, respectively, and this in order to

create stressful conditions promoting biofilm formation rather than planktonic growth (Campanac *et al.*, 2002; Khalilzadeh *et al.*, 2010).

II.1 CH and EA extracts derived from the green alga U. lactuca: two extracts with promising antibiofilm activity against P. aeruginosa exhibiting two distinct mechanisms of action

The evaluation of the antibiofilm activity of extracts against *P. aeruginosa* revealed the ability of the green alga *U. lactuca*, especially CH and EA extracts, to offer a promising source of effective antibiofilm agents. Interestingly, two distinct antibiofilm modes of action have been observed for these two active extracts. On the one hand, CH extract was able to reduce the number of adherent cells resulting in an unstructured biofilm formed of separated bacterial aggregates with associated matrix. On the other hand, both antibiofilm assays and epifluorescence microscopic analysis revealed a potential effect of EA extract on the EPS matrix of *P. aeruginosa* biofilm leading to inhibition of its production and/or to its degradation. This hypothesis was supported by the detection of a synergistic antibiofilm activity between EA extract and tobramycin, a polycationic antibiotic which is highly retained by the EPS matrix, thus limiting its diffusion through the biofilm.

Considering the implication of QS communication system in formation, maintenance, and resilience of *P. aeruginosa* biofilm (Moradali *et al.*, 2017) and in an attempt to go further in deciphering the possible antibiofilm mode of action of the active extracts, the potential ability of seaweed extracts to interfere with AHL-dependent QS system, the most common QS system found in Gram-negative bacteria, was assessed by biosensor-based assay. Regarding the two extracts (CH and EA extracts derived from the green alga *U. lactuca*) showing an interesting antibiofilm activity against *P. aeruginosa*, they were able to significantly (***, P -value < 0.001) hinder the detection and the response to both the short-chain AHLs (C₆-HSL) and the long-chain AHLs (oxo-C₁₀-HSL). However, the anti-QS activity exhibited by some extracts such as MeOH extracts derived from both the green and the brown alga that did not show an antibiofilm activity makes us reconsider the involvement of this system in the antibiofilm activity of the active extracts. In fact, given the complexity of QS circuit as well as the myriad of functions controlled by this communication system (virulence factors production, motility...) (Moradali *et al.*, 2017; Pena *et al.*, 2019), the demonstration of a direct link between the detected antibiofilm effect and an anti-QS activity needs further experiments.

II.2 The green alga *U. lactuca* and the brown alga *S. scoparium*: promising gateway in the search for novel antibiofilm agents against *S. aureus*

The evaluation of the antibiofilm activity of extracts against *S. aureus* revealed the ability of four extracts (CH and DCM extracts derived from the green alga *U. lactuca* and CH and EA extracts derived from the brown alga *S. scoparium*) to inhibit the initial adhesion and/or to delay biofilm proliferation. In fact, regarding CH and DCM extracts derived from the green alga, their demonstrated antibiofilm activity which was restricted to the early stages of biofilm development can be attributed in part to a significant reduction (**, P -value < 0.01; ***, P -value < 0.001, respectively) in the hydrophobicity of *S. aureus* cells, a trait that is strongly involved in the adhesion (Kouidhi *et al.*, 2010; Krasowska & Sigler, 2014). In fact, this hydrophobicity reduction may be due to direct interaction of some extract components with the extra-cellular part of *S. aureus* but also to modification in the cell wall composition. Nevertheless, further experiments, particularly through molecular analysis, are required in an attempt to accurately decipher the targets of these selected active extracts which can drive their potential use.

Following the evaluation of the antibiofilm activity of extracts against the two pathogenic bacteria *P. aeruginosa* and *S. aureus*, several extracts have exhibited a significant effect by presenting different mechanisms of action. Interestingly, while some extracts such as CH and DCM extracts derived from the brown and the green alga, respectively, have exhibited a significant effect only against *S. aureus*, the antibiofilm activity of CH extract derived from the green alga *U. lactuca* was recorded against both bacteria, which suggests the richness of these algae in bioactive compounds with a broad spectrum of action. In an attempt to identify the bioactive molecules responsible for the demonstrated antibiofilm effect of the active extracts, an analysis of the chemical composition was initiated by GC-MS first and then by LC-MS (ongoing). For the moment, the identified molecules do not permit us to predict the active compound(s) since most of them were also detected in the non-active extracts, such as the extracts derived from the red alga.

