Analysis of the bioactive compounds of seagrasses and mangroves: composition, identification of compounds and their role in biofilm inhibition

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Die voranzustellende ausführliche Darstellung ist in dieser Arbeit aufgeteilt in die Kapitel 1 und 6.

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Publikation (Kapitel 2)

Glasenapp, Y., Korth, I., Nguyen, X.-V., Papenbrock, J. (2019): Sustainable use of mangroves as sources of valuable medicinal compounds: Species identification, propagation and secondary metabolite composition. South African Journal of Botany, 121: 317–328. doi.org/10.1016/j.sajb.2018.11.020

Die Idee für die Experimente stammt von J. Papenbrock und Y. Glasenapp. Die Experimente mit den Pflanzen sowie die Analyse der Inhaltsstoffe wurden von Y. Glasenapp durchgeführt. Informationen zu den optimalen Wachstumsbedingungen der Mangroven stammen von Y. Leye, ebenso wie die Daten zur Vermehrung. DNA Isolation der Gewächshauspflanzen und ein Teil der Datenanalyse wurde von I. Korth ausgeführt. Die Elementanalyse wurde von I. Rupp-Schröder durchgeführt. Die Auswertung aller Daten sowie das Erstellen von Graphen und Tabellen wurde von Y. Glasenapp, und im Fall der Stammbaumanalyse, von X-V. Nguyen unternommen. Der Methodenteil der DNA Analyse und Datenauswertung wurde von X-V. Nguyen, das gesamte weitere Manuskript von Y. Glasenapp und J. Papenbrock geschrieben.

Manuskript in Vorbereitung (Kapitel 3)

Glasenapp, Y., Papenbrock, J. Effect of tea varieties and the flavonoid taxifolin on *Escherichia coli* colony growth and on the formation of extracellular polymeric substances. (In preparation)

Die Idee für die Experimente stammt von J. Papenbrock und Y. Glasenapp. Die Experimente mit den Agarplatten wurden von Y. Glasenapp mit technischer Unterstützung von P. v. Trzebiatowski durchgeführt. Die Auswertung aller Daten sowie das Erstellen von Graphen und Tabellen wurde von Y. Glasenapp, und im Fall der Bildauswertung, mit Unterstützung von P. v. Trzebiatowski unternommen. Das gesamte Manuskript wurde von Y. Glasenapp geschrieben, mit hilfreichen Hinweisen und Verbesserungen von J. Papenbrock.

Publikation (Kapitel 4)

De Vincenti, L., Glasenapp, Y., Cattò, C., Villa, F., Cappitelli, F. (2018): Hindering the formation and promoting the dispersion of medical biofilms: non-lethal effects of seagrass extracts. BMC Complementary and Alternative Medicine, 18: 168. doi.org/10.1186/s12906-018-2232-7

Die Idee für die Experimente stammt von J. Papenbrock und F. Cappitelli. Extrakte aus Seegrasmaterial wurden von Y. Glasenapp und C. Cattò hergestellt. Mikrobiologische Tests wurden von L. de Vincenti und C. Cattò durchgeführt. Die Bestimmung der gesamten Flavonoid- und Phenolgehalte, ORAC sowie LC-MS-Analysen wurden von Y. Glasenapp durchgeführt. Die Auswertung der Daten sowie das Erstellen von Graphen und Tabellen wurde von Y. Glasenapp, L. de Vincenti, C. Cattò und F. Villa unternommen. Der Methoden- und Ergebnisteil zur Inhaltsstoffanalyse wurde von Y. Glasenapp geschrieben, das restliche Manuskript von L. de Vincenti und F. Villa mit intensiver Korrektur aller Autorinnen.

Publikation (Kapitel 5)

Glasenapp, Y., Lucas, C., Wöltje, T., Fohrer, J., Papenbrock, J. (2019): Anti-adhesion activity of tannins isolated from the mangrove *Laguncularia racemosa*. Chemistry & Biodiversity 16: e1800632. doi.org/10.1002/cbdv.201800632

Die Idee für die Experimente stammt von J. Papenbrock und Y. Glasenapp. Extrakte und Fraktionen aus Mangrovenmaterial wurden von Y. Glasenapp hergestellt. Mikrobiologische Tests wurden von Y. Glasenapp durchgeführt, mit technischer Unterstützung von P. v. Trzebiatowski. Die Analysen der Inhaltsstoffe mittels LC-MS wurden vor allem von Y. Glasenapp und teilweise von C. Lucas durchgeführt. Die NMR Experimente und deren Auswertung wurden von J. Fohrer ausgeführt. Die Auswertung aller Daten sowie das Erstellen von Graphen und Tabellen wurde von Y. Glasenapp unternommen. Das gesamte Manuskript wurde von Y. Glasenapp geschrieben, mit hilfreichen Hinweisen und Verbesserungen von J. Papenbrock.

Summary

In this work, plant extracts and compounds as a source of biofilm inhibiting substances were analyzed, with a focus on seagrasses and mangroves. To have access to fresh plant material, and to limit plant collection in the wild, mangrove cultivation in the greenhouse was studied. Good growth and successful propagation of *Avicennia germinans* and *Laguncularia racemosa* was achieved. *Bruguiera cylindrica* was growing very slowly and could not be propagated. The composition of secondary metabolites present in greenhouse grown *A. germinans* was comparable to plants collected outdoors in Guatemala. The internal transcribed spacer (ITS) as a genetic marker was shown to be a useful tool in the clear species identification of mangroves.

Different microbial biofilm assays were carried out to study biofilm inhibitory actions of plant extracts. In a biofilm assay specific for *Escherichia coli* macrocolony growth and extracellular polymeric substance (EPS) production, different tea varieties and one flavonoid were screened in a first approach. Green tea and hawthorn tea as well as the flavonoid taxifolin showed good inhibitory activities. Three seagrass species, namely *Enhalus acoroides*, *Halophila ovalis* and *Halodule pinifolia* were tested in different biofilm assays on *E. coli* and *Candida albicans*. *E. acoroides* showed to be a promising source of biofilm inhibiting compounds, which are also able to induce cell dispersion from *C. albicans* biofilms.

In preliminary experiments with mangrove extracts on biofilm inhibition, extracts of *L. racemosa* were most effective. Crude extracts of *L. racemosa* were able to reduce biofilm formation of *E. coli*, *C. albicans* and *Candida glabrata* in microtiter-based assays. To identify the active compounds, fractions of the crude extract enriched for phenolic compounds were tested. Here, two fractions inhibited *C. albicans* biofilm adhesion to 51 and 57%, respectively, compared to the positive control. The substances in the fractions were identified as ellagitannins and one gallotannin by liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR) spectroscopy. Obtained mass-spectrometric fragmentation patterns were compared to databases and the literature. According to this study, mangroves and seagrasses can be considered as a source of biofilm inhibiting compounds.

Keywords: biofilm**,** mangroves, natural products, phylogeny, seagrasses, secondary metabolites, tannins

Zusammenfassung

In dieser Arbeit wurden Pflanzenextrakte und -substanzen als eine Quelle von Biofilm inhibierenden Substanzen analysiert, mit einem Fokus auf Seegräser und Mangroven. Für einen Zugang zu frischem Pflanzenmaterial, und um die Entnahme aus Wildbeständen zu limitieren, wurde die Mangrovenanzucht im Gewächshaus etabliert. Ein gutes Wachstum und erfolgreiche Vermehrung von *Avicennia germinans* und *Laguncularia racemosa* wurden erreicht. *Bruguiera cylindrica* wuchs langsam und konnte nicht vermehrt werden. Die Zusammensetzung von Sekundärmetaboliten in *A. germinans* Gewächshauspflanzen war vergleichbar zu natürlich vorkommenden Pflanzen aus Guatemala. Die interne transkribierte Spacer- Sequenz (ITS) wurde als Werkzeug für eine eindeutige Artenbestimmung von Mangroven verwendet.

Verschiedene mikrobielle Testsysteme wurden zum Studieren von biofilminhibierenden Aktivitäten von Pflanzenextrakten eingesetzt. In einem Biofilmtest spezifisch für *Escherichia coli* Makrokoloniewachstum und die Produktion von extrazellulären polymerischen Substanzen (EPS) wurden mehrere Teesorten und ein Flavonoid in einem Screening getestet. Grüner und Weißdorntee und das Flavonoid Taxifolin zeigten gute Inhibition. Drei Seegrasarten, *Enhalus acoroides*, *Halophila ovalis* und *Halodule pinifolia*, wurden in diversen Biofilmtests an *E. coli* und *Candida albicans* getestet. *E. acoroides* hat sich als vielversprechende Quelle von biofilminhibierenden Substanzen herausgestellt, welche auch die Dispersion von *C. albicans* Biofilmzellen induzieren.

In ersten Experimenten mit Mangrovenextrakten hat sich *L. racemosa* als die effektivste Art zur Biofilminhibierung herausgestellt. Rohextrakte reduzierten die Biofilmbildung von *E. coli*, *C. albicans* und *Candida glabrata* in Mikrotitertests. Um die aktiven Substanzen zu identifizieren, wurden Fraktionen des für phenolische Substanzen angereicherten Rohextraktes getestet. Zwei Fraktionen inhibierten die Biofilmadhäsion von *C. albicans* um 51 und 57% im Vergleich zur Positivkontrolle. Die in den Fraktionen enthaltenen Substanzen wurden als Ellagitannine und ein Gallotannin durch Flüssigchromatographie-Massenspektrometrie (LC-MS) und Kernspinresonanz (NMR) identifiziert. Aufgenommene massenspektrometrische Fragmentierungsmuster wurden mit Datenbanken und der Literatur abgeglichen. Nach den Erkenntnissen dieser Arbeit können Mangroven und Seegräser als eine Quelle von biofilminhibierenden Substanzen dienen.

Schlüsselwörter: Biofilm**,** Mangroven, Naturprodukte, Phylogenie, Seegräser, Sekundärmetaboliten, Tannine

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List of abbreviations

AB	Autoinducer bioassay
AHL	*N*-acyl homoserine lactone
AI-2	Autoinducer 2
ANOVA	Analysis of variance
CE	Catechin equivalent
CDC	Center for disease control
COSY	Correlation spectroscopy
DAPI	4',6-diamidino-2-phenylindole
DM	Dry mass
EPS	Extracellular polymeric substances
EGCG	Epigallocatechin gallate
FB28	Fluorescent brightener 28
GAE	Gallic acid equivalent
HHDP	Hexahydroxydiphenoyl
HMBC	Heteronuclear multiple bond correlation
HPLC	High performance liquid chromatography
HSD	Honestly significant difference test
HSQC	Heteronuclear single quantum coherence
IDA	Information dependent acquisition
ITS	Internal transcribed spacer
LB	Luria Bertani broth
LC-MS	Liquid chromatography mass spectrometry
MeOH	Methanol
NaCl	Sodium chloride
NMR	Nuclear magnetic resonance
NOESY	Nuclear Overhauser effect spectroscopy
OD	Optical density
ORAC	Optical radical absorbance capacity
PBS	Phosphate buffered saline
qTOF	Quadrupole time of flight

RFU	Relative fluorescence units
ROS	Reactive oxygen species
RT	Room temperature
SPE	Solid phase extraction
TE	Trolox equivalent
TFC	Total flavonoid content
TOCSY	Total correlation spectroscopy
TPC	Total phenolic content
YNB	Yeast nitrogen base
YNBG	Yeast nitrogen base supplemented with 0.5% glucose

Chapter 1

Introduction

Plants as a source of valuable secondary compounds

Plants are able to synthesize a broad range of metabolites. Those, which are essential for plant growth and development, are called primary metabolites. These are e.g. sugars, amino acids and nucleotides. Metabolites, which fulfil certain purposes, but are not essential for plant growth, are the secondary metabolites (Croteau et al. 2000). They play an important role in plant defence, signalling, attraction of pollinators and stress response (Figure 1). A number of these secondary metabolites possess favourable effects when consumed by humans, e.g. in the form of tea. Because of this, plant parts rich in secondary metabolites are widely used in traditional medicine. In ethnobotany, many applications of different plant parts are passed on to the next generation by tradition. The diseases treated with plant medicines are diverse, including various infections and inflammations (Bussmann & Sharon 2006). A famous example is aspirin (acetylsalicylate), as its precursor salicin was originally isolated from *Salix* spp. bark and the salicylate from *Spiraea ulmaria* (Zaugg et al. 1997, Bourgaud et al. 2001). The importance of plant-derived compounds is still high today, as there is the need for new pharmaceuticals in many medical fields. The richness in plant species and the variability in their metabolic profile make them a valuable source of interesting compounds (Heilmann 2009). One interesting group of plants are salt-tolerant species, so-called halophytes, which are able to grow and reproduce in saline environments and produce secondary compounds as part of their adaptation to salt stress (Ksouri et al. 2012).

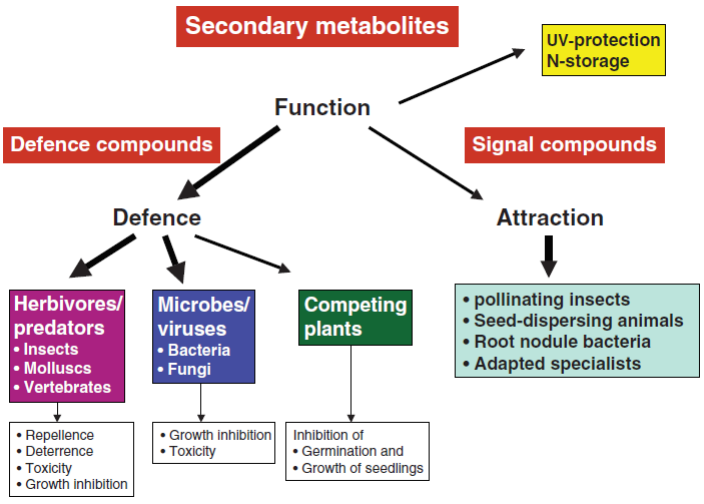

Figure 1. Functions of plant secondary metabolites. Taken from Wink 2009.

The specific characteristics of halophytes

The distinctive feature of halophytic plants is their ability to grow and reproduce in saline environments, in which other glycophytic plants cannot survive. The rate of salt tolerance defined for halophytes is 200 mM sodium chloride or higher in the medium or substrate (Flowers et al. 1986). Main habitats of halophytes are seashores and marshes as well as saline deserts and lakes (Flowers & Colmer 2015). Through intensive crop production with artificial irrigation, the area of salt-affected soils is increasing worldwide, so the importance of halophytic crop plants is growing (Pessarakli & Szabolcs 2010). Plants of this interesting class are producing numerous secondary metabolites, which are part of their adaptation mechanisms to the saline conditions (Ksouri et al. 2012). The cultivation of halophytes can be conducted in the field or in greenhouses. To grow tropical halophytic plants in the temperate zone, greenhouse cultivation is the only possibility. Since many secondary metabolites play a role in stress defence, their content can vary with the growth site and synthesis might change in a greenhouse setting (Ksouri et al. 2008). On the other hand, controlled growth conditions can provide a steady supply with nutrients and the stable environment might lead to a less variable composition of secondary compounds. A better understanding of halophyte secondary metabolite content and their cultivation can promote their use in further applications, e.g. the search for bioactive compounds.

Mangroves: Halophytic plants with high importance in local ecosystems

Mangroves consist of plant families habituated in tropical and subtropical coastal areas worldwide. The name “mangrove” describes a group of plant species as well as the complete ecosystem of a mangrove forest. They belong to the group of halophytic plant species and are reported to contain a high variety and concentration of secondary metabolites, which are responsible for their use in traditional medicine (Bandaranayake 1998). There are 73 species and hybrids considered as true mangroves and a number of mangrove-associated plant species (Spalding et al. 2010). They all share the ability to thrive in muddy or sandy coastal soils with varying salt concentrations in the water. Mangroves developed different adaptation mechanisms to cope with these conditions. As the soil is very muddy, genera like *Bruguiera* and *Heritiera* grow stilt or buttress roots to increase the stability. During tidal

flooding, the oxygen content in the soil is strongly decreased, leading to anoxic stress on the roots. Some genera, including *Avicennia* and *Sonneratia* spp., produce air roots, so-called pneumatophores, which grow upwards and above the ground, so they can deliver oxygen to the flooded roots.

All mangroves are halophytic plants and can tolerate a broad spectrum of salinity. The salt concentration of sea water is around 35 g L^{-1} (Antonov et al. 2010), but when the sea water evaporates, the concentration can reach much higher values. Different strategies have evolved in mangrove species to cope with the saline water. One is the limitation of salt uptake, which is achieved by filtering systems in the epidermis of the roots. Another mechanism is salt secretion, either through salt glands on the leaf surface, as it is known for *Avicennia* species, or at glands close to the petiole like for *Laguncularia* species. Salt can also be deposited in root and stem bark, or in case of *Xylocarpus* and *Exoecaria* genera be actively transported in senescent leaves.

Growing in regularly flooded soils close to the sea, mangroves have developed specialized ways of propagation (Kathiresan & Bingham 2001). Most mangrove seeds can float for at least a short time, or even remain floating and viable for over a year in case of *Rhizophora harrisonii*. A common feature of five major mangrove families is the development of viviparous propagules instead of seeds. In this way, the propagules take root fast enough, so they are not washed away by the tide.

Mangrove forests have a diverse appearance, depending on the geographical features (Lugo & Snedaker, 1974). They are found in river deltas, estuaries, coastal lagoons and open coastlines. In each mangrove forest, different species grow in their preferred zone, depending on their tolerance to salt, inundation and soil quality. In highly arid and saline conditions, only extremely salt tolerant species can survive, and grow only in a dwarf or scrub form. In wet and tropical conditions like in Brazil, mangrove trees are able to grow up to a height of 40 m (Schaeffer-Novelli et al. 1990).

Mangrove ecosystems are highly productive concerning biomass production and as carbon dioxide sinks. Many species of other plants, animals, bacteria and fungi are directly linked to and interacting with mangroves. They provide an environment for the growth of molluscs, crustaceans, insects, and a breeding ground for fish (Nagelkerken et al. 2008). Reptiles and amphibians as well as birds also benefit from the mangrove forest as habitat. Only few mammals live permanently in mangroves, among them primates, deer, otters and tigers.

Seagrasses: Specialized hydrophytes

Seagrasses are salt tolerant hydrophytes, which are distributed along tropical and subtropical coasts (den Hartog, 1970). Like other halophytes, they are known to contain numerous secondary metabolites as part of their adaptation to the marine environment (Papenbrock 2012). They grow on sandy soils and in clear waters with low turbidity. Interestingly, seagrasses derived from terrestrial plants during evolution. They "re-invaded" the sea from land-grown angiosperms, which have evolved from primitive water plants in the first place (Lee et al. 2018). At least three independent events of this kind, which lead to the existing seagrass species of today, have taken place (Kato et al. 2003, Janssen & Bremer 2004). There are 12 genera known, which are grouped into four families: Cymodoceaceae, Hydrocharitaceae, Posidoniaceae and Zosteraceae (Kuo & den Hartog, 2001). They live fully submerged under water, which led to similar morphological features adapted to this lifestyle. The leaves of some species have a long, strap-like shape, while others present oval leaves. They lack stomata and have a thin cuticle, and chloroplasts are present in the epidermal cell layers (Kuo & den Hartog 2006). Roots, rhizomes and underground stems have an important function as an anchor in the soft ground. In addition, the roots can tolerate an anoxic soil environment, in which oxygen is supplied from the leaves (Larkum et al. 2006).

Propagation is achieved by a pollination system adapted to the aquatic habitat, which is called hydrophily. Flowers present a simplified morphology compared to their terrestrial relatives. In addition, vegetative reproduction is an important factor for the extension of a seagrass bed through spreading rhizomes (Marbà & Duarte 1998). A limiting factor for seagrass growth and distribution is the light intensity reaching the sea sediment. Most seagrasses require 10% of surface light at an average, while some species like *Halophila* spp. can also thrive in deeper waters with around 5% of light (Dennison et al. 1993). Increased turbidity in shallow coastal waters, often caused by anthropogenic activities, leads to a decline in seagrass growth. Worldwide, seagrass populations are declining in size and number, and some species are at an elevated risk of extinction (Orth et al. 2006).

Seagrass beds fulfil an important role in coastal ecosystems. They provide a breeding ground for fish and other marine species (Beck et al. 2001). The root systems stabilize the loose sediment, and dead leaves provide a carbon source. Large sea herbivores such as sea turtles, manatees and dugongs feed on seagrass meadows.