II.3 Perspectives

Overall, these findings present a promising gateway in the search for novel and effective antibiofilm agents and emphasize the suitability of seaweed to be valorized in this field as preventive and/or curative agent. In light of the encouraging results observed and in order to go further in this study towards real applications, additional experiments are to be considered:

– *Molecular biology analyses*

Concerning EA extract which potentially alters the protective matrix of *P. aeruginosa*, it will be interesting to confirm this hypothesis and to evaluate the potential effect of this extract on the expression of genes that code for *P. aeruginosa* matrix (alginate operon, *pslA*, *pelA*...) by RT-qPCR. It will be also interesting to examine the potential impact of the two selected extracts (CH and EA extracts derived from the green alga *U. lactuca*) on the expression of *P. aeruginosa* genes (DNA microarray) including those involved in QS system (potential variation in genomic expression of QS inducers or of some factors under the regulation of QS) in an attempt to accurately decipher their mechanism of action.

In addition, the application of this technique will also be of great interest in the exploration of the genes targeted by the extracts that have exhibited a significant antibiofilm activity against *S. aureus*. It should be noted that the mechanism of action exhibited by CH extract derived from the green alga *U. lactuca* which showed a significant antibiofilm activity against both *P. aeruginosa* and *S. aureus* cannot be limited to a simple effect on QS system given the difference of this communication system between Gram-negative and Gram-positive bacteria.

– *Chemical analyses*

In order to isolate highly active molecules, it will be necessary to go further in the analysis of the chemical composition by combining it with a bio-guided fractionation. However, the isolation and the identification of an active molecule remains a challenging step due to the wide variety of compounds present in the extract. Moreover, the activity of an extract may come from a mixture of compounds rather than from a single active molecule.

– *Additional experiments*

- Effect on *P. aeruginosa*: In order to evaluate the potential implication of QS system in the mechanism of action exhibited by the two active extracts, it will be interesting to check as previously done (Khalilzadeh *et al.*, 2010), their possible competition with natural *P. aeruginosa* HSL (or other QS inducers as PQS). In addition, it will be also interesting to evaluate the synergistic antibiofilm activity between the two selected active extracts and cleaning and/or antimicrobial agents i.e. detergents, disinfectants or antibiotics in an attempt to improve their efficiency and reduce their consumption.
- Effect on *S. aureus*: To gain insight into the antibiofilm activity of some extracts that was restricted to the early stages of *S. aureus* biofilm formation as well as to evaluate their potential ability to inhibit the initial adhesion and/or to stimulate cells detachment, it will be interesting to conduct an experiment dedicated to this step in a flow cell by adding extract at different times point (de la Fuente-Nunez *et al.*, 2012; Soumbo, 2019). In the same way, other materials could be evaluated as surface for adhesion.

On the other hand, we have currently developed an approach to select the conditions to obtain multi-species biofilm, especially regarding *P. aeruginosa* and *S. aureus*. This combination is of particular interest for various approaches:

- For prevention or treatment of deleterious biofilms formed in industries
In this way, it will be interesting to demonstrate if some extracts (or molecules) express a ubiquitous antibiofilm activity (Gram-negative and Gram-positive bacteria). This type of product is considered under BPR (Biocidal Product Regulation) and its development needs further experiments regarding toxicological and ecotoxicological assays as the exploration of acquired resistance risk.
- For prevention or treatment of deleterious biofilms formed *in vivo*, during lung colonization/infection in cystic fibrosis patients
In this way, the antibiofilm activity of extracts can be evaluated using the proposed dual-species model. This pharmaceutical approach may be dedicated to the development of active molecule(s) (alone or in combination with current antibiotic treatments).

In conclusion, this study presents new insight into the exploration of seaweed as a valuable source of bioactive compounds that can be valorized in the agricultural area as an alternative to chemical insecticides as well as in the industrial/pharmaceutical field as a promising source of antibiofilm agents. It should be noted that in view of the implication of bacterial biofilms (*Pseudomonas* species) in plant infections, the antibiofilm activity of these algae can also fall within the scope of biopesticides.

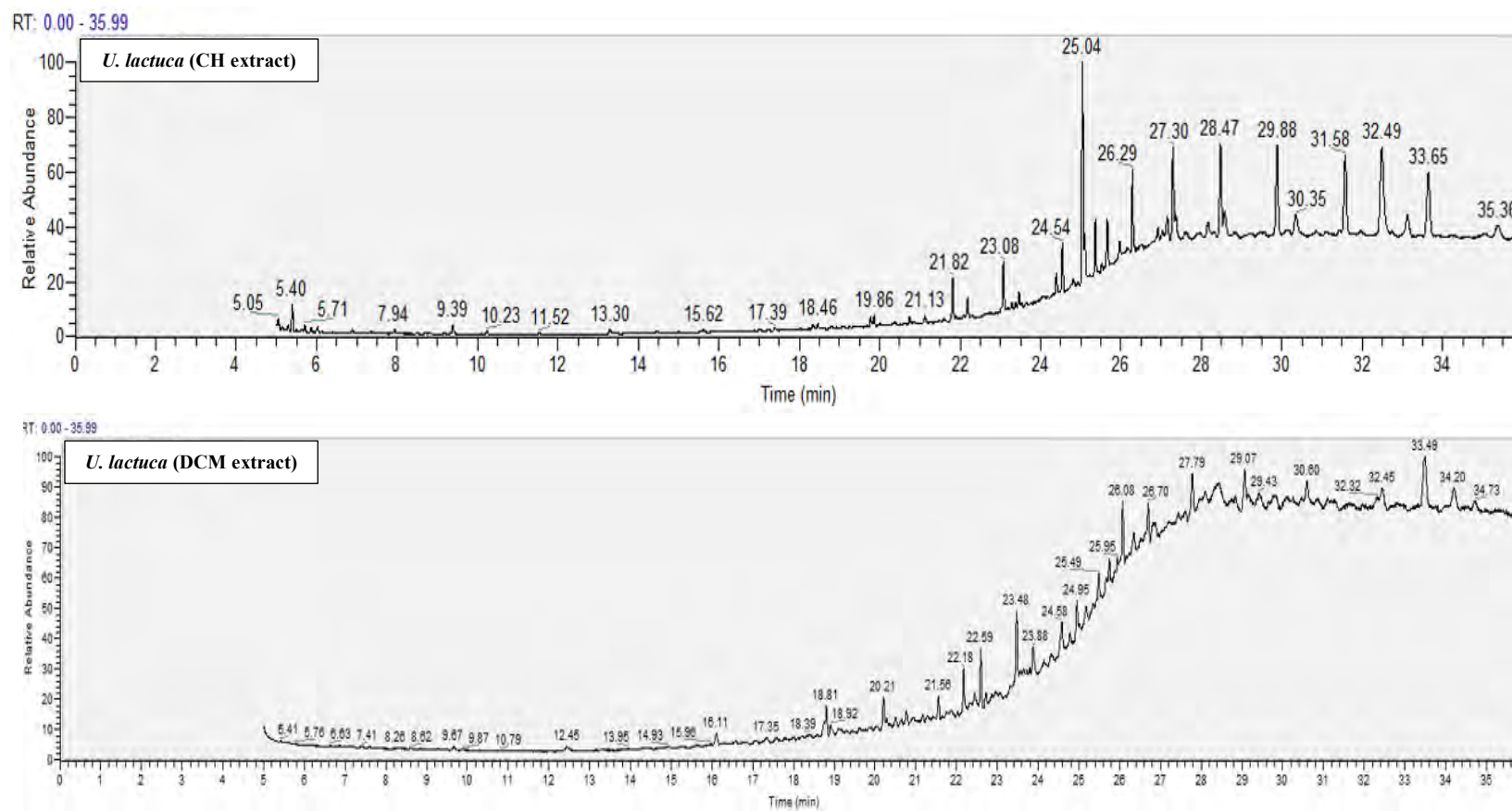
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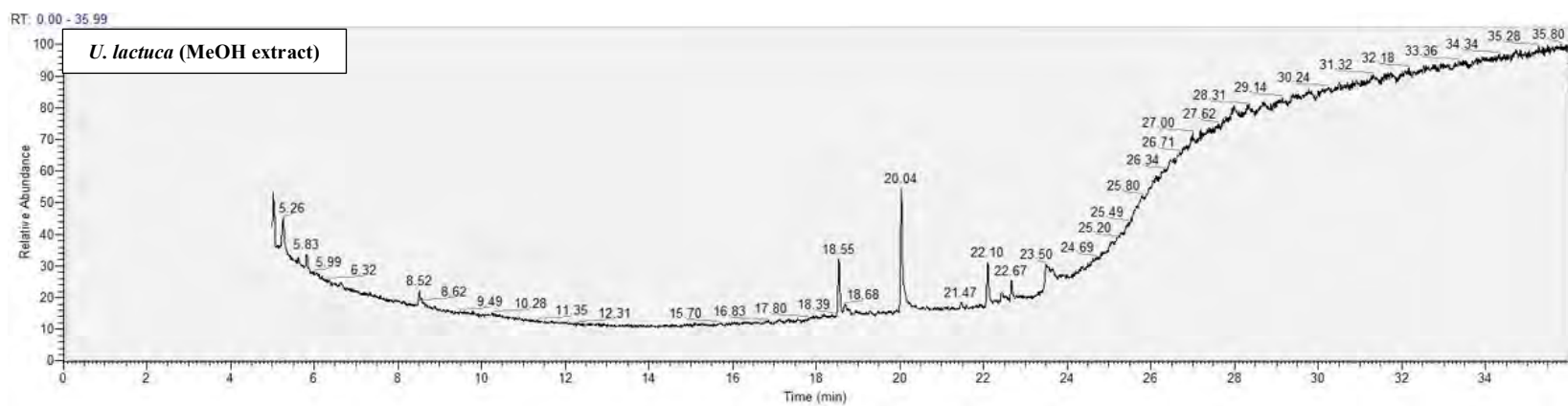