Phylogeny and DNA barcoding

Clear identification of plants on the species level is often challenging and leads to misidentification of samples. Closely related mangrove and seagrass species often share physiological features and are hard to distinguish by eye. In case of seagrasses, flowers are often unavailable, whereas they can be a good identifier for mangroves. For both plant groups, the exact distribution, growth density and number of individual species in one area is often unclear. As the habitat of these plants is endangered by the increasing numbers of aquacultures and building density, more detailed assessments of mangrove and seagrass appearance and their decline is needed (Orth et al. 2006, Ragavan et al. 2014). To achieve this, a fast and reliable identification method needs to be established.

DNA barcoding is a method to identify species by the comparison of short orthologous DNA sequences. These “barcodes” are compared to a database of all collected sequence data and in this way, the species can be assigned (Figure 2). The barcode sequence needs to be distributed in a wide range of taxa and should be conserved within species, but at the same time have a high inter-species variation (Hollingsworth et al. 2009).

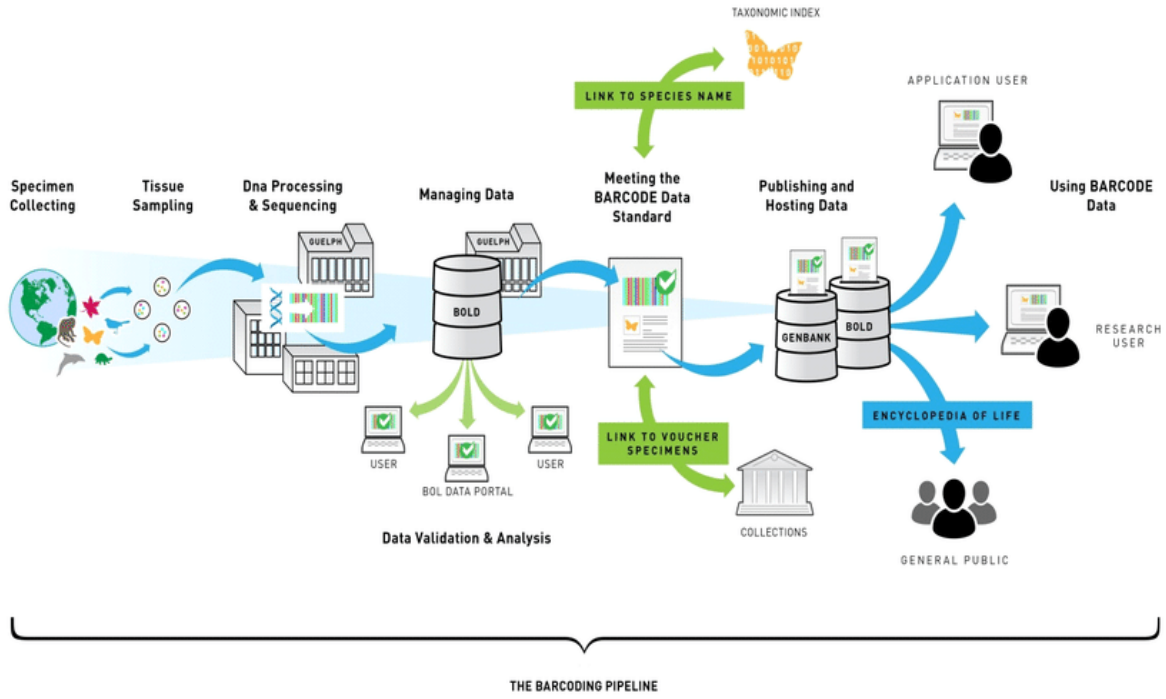

Figure 2. Principle of DNA barcoding of plant and animal specimen for public use. Taken from barcodeoflife.org.

Phenolic acids and flavonoids in seagrasses and mangroves

In their natural habitat, plants like mangroves and seagrasses are exposed to a number of biotic and abiotic stress factors. These are e.g. strong solar radiation, salinity, herbivores, wind and strong tides. Especially UV radiation causes the build-up of reactive oxygen species (ROS), which damage the cells and have to be neutralized by the plant (Figure 3). Phenolic compounds have the ability to react with ROS due to their antioxidant activities (Selmar & Kleinwächter 2013). There is the possibility to induce the synthesis of secondary metabolites by applying stress, e.g. salt stress on mangrove and seagrass plants. Since these plants are rich in phenolic compounds like flavonoids, phenolic acids and tannins, the focus of this work lays on the analysis of these substances.

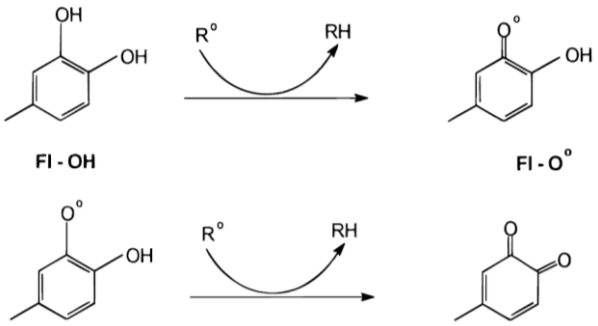

Figure 3. Reaction scheme of ROS scavenging by flavonoids. Taken from Pietta 2000.

Phenolic and polyphenolic compounds in plants derive from either the shikimate/phenylpropanoid or the acetate/malonate pathway (Lattanzio 2013). There is a broad structural variety of these compounds, which are classified in the main groups of flavonoids and non-flavonoid polyphenols (de la Rosa et al. 2010). Flavonoids share a basic structure of two benzene rings connected by a pyran ring formed by a C3-chain and one benzene ring (Pietta 2000). They are subdivided in flavanones, flavones, dihydroflavonols, flavonols, flavan-3-ols, anthocyanidins, isoflavones, and proanthocyanidins. The non-flavonoid polyphenols contain one aromatic ring, as in the case of simple phenols, benzoic acids, acetophenones and phenylacetic acids, cinnamic acids and coumarins, or at least two, like hydrolyzable tannins, benzophenones, xanthones, stilbenes, chalcones, lignans, and secoiridoids (de la Rosa et al. 2010). In addition, these compounds can occur as polymers, or in case of flavonoids, are often glycosylated. Bioactivities are reported for many plant-derived phenolic compounds, which makes them an interesting group of chemicals to test for new bioactive functions.

Importance of biofilms and the need for new bioactive compounds

The relevance of bacterial and fungal biofilms in infections and diseases of humans is a more recent discovery in medical biology. In the 1970s, the formation of bacteria in sessile communities was first described (Costerton et al. 1978). Clinical relevant biofilms are most likely to form on dead cell tissue or implants and medical devices, but also on living tissue as e.g. in an endocarditis (Andes et al. 2004). Most endangered are immune compromised and hospitalized individuals, which are prone for infections with the opportunistic skin bacterium *Staphylococcus epidermidis* or the infectious bacterium *Pseudomonas aeruginosa* (Singh 2000, Vuong & Otto 2002).

Once an infectious biofilm is established, its treatment is very challenging, as biofilms are highly resistant to antibiotics and fungicides compared to planktonic cells (Hall & Mah 2017, Ramage et al. 2002). This resistance is achieved in different ways. One is the shielding of microbial cells by the biofilm matrix, which cannot be penetrated completely by the antimicrobial agents (Chiang et al. 2013). This effect is dependent on the biofilm building species and the antimicrobial agent used. Another factor gaining higher antimicrobial resistance is the heterogeneity of cells. As a biofilm consists of different specialized sections, cells occur in various developmental stages. In the lower layers, less nutrients are available, so cells convert into a non-growing, dormant state (Stewart & Franklin 2008). These are less susceptible than the active, growing cells on the outer layer, which are more prone to antibiotics. From these dormant cells, the biofilm can re-establish after the antimicrobial treatment. In fungal *Candida* biofilms, these cells are called “persister-cells” and are a phenotypical variation of *Candida* cells, which lead to relapsing infections (Ramage et al. 2012).

Ongoing research on the detailed mechanisms of biofilm formation and antimicrobial resistance aims to identify new targets for the prevention and treatment of bacterial and fungal biofilms. In addition, there is a need for new active anti-biofilm compounds, such as secondary metabolites from plants, which have higher success rates and a better tolerability than currently available products.

Biofilm structure

In the life of bacteria and unicellular fungi, the predominant form is not the well-known planktonic state of free-swimming cells, but the more complex structure called biofilm. It describes microorganisms attached to a surface, which develop a three dimensional structure with specialized phenotypic elements (O'Toole et al. 2000).

The first step towards a biofilm is the attachment of the microbial cells to a surface (Figure 4). Bacteria first connect to a surface through reversible bonds. Triggers for attachment are e.g. environmental conditions, nutrient availability and temperature (Garrett et al. 2008). If the surface conditions are favourable, cells bind permanently through appendages such as flagella and pili. In the second step, the development of the biofilm matrix takes place. A big part of the biofilm consists of this matrix, which is build up by extracellular polymeric substances (EPS). These are polysaccharides, proteins, lipids and extracellular DNA in variable parts (Flemming & Wingender 2010). Cells together with EPS form three-dimensional structures, which can have a mushroom-like shape as for *Pseudomonas aeruginosa* (O'Toole et al. 1998). In this state, the microorganisms are protected from mechanical forces and have an increased resistance against microbial agents (Høiby et al. 2010). In the last step of the biofilm life cycle, planktonic cells disperse from the biofilm to colonize new habitats. Fungi can develop biofilms in a similar way. Well known for their ability to form biofilms are *Candida* species, but also e.g. *Aspergillus* (Fanning & Mitchell 2012). An important element in the fungal biofilm is the transition from yeast cells to hyphae growth (Chandra et al. 2001). A reduction of cell attachment, the hindered development of a functional matrix or a promoted dispersion of biofilm cells are possible working mechanisms of anti-biofilm compounds, which could be present in plant extracts.

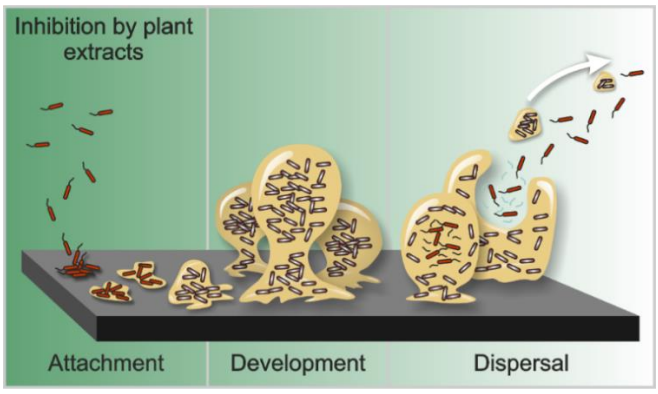

Figure 4. Graphic showing the three steps of biofilm formation. Adapted from P. Dirkx, Center of Biofilm Engineering, Montana State University 2003.

Different biofilm test systems

To determine, whether a compound shows anti-biofilm activity, different test systems can be applied. In general, 96-well microtiter plates offer a growth vessel, since it is possible to analyse many samples at the same time. The plates are available with different surface conditions, e.g. hydrophilic and hydrophobic, or with a clear glass or synthetic material for microscopic analysis. The choice of the plate surface condition has an influence on the cell attachment and biofilm growth, so variable results can be obtained on different plates (Villa et al. 2010). Therefore, the biofilm growth of *E. coli* and *C. albicans* will be compared on hydrophilic and hydrophobic surfaces. In addition, polystyrene plates are used, with an untreated hydrophobic surface comparable to many daily life materials and products.

In the crystal violet assay, bacterial cells attached to the walls of the plate are stained with crystal violet. Then, the staining is solubilized in ethanol and the optical signal is measured and compared across samples (O'Toole 2011). This test provides information about the density of biofilm cells between treated samples and a control. A similar method uses a fluorescent dye to stain attached cells, and subsequently compares the intensity of the fluorescent signal (Burton et al. 2007, Villa et al. 2010). Both of these tests give a general overview, if the tested substances affect biofilm adhesion and formation. More detailed results can be achieved in flow chambers, in which the biofilm grows on glass slides covered with the growth medium flow (Thormann et al. 2004). The biofilm can be maintained for longer times than in the small wells of the microtiter plate. In highly advanced systems, these slides can be monitored automatically with a microscope during all developmental stages. These microscope pictures can provide insights into changes of the biofilm structure under specific treatments, so possible modes of action become visible. To test the activity on individual biofilm development mechanisms, like quorum sensing or protein synthesis, other specialized methods have to be applied (Larsen et al. 2007, Vandeputte et al. 2011).

For this work, the main method chosen for biofilm tests is the microtiter test with fluorescent staining. It allows a high throughput of samples and is sufficient to identify promising compounds. Mangrove and seagrass extracts and fractions are screened on *E. coli* and *C. albicans* for their potential biofilm inhibitory activities. In addition, a more specified test method focusing on curli fiber production in *E. coli* colonies is applied for the analysis of tea varieties and the flavonoid taxifolin as possible sources of biofilm inhibiting compounds.

Modern techniques in drug development from natural sources

The discovery and identification of bioactive compounds from natural sources like fungi, plants and animals is achieved by modern analytical techniques. The first step from a natural source to a pure substance is the extraction of the active metabolite. Here, various extraction devices and conditions can be applied. A crucial factor is the choice of solvent: depending on the compound's polarity, it can or cannot dissolve in solvents like water or hexane (Sticher 2008). Other factors like extraction time and temperature can also exclude certain compounds. In the second step, pure compounds are isolated from the extract, which usually consists of a compound mixture. Common methods for the separation of compound groups are column-based solid-phase chromatography, or liquid-liquid partitioning (Månsson et al. 2010, Ignat et al. 2011). Finally, the compound needs to be isolated and its structure determined. A fast and high-throughput method is high performance liquid chromatography (HPLC) coupled to mass spectrometry (MS) and tandem mass spectrometry (MS/MS) (Hoffmann et al. 2014). More time-consuming is the nuclear magnetic resonance spectroscopy (NMR), which is used to assign the structure of a molecule (Molinski 2010). After a new compound has been isolated and identified, its bioactivity is analysed and quantitatively described. Another approach is the bioactivity-guided fractionation. Here, extracts are analysed for their bioactivity and based on these results, promising extracts or extract fractions are further purified to isolate the active component (Nothias et al. 2018). In accordance with this procedure, mangrove and seagrass extracts will be screened first, and the most promising extract will be subjected to a more detailed identification and more specialized biofilm assays.

Natural anti-biofilm compounds

Since the treatment of medical relevant biofilms with conventional antibiotics and antifungals is suboptimal, recent research focuses on the development of specific anti-biofilm agents.

In the year 2014, around 23% of all newly approved drugs were derived from a natural origin (Newman & Cragg 2016). The high relevance of natural products in drug research has led to the discovery of anti-biofilm products with a natural source. These can either inhibit the

permanent attachment of biofilm forming cells, or induce the disruption of preformed mature biofilms. Indole derivatives are potent in inhibiting biofilm formation in *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus* (MRSA) (Rogers et al. 2011). Their functional group 2-aminoimidazole originates from marine sponges (Akihiro et al. 1997). Numerous compounds are derived from plants as well. These are the anthraquinone emodin, the flavonoid phloretin and several ellagic acid derivatives, to name just a few (Rabin et al. 2015). Another flavonoid, quercetin, inhibits the alginate production in *S. aureus*, thus leading to a decreased adherence of cells (Lee et al. 2013). A different target for biofilm-inhibiting agents is the quorum sensing of bacteria. Furanones as well as plant derived gallic acid derivatives increase biofilm dispersion in *P. aeruginosa* and *Escherichia coli*, respectively, due to the interference with *N*-acyl homoserine lactone (AHL)-mediated quorum sensing (Hentzer et al. 2002, Huber et al. 2003). An example for disruption-promoting substances of *P. aeruginosa* biofilms is lauroyl glucose (Dusane et al. 2008). There is a high chance to discover more active anti-biofilm compounds in plants, which can have specific modes of action in the prevention or disruption of biofilm formation. Plants with high contents in secondary metabolites, such as halophytes, constitute a promising source of bioactive compounds.

Seagrasses and mangroves as a source of bioactive compounds

There is a general consent that mangroves and seagrasses are rich in secondary metabolites, but detailed analysis of their composition and concentrations exists only in parts. Mangroves have a long history as a drug in traditional medicine, which is connected to their secondary metabolite content (Bandaranayake 1998).

The diseases and conditions that are treated with mangrove products are very diverse. They reach from skin diseases and parasites to asthma, leukaemia and cancer (Bandaranayake 1998). This broad usage is also due to the unavailability of other medical care in rural areas of developing countries. On some of these reported activities, research has been carried out. Premnathan et al. (1992) have analysed extracts from 51 mangrove species for their antiviral activities, of which 29 showed activity (> 50%) against at least one virus (Premnathan et al. 1992). Bark extracts of *Xylocarpus granatum* and *X. moluccensis* exhibited fever-curing activities (Alvi et al. 1994). Extracts from *Avicennia marina* and *Rhizophora mucronata* have

shown anti-biofilm activity and influence on AHL signalling tested on *Vibrio* spp. (Deepa et al. 2014).

Dried seagrass leaves have a long tradition as valuable material for various purposes (Wyllie-Echeverria et al. 2000). It was used to fill matrasses, as padding material and insulation, since it is very mould-resistant. Seagrass detritus is poor in nutrients and contains phenolic compounds, which inhibit microbial growth (Harrison 1998). Seagrasses contain various phenolic compounds, including phenolic acids, sulphated phenolic acids, flavones, condensed tannins, and lignins (Vergeer et al. 1995). In the marine environment, these compounds protect the seagrass from amphipod grazing, microbial growth and epiphytic diatoms (Harrison 1982, Harrison & Durance 1985) Phenolic acid sulphate esters from *Zostera marina* reduced the attachment of marine bacteria and barnacles to artificial surfaces (Todd et al. 1993). One of these compounds, called zosteric acid, has shown to act inhibitory against non-marine bacterial and fungal biofilms (Villa et al. 2010). Flavonoids have been reported to show antimicrobial activities. These are luteolin, apigenin, luteolin-3-glucoronide and luteolin-4-O-glucoronide, isolated from an ethanolic *Enhalus acoroides* extract (Qi et al. 2008). Uncommon sulphated flavones are found in some seagrass species, e.g. *Thalassia* and *Zostera* (Enerstvedt et al. 2016, Hawas & El-Kassem 2017). Those findings indicate the presence of numerous bioactive compounds in mangroves and seagrasses. Therefore, this work focuses on the investigation of the active compounds from plants, with a focus on the halophytic mangroves and seagrasses. They have a high potential to contain secondary metabolites, possibly not described yet, which possess bioactive properties against microorganisms. Since there is a need for new agents inhibiting biofilm formation, their biofilm inhibitory activities on bacteria and fungi are analysed.

Aims of the thesis

The aim of this work is to explore plants, especially halophytes, as a resource of bioactive compounds, with a focus on the potential use of mangrove and seagrass species as sources of anti-biofilm compounds.

The specific objectives are:

To evaluate the potential of greenhouse grown mangroves as a resource of secondary metabolites to be utilized in the search of bioactive compounds. It is hypothesized, that secondary metabolite production can be induced by salt stress, the identification of species can be achieved by DNA barcoding and that greenhouse grown and naturally grown mangroves are comparable in their phenolic compound composition.

To investigate the effect of tea varieties and individual phenolic compounds on biofilm formation and extracellular matrix production of *Escherichia coli* colonies. Traditionally used natural remedies like teas, and phenolic compounds from plants, represent a source of bioactive compounds.

To analyse anti-biofilm properties of methanolic extracts from seagrasses on biofilm formation. Seagrasses contain compounds, which are able to interfere with biofilm formation during different developmental stages.

To evaluate anti-biofilm activities of methanolic extracts from mangroves and to identify the bioactive compounds present in the extract. Phenolic compounds present in mangrove leaves are able to inhibit biofilm adhesion.

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Chapter 2

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Sustainable use of mangroves as sources of valuable medicinal compounds: Species identification, propagation and secondary metabolite composition

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ABSTRACT

Mangroves are able to withstand a number of stress factors, such as high salt concentrations, tidal flooding, strong wind, solar radiation and heat. Their ability to grow under these circumstances is based on morphological and physiological adaptations, among them the high abundance of plant secondary metabolites. We are interested to investigate and exploit their medicinal and biotechnological potential for new bioactive compounds, without collecting material in the countries of origin and in a sustainable way. Therefore, a simple identification system based on molecular marker analysis, and a sustainable greenhouse propagation protocol for the continuous supply of fresh plant material, were established. DNA barcoding of the internal transcribed spacer (ITS) including ITS1, the 5.8S rRNA region and ITS2 as a molecular marker was applied for several mangrove species. The obtained data and GenBank sequences were used for species identification. Three mangrove species are cultivated in our greenhouse and propagated in different ways: *Avicennia* species produced many propagules in the greenhouse, however, further propagation by cuttings was not successful. *Laguncularia racemosa* was propagated by cuttings in a fog house whereas *Bruguiera cylindrica* was difficult to cultivate and propagation was not successful. Finally, the concentration of secondary phenolic compounds, including flavonoids, and the content of major elements were compared among naturally and greenhouse-grown mangroves indicating comparable amounts and composition.