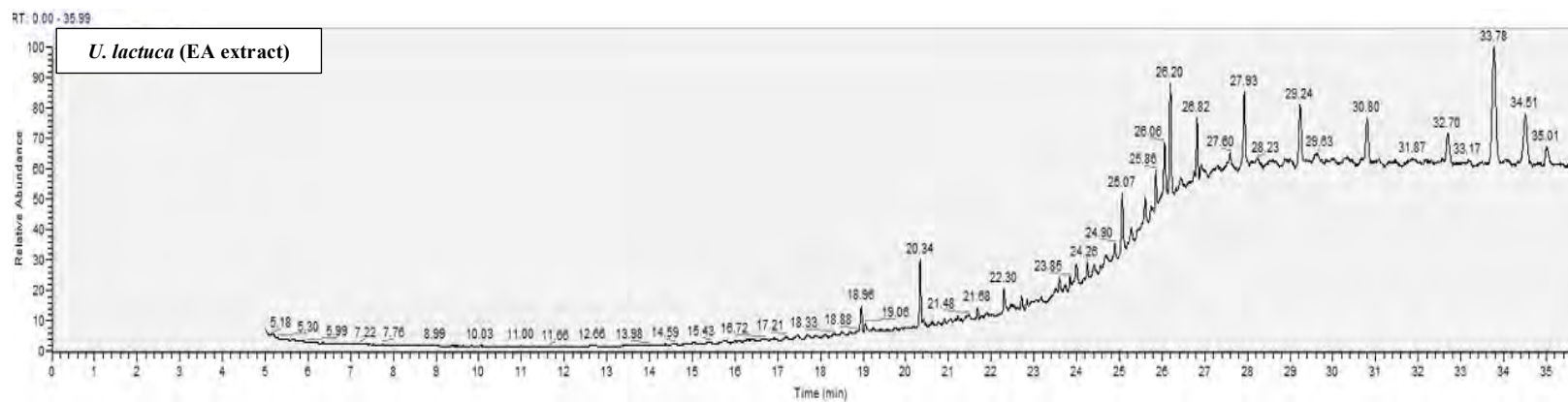
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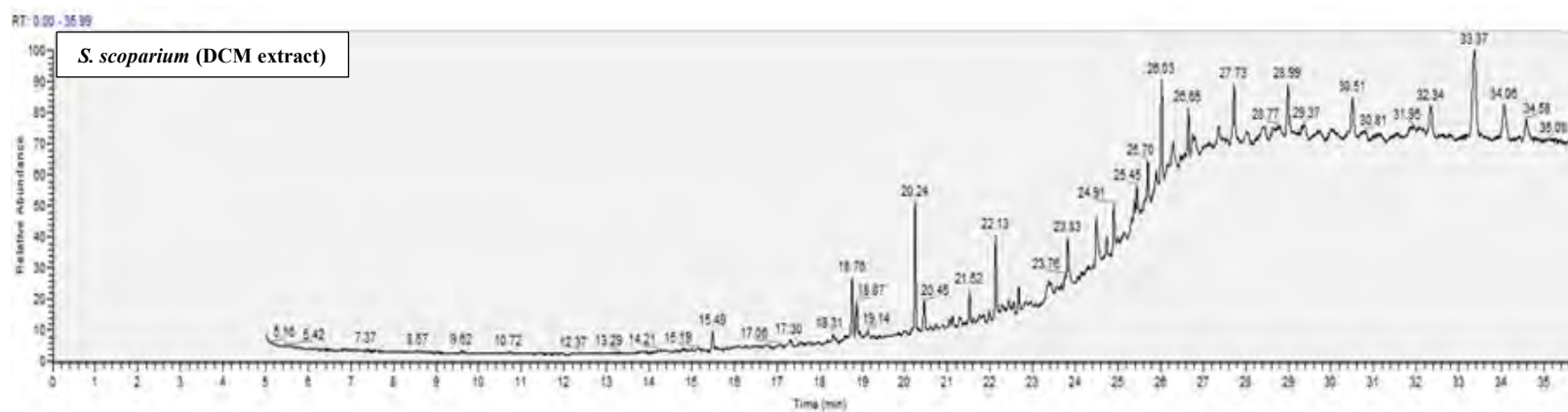
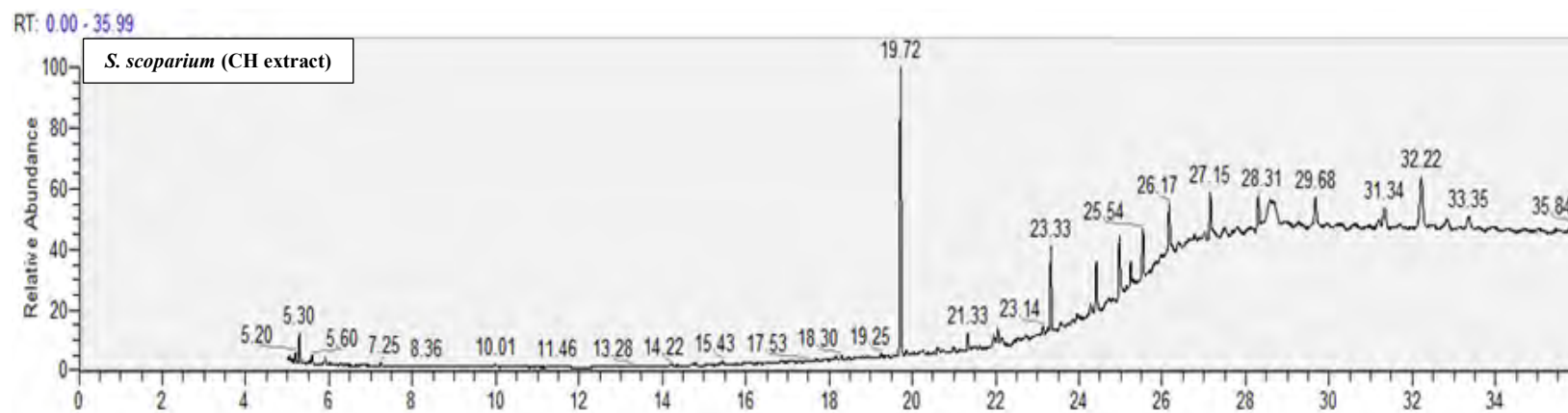
CHROMATOGRAMS OF SEAWEED EXTRACTS – GC/MS