1. Introduction

Mangroves are trees or shrubs that are able to grow in saline water along tropical and sub-tropical coasts around the world (Kathiresan and Bingham, 2001). These plants are able to withstand a number of environmental stress factors: high salt concentrations, tidal flooding, strong wind, solar radiation and heat (Spalding et al., 2010). Their ability to grow under these circumstances is linked to various morphological, physiological and biochemical adaptations, such as stilt and air roots, salt excretion systems and secondary metabolites.

A mangrove forest provides protection against erosion and high waves for coastal regions (Alongi, 2008). Mangroves also offer an important habitat for many species. For example, many fish species use the sheltered root systems to breed, which is important for local fisheries (Phillips et al., 1993). However, the area of mangrove forests worldwide is decreasing at a rate of 1%–2% every year (FAO, 2003). In Asia, almost half of the area used for aquacultures (42%) was previously covered by mangroves (ADB/NACA, 1998). In a counter movement, there are several reforestation programs, e.g. in India, Vietnam and Bangladesh (Benthem et al., 1999; MFF Vietnam, 2015; Chow, 2018). Studies of the economic value of replanted mangrove forests show that the benefits outweigh the input costs (Tuan and Tinh, 2013).

In general, woody plant species can be propagated in different ways. An easy, low cost method is the use of seeds. In some woody plant species, seed development and germination are time-consuming processes, which limits the availability. In that case, preparation of cuttings or in vitro culture can be applied. Cuttings are branches of a tree, which can have a variable length and can be directly put in soil or growth media to generate roots at the cutting site. The plant hormone auxin can be used to improve root growth, and a humid atmosphere supports the rooting of vegetative cuttings (Milbocker, 1983; Dirr, 1992). In vitro propagation requires more equipment and is more cost-intensive, but is independent of seasons.

Species determination and taxonomy of mangroves is not fully resolved (Ragavan et al., 2014). Even the taxonomy on the family level is not conclusively determined. For example, the taxonomic placement

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of the genus *Avicennia* is controversial. In some classifications, it has been placed in the family Verbenaceae (Moldenke and Moldenke, 1980; Li et al., 2016) but recent phylogenetic studies have suggested that *Avicennia* is derived from within the Acanthaceae (The Angiosperm Phylogeny Group, 2016). In addition, the designation of species remains difficult due to the great variability and morphological plasticity, such as within *Avicennia marina*. Therefore, the application of an identification system that is based on molecular markers could support the correct identification of species and even subspecies, as was previously shown for other taxa (Lucas et al., 2012; Nguyen et al., 2015). Recently, the efficacy evaluation of a multilocus marker system for delineating mangrove species from the West Coast of India could successfully demonstrate the mangrove species resolution based on several genes (Saddhe et al., 2016, 2017). However, for most applications such as a fast species identification, a simpler DNA barcode system is preferable.

Mangroves have a long tradition of medicinal use and are rich in secondary metabolites (Bandaranayake, 1998, 2002). Some of these secondary compounds from mangrove species have been reported to have antimicrobial, antioxidant and other effects, which is mainly based on ethnobotanical reports (Patra and Thatoi, 2011). For researchers in the temperate zones, it is only possible to have access to fresh plant material when mangroves are cultivated successfully in the greenhouse. In addition, the use of greenhouse plants makes it unnecessary to take plant material out of the wild. Prerequisites for the successful use of greenhouse-grown plants are high contents of secondary metabolites and a similar composition as in naturally grown plants.

For our study, several mangrove species were selected from three main mangrove families, which is based on ethnobotanical studies. The species *Avicennia marina* (Forssk.) Vierh. and *A. germinans* (L.) L. (Acanthaceae) use different strategies to survive in high salinities such as salt rejection, elimination, and a slight concentration. *Bruguiera cylindrica* (L.) Blume (Rhizophoraceae) uses rejection, concentration, and ultrafiltration. The species develops salt glands and accumulation of salt in old leaves has been observed. For *Laguncularia racemosa* (L.) C.F. Gaertn. (Combretaceae), several bioactive effects have been reported, such as protein kinase inhibition and insecticidal activity (Shi et al., 2010). The high salt tolerance is based on salt excretion through glands close to the petiole and conservative water use at high salinities (Sobrado, 2005).

The aims of this study were to clearly identify mangrove species for further propagation and as a resource of valuable secondary compounds. ITS including ITS1, the 5.8S rRNA region and ITS2 were tested as a phylogenetic marker for fast and simple species determination for mangroves. Methods for propagation in the greenhouse in temperate zones were determined. Secondary metabolites were characterized in mangroves grown in the greenhouse and outdoors, to reveal if greenhouse-grown mangroves can be considered for further exploitation of secondary compounds.

2. Material and methods

2.1. Plant material

The mother plants used for propagation in the greenhouse have different origins. A small tree of the species *Avicennia marina* (Forrsk.) Vierh., Acanthaceae, was donated by Prof. Dr. H. Lieth, University of Osnabrück, in 1999, but is of unknown origin. In the course of this study, the identification as *A. marina* was found to be incorrect. Five ca. 15-year-old plants of *Avicennia germinans* (L.) L. (Acanthaceae), and a ca. 15-year-old plant of the species *Laguncularia racemosa* (L.) C.F. Gaertn. (Combretaceae), originally collected in South America, were donated by Prof. R. and Dr. M. L. Schnetter, University Gießen, Germany. *Bruguiera cylindrica* (L.) Blume (Rhizophoraceae) plants were purchased as propagules originally collected in Indonesia (Marek Mangroven, Wien, Austria). The plants were cultivated in the greenhouse of the Institute of Botany, Leibniz University Hannover, Hannover, Germany. Further plant material used for molecular analysis was collected in Bangladesh, Cuba, Guatemala, Egypt, India and Vietnam at various collection sites (Fig. 1, Table 1).

2.2. Internal transcribed spacer (ITS) analysis

2.2.1. DNA extraction and PCR

DNA was extracted from leaf material dried at ambient temperature using the Plant Nucleospin II Kit (Macherey & Nagel, Düren, Germany). The procedure followed the manufacturer's instruction modified according to Lucas et al. (2012). The success of DNA extraction was determined by visualizing bands on 1% agarose gels stained with Midori green (Biozym-Diagnostik GmbH, Hess. Oldendorf, Germany). The concentration of DNA was measured on micro-volume plates with a microplate reader (Synergy Mx Multi-Mode, BioTek, Bad Friedrichshall, Germany).

The nuclear ITS region including ITS1, the 5.8S rRNA region and ITS2, with a size of 700 to 720 bp, was amplified by PCR. The primers P674 5′-CCTTATCATTTAGAGGAAGGAG-3′ (ITS5a) (Stanford et al., 2000) and P675 5′-TCCTCCGCTTATTGATATGC-3′ (ITS4) (White et al., 1990) were used. A PTC 200 thermocycler (Biozym-Diagnostik GmbH) with lid heating was used for the PCR reactions, with following: 0.2 mM dNTPs, 2 mM $MgCl_2$, 1 μl Dream *Taq* polymerase (Thermo Fisher Scientific, Waltham, USA), 1 × Dream *Taq* Green buffer, 10–30 ng template DNA, 1 pmol of each primer in a total volume of 25 μl.

An initial denaturation step at 95 °C for 4 min was followed by 30 cycles with the following steps: denaturation at 95 °C for 25 s, primer annealing at 52 °C for 30 s and primer extension at 72 °C for 35 s. The reaction was terminated with a final hold at 10 °C. To avoid possible errors in the final consensus sequence (due to the *Taq* polymerase) each PCR reaction for every specimen was repeated two to four times independently. Sequencing of the PCR products was done by GATC Biotech (Konstanz, Germany) using the primers P674 5′-CCTTATCATTTAGAGGAAGGAG-3′ and P675 5′-TCCTCCGCTTATTGATATGC-3′.

2.2.2. Data analysis

In this study, 27 sequences of Acanthaceae, 3 sequences of Rhizophoraceae and 5 sequences of Combretaceae were retrieved from the mangrove species collected in Bangladesh, Cuba, Egypt, Guatemala, India, Vietnam and the greenhouse (Table 1). For comparison, known ITS sequences of Acanthaceae, Combretaceae and Rhizophoraceae species retrieved from GenBank were added to the dataset. These sequences were aligned by CLUSTAL X (Thompson et al., 1997) in MEGA 7 (Kumar et al., 2016) and the alignment controlled visually. Gaps between nucleotides in the alignment were considered as missing data. Identical sequences within each species were excluded from the alignment. Additional in-group sequences were obtained from GenBank (Table 1), and included in the alignment. The total length of the alignment was 604 bp. jModelTest version 2.1.6 (Darriba et al., 2012) and the corrected Akaike Information Criterion was used to find the best model for the analysis. Phylogenetic analyses were performed using Maximum Likelihood (ML) in RAxML version 8.1 (Stamatakis, 2014) with the model General Time Reversible (GTR) (Lanave et al., 1984). Maximum Parsimony (MP) (Felsenstein, 1978) and Neighbor Joining (NJ) (Saitou and Nei, 1987) with the GTR model were estimated using MEGA 7 (Kumar et al., 2016). Bayesian inference, using the Metropolis coupled Markov-chain Monte-Carlo method, was performed in MrBayes v.3.2.2 (Ronquist et al., 2012). Two parallel runs with four chains each (three heated and one cold) were performed for 3 million generations, sampling a tree every 1000 generations. The 530,000 burn-in period was identified graphically using Tracer 1.7.1 software (Rambaut et al., 2018) by tracking likelihoods at each generation to determine whether the likelihood values had reached a plateau, and the average deviation of split frequencies fell below 0.01. The 7952 trees sampled at stationary were used to infer Bayesian posterior. The

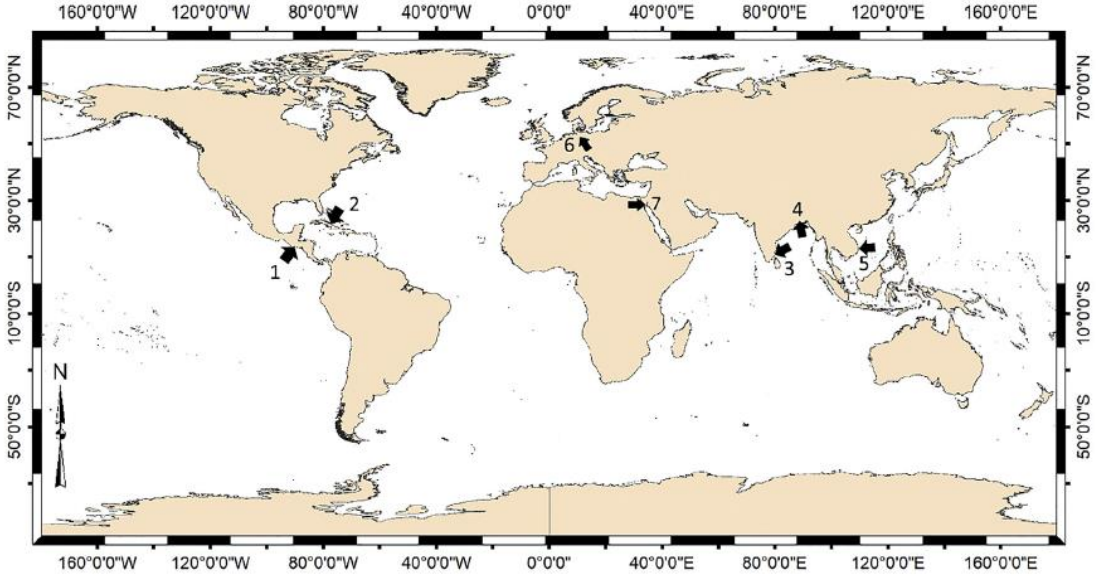

Fig. 1. The world map (Source: The National Oceanic and Atmospheric Administration (NOAA), USA) shows the sampling sites (solid arrows). Thirty five samples were collected at 1 (Guatemala, six samples), 2 (Cuba, one sample), 3 (India, three samples), 4 (Bangladesh, thirteen samples), 5 (Viet Nam, six samples), 6 (at greenhouse, LUH, Germany, five samples) and 7 (Egypt, one sample). The map was processed by MapInfo Pro™, version 12.5.5 (Pitney Bowes Software Inc., NY, USA).

consensus tree based on four different trees (achieved from the four methods) was constructed by Dendro Scope software, version 3.2.10 (Huson and Scornavacca, 2012).

2.3. Greenhouse experiments

2.3.1. Greenhouse conditions

The illumination by sunlight was supported by sodium vapor lamps (SON-T Agro 400, Philips, Amsterdam, Netherlands) to raise the quantum fluence rate to approximately 350 µmol m^{-2} s^{-1} over the day period of 12 h. The average temperature was 25 °C. Water used for irrigation was tap water, which was occasionally enriched with artificial sea salt (Seequasal GmbH, Münster, Germany). Plants were grown either in soil (Einheitserde, Einheitserdewerk Hameln-Tündern, Germany), sand (0–2 mm grain size, Hornbach, Hannover, Germany) or a mixture of both. Macro- and micronutrients were added within the irrigation solution with 0.8% (winter) or 1% (summer) liquid fertilizer (Wuxal Top N, Manna, Düsseldorf, Germany) once per week. The fertilizer is composed of the following nutrients: 12.0% N, 4.0% P_2O_5, 6.0% K_2O, 0.01% B, 0.004% Cu, 0.02% Fe, 0.012% Mn, 0.001% Mo, 0.004% Zn.

2.3.2. Propagation and growth conditions

For each species, cuttings for propagation were taken from mother plants of 5–15 years old and approximately 2 m high. Propagation was tested in the greenhouse, in a fog house with a high humidity of 90%, and subsequently covered with a hood for eight weeks to acclimatize in the greenhouse. In addition, root growth promoting methods were applied to increase rooting, such as covering with humid sphagnum moss and the application of different concentrations of the plant hormone auxin.

Plants of *Avicennia germinans* were grown in soil and watered with tap water, which was enriched every 4 weeks with 10 Practical Salinity Units (PSU) with sea salt. During a biofilter experiment, 1.5-year-old *A. germinans* plants were grown in sand and the salt concentrations were increased to 15 and 30 PSU for 4 weeks. In another biofilter experiment using about 9-month-old *Laguncularia racemosa* plants the influence of salt at 15, 30 and 45 PSU was followed for 6 weeks.

2.3.3. Analysis of secondary compounds

The content of secondary compounds in plants from natural populations and greenhouse-grown plants of *Avicennia germinans* were compared using liquid chromatography coupled to a mass spectrometer (LC–MS). From six plants grown in the greenhouse, leaves growing at the top, middle and bottom of each plant were harvested and dried at 30 °C for five days. Already dried samples from Guatemala and the dried greenhouse samples were milled with a bead mill (Retsch, Haan, Germany). For extraction of secondary metabolites, 20 mg of milled material was weighed into a reaction tube and 800 µl of 80% methanol (MeOH) was added. After 10 min incubation with regular vortexing, the tubes were centrifuged for 5 min at 7800*g*. The supernatant was transferred into a new reaction tube with a pipette. These steps were repeated three times with 400 µl MeOH 80% each. The extracts were stored at −20 °C over night and centrifuged again the next day. In a glass vial, 500 µl of the supernatant was diluted with 500 µl MeOH 80% for LC–MS analysis. Standards for quantification (rutin and naringenin) were dissolved in ethanol and MeOH, respectively, and thereafter diluted in 80% MeOH. Concentrations of 0.001, 0.01, 0.1, 0.5, 1 and 10 µmol L^{-1} for naringenin and 0.5, 1, 10 and 100 µmol L^{-1} for rutin were measured. The LC–MS consisted of a HPLC (Shimadzu, Darmstadt, Germany) with a controller, two pumps, an auto sampler, column oven and photo diode array detector (PDA) and a time-of-flight mass spectrometer (AB Sciex TripleTOF 4600, Canby, USA). A Knauer Vertex Plus column (250 × 4 mm, 5 µm particle size, packing material ProntoSIL 120–5 C18-H) with pre-column (Knauer, Berlin, Germany) was used for sample separation, with 10 µl injections. Water and MeOH supplemented with 2 mM ammonium acetate and 0.01% acetic acid were used as solvents. The flow rate was 0.8 ml min^{-1} with a linear gradient from 10% to 90% MeOH over 35 min, 2 min of 90% MeOH, switch to 10% MeOH in 1 min and subsequent equilibration at 10% MeOH for 2 min. In the photodioide array, UV–vis spectra between 190 and 800 nm were recorded. Mass spectrometry was conducted in negative ionization mode at a nebulizer temperature of 600 °C and an ion spray voltage floating of −4500 V. Masses from 100 to 800 Da

Table 1
List of taxa, locations, and GenBank number used for the analysis. *: First recorded as *Avicennia marina*. **: First recorded as *A. marina*. # First recorded as *Rhizophora x annamalayana*. ##: First recorded as *Rhizophora apiculata*. LUH: Leibniz University Hannover. —/—: as above. na: not available.