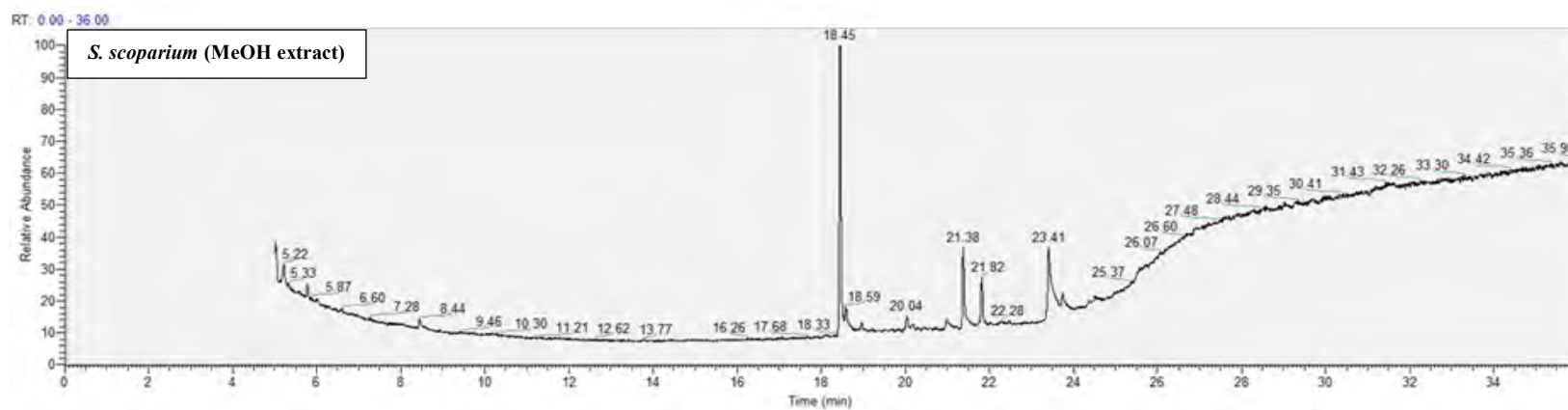
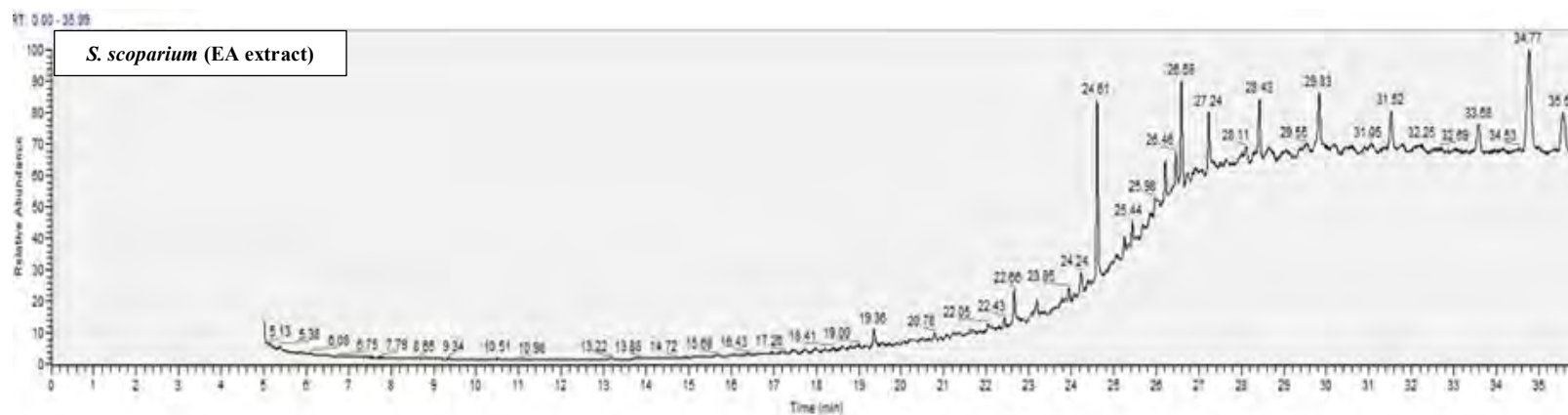
1. Green alga *U. lactuca*



Annex 1: Chromatograms of extracts derived from the green alga *U. lactuca*.

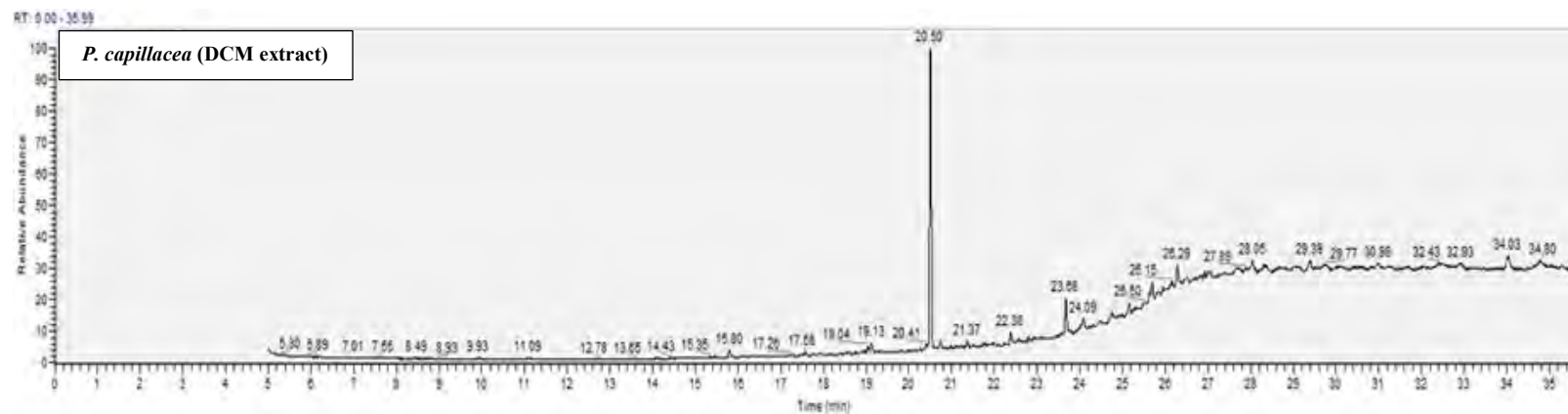
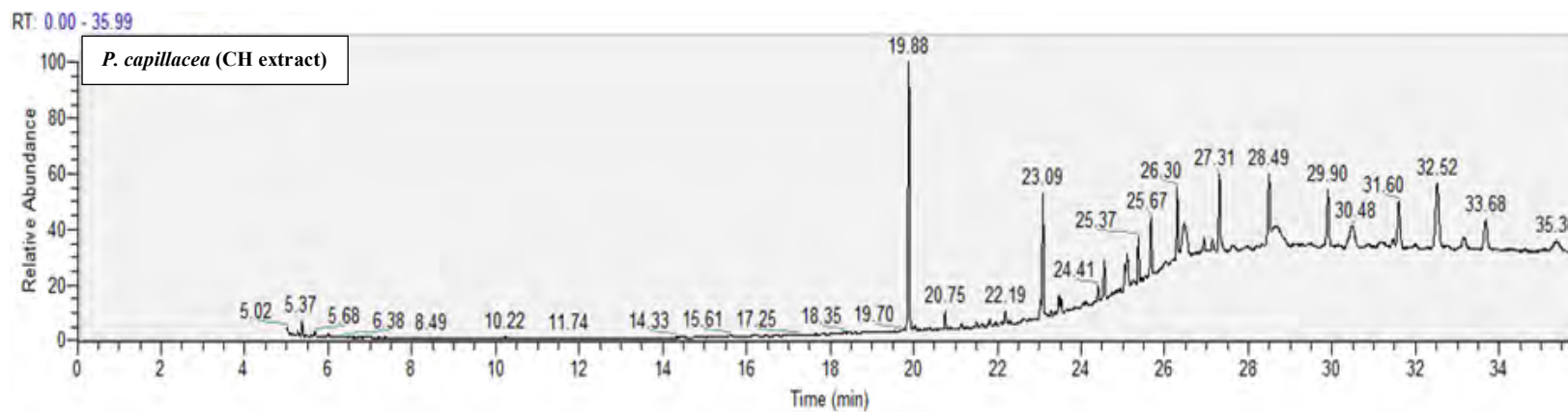
2. Brown alga *S. scoparium*