No	Taxa	Family	Country	Location	GenBank accession number	Source
1	*Avicennia alba* Blume	Acanthaceae	India	na	KF848261	Direct submission
2	*Avicennia alba* Blume	—/—	Thailand	na	KX641594	Li et al. (2016)
3	*Avicennia alba* Blume	—/—	Indonesia	na	EF540977	Nettel et al. (2008)
4	*Avicennia alba* Blume	—/—	Bangladesh	Patakhali	MG880028	This study
5	*Avicennia alba* Blume	—/—	Bangladesh	Patakhali	MG880029	This study
6	*Avicennia alba* Blume	—/—	Bangladesh	Patakhali	MG880030	This study
7	*Avicennia alba* Blume	—/—	Bangladesh	Patakhali	MG880031	This study
8	*Avicennia alba* Blume*	—/—	Bangladesh	Patakhali	MG880032	This study
9	*Avicennia alba* Blume*	—/—	Bangladesh	Patakhali	MG880033	This study
10	*Avicennia alba* Blume*	—/—	Bangladesh	Patakhali	MG880034	This study
11	*Avicennia alba* Blume*	—/—	Bangladesh	Patakhali	MG880035	This study
12	*Avicennia alba* Blume	—/—	Viet Nam	Cam Hai Dong	MG880036	This study
13	*Avicennia germinans* (L.) L.	—/—	Dominica	na	EF136923	Nettel and Dodd (2007)
14	*Avicennia germinans* (L.) L.	—/—	—/—	na	DQ469854	Nettel and Dodd (2007)
15	*Avicennia germinans* (L.) L.	—/—	Guadeloupe	na	EF136925	Nettel and Dodd (2007)
16	*Avicennia germinans* (L.) L.	—/—	Costa Rica	na	EF540979	Nettel et al. (2008)
17	*Avicennia germinans* (L.) L.	—/—	Angola	Soyo	DQ469860	Nettel and Dodd (2007)
18	*Avicennia germinans* (L.) L.	—/—	Germany	Greenhouse Hannover	MG880037	This study
19	*Avicennia germinans* (L.) L.**	—/—	Germany	Greenhouse Hannover	MG880038	This study
20	*Avicennia germinans* (L.) L.	—/—	Germany	Greenhouse Hannover	MG880039	This study
21	*Avicennia germinans* (L.) L.	—/—	Germany	Greenhouse Hannover	MG880040	This study
22	*Avicennia germinans* (L.) L.	—/—	Cuba	Cayo Coco	MG880041	This study
23	*Avicennia germinans* (L.) L.	—/—	Guatemala	Iztapa	MG880042	This study
24	*Avicennia germinans* (L.) L.	—/—	Guatemala	Iztapa	MG880043	This study
25	*Avicennia germinans* (L.) L.	—/—	Guatemala	Manchón-Guamuchal	MG880044	This study
26	*Avicennia germinans* (L.) L.	—/—	Guatemala	Manchón-Guamuchal	MG880045	This study
27	*Avicennia germinans* (L.) L.	—/—	Guatemala	San Andrés Villa Seca	MG880046	This study
28	*Avicennia germinans* (L.) L.	—/—	Guatemala	Tulate	MG880047	This study
29	*Avicennia integra* N.C. Duke		Australia	na	KX641598	Li et al. (2016)
30	*Avicennia marina* (Forssk.) Vierh.	—/—	China	na	AF477771	Shi et al. (2003)
31	—/—	—/—	—/—	na	AF477770	—/—
32	*Avicennia marina* (Forssk.) Vierh.	—/—	Egypt	Nabq Nature Reserve	MG880048	This study
33	*Avicennia marina* (Forssk.) Vierh.	—/—	Viet Nam	Cam Hai Dong	MG880049	This study
34	*Avicennia marina subsp. australasica* (Walp.) J. Everett	—/—	Australia	na	AF365978	Schwarzbach and McDade (2002)
35	*Avicennia marina* (Forssk) Vierh. subsp. *marina*	—/—	China	na	KX641593	Li et al. (2016)
36	*Avicennia marina subsp. eucalyptifolia* (Valeton) J. Everett		Australia	na	KX641592	Li et al. (2016)
37	*Avicennia officinalis* L.	—/—	Thailand	na	KX641597	Li et al. (2016)
38	*Avicennia officinalis* L.	—/—	Bangladesh	Patakhali	MG880050	This study
39	*Avicennia officinalis* L.	—/—	Bangladesh	Patakhali	MG880051	This study
40	*Avicennia officinalis* L.	—/—	Bangladesh	Patakhali	MG880052	This study
41	*Avicennia officinalis* L.	—/—	Bangladesh	Patakhali	MG880053	This study
42	*Avicennia officinalis* L.	—/—	Bangladesh	Patakhali	MG880054	This study
43	*Avicennia rumphiana* Hallier f.	—/—	Malaysia	na	KX641595	Li et al. (2016)
44	*Avicennia schaueriana* Stapf & Leechm. ex Moldenke	—/—	Brasil	na	AB861236	Mori et al. (2015)
45	*Avicennia schaueriana* Stapf & Leechm. ex Moldenke	—/—	Guadeloupe	na	EF540986	Nettel et al. (2008)
46	*Laguncularia racemosa* (L.) C.F. Gaertn.	Combretaceae	China	na	AF425685	Tan et al. (2002)
47	*Laguncularia racemosa* (L.) C.F. Gaertn.	—/—	Germany	Greenhouse Hannover	MG880055	This study
48	*Lumnitzera littorea* (Jack) Voigt	—/—	China	na	AF160468	Tan et al. (2002)
49	*Lumnitzera littorea* (Jack) Voigt	—/—	Vietnam	Thuan An	MG880056	This study
50	*Lumnitzera littorea* (Jack) Voigt	—/—	Vietnam	Cam Hai Dong	MG880057	This study
51	*Lumnitzera x rosea* C. Presl	—/—	Vietnam	Thuan An	MG880058	This study
52	*Lumnitzera racemosa* Willd.	—/—	China	na	AF160467	Tan et al. (2002)
53	*Lumnitzera racemosa* Willd.	—/—	Vietnam	Ninh Ich	MG880059	This study
54	*Bruguiera cylindrica* (L.) Blume	Rhizophoraceae	Australia	na	HM366078	Sun and Lo (2011)
55	*Bruguiera cylindrica* (L.) Blume	—/—	Australia	na	HM366079	—/—
56	*Bruguiera gymnorrhiza* (L.) Savigny	—/—	Indonesia	na	HM366082	Sun and Lo (2011)
57	*Bruguiera gymnorrhiza* (L.) Savigny	—/—	China	na	HM366083	Sun and Lo (2011)
58	*Bruguiera parviflora* (Roxb.) Wight & Arn. ex Griff.	—/—	Australia	na	HM366110	Sun and Lo (2011)
59	*Bruguiera parviflora* (Roxb.) Wight & Arn. ex Griff.	—/—	Australia	na	HM366111	Sun and Lo (2011)
60	*Bruguiera sexangula* (Lour.) Poir.	—/—	China	na	HM366122	Sun and Lo (2011)
61	*Bruguiera sexangula* (Lour.) Poir.	—/—	Indonesia	na	HM366134	Sun and Lo (2011)
62	*Ceriops tagal* (Perr.) C.B. Rob.	—/—	na	na	AF130329	Schwarzbach and Ricklefs (2000)
63	*Ceriops tagal* (Perr.) C.B. Rob.	—/—	na	na	EF119031	Direct submission
64	*Kandelia candel* (L.) Druce	—/—	na	na	AF130327	Schwarzbach and Ricklefs (2000)
65	*Kandelia candel* (L.) Druce	—/—	na	na	EF119071	Direct submission
66	*Rhizophora apiculata* Blume	—/—	Malaysia	na	HQ337923	Lo et al. (2014)
67	*Rhizophora apiculata* Blume	—/—	Micronesia	na	HQ337918	Lo et al. (2014)
68	*Rhizophora x annamalayana* Kathiresan	—/—	India	na	KF848256	Direct submission
69	*Rhizophora mangle* L.	—/—	Mexico	na	HQ337958	Lo et al. (2014)

Table 1 (*continued*)

No	Taxa	Family	Country	Location	GenBank accession number	Source
70	*Rhizophora mangle* L.	−/−	na	na	AF130332	Schwarzbach and Ricklefs (2000)
71	*Rhizophora mucronata* Poir.	−/−	Kenya	Gazi Bay	HQ337949	Lo et al. (2014)
72	*Rhizophora mucronata* Poir.	−/−	Kenya	Mida Creek	HQ337948	Lo et al. (2014)
73	*Rhizophora mucronata* Poir.	−/−	India	Parangipettai, Vellar Estuary	MG880060	This study
74	*Rhizophora mucronata* Poir.#	−/−	India	Parangipettai, Vellar Estuary	MG880061	This study
75	*Rhizophora mucronata* Poir.##	−/−	India	Parangipettai, Vellar Estuary	MG880062	This study
76	*Rhizophora stylosa* Griff.	−/−	Taiwan	na	HQ337934	Lo et al. (2014)
77	*Rhizophora stylosa* Griff.	−/−	Malaysia	na	HQ337935	Lo et al. (2014)

were measured in TOF mode. In addition, MS/MS spectra from 50 to 800 Da at a collision energy of −30 eV were recorded. The resulting peaks of extracted secondary compounds were evaluated using PeakView and MultiQuant (AB Sciex, Canby, USA). Masses and MS/MS spectra were compared to database entries from MassBank (Horai et al., 2010) and ReSpect (Sawada et al., 2012). Calibration curves and quantifications were calculated with MultiQuant.

2.3.4. Determination of the elemental composition of mangrove leaves

Dry plant material was ground to a fine powder (MM 400 grinder, Retsch GmbH, Haan, Germany) and 38 mg was incinerated for 8 h in a muffle furnace at 480 °C (M104, Thermo Fisher Scientific Corporation, Waltham, Massachusetts, USA). After cooling the samples to about 22 °C, 1.5 ml of 66% nitric acid was added and, after 10 min, 13.5 ml of ultra-pure water. The solutions were filtered (0.45 µm pore size, Carl Roth, Karlsruhe, Germany) and stored in vials at 4 °C before final analysis. The samples were analyzed by inductively coupled plasma optical emission spectrometry (ICP-OES) (iCAP 6000 ICP Spectrometer, Thermo Fisher Scientific).

3. Results

3.1. Phylogenetic analysis

Results of the four phylogenetic analyses (maximum likelihood, neighbor joining, maximum parsimony and Bayesian inference) showed that all sequences were distributed into three main clades consisting of Acanthaceae, Rhizophoraceae and Combretaceae (Fig. 2). For the Acanthaceae family, four samples labeled as *Avicennia marina* collected in Bangladesh clustered with the *A. alba* branch instead of the *A. marina* group. This result indicated the misidentification of *A. marina* samples from Bangladesh. Samples of *A. officinalis* collected in Bangladesh clustered with known *A. officinalis* samples. Interestingly, *A. germinans* collected in Cuba clustered with *A. germinans* "Caribbean sea clone" and the materials collected in Guatemala grouped together with the "Pacific clone" with very high posterior probability and bootstrap values of 1.0, 99%, 93% and 72%, respectively. As illustrated in Fig. 2, it became obvious that *A. germinans* cultivated in the greenhouse of the Institute of Botany, Leibniz University Hannover, Hannover, Germany, was grouped within the *A. germinans* "Caribbean sea clone". Since the origin of the *A. germinans* mother plant was hitherto unknown, it can now be concluded that only areas of the Central and South American North Atlantic coast are possible origins. The putative *A. marina* growing in the greenhouse of the Institute of Botany clustered with *A. germinans*. Based on the ITS analysis results, two more misidentifications were detected. Two samples identified as *Rhizophora x annamalayana* and *R. apiculata* (Rhizophoraceae) were found to have closer affinities to *R. mucronata* instead.

3.2. Different ways of propagation

A trial of 200 *Avicennia germinans* cuttings were prepared and grown under the same conditions as the mother plant. In addition, longer cuttings (30 cm) were taken and grown in the same way. To improve the survival rate of the cuttings, the fresh cut branches were grown in a fog house for 13 weeks with a humidity higher than 90%. Afterwards, the plants were transferred back to the greenhouse and covered with a hood for eight weeks to acclimatize. Rooting experiments with cuttings of different lengths were performed at high humidity, based on rooting results obtained with other woody species in a fog house experimenting with different length of cuttings (Mateja et al., 2007). Cuttings showed a low survival rate of only 10 rooted cuttings among the 200 cuttings tested (Table 2). Also longer cuttings of 30 cm and keeping the fresh cuttings in a fog house with high humidity did not lead to better results. The treatment with different concentrations of the growth hormone auxin did not induce root formation, neither the application of the sphagnum moss method (data not shown). Plants of *A. germinans* are flowering in the greenhouse and produce viable seeds. These seeds were laid on wet soil without any further treatment and had a high germination rate. In comparison to propagation by cuttings, for *A. germinans*, the propagation by seeds was the easier and more effective strategy.

Propagules from *Bruguiera cylindrica*, which come from Indonesia, grew slowly and 9 of 23 plants were put in the fog house to regenerate. The propagation of *B. cylindrica* by cuttings was also not very productive (2 of 10 cuttings rooted) (Table 2). For *Lumnitzera racemosa*, 66 cuttings were prepared and placed in the fog house for 13 weeks, and 18 cuttings were grown in the green house without further treatment. Cuttings of *L. racemosa* from the fog house showed a much higher rooting rate (95%) than cuttings grown in the normal greenhouse (50%) (Table 2).

3.3. Identification of suitable culturing conditions and influence of salinity

For *Avicennia germinans* and *Laguncularia racemosa* sand, soil or a mixture of both, provided good growing media. Watering the plants without letting the soil dry worked well for *A. germinans* and *L. racemosa*. *Bruguiera cylindrica* plants grew best in a sand/soil mixture of 1:2 when watered only 1–2 times per week, depending on the ambient temperature. Nonetheless, *B. cylindrica* plants grown from propagules showed slow growth and yellow leaves, even though the plants were supplied with nutrients through regular fertilization.

All mangrove plants were regularly watered with saline water (10 PSU) every 4 weeks, which lead to typical morphological adaptations to salinity. On *Avicennia* leaves, small salt crystals were visible on the top of the leaves, whereas *L. racemosa* excretes salt-enriched sap through salt glands close to the petiole. To find out more about the influence of higher salinity on the plant growth, different salt concentrations

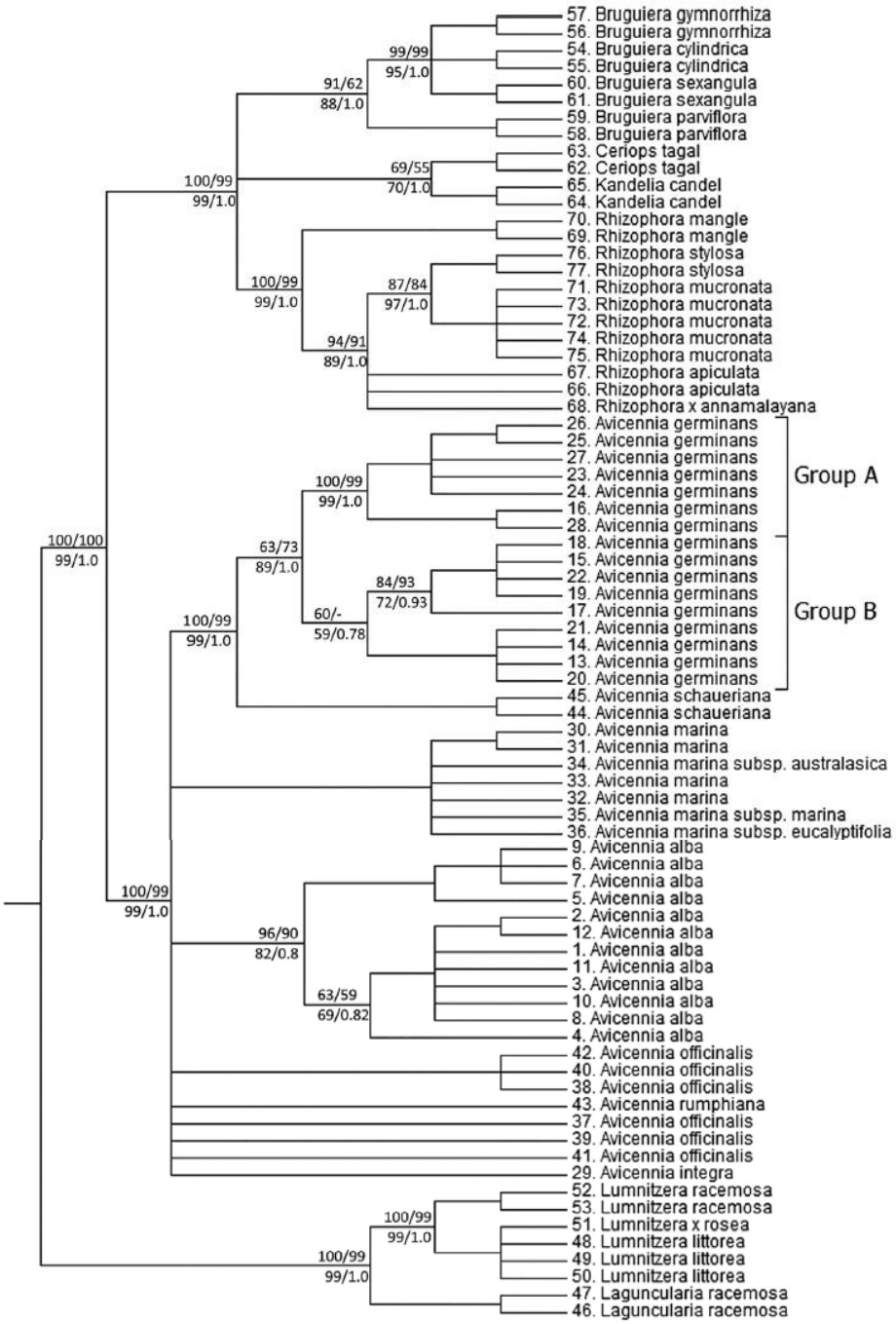

Fig. 2. Phylogeny of members of mangroves inferred from Bayesian Inference, maximum likelihood, neighbor joining and maximum parsimony. The data set based on 600 bp (including gaps) of nrDNA sequences comprising ITS-1, 5.8SrDNA and ITS-2. The posterior probability and bootstrap values of each method are shown in each node: above nodes, left: Bayesian Inference, right: Maximum Likelihood; below nodes, left: Maximum Parsimony, right: Neighbor Joining. Species names printed in bold were this study. Group A: Pacific clone; Group B: North Atlantic clone. See Table 1 for the number in front of each taxon.

were tested on *A. germinans* (15, 30 PSU) and *L. racemosa* (15, 30, 45 PSU). Leaves and shoots of *A. germinans* plants showed a significantly lower growth rate at 30 PSU compared to 15 PSU (Fig. 3A). Complete plant biomass of *L. racemosa* showed no difference after two weeks of growth. After four weeks, plants grown at 15 PSU had a significantly higher biomass weight compared to 30 and 45 PSU. At the last measurement after 6 weeks, plants treated with 15 and 30 PSU both had a higher weight than when grown at 45 PSU (Fig. 3B).

Table 2
Growth conditions and rooting rates of cuttings from *A. germinans*, *L. racemosa* and *B. cylindrica*.

Species	Cuttings	Fog House [weeks]	Hood [weeks]	Rooted	% rooting
A. germinans	200	–	–	10	5
A. germinans	52	13	8	6	12
A. germinans	9 (long)	–	–	3	33
A. germinans	9 (long)	13	8	5	56
L. racemosa	18	–	–	9	50
L. racemosa	66	13	8	63	95
B. cylindrica	10	8	8	2	20

3.4. Comparison of secondary compounds from naturally and greenhouse-grown mangrove plants

Leaf extracts from *Avicennia germinans* plants grown in the greenhouse were compared to those from naturally grown plants from Guatemala. The extracted secondary metabolites were analyzed by LC–MS and the resulting peaks identified with database comparison (Figs. 4 and 5, Table 3). A total of 32 compounds were detected. For the compounds producing peaks 2, 20 (kaempferol-3-glucoside), 21 (kaempferol-3-glucoside-3-rhamnoside) and 29 (kaempferide) the content in plants from Guatemala was significantly higher than in greenhouse-grown plants. The content of the compounds producing peaks 6, 9 and 23 was higher in greenhouse plants than in the samples from Guatemala. The compound concentration in the six individual samples of *A. germinans* grown outdoors or in the greenhouse was variable, resulting in high standard deviations like for peak 22.

Eight compounds were found only in extracts from greenhouse plants. Peaks were considered as present if more than four samples presented the peak at a height at least 3× above the background value. Two were identified as sugars, namely galactinol and modified palatinose monohydrate. The flavonoids flavanomarein, prunin and a modified form of marein were found. Two peaks were identified as modified forms of eriodyctiol-7-O-glucoside. One compound was identified as a gentisic acid derivate. Leaf extracts from Guatemalan samples comprised three unknown compounds, which did not occur in greenhouse samples that could not be further identified with the help of the databases.

The quantification of the two flavonoids naringenin and rutin is shown in Fig. 6. Calibration curves for naringenin and rutin were linear through zero with $y = 8.714e^5$ x, r = 0.999 and $y = 1.241e^5$ x, r = 0.999, respectively. Naringenin showed very low concentrations in samples from Guatemala, with values between 0.001 and 0.006 µmol g^{-1} DW. In greenhouse plants two and three, the values were comparably low. Greenhouse plants one, four, five and six contained concentrations between 0.191 and 1.234 µmol g^{-1} DW. Rutin was present in low concentrations in half of the samples from Guatemala (0.018–0.025 µmol g^{-1} DW) and in higher concentrations in the other half (0.553–1.231 µmol g^{-1} DW). In the greenhouse plants, all samples had higher rutin concentrations between 0.169 and 0.835 µmol g^{-1} DW.

3.5. Elemental composition

Results of the elemental analysis using ICP-OES are shown for twelve essential elements (Fig. 7 A, B). In leaf samples collected in Guatemala, iron, sodium, sulfur and magnesium were detected in higher amounts compared to the greenhouse samples, but only the latter being statistically significant (2.54/0.89 mg g^{-1} DM). The leaf samples from the greenhouse were significantly richer in calcium (16.93/6.38 mg g^{-1} DM), manganese (0.15/0.07 mg g^{-1} DM), phosphorus (7.21/3.97 mg g^{-1} DM), and zinc (0.04/0.01 mg g^{-1} DM).

4. Discussion

The use of a DNA marker is an advantageous method to identify the phylogenetic affiliation of an organism. In this work, phylogenetic analysis using just one molecular marker was applied for characterizing different mangrove species collected at various sites. The aim was to apply a fast and cheap identification method of plant species already identified based on morphological characters. The different mangrove species analyzed were chosen in a way that the broad distribution of *Avicennia* species is covered, including the Atlantic Caribbean East-Pacific (ACEP) and the Indo West-Pacific (IWP) region. Samples from the Acanthaceae family were misidentified in some cases based solely on the analysis of morphological traits. *A. marina* collected in Bangladesh was found to be *A. alba* instead. One plant from the greenhouse in the Institute of Botany, which was designated as *A. marina*, was also misidentified and belongs to *A. germinans* "Caribbean sea clone". *A. marina* and the closely related species *A. alba*, *A. officinalis* and *A. germinans* have a very similar leaf and flower morphology, which makes them not easy to distinguish. Similarly, two *Rhizophora* subspecies from India were identified as *R. mucronata* instead of *R. x annamalayana* and *R. apiculata*. However, the use of a multilocus marker system (Saddhe et al., 2017) or a different marker system, such as amplified fragment length polymorphisms (AFLP) (Garcia et al., 2004), might result in a better resolution but is also more time consuming and expensive. The ITS analysis clearly

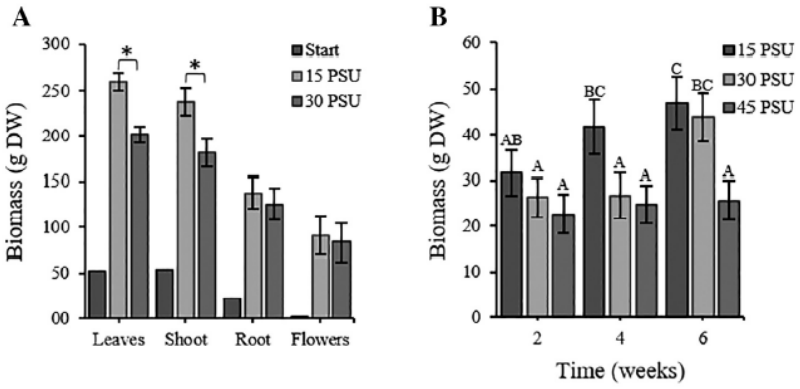

Fig. 3. Growth of *A. germinans* plants growing in different salt concentrations (A). One plant was harvested in the beginning of the experiment and the dry weight of leaves, shoot, root and flowers was measured (Start). For each treatment, three plants were grown in containers at 15 and 30 PSU. After 20 weeks, the dry weight was also measured. A star above the bar indicates a significant difference ($p < .05$) tested by an ANOVA analysis. Biomass of *L. racemosa* grown at different salt concentrations (B). The plants have been grown at 15, 30 and 45 PSU and three plants of each treatment were harvested after 2, 4 and 6 weeks. The plant material was dried for 4 d at 85 °C and then the dry weight was measured. Means with a common letter are not significantly different ($p > .05$) according to ANOVA results with Duncan alpha test.

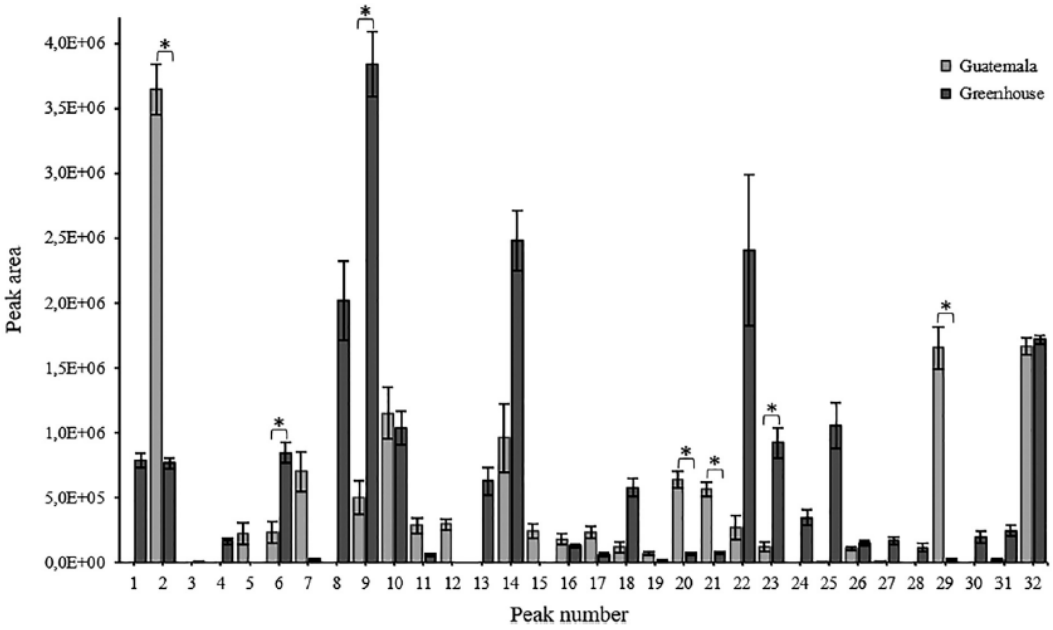

Fig. 4. Relative content of metabolites in *A. germinans* leaf samples from Guatemala and the greenhouse. Six samples each were extracted with MeOH 80% and analyzed by LC–MS. The mean peak area and standard error are shown. Statistically significant difference with $p < .05$ tested by t-test is indicated with a star above the bar.

shows also the division of *A. germinans* into two groups: the Pacific and the Caribbean sea clone. These results provide valuable information about the origin of mangroves grown in the greenhouse and from different collection sites, as it was found that on-site species identification could be incorrect.

The natural habitat of mangroves is characterized by complex environmental factors. Tide, water currents, strong winds and high solar radiation cannot be imitated in a greenhouse. Nevertheless, it was shown that cultivation of mangroves in the greenhouse in temperate zones is possible: *A. germinans* and *L. racemosa* were successfully grown and propagated in our greenhouse. Plants grown from propagules of *Bruguiera cylindrica* can also be grown in a greenhouse in the temperate zone, but further optimization is necessary.

Many findings in the search for optimized growth conditions for mangroves in the greenhouse were found by chance and observation of plant growth over the last 10 years. For all three species examined

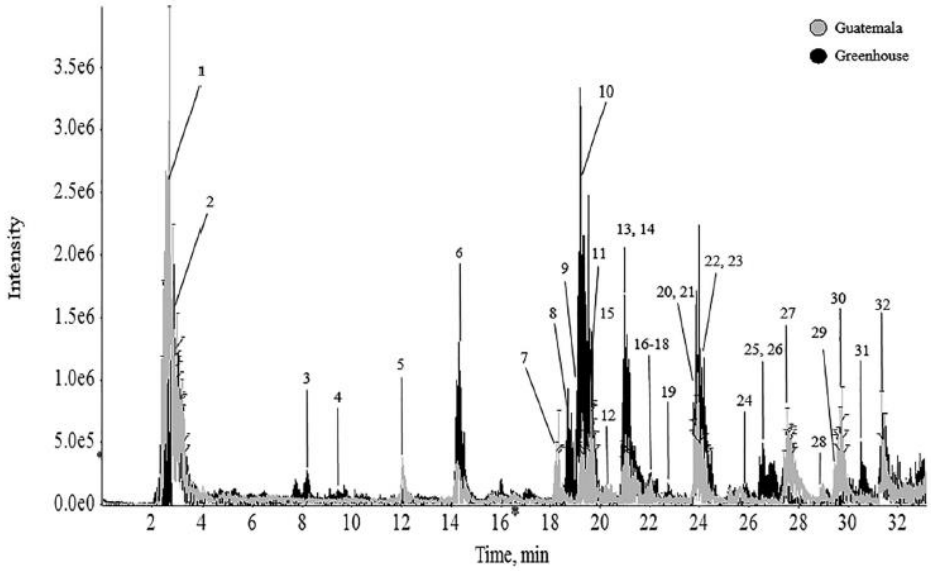

Fig. 5. Total ion current (TIC) chromatogram from LC–MS in negative electrospray ionization. Individual chromatograms for six samples from Guatemala and the greenhouse were summed up. Numbers indicate peaks described in Table 3.

Table 3
Compounds identified in *A. germinans* leaf extracts by database comparison of MS/MS fragment masses. No = number of peak, RT = retention time, Mass = mass of precursor ion in Da, MS/MS = fragment masses obtained at −30 eV collision energy in Da, n. i. = not identified, mod. = modified.

No	RT	Mass	MS/MS	Name	Accession	Database
Guatemala and greenhouse						
2	3.1	201.02	157.02, 59.01	n. i.	–	–
6	14.2	703.16	623.21, 541.13, 461.17	n. i.	–	–
7	18.2	461.07	285.04, 175.03, 133.02	Luteolin mod.	PT204040	ReSpect
9	19.2	623.21	461.17, 161.02,	n. i.	–	–
10	19.6	521.17	357.12, 213.07, 163.04, 145.02, 119.04	n. i.	–	–
11	20.2	475.09	299.04, 284.02, 175.01, 113.02	Kaempferide mod.	PS040309	ReSpect
14	21.2	623.21	461.17, 161.02	n. i.	–	–
16	21.8	609.15	300.01	Rutin	PS045410	ReSpect
17	22.0	463.09	300.01, 271.01, 255.01	Hyperoside	PT204320	ReSpect
18	22.1	535.15	329.08, 179.02, 135.04	Caffeic acid derivative	PS044608	ReSpect
19	23.0	489.10	285.02, 255.01, 227.02	Kaempferol-3-glucoside mod.	PT209270	ReSpect
20	23.8	447.09	285.02, 255.01, 227.02	Kaempferol-3-glucoside	PT209270	ReSpect
21	23.8	593.15	285.02, 255.02	Kaempferol-3-glucoside-3-rhamnoside	PT209200	ReSpect
22	24.0	519.16	313.11, 193.05, 163.04, 149.06	Sinapic acid derivative	PT210880	ReSpect
23	24.2	549.17	343.12, 325.11, 193.05, 175.04, 149.06	Sinapic acid derivative	PT210880	ReSpect
25	26.5	271.06	177.01, 151.00, 119.04, 107.01	Naringenin	PS040709	ReSpect
26	26.5	301.03	285.02, 257.03	Hesperetin	PS078009	ReSpect
27	27.5	541.24	379.18, 355.10, 335.18, 193.05, 185.11, 175.04, 149.06	Sinapic acid derivative	PT210880	ReSpect
29	29.6	299.06	284.02, 256.02	Kaempferide	PS040309	ReSpect
31	30.5	531.19	357.10, 195.05, 173.05, 151.07	n. i.	–	–
32	31.6	293.18	236.10, 221.15, 192.11	n. i.	–	–
Only greenhouse						
1	2.8	341.11	179.05, 119.03, 89.02, 71.01	Galactinol	PT211910	ReSpect
3	8.2	373.11	211.06, 167.07, 149.06, 123.04	Gentisic acid derivative	PS055907	ReSpect
4	9.4	353.14	221.1, 179, 161.04, 101.02	Palatinose monohydrate mod.	PT212460	ReSpect
8	18.7	449.11	287.05, 151.00, 135.04	Flavanomarein	PS084609	ReSpect
13	21.1	433.12	271.06, 151.00, 119.05	Prunin (naringenin-7-O-glucoside)	PR040149	MassBank
24	25.8	597.17	287.05, 151.00, 135.04	Marein mod.	PR100806	MassBank
28	29.1	615.22	449.11, 287.05, 151.00	Eriodyctiol-7-O-glucoside mod.	PR040090	MassBank
30	29.9	617.23	287.06, 151.00	Eriodyctiol-7-O-glucoside mod.	PR040090	MassBank
Only Guatemala						
5	12.0	637.08	351.04, 285.03	n. i.	–	–
12	20.5	291.13	96.96	n. i.	–	–
15	21.3	289.11	96.96	n. i.	–	–

for this work, it was found that both sand and soil or a mixture of both can be used as a base. Soil offers more stability for the plants, while sand is useful for experiments in which the roots are weighed, as the sand can be washed off more easily. Websites for commercially available mangroves also recommend sand mixtures rather than soil (mangrove.at).

Adding low amounts of salt did not affect plant growth, only concentrations higher than 15 PSU lead to a reduced growth rate for *A. germinans* and *L. racemosa* plants. In a study on *R. mangle*, it was shown that salinity of 15 ppt had no significant influence on plant growth compared to fresh water (Doyle, 2003). In another study, *A. germinans* plants had a lower photosynthesis rate when grown at increasing salinities (López-Hoffman et al., 2007). Stress is known to induce the biosynthesis of plant secondary metabolites and increases the overall yield of secondary compounds (Boestfleisch and Papenbrock, 2017).

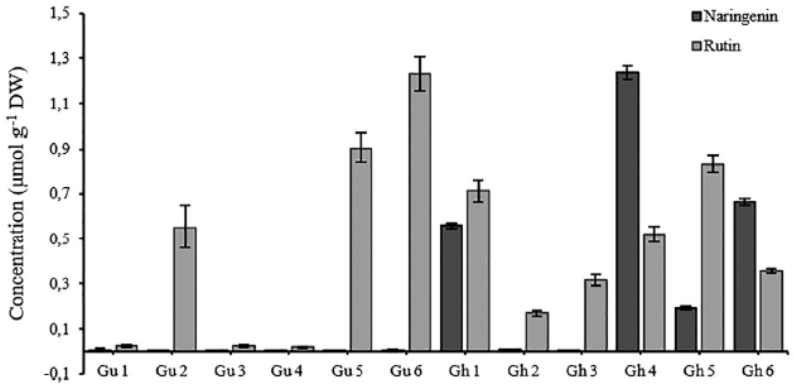

Fig. 6. Quantification of rutin and naringenin in *A. germinans* leaf extracts from plants collected in Guatemala (Gu) and the greenhouse (Gh) given in µmol g^{-1} dry weight (DW). Means and standard deviation are shown from three individual measurements in negative electrospray ionization mode. Concentrations of rutin and naringenin were calculated according to calibration curves.

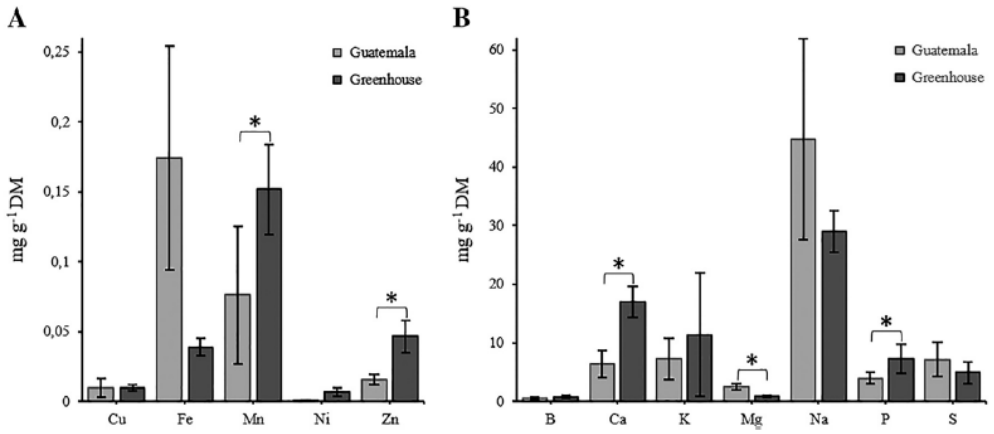

Fig. 7. Elemental composition of leaf material from samples collected in Guatemala and in the greenhouse measured by ICP-OES in mg g^{-1} dry mass. Mean values of six individual *A. germinans* plant samples from Guatemala and the greenhouse in Hannover, Germany, are shown for elements with low concentrations (A) and high concentrations (B). A star above the bar indicates statistically significant difference with $p < .05$ analyzed by *t*-test.

Reforestation of mangrove forests is achieved by growing plants in nurseries from seeds or by cuttings from mature trees (Eganathan et al., 2000). The costs of this investment are outweighed by the benefits of re-planted mangrove forests (Tuan and Tinh, 2013). For the propagation of greenhouse-grown mangroves, rooting rates of up to 95% can be obtained (in this study, for *L. racemosa* cuttings grown in a fog house for 13 weeks before transfer into the greenhouse). The rooting rate of *A. germinans* was also improved by growing cuttings in the fog house (12/56%). In contrast, *B. cylindrica* cuttings showed a low survival rate of 20%, despite also being grown in the fog house. The fog house helped to provide the high humidity, which is found in the natural habitat of mangroves. The use of fog to improve the rooting and survival of cuttings has been described in the literature and is a recommended procedure for woody species (Milbocker, 1983). The sensitive cuttings can build roots more easily and can later be adapted to the greenhouse conditions with less humidity.

Vegetative propagation or in vitro multiplication are likely to be more reliable than seed propagation of mangroves. Reproduction of *A. germinans* was also achieved by seeds, the plants are able to cross-pollinate via insects in the greenhouse (flies and bees) or possibly self-pollinate (Kathiresan and Bingham, 2001). *Laguncularia racemosa* has a variable pollination system: plants can be either male or hermaphroditic, so that self-pollination can occur (Landry et al., 2009). The species flowered in the greenhouse but did not set seed, suggesting that the plants have male flowers or the physiological conditions are suboptimal. *Bruguiera cylindrica* plants did not flower in the greenhouse during this study. The overall low growth rate and yellowing of leaves could be caused by a suboptimal nutrient composition, even though essential macro- and micronutrients were supplied by fertilization or the absence of essential endophytic or mycorrhizal fungi, which could be essential for nutrient uptake and availability (D'Souza and Rodrigues, 2013). In vitro propagation remains a possibility to reproduce mangroves, if cuttings have a low survival rate and there are no seeds available. Cousins and Saenger (2002) have investigated in vitro propagation of *A. marina* on different media, but only with minor success, and further tests on a variety of species are needed.

Mangrove species are valuable sources of secondary metabolites with potential for new drug development (Wu et al., 2008). Diseases like rheumatism and throat pains are treated with *A. germinans* bark and leaves in folk medicine (Bandaranayake, 2002; Table A.1). The active compounds, which are responsible for the effectiveness, are in many cases still unknown (Spalding et al., 2010). LC–MS analysis of methanolic extracts from *A. germinans* grown in the greenhouse and from a natural habitat in Guatemala revealed the presence of mainly phenolic secondary metabolites. One caffeic acid derivative and three sinapic acid derivatives could not be identified. Several polyphenolic compounds were detected, including eight flavonols (kaempferide modified, kaempferol-3-glucoside modified, kaempferol-3-glucoside-3-rhamnoside, kaempferide, rutin, hyperoside), two flavanones (naringenin and hesperetin) and one flavone (luteolin modified). Five peaks of the chromatograms could not be identified in this way.

Two of the substances identified in both wild and greenhouse-grown sample groups of *A. germinans*, namely naringenin and rutin, show a high discrepancy in their content in individual plant samples. In the class of flavonoids, naringenin belongs to the subgroup of flavanones and rutin to the flavonols as a glycosidic form of quercetin (Zhang et al., 2013). These compound classes play an important role for plants, e.g. in UV protection and plant defense against herbivores (Simmonds, 2003). The concentration of quercetin and rutin as well as their biosynthesis activity has been found to be increased by water, salt and UV-B stress (Lucci and Mazzafera, 2009; Kreft et al., 2002). In this context, differing concentrations can be explained by the stress factors affecting each individual plant or the collected leaves. In the greenhouse, those factors are more consistent, which is revealed by a more comparable content of the two metabolites. Here, naringenin was present in four out of six samples and rutin in all, whereas in samples from Guatemala rutin was only present in three out of six samples. Naringenin is a precursor in the biosynthesis of flavones and flavonols such as apigenin and kaempferol (Zhang et al., 2013). The low concentration of naringenin in samples from Guatemala matches to higher concentrations of kaempferol derivatives represented by peaks 19, 20, 21 and 29.

Eight compounds were detected only in the samples of *A. germinans* plants grown in the greenhouse. Two of these were identified as sugars (galactinol and palatinose monohydrate modified), whereas the other six belong to the group of flavanones (flavanomarein, prunin, eriodyctiol-7-O-glucoside modified) and chalcones (marein). This indicates a higher diversity of flavanones in the greenhouse plants compared to naturally growing plants, which comprise only of two flavanones. In the plant samples from Guatemala, three unidentified peaks might belong to a group of phenolic compounds as well, and are possibly not yet characterized in the literature. In total, the majority of compounds was found in the greenhouse samples as well as in samples collected in Guatemala, which speaks for a comparable composition even though they grow in different environments. An advantage of plant material from the greenhouse is the stable production of secondary metabolites under controlled conditions, as shown for the medicinal plant perilla (Lu et al., 2017).

The elemental composition of *A. germinans* leaf samples from Guatemala and the greenhouse was measured by ICP-OES for 25 elements. Of these, 13 were detected only in trace amounts and are not discussed. From the remaining 12, 5 showed significant differences between the two sample groups. Magnesium was more abundant in the samples collected in Guatemala. In saline soils, magnesium belongs to the main cations, together with sodium and calcium (Szabolcs, 1989). This can explain the higher concentration of magnesium, even though the calcium content is lower compared to greenhouse plants. The nutrient availability can also vary strongly in individual mangrove forests, depending on, e.g. soil texture, microbial activity and plant species (Boto and Wellington, 1984; Reef et al., 2010).