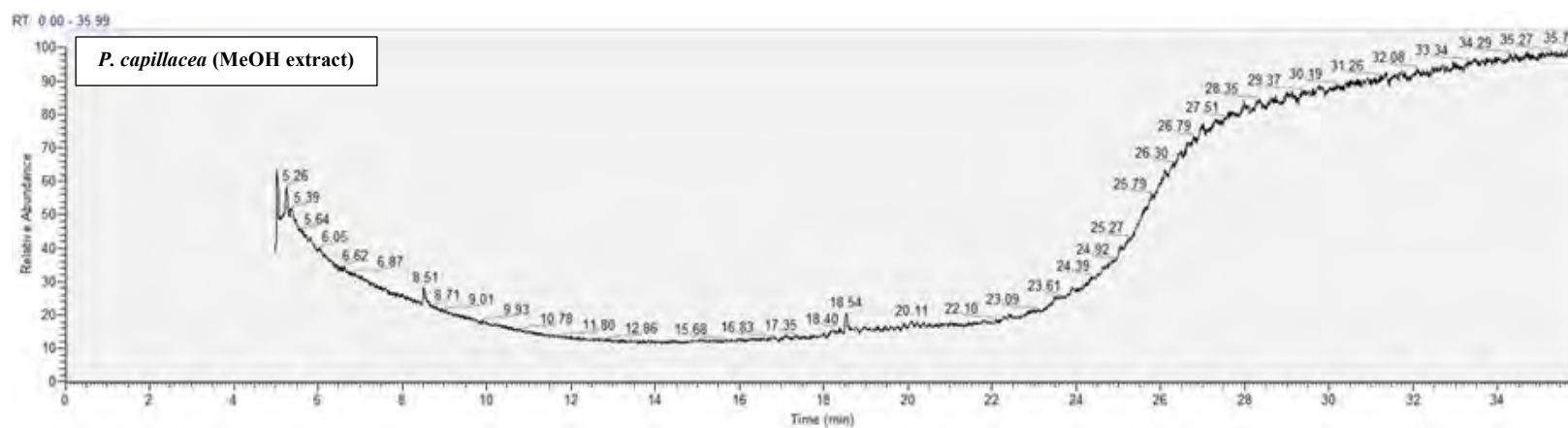
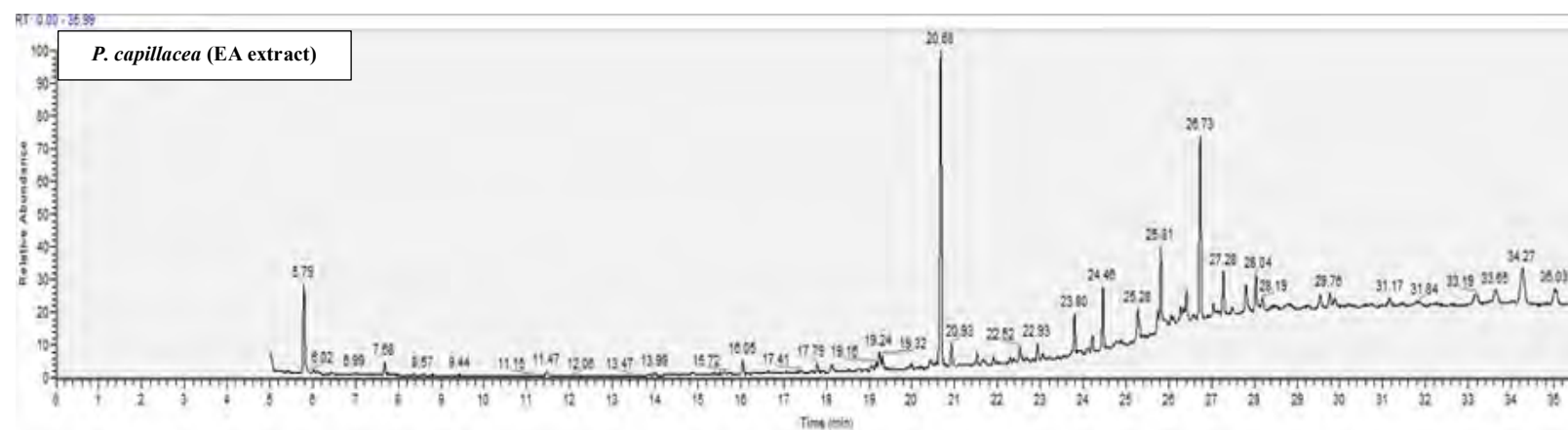




Annex 2: Chromatograms of extracts derived from the brown alga *S. scoparium*.

3. Red alga *P. capillacea*





Annex 3: Chromatograms of extracts derived from the red alga *P. capillacea*.