In greenhouse leaf material, the four elements manganese, zinc, calcium and phosphorus were found in significantly higher concentrations compared to the samples from Guatemala. Mangrove soils in general have a low nutrient content (Alongi et al., 1992). In the greenhouse experiments, all elements are provided in high amounts in regular fertilization. In addition, sodium was found in higher concentrations in outside grown mangroves (44.7 compared to 29.0 mg g^{-1} DM) but with no statistical significance between the two sample groups. This can be explained by the growth in saline water, as *A. germinans* takes up salt and excretes it through salt glands on the upside of the leaves. The stable supply of nutrients is an advantage for the propagation of mangroves in the greenhouse. Together with the gained knowledge about optimal growth parameters, mangrove plant material can be harvested throughout the whole year for further studies on mangrove physiology and secondary metabolites.

Contributions

YG cultivated the mangroves and performed the secondary compound analysis. YG, IK and XVN did the phylogenetic analysis. YG, IK, XVN and JP evaluated the data. YG, XVN and JP wrote the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sajb.2018.11.020.

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Sustainable use of mangroves as sources of valuable medicinal compounds: Species identification, propagation and secondary metabolite composition- Appendix A

South African Journal of Botany

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Table A.1 Mangrove families analyzed in this work, total number of species in each, distribution, traditional use and conservation status.

Mangrove Family	No of species [1]	Distribution [1]	Traditional use	IUCN Red List status [5]
Acanthaceae	8	Mangroves throughout the tropics, but also warm temperate	Skin diseases, tumors, ulcers, rheumatism, parasites[2]	Least concern Trend: decreasing or unknown *A. bicolor*: Vulnerable *A. integra*: Vulnerable *A. rumphiana*: Vulnerable *A. lanata*: Vulnerable
Rhizophoraceae	17	Pantropical, centred on the eastern Indian Ocean, introduced into the central Pacific and Hawaii	Bowel disorders, astringent effect [3]	Least concern Trend: decreasing *Bruguiera hainesii*: Critically Endangered *Rhizophora samoensis*: Near threatened
Combretaceae	8	Tropical, often mangroves, esp. N. Australia	Herbicide, treatment of thrush, asthma, diabetes [4]	Least concern Trend: decreasing

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Chapter 3

Glasenapp, Y., Papenbrock, J. Effect of tea varieties and the flavonoid taxifolin on *Escherichia coli* colony growth and on the formation of extracellular polymeric substances. (In preparation)

Abstract

Microbial biofilms have been recognized as a major issue in medicine. The pathogenicity of bacteria like *Escherichia coli* is increased due to their ability to form biofilms. One important element in *E. coli* cell attachment and biofilm formation are amyloid curli fibers as one part of the extracellular polymeric substances (EPS). In the search for new treatments of biofilms, agents influencing different mechanisms and stages in biofilm development are of interest. Teas have a long tradition as a health promoting beverage and contain various plant secondary metabolites, of which many possess favorable properties. Of these, phenolic compounds like flavonoids are known to be bioactive, including antimicrobial activity. An extract from the mangrove *Laguncularia racemosa* showed biofilm inhibiting action against *Escherichia coli*, and taxifolin was identified as one of the phenolic constituents. The effect of different tea varieties and the flavonoid taxifolin on *E. coli* biofilm formation and curli fiber synthesis was analyzed. Reduction of curli production and biofilm growth was observed on agar plates supplemented with Congo red. The highest reduction of colony growth and curli fiber development was achieved with green tea. After six days of growth, hawthorn tea also presented biofilm reducing and curli inhibiting properties. Mate and peppermint tea showed moderate effects on curli production and no effect on colony size. The flavonoid taxifolin was able to reduce the colony size and intensity of curli staining, too. Tested on a curli negative *E. coli* mutant, all tea varieties and taxifolin inhibited growth as well, indicating an activity not limited to curli synthesis. These results underline the importance of natural plant products like teas and plant secondary metabolites as sources of bioactive compounds against biofilm formation.

1. Introduction

Biofilms are formations of microbial cells and an extracellular matrix attached to a solid surface. This form of growth provides protection from external influences and cells detaching from it can colonize new habitats. The matrix consists of proteins, lipids, polysaccharides and extracellular DNA (Flemming & Wingender 2010). The relevance of these structures in medicine and industry is high, since they have an increased resistance to antimicrobial agents and other techniques of removal (Hall-Stoodley et al. 2004).

Ongoing research provides more and more insights into individual biofilm formation mechanisms in bacteria and fungi. The biofilm development is divided into three main parts: Attachment, development and dispersal (O'Toole et al. 2000). In each stage, different physiological mechanisms are activated. In *Escherichia coli* commensal and pathogenic strains, one important structural element in biofilm formation is the production of curli fibers (Cegelski et al. 2009). Curli fibers are amyloid proteins consisting of a major and a minor subunit, encoded by the genes CsgA and CsgB (Evans & Chapman 2014). Curli fibers are located on the cell surface and mediate the attachment of bacterial cells to potential host cells and surfaces (Barnhart & Chapman 2006). They are an important virulence factor, as they are able to bind to many different host proteins, and therefore reach a better spreading in the host tissues (Bian et al. 2000). In the development to a mature biofilm they are functioning as a structural element as an extracellular polymeric substance (EPS).

In the search for new ways of treating infectious biofilms, different biofilm formation mechanisms can be targeted. One is the microbial cell-cell communication, which can induce the dispersion of planktonic cells and therefore prevent quorum sensing. Another target is the biofilm matrix, which is essential for a stable biofilm growth. Inhibition of curli synthesis in *E. coli* leads to a reduced attachment ability and influences the structure of the mature biofilm (Kikuchi et al. 2005). Therefore, curli inhibiting substances are of interest in the treatment of *E. coli* biofilm formation.

Bacteria producing curli cause a red staining on agar plates supplemented with the dye Congo red and in this way effects on curli synthesis can easily be monitored experimentally (Collinson et al. 1993).

Plant derived phenolic compounds are known to possess diverse medicinal properties, including anti-inflammatory and anti-biofilm activities (Huber et al. 2003, Cicerale et al. 2012). These active substances are secondary metabolites, fulfilling diverse tasks in the

plant: defense against herbivores, protection from UV radiation, attraction of pollinators and more (Selmar & Kleinwächter 2013). Flavonoids are a group of phenolic secondary metabolites widespread in plants, consisting of two aromatic rings connected by a C-3 chain (Pietta 2000). In tea prepared from plant leaves, bark, flowers or roots, the contained secondary compounds dissolve and are consumed. There are various positive effects associated with the consumption of different tea varieties. Polyphenols from black and green tea protect blood cells from oxidative damage (Grinberg at al. 1997). The positive effects of green tea on medical conditions like cancer and cardiovascular diseases have been studied intensively (Jian et al. 2004, Kuriyama et al. 2006). Yerba mate tea, prepared from the tree *Ilex paraguariensis* A.St.-Hil., is widely consumed in South America and has gained some popularity in Europe and other countries as well. It contains a high concentration of polyphenols, leading to an antioxidant activity and health promoting effects. While in green and black tea catechins are the major polyphenol constituents, mate contains no catechins, but a high concentration of chlorogenic acid (Heck & De Mejia 2007).

Individual secondary metabolites can possess very specific activities against bacterial growth and biofilm formation. The flavonoids naringenin, kaempferol, quercetin and apigenin have been reported to inhibit bacterial cell-cell signaling in *E. coli* and therefore inhibit biofilm formation (Vikram et al. 2010). The green tea polyphenol epigallocatechin gallate (EGCG) shows a high reduction of biofilm formation and curli synthesis in different commensal and pathogenic *E. coli* strains (Serra et al. 2016). In preliminary experiments with different mangrove extracts, the phenolic metabolites in the most active extract were analyzed. A number of flavonoids were identified, including taxifolin. This compound has also been reported to act biofilm-inhibitory by inhibiting quorum sensing in *Pseudomonas aeruginosa* and reducing biofilm adhesion in *E. coli* (Vandeputte et al. 2011, Glasenapp et al. 2018). In this work, the effect of different tea varieties and of the flavonoid taxifolin on *E. coli* biofilm colony growth and amyloid curli fiber production is evaluated.

2. Material and Methods

2.1 Bacterial strains

The *E. coli* strains were provided by Prof. Dr. Regine Hengge, Humboldt-Universität zu Berlin. As biofilm former and curli fiber positive strain, the K-12 strain W3110 (Hayashi et al. 2006) was used. In addition, the curli-fiber negative *E. coli* mutant AP303 csgBA::kan was used.

2.2 Test compounds

Different tea varieties and the flavonoid taxifolin were tested for their activity on *E. coli* W3110 colony growth and curli fiber production. Taxifolin was purchased from Sigma-Aldrich (St. Louis, USA). Hawthorn tea bags from the brand Bad Heilbrunner contained 2.0 g of finely cut hawthorn leaves and flowers. Mate tea from the same brand weighed 1.8 g per bag and consisted of green mate leaves. Green tea from Fujian Tea Import & Export Co, Ltd., contained 2.0 g per bag. Peppermint tea from Meßmer comprised of 2.25 g peppermint leaves.

2.3 Macrocolony growth and curli fiber assay

This method was adapted from Serra et al. (2016). An overnight culture of *E. coli* was prepared in 3 ml liquid Luria Bertani (LB) medium (Miller, 1972) and incubated at 37°C with constant shaking at 180 rpm. For *E. coli* AP303, the medium was supplemented with 25 µg/ml kanamycin. For each test condition 100 ml salt-free LB medium with 1.5% agarose was autoclaved and supplemented with 40 µg/ml Congo red (Sigma-Aldrich, St. Louis, USA) and 20 µg/ml Coomassie brilliant blue (Applichem, Darmstadt, Germany). Before pouring the medium into petri dishes, the defined volume of taxifolin dissolved in ethanol was added. Control plates were prepared without further modifications. For analyzing the effects of teas on colony growth, three bags of tea were incubated in 100 ml of boiling water for 10 min. Then, the bags were removed and the tea filtered through a 22 µm syringe filter (Roth, Karlsruhe, Germany). Ten milliliters of filtered tea were added to 100 ml LB medium. Three plates of each condition were spotted with 5 µl overnight culture, with three spots of *E. coli* W3110 and AP303 each. Plates were incubated at 28°C and pictures of the colonies were

taken at day one, two, three and six with a Panasonic Lumix DMC-FZ45 camera (Panasonic Corporation, Kadoma, Japan) fixed on a stand.

2.4 Evaluation of macrocolony development

Size and color intensity of the macrocolonies were analyzed using ImageJ (Schneider et al. 2012). To measure the colony size, the picture of a single colony was transferred into a binary picture and the outline of the colony was determined by a selection tool. The area was measured by a previously set scale according to a ruler lying on the plate. The color intensity of the Congo red stained curli fibers was analyzed by measuring the mean gray value of each colony and the background close to each colony. The mean value of three macrocolonies per plate was subtracted from the mean of all background values und divided by 2.55 (255 = white, 0 = black) to gain the result in percent absorbed light.

2.5 Statistical analysis

Statistical differences were analyzed with a two-tailed student t-test in SigmaPlot 13.0. Significant differences were characterized with $p<0.05$.

3. Results

3.1 Tests on *E. coli* curli positive strain W3110

The development of *E. coli* W3110 and AP303 macrocolonies was observed on Congo red agar plates. A red staining of the colonies shows the presence of amyloid curli fibers, and therefore indicates biofilm formation. Different tea varieties and the flavonoid taxifolin were tested for their influence on the growth, detected as colony size, and biofilm formation, as seen by the staining intensity. Green tea significantly reduced colony size and color intensity of *E. coli* W3110 compared to the positive control, showing a strongly reduced biofilm growth (Figure 1). After six days, control colonies reached a size of 1.68 ± 0.07 cm^2, whereas green tea treated colonies were 0.49 ± 0.01 cm^2 big.

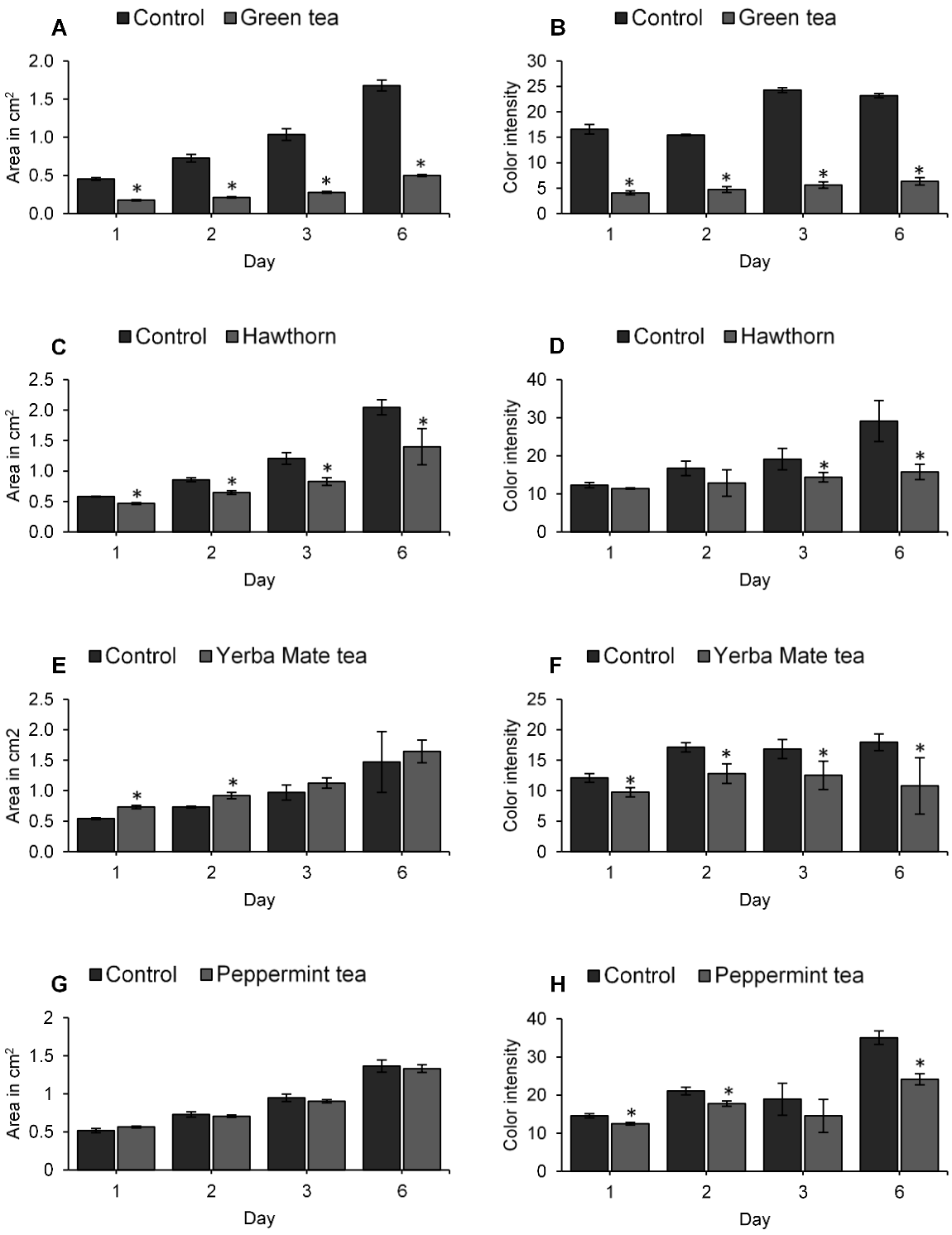

Figure 1. Macrocolony area in cm^2 (A, C, E, G) and color intensity in % absorbed light (B, D, F, H) of *E. coli* W3110 colonies in the presence of different tea varieties on agar plates supplemented with Congo red. On each plate, 5 µl of *E. coli* overnight culture were spotted three times. Four plates were prepared in three biological replicates. Plates were incubated at 28°C and pictures taken at day one, two, three and six.

Hawthorn tea reduced the colony size in all days of treatment. The biggest difference to the control conditions was observed on day 6 with a 31.6% smaller colony area. Reduction of curli fiber synthesis was observed on days three and six with a reduction of 24.7 and 45.8%, respectively, coloring intensity. Mate and peppermint tea did not influence the size of *E. coli* macrocolonies. The color intensity of colonies was significantly reduced on all days. Peppermint tea led to a reduced curli fiber production on day one, two and six.

The flavonoid taxifolin was tested in concentrations of 100 and 200 µg/ml on *E. coli* W3110 macrocolony biofilm formation and Congo red staining. Both concentrations reduced the colony area significantly (Figure 2). Curli fiber intensity was not affected on day one, two and three, but on day six a reduction of 19.4 and 19.7% at 100 and 200 µg/ml, respectively, was observed compared to the positive control.

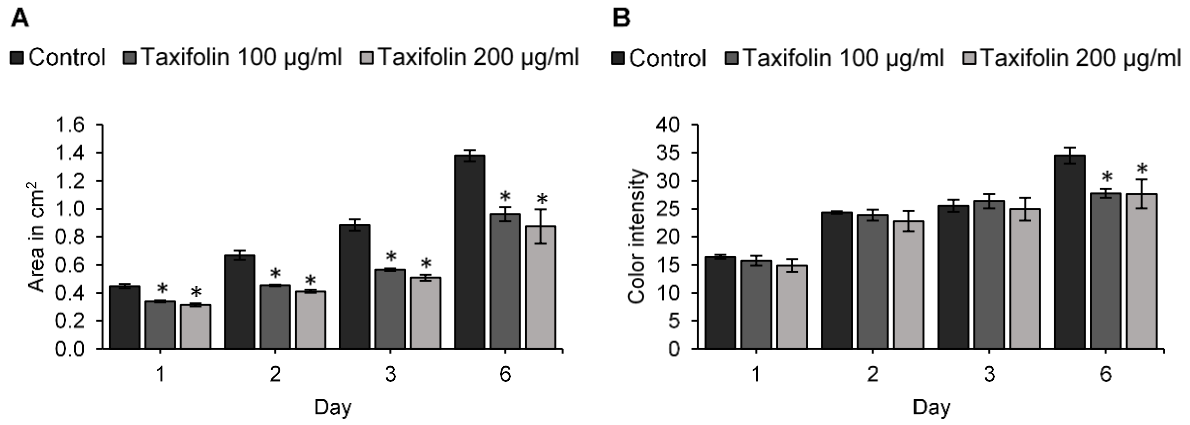

Figure 2. Macrocolony area in cm^2 (A) and color intensity in % absorbed light (B) of *E. coli* W3110 colonies in the presence of 100 and 200 µg ml^{-1} taxifolin on agar plates supplemented with Congo red. On each plate, 5 µl of *E. coli* overnight culture were spotted three times. Three plates were prepared in three biological replicates. Plates were incubated at 28°C and pictures taken at day one, two, three and six.

Pictures of the macrocolonies were taken on day one, two three and six. After six days of growth at 28°C, *E. coli* W3110 control colonies appeared in a deep red color. In the center of the colony, a small ring was visible, marking the drop of the 5 µl *E. coli* culture brought up on the plate. From this inoculation area, the biofilm spread out circular and ridges are visible in case of the positive control. As an example, colonies grown on plates in the presence of taxifolin in concentrations of 100 and 200 µg/ml are shown in Figure 3. A concentration of 100 µg/ml led to a reduced size and a less deep coloring. The effect on colony morphology was stronger at a concentration of 200 µg/ml. Here, the size was reduced to about 50% and

color intensity was very dim, with a light-orange tone. Interestingly, the center of the colony remained in a slightly stronger color, whereas the outer ring showed the lightest color.

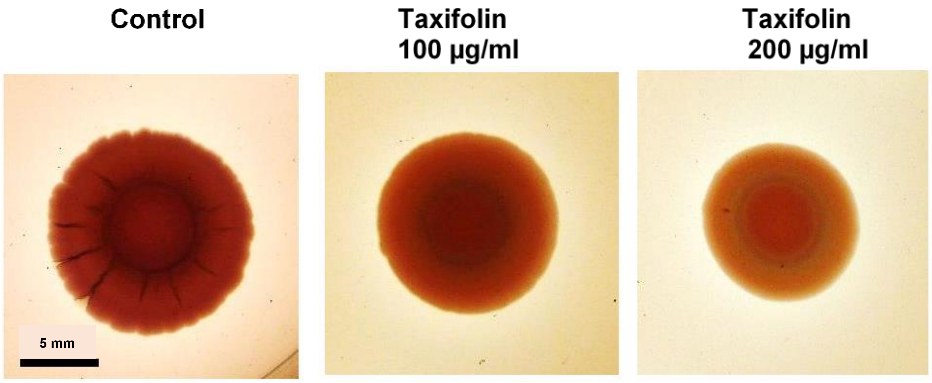

Figure 3. *E. coli* W3110 macrocolonies on Congo red supplemented agar plates in the presence of 100 and 200 µg/ml taxifolin after 6 days of growth at 28°C.

3.2 Tests on *E. coli* curli negative mutant AP303

The same tea varieties and the flavonoid taxifolin were tested on the curli-fiber negative *E. coli* mutant AP303 csgBA::kan. The results of green tea supplemented plates showed comparable results as tested on *E. coli* W3110 (Figure 4). After six days of growth, colony size of treated colonies had a size of 0.61 ± 0.02 cm^2, control colonies reached 2.02 ± 0.19 cm^2. Similarly, hawthorn tea also led to a reduction in colony size on all days of growth, and a significant lower coloring intensity was observed on day six with 15.92 ± 1.76% absorbed light compared to 21.78 ± 3.20 in the positive control. On day one and two of growth, yerba mate tea increased the colony size compared to the positive control. The intensity of color was reduced on all days of growth. Peppermint tea did not influence the colony area, but led to a lower color intensity on all days. The maximal effect on color intensity was observed on day six, with 12.22 ± 1.80% absorbed light compared to 20.05 ± 0.82% in the control.

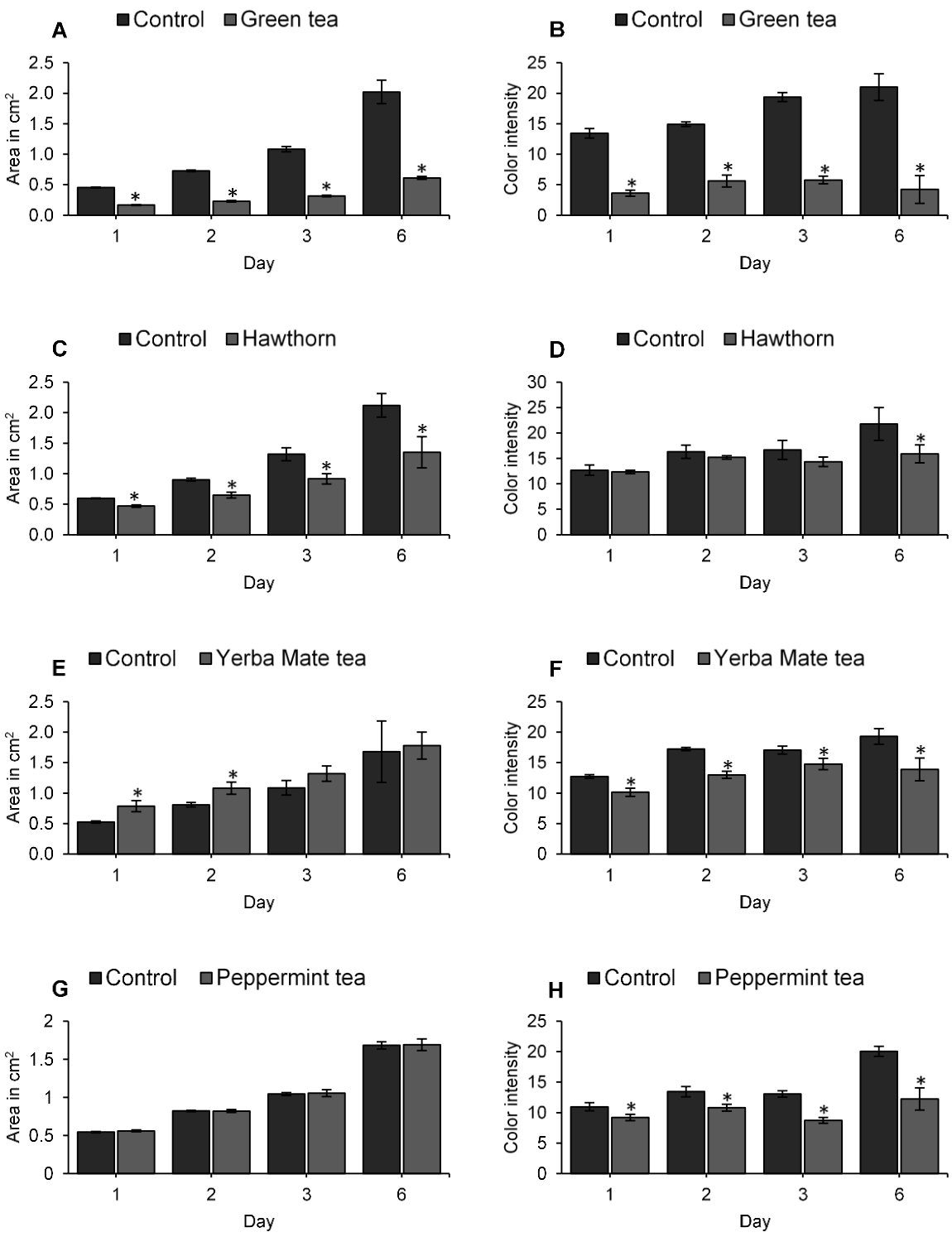

Figure 4. Macrocolony area in cm^2 (A, C, E, G) and color intensity in % absorbed light (B, D, F, H) of *E. coli* AP303 colonies in the presence of different tea varieties on agar plates supplemented with Congo red. On each plate, 5 µl of *E. coli* overnight culture were spotted three times. Four plates were prepared in three biological replicates. Plates were incubated at 28°C and pictures taken at day one, two, three and six.

Taxifolin reduced the colony area during the complete time of the experiment. After six days, colony size on plates with 100 µg/ml taxifolin was 1.05 ± 0.05 cm^2 and 0.90 ± 0.05 cm^2 on plates with 200 µg/ml, whereas the control colonies grew to 1.70 ± 0.15 cm^2 (Figure 5). The intensity of colony coloring was reduced by 100 µg/ml taxifolin on day one and by both concentrations on day six.

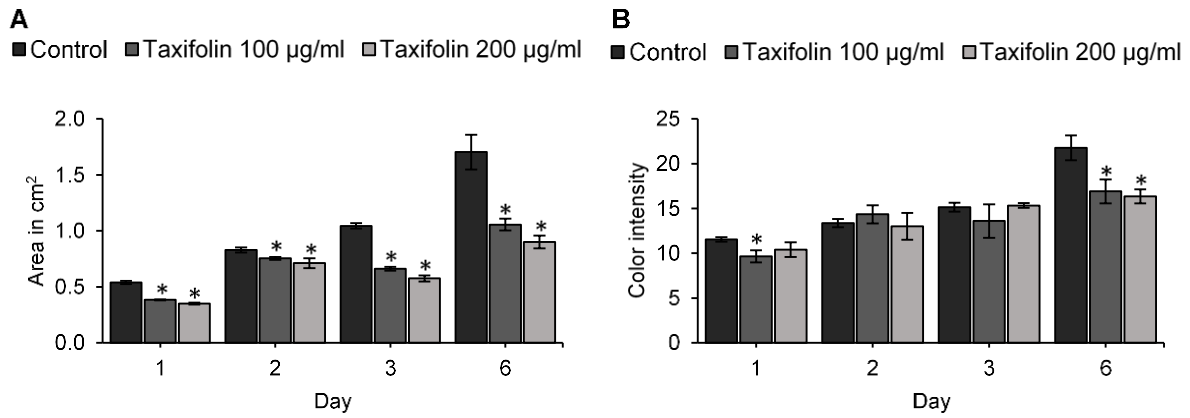

Figure 5. Macrocolony area in cm^2 (A) and color intensity in % absorbed light (B) of *E. coli* AP303 colonies in the presence of 100 and 200 µg ml^{-1} taxifolin on agar plates supplemented with Congo red. On each plate, 5 µl of *E. coli* overnight culture were spotted three times. Three plates were prepared in three biological replicates. Plates were incubated at 28°C and pictures taken at day one, two, three and six.

Pictures of *E. coli* AP303 macrocolonies were taken on day one, two, three and six, in parallel with *E. coli* W3110 colonies. In the center of the colony, an orange-brown ring was visible, marking the drop of the 5 µl culture brought up on the plate (Figure 6). From this inoculation area, the biofilm spread out, forming prominent waveforms in the positive control and smaller ones with slight notches on the treated plates. As an example, colonies grown on plates in the presence of taxifolin in concentrations of 100 and 200 µg/ml are shown in Figure 6. A concentration of 100 µg/ml led to a reduced size and the ring in the center of the colony was just slightly darkened. At 200 µg/ml taxifolin, the size was reduced and color intensity in the center was even lower.

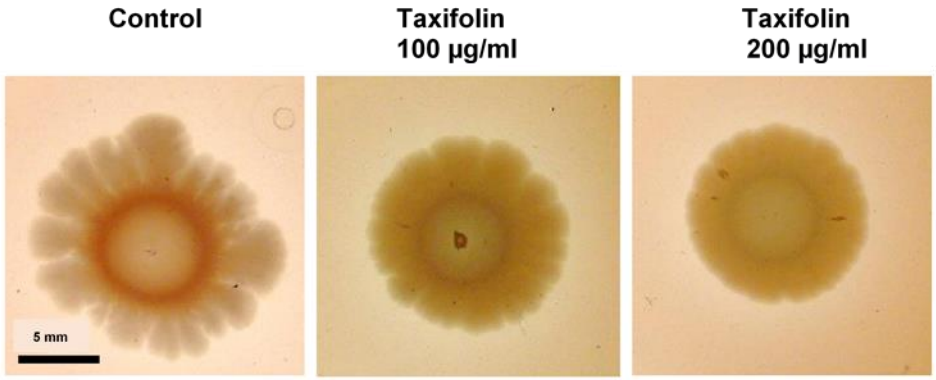

Figure 6. *E. coli* AP303 macrocolonies on Congo red supplemented agar plates in the presence of 100 and 200 µg/ml taxifolin after 6 days of growth at 28°C.

4. Discussion

The tested tea varieties presented a variable efficacy against *E. coli* W3110 biofilm formation and amyloid curli fiber production. Highest reduction rates were achieved with green tea. Many articles have already described the activity of green tea against *E. coli* biofilm formation, but also on other bacterial species. The active compounds causing this effect are catechins. Serra et al. (2016) have compared different catechins for their inhibitory activity, and found epigallocatechin gallate and epicatechin gallate to be the most efficient catechins. Compared to the other catechins present, epigallocatechin and epicatechin, the active compounds possess a gallate moiety, which was demonstrated to be needed for an inhibitory effect.

Hawthorn tea was found to reduce biofilm size on all days of growth, and significant reduction in curli synthesis was observed after three and six days. The main components of plant secondary metabolites in hawthorn are various flavonoids, chlorogenic acids and triterpenes (Nabavi et al. 2015). Preparations from hawthorn leaves and fruits are traditionally used to treat cardiovascular conditions (Rigelsky & Sweet 2002). The positive effect is caused by a reduction of inflammation, hypertension and thrombosis, which can be related to the contained antioxidants such as epicatechin, hyperoside, and chlorogenic acid (Kirakosyan et al. 2003). These compounds are water soluble and therefore present in the hawthorn tea. Ethanolic extracts of hawthorn berries have shown to act moderately bactericidal against the Gram-positive bacteria *Micrococcus flavus*, *Bacillus subtilis*, and *Lysteria monocytogenes* (Tadić et al. 2008). The extract contained 3.54% phenolic

compounds, 0.44% procyanidins, 0.18% flavonoid aglycones and 0.14% of hyperoside, the main flavonoid. The bioactive compound with antibacterial properties was not identified. In another study by Kostić et al. (2012), antimicrobial activity of an ethanolic fruit extract against *E. coli*, *P. aeruginosa*, *Salmonella abony* and *Candida albicans* (Kostić et al. 2012) was observed. The identification of possible antimicrobial substances remains to be achieved.

Mate and peppermint tea were not able to reduce *E. coli* macrocolony size. In case of mate, in the first two days of colony growth there was an increase in colony area compared to the control. The production of curli fibers as visible from a reduced coloring intensity was achieved on all days of growth in case of mate tea and on days one, two and six in case of peppermint tea. Mate tea is rich in polyphenols, xanthines and saponins. The main polyphenols are chlorogenic acid and its derivatives, caffeic acid, and flavonoids like quercetin, rutin and kaempferol (Heck et al. 2008). Xanthines include theophylline, theobromine, and caffeine (Athayde et al. 2000). The latter is responsible for the stimulating effect of mate tea. The bitter taste is caused by saponins, the most prominent ones are called matesaponins 1, 3, 3, 4 and 5 (Gnoatto et al. 2005). In contrast to the results obtained in this work, an antimicrobial activity of Yerba Mate aqueous extracts against *E.coli* was described by Burris et al. (2011). On the other hand, Martin et al. (2012) did not find activity of alcoholic mate extracts against *E. coli* growth. Differences in these studies are the strains of *E. coli* and the antimicrobial assay used. Burris et al. (2011) used a time kill assay to determine the antibacterial activity of Yerba Mate extracts, in which *E. coli* was grown in a suspension culture in the presence of the extracts. In the study of Martin et al. (2012), an agar diffusion assay was performed and showed no inhibition zone on *E. coli* plates treated with mate extracts. In this study, *E. coli* colonies were grown on solid medium as well and not in liquid culture. Still, both the different strains used or the cultivation in suspension or in/on agar plates can be responsible for the different results on antibacterial activity of mate. Also, an antibiotic activity in a planktonic experimental setup does not necessarily lead to an activity against biofilm formation, as biofilms have a higher resistance to antibiotics. Since the inhibitory activity on *E. coli* is uncertain and not as concise as for other teas tested, a further investigation of bioactive compounds from mate tea seems to be not promising.

The same might be true for peppermint tea. Macrocolony growth was not affected, and curli inhibition was lower compared to green tea and hawthorn tea. Traditionally, peppermint tea is used as a remedy for different conditions, including dyspepsia and enteritis (McKay &

Blumberg 2006). Secondary plant metabolites present in peppermint leaves are essential oils, including menthol and menthone, which are responsible for the refreshing taste, and polyphenolic compounds as well. The content of phenolic substances ranges between 19 and 23%, of which flavonoids comprise 12%. The main compounds are the flavonoid eriocitrin and the phenolic acid rosmarinic acid. Luteolin 7-O-rutinoside, hesperidin, 5,6-dihydroxy-7,8,3',4'-tetramethoxyflavone, pebrellin, gardenin B and apigenin are present in smaller quantities (Areias et al. 2001, Zheng & Wang 2001). In studies analyzing potential antimicrobial activities of peppermint, in most cases the oil was tested. Regarding the effect on *E. coli*, results were diverse, probably due to different strains and assays used (McKay & Blumberg 2006). In a study by Yap et al. (2013), peppermint oil showed synergistic effects paired with antibiotics and resulted in increased minimum inhibitory concentrations. This demonstrates a possible application for natural compounds, even if they do not possess strong inhibitory activities tested on their own. A reduced antibiotic usage is an important goal in the prevention of the emergence of resistant bacteria.

To gain more information about individual bioactive compounds, single compounds need to be analyzed. This was done for the flavonoid taxifolin. Both concentrations tested reduced total biofilm growth, curli inhibition was only observed after 6 days of growth. It belongs to the subgroup of flavonols and is found in high concentrations in the Siberian larch *Larix sibirica*, but also in many other plant species (Loers et al. 2014). It is available as a supplement, as many chemopreventive activities are proposed for it due to its antioxidant capacity (Trouillas et al. 2004). Activity against bacterial growth and biofilm formation has been shown against *P. aeruginosa* due to quorum sensing inhibition (Vandeputte et al. 2011). In a microtiter-based assay, it presented biofilm inhibitory action, but also a slight reduction of planktonic cell growth in *E. coli* (Glasenapp et al. 2018).

As a control, the same experiments as described above were conducted with the *E. coli* mutant AP303 csgBA::kan deficient for curli fiber production. The genes for the minor and the major curli subunits csgA and csgB are deleted and kanamycin resistance is inserted as a selection tool. If the extracted tea compounds or taxifolin were curli specific, no changes in colony size should be visible. Interestingly, very similar effects on the growth and the coloring intensity were observed on *E. coli* W3110 and AP303. The absence of curli fibers as a structural element led to a different colony morphology. On the control plates, AP303 colonies grew light-yellow colored with corrugated edges, whereas W3110 presented

circular, red-violet colonies. The effects of the tea varieties and taxifolin were a slightly lighter coloring, especially in the center ring of the colony, and smaller waveforms on the colony edge. This indicates a more general effect of the tested compounds on the growth of *E. coli*, which is not limited to curli fiber synthesis. Other biofilm matrix components could be reduced in their production. Another reason can be a lower cell reproduction by an antibiotic activity of the tested substance. For all tea varieties, antibiotic activities are described in the literature (Taylor et al. 2005, Kostić et al. 2012, Burris et al. 2011, Yap et al. 2013). For taxifolin, a reduction of planktonic cell growth was reported as well (Glasenapp et al. 2018).

In summary, plant-derived products and compounds have shown to be promising agents in the prevention of biofilm formation, probably combined with a general antibiotic effect. The next step from a complex extract as a tea preparation to active compounds is the testing of individual components. Hawthorn tea is a possible source of active components, so individual substances should be tested.

Conclusions

Plant derived secondary metabolites are a rich source of bioactive compounds. Traditionally used plant products such as tea are a possible source of non-toxic agents against e.g. biofilm formation. The activity of green tea against *E. coli* biofilm formation was demonstrated in this work in agreement with results described in the literature. Of other tested tea varieties, hawthorn has shown the most promising results and individual compounds present in hawthorn leaves and flowers could be objects of future research. The flavonoid taxifolin has potential for further applications in prevention and treatment of *E. coli* biofilms.

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Chapter 4

De Vincenti, L., Glasenapp, Y., Cattò, C., Villa, F., Cappitelli, F. (2018): Hindering the formation and promoting the dispersion of medical biofilms: non-lethal effects of seagrass extracts. BMC Complementary and Alternative Medicine, 18: 168. doi.org/10.1186/s12906-018-2232-7

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RESEARCH ARTICLE Open Access

Hindering the formation and promoting the dispersion of medical biofilms: non-lethal effects of seagrass extracts

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Abstract

Background: Biofilms have great significance in healthcare-associated infections owing to their inherent tolerance and resistance to antimicrobial therapies. New approaches to prevent and treat unwanted biofilms are urgently required. To this end, three seagrass species (*Enhalus acoroides*, *Halophila ovalis* and *Halodule pinifolia*) collected in Vietnam and in India were investigated for their effects in mediating non-lethal interactions on sessile bacterial (*Escherichia coli*) and fungal (*Candida albicans*) cultures. The present study was focused on anti-biofilm activities of seagrass extracts, without killing cells.

Methods: Methanolic extracts were characterized, and major compounds were identified by MS/MS analysis. The antibiofilm properties of the seagrass extracts were tested at sub-lethal concentrations by using microtiter plate adhesion assay. The performance of the most promising extract was further investigated in elegant bioreactors to reproduce mature biofilms both at the solid/liquid and the solid/air interfaces. Dispersion and bioluminescent assays were carried out to decipher the mode of action of the bioactive extract.

Results: It was shown that up to 100 ppm of crude extracts did not adversely affect microbial growth, nor do they act as a carbon and energy source for the selected microorganisms. Seagrass extracts appear to be more effective in deterring microbial adhesion on hydrophobic surfaces than on hydrophilic. The results revealed that non-lethal concentrations of *E. acoroides* leaf extract: i) reduce bacterial and fungal coverage by 60.9 and 73.9%, respectively; ii) affect bacterial biofilm maturation and promote dispersion, up to 70%, in fungal biofilm; iii) increase luminescence in *Vibrio harveyi* by 25.8%. The characterization of methanolic extracts showed the unique profile of the *E. acoroides* leaf extract.

Conclusions: *E. acoroides* leaf extract proved to be the most promising extract among those tested. Indeed, the selected non-lethal concentrations of *E. acoroides* leaf extract were found to exert an antibiofilm effect on *C. albicans* and *E. coli* biofilm in the first phase of biofilm genesis, opening up the possibility of developing preventive strategies to hinder the adhesion of microbial cells to surfaces. The leaf extract also affected the dispersion and maturation steps in *C. albicans* and *E. coli* respectively, suggesting an important role in cell signaling processes.

Keywords: Seagrass extracts, Non-lethal concentrations, Antibiofilm activity, *Escherichia coli*, *Candida albicans*

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Background

The ability of microorganisms to colonize surfaces and develop into highly organized communities enclosed in a self-produced polymeric matrix is the predominant growth modality in both nature and artificial systems. Such lifestyle is called biofilm and it is characterized by alterations in microbial phenotypes with respect to growth rates and gene transcriptions [1–3].

Biofilms have great significance for public health, representing 65–80% of microbial diseases currently treated by physicians in the developed world [4, 5]. The presence of indwelling medical devices further increases the risk for biofilm formation and subsequent infection [6]. The bacterium *Escherichia coli* and the polymorphic fungus *Candida albicans* are among the most frequent cause of bloodstream infections, and the predominant microorganisms isolated from infected medical devices [7, 8]. These biofilms, as any other biofilm, exhibit dramatically decreased susceptibility to antimicrobial agents and resistant to the host immune clearance, which increases the difficulties for the clinical treatment of infections [9–11]. Furthermore, the antimicrobial arena is experiencing a shortage of lead compounds, and growing negative consumer perception against synthetic products has led to the search for more natural solutions [12].

In this context, it has been reported that plant-derived extracts exhibit good antibiofilm properties against a range of microorganisms [13–15]. However, in the past, these extracts were mainly screened by focusing on their lethal effects [16–18] disregarding their activity at non-lethal concentrations. At these concentrations, plant-derived extracts may reveal elegant mechanisms to sabotage the sessile lifestyle, manipulating the expression of stage-specific biofilm phenotypes [19]. For instance, by affecting the cellular ability to attach to surfaces and by mystifying intercellular signals, the biofilm cascade might be hampered. Thus, non-lethal concentrations of plant-derived extracts can inspire innovative, eco-friendly and safe strategies aim at treating deleterious biofilms. Interfering with specific key steps that orchestrate biofilm genesis might offer new ways to disarm microorganisms without killing them, sidestepping drug resistance [4].

Seagrasses, which belong to the halophytes, represent a functional group of underwater marine flowering plants that have developed several strategies to survive and reproduce in environments where the salt concentration is around 200 mM NaCl or more [20]. As these plants grow in very high saline conditions, it is predicted that they could possess rare and new activities not reported for their terrestrial relatives [21, 22]. Indeed, metabolomic studies have shown that increased salinity leads to changes in conserved and divergent metabolic responses in halophytes [23–25]. Moreover, interesting activities of seagrass extracts, including antibacterial, antifungal, antialgal, antioxidant, anti-inflammatory, insecticidal, antimalarial and vasoprotective properties, have been reported [26–28].

Thus, the well described properties of seagrasses extracts offer a promising framework for investigating novel antibiofilm activities at non-lethal concentrations.

The present study explores, for the first time, the effect of extracts from different seagrasses (namely, leaves and roots from *Enhalus acoroides* Rich. ex Steud., Hydrocharitaceae, leaves of *Halophila ovalis* (R.Br.) Hook.f., Hydrocharitaceae, and leaves of *Halodule pinifolia* (Miki) Hartog, Cymodaceaceae) in mediating non-lethal interactions on sessile *Candida albicans* and *Escherichia coli* cultures, selected as model systems for fungal and bacterial biofilm infections, respectively. The work focuses on investigating the antibiofilm performance of seagrass extracts at sub-inhibitory concentrations, studying how they affect biofilm functional traits (such as adhesion, biofilm maturation, dispersal and quorum sensing), and induce cellular responses other than those associated with antimicrobial activities.

Methods

Plant material and extraction

Three species of seagrasses (leaves and roots from *Enhalus acoroides* Rich. ex Steud., Hydrocharitaceae, leaves of *Halophila ovalis* (R.Br.) Hook.f., Hydrocharitaceae, and leaves of *Halodule pinifolia* (Miki) Hartog, Cymodaceaceae) were collected in Vietnam and India and air-dried in a dark place (Table 1). *Enhalus acoroides* and *Halophila ovalis* were collected and identified by Xuan-Vy Nguyen, Department of Marine Botany, Institute of Oceanography, Vietnam Academy of Science and Technology, Nha Trang City, Vietnam, based on morphological characters and controlled by ITS molecular marker analysis [29]. Specimens of *Enhalus acoroides* are stored in the herbarium of the Institute of Botany, Hannover, Germany (Specimen number: EA20130301). *Halodule pinifolia* was collected by Jutta Papenbrock and further identified by Thirunavakkarasu Thangaradjou, Centre of Advanced Study in Marine Biology, Annamalai University, Parangipettai, Tamilnadu, India, based on morphological characters and controlled by ITS molecular marker analysis [30]. Specimens are stored in the herbarium of the Annamalai University, Parangipettai, Tamilnadu, India.

The plants were separated into different organs (leaves and roots), and samples were cooled with liquid nitrogen and ground to a fine powder using a bead mill (Retsch), three times for 10 s at a frequency of 30/s. The samples were stored at − 80 °C prior to analysis. Crude extracts were obtained using 80% methanol (MeOH) as solvent. Around 50 mg of powdered seagrass material was weighed in a reaction tube and extracted with 800 μl 80% MeOH

Table 1 Seagrass species and information about collection sites

Species	Plant organ	Collection site	GPS	Collection date
Enhalus acoroides	Leaf	Nha Trang Bay, Vietnam	109.209208°E 12.158073°N	19.04.2011
Enhalus acoroides	Root	Nha Trang Bay, Vietnam	109.209208°E 12.158073°N	19.04.2011
Halophila ovalis	Leaf	Nha Trang Bay, Vietnam	109.209208°E 12.158073°N	19.04.2011
Halodule pinifolia	Leaf	Chilika Lagoon, India	85.418015°E 19.775105°N	16.02.2010

for 10 min with regular shaking. Then the extract was centrifuged for 5 min at 18000 x g and the supernatant transferred into a new reaction tube. These steps were repeated three times with 400 µl 80% MeOH each. The supernatants were collected in the same reaction tube and stored at − 20 °C. Phosphate buffered saline (PBS, 0.01 M phosphate buffer, 0.0027 M potassium chloride 0.137 M, sodium chloride, Fisher Scientific) was used to obtain several concentrations of each crude extract: 100, 10, 1, 0.1, 0.01 and 0.001 mg/l.

Microbial strains and growth media

The microbial strains *Candida albicans* SC5314 (ATCC MYA-2876) and *Escherichia coli* K-12 wild-type strain (ATCC 25404) were selected as model systems for fungal and bacterial biofilms respectively. *C. albicans* and *E. coli* strains were stored at − 80 °C in suspensions containing 50% glycerol and 2% peptone, and were routinely grown in amino acid-free yeast nitrogen base (YNB, Sigma-Aldrich) supplemented with 0.5% glucose (YNBG, Conda) and Luria-Bertani broth (LB, Sigma-Aldrich), respectively, for 16 h at 30 °C.

Quantification of total flavonoid contents (TFC)

The total flavonoid content of the seagrass extracts was measured in 96-well plate according to a modified protocol from Dudonné et al. [31]. The wells were filled with 150 µl H_2O each. Dilutions of the methanolic seagrass extracts (1:2) were prepared and 25 µl of sample were filled in one well, with four replicates. A calibration curve with catechin hydrate with the following concentrations was prepared in 80% MeOH: 0, 10, 25, 50, 100, 125, 250 and 400 µg/ml. The calibration curve was placed on the plate in triplicate. In the next step, 10 µl $NaNO_2$ 3.75% were added into each well and incubated for 6 min. Afterwards, 15 µl of $AlCl_3$ 10% were added and incubated for 10 min. In the last step, 50 µl of NaOH 1 M were added and the absorption was measured at 510 nm in a microplate reader (Biotek, Winooski, USA). The slope of the calibration curve was used to calculate the total flavonoid content in mg catechin equivalent.

Quantification of total phenolic contents (TPC)

To measure the total phenolic acid content, a modified protocol after Dewanto et al. [32] was used with the same extracts described above. 96-well microtiter plate were filled with 100 µl H_2O each. From each sample, 10 µl were added; seagrass extracts were diluted 1:2. A gallic acid calibration curve with the following concentration was used: 0, 5, 10, 25, 50, 75, 100, 125 and 250 µg/ml. Next, 100 µl Na_2CO_3 7% were added and the plate was incubated for 100 min in the dark. The absorption was measured at 765 nm in a microplate reader. With the slope of the gallic acid calibration curve, the concentration of phenolic acids was calculated in mg gallic acid equivalent.

Determination if the oxygen radical absorbance capacity (ORAC)

The analysis of the oxygen radical absorbance capacity (ORAC) was conducted according to a protocol based on Huang et al. (2002) [33] and Gillespie et al. [34] with the same extracts. A black 96-well microtiter was used and the wells were filled with 120 µl fluorescein (112 nM) in phosphate buffer (75 mM, pH 7.4). Of each sample and the standard curve, 20 µl were added in each well. The standard curve of 6-hydroxy-2, 5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was prepared in phosphate buffer with the following concentrations: 6.25, 12.5, 25 and 50 µM. Seagrass extracts were diluted 1:200 with methanol 80%. The microtiter plate was incubated for 15 min at 37 °C. The fluorescence was then measured at 485/520 nm as time point zero. Next, 80 µl of 2,2′-azobis(2-amidino-propane) dihydrochloride (62 mM) were added and the fluorescence was measured every minute for 80 min. The ORAC value was calculated as the difference between time point zero and 80 min and quantified with the Trolox standard curve.

LC-MS analysis

LC-MS analysis was performed on a Shimadzu HPLC system (controller CBM-20A, two pumps LC-20 AD, a column oven CTO-20 AC and a photo diode array detector SPD-M20A; Shimadzu, Darmstadt, Germany)

coupled to a Triple Tof 4600 mass spectrometer (AB Sciex, Canby, USA). The separation of extracted compounds was realised on a Knauer Vertex Plus column (250 × 4 mm, 5 μm particle size, packing material ProntoSIL 120–5 C18-H) with precolumn (Knauer, Berlin, Germany). The column oven temperature was set to 30 °C and 25 μl of undiluted methanolic seagrass extract prepared as described above was injected. The solvent flow rate was 0.8 ml/min. In this time, a gradient was run from 10 to 90% B from minute 0 to 35, 2 min of 90% B, switch to 10% B in 1 min and subsequent equilibration at 10% B for 2 min. Solvent A (water) and B (methanol) were both supplemented with 2 mM ammonium acetate and 0.01% acetic acid. Mass spectra were monitored between 100 and 800 Da in negative ionisation mode. In addition, MS/MS spectra were generated with a collision energy of − 30 eV and measured between 50 and 800 Da. Spectra for the most prominent peaks were compared to database entries in MassBank [35] and ReSpect [36] for identification.

Planktonic growth in the presence of seagrass extracts as the sole source of carbon and energy

The ability of *C. albicans* and *E. coli* planktonic cells to grow in the presence of each extract as the sole carbon and energy source was tested using YNB and M9 (Sigma-Aldrich) mineral medium, respectively, supplemented with the highest working extract concentration: 100 mg/l. Then a 100 μl mix of mineral medium together with 45 μl (3% *v*/v) of the overnight culture (final concentration 10^8 cells/ml) and the highest concentration of each marine plant extract were used to fill each well of 96-well plates (Thermo Fisher Scientific) and incubated for 48 h at 30 °C. A medium complemented with cells and glucose (5 g/l), and medium without cells, were used as positive and negative controls, respectively. Microbial growth was monitored using the PowerWave XS2 microplate reader (Biotek) measuring the absorbance at 600 nm (A_{600}) every 10 min. Six biological replicates of each treatment were performed. The obtained data were normalized to the negative control and reported as the mean of these.

Growth inhibition assay in the presence of seagrass extracts

The ability of the seagrass extracts to inhibit the planktonic growth of the selected microorganisms was investigated. For this, *C. albicans* and *E. coli* were grown YNBG and LB broth respectively without (positive control) and with the highest working concentrations (10 and 100 mg/l) in 96-well plates (Thermo Fisher Scientific). Growth curves at 30 °C were generated using Infinite® F200 PRO microplate reader (TECAN, Mannedorf, Switzerland) by measuring the optical density at 600 nm (OD_{600}) every 60 min for 30 h in wells inoculated with 45 μl (3% vol/vol) of an overnight culture (approximately 10^8 cells/ml). The negative control was represented by PBS supplemented with 45 μl (3% vol/vol) of the overnight culture. The polynomial Gompertz model [37] was used to fit the growth curves to calculate the maximum specific growth rate (A_{600}/min), using GraphPad Prism software (version 5.0, San Diego, CA, USA). Five biological replicates of each treatment were performed.

Microplate-based biofilm assay

The antibiofilm activity of seagrass extracts was assessed quantitatively as previously reported by Villa et al. [38]. Briefly, 200 μl of PBS containing 10^8 cells/ml supplemented with 0 (positive control), 100, 10, 1, 0.1, 0.01, and 0.001 mg/l of each crude extract were placed in hydrophobic and hydrophilic 96-well polystyrene-based microtiter plates (Thermo Fisher Scientific). After an incubation time of 24 h at 20 °C, *C. albicans* and *E. coli* planktonic cells were removed and adhered cells were stained using 0.1 mg/ml of Fluorescent Brightener 28 vital dye (Sigma-Aldrich) or 4′, 6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) in PBS, respectively. After 20 min staining in the dark at room temperature the microtiter plates were washed twice with 200 μl PBS and the fluorescence intensity due to adhered cells was measured using a fluorescence microplate reader (TECAN, Manneford, Switzerland) at excitation wavelength of 335 nm and emission wavelength of 433 nm. A standard curve of fluorescence intensity versus cell number was determined and used to quantify the antibiofilm performance of the crude extracts. Percentage reduction with respect to the positive control is calculated as (treated data −control data) × 100 / control data. Cattò et al. [39] proposed the following anti-adhesion ranges computing the percentage reduction in comparison to the negative control: ≤20% without anti-adhesion activity; between 20 and 30% and 30 and 40% low anti-adhesion activity and with moderate anti-adhesion activity respectively; ≥40% adhered cells with excellent anti-adhesion activity. Five biological replicates were performed for each condition and a percentage reduction in comparison to the negative control was calculated as (treated data − positive control data) × 100/ positive control data. The experiment was repeated three times.

Biofilm growth at the solid/liquid interface

The most promising plant extracts were screened for their effects on biofilm development. *C. albicans* biofilm was grown in the CDC biofilm reactor (Biosurface Technologies, Bozeman, MT, USA) as previously described by Villa et al. [40]. Briefly, two bioreactors hosting 24 polycarbonate coupons (to simulate a hydrophobic surface) were filled with YNBG and 1 ml of overnight

planktonic culture (approximately 10^8 cells/ml) and, in one of them, 0.01 mg/l of *E. acoroides* leaf extract was added. Bioreactors were maintained under static conditions (no flow) for 24 h under mild stirring at 37 °C, promoting fungal adhesion to the surface of the removable polycarbonate coupons. After that, the dynamic phase was initiated and diluted YNGB was fluxed for 48 h at flow rate of 250 ml/h. Biofilm growth in the absence (positive control) and presence of the extract was evaluated by quantification of the biomass. At different time steps (24, 48 and 72 h) some polycarbonate coupons were collected in aseptic conditions and resuspended in 3 ml of PBS each. Subsequently, serial dilutions were carried out, and 10 μl were inoculated in petri dishes containing Tryptic Soy Broth medium (TSB, Sigma-Aldrich) complemented with agar (Merck) following the drop counting method. After 12 h at 30 °C, *C. albicans* colonies were counted and the data obtained were normalized to the coupon area, and means were reported. The same protocol was used to obtain mature biofilm of *E. coli*, using LB as a medium, and evaluating 10 mg/l of *E. acoroides* leaf extract. Each experiment was repeated three times.

Biofilm dispersion assay

Mature *C. albicans* biofilm was grown in the CDC reactor in the absence (positive control) and presence and of 0.01 mg/l of *E. acoroides* leaf extract as reported below. As previously described by Cattò et al. [41], after 72 h polycarbonate coupons were collected, immersed in 27 ml of PBS for one minute at room temperature, serial dilutions were carried out and 10 μl were inoculated in petri dishes containing TSB supplemented with agar (Merck) following the drop counting method. After 12 h at 30 °C, *C. albicans* colonies were counted and the percentage of biofilm dispersion was calculated as (number of viable cells from bulk PBS × 100) / (number of viable cells from bulk PBS + number of viable cells from the coupon biofilm) and means were reported. Three biological replicates were performed for each treatment and six technical replicates were performed for each experiment. The experiment was performed three times.

Biofilm growth at the solid/air interface

E. coli biofilm was grown on a sterile polycarbonate membrane (PC, Whatman Nucleopore, diameter 2.5 cm, pore diameter 0.2 μm) as previously described by Garuglieri et al. [42]. Briefly, 0.05 ml of an overnight culture (approximately 10^6 cells/ml) were inoculated at the center of a sterile polycarbonate membrane and, when the inoculum was completely dried, the membrane was carefully put inside a transwell structure (ThinCert™ Cell Culture Inserts with translucent PET membrane – Greiner bio-one) inlaid in a 6 well culture plate (Greiner bio-one). One ml of LB medium was inoculated in the basolateral compartment (plate well). Biofilm formation was performed at 37 °C in aerobic conditions for 16 h. At different time points (0, 4, 6, 8, 16 h) some membranes were removed, biofilm was scraped off using a sterile loop, put inside a tube containing 1 ml of PBS and then homogenized twice using a homogenizer (IKA T10 basic Ultra-Turrax – Cole-Parmer Instrument Company). Then serial dilutions were prepared and 10 μl were inoculated in petri dishes containing LB with agar following the drop counting method. After 12 h at 37 °C, *E. coli* colonies were counted and the biomass was quantified. This assay was assessed under three experimental conditions: i) treatment 1: growth in contact with 1 ml of LB with 10 mg/l of *E. acoroides* leaf extract for 16 h; ii) treatment 2: overnight culture grown with 10 mg/l of *E. acoroides* leaf extract, and then growth in contact with 1 ml of LB for 16 h; iii) treatment 3: overnight culture grown with 10 mg/l of *E. acoroides* leaf extract, and then growth in contact with 1 ml of LB with 10 mg/l of *E. acoroides* leaf extract for 16 h. In the positive control, the microorganisms grew in 1 ml LB inside a basolateral well for 16 h without the extract. The data obtained were divided by the area of the membrane, and the means were reported. The experiment was repeated three times.

B2ioluminescence assay using *Vibrio harveyi*

Two hundred μl of autoinducer bioassay (AB) mineral medium (0.3 M NaCl, 0.05 M $MgSO_4$, 0.5% casein hydrolysate, 10 μM KH_2PO_4, 1 μM L-arginine, 50% glycerol, 0.01 μg/ml riboflavin, 1 μg/ml thiamine. pH 7. Sigma-Aldrich) containing 10% (*V*/V) of a tenfold dilution of an overnight culture of *Vibrio harveyi* BB170 (ATCC BAA-1117) grown in AB medium were supplemented with 10 mg/l of *E. acoroides* leaf extract respectively, and were placed in hydrophobic 96-well polystyrene-based microtiter plates (Thermo Fisher Scientific) with transparent bottom. The positive control was an AB mineral medium supplemented with 10% (V/V) tenfold dilution of the overnight culture. Absorbance (OD_{600nm}) and luminescence were measured using a microplate reader (VICTOR™X, Perkin Elmer, USA) every 8 h for 24 h, incubating the microtiter plate at 30 °C during the experiment. The data obtained were normalized to the number of viable cells, divided by the area of the membrane, and the means reported. The experiment was repeated three times.

Statistical analysis

To evaluate statistically significant differences among samples, analysis of variance (ANOVA) via MATLAB software (Version 7.0, The MathWorks Inc., Natick, USA) was applied. Tukey's honestly significant different test (HSD) was applied for pairwise comparison to

establish the significance of the data. Statistically significant results were represented by P values ≤0.05.

Results

Seagrass extracts contain phenolic compounds and show antioxidant capacities

The methanolic extracts from the seagrass material contained phenolic acids as well as flavonoids (Fig. 1a-b). The content of phenols and flavonoids was highest in *H. pinifolia* leaf extracts with 18.0 ± 0.25 and 14.3 ± 0.25 mg/g dry mass (DM), respectively. In *E. acoroides*, the root material showed higher amounts of total flavonoids and phenols than the leaf material. For all seagrass species, the content of phenolic acids was higher than the flavonoid content with respect to the DM.

Methanolic extracts from the four seagrass species were analyzed for their antioxidant capacity (Fig. 1c). All tested extracts had the ability to absorb oxygen radicals. *H. pinifolia* showed the highest activity with 97.7 ± 2.7 mg Trolox equivalents (TE)/g DM. *E. acoroides* and *H. ovalis* leaf extracts showed similar antioxidant capacities with 70.2 ± 4.1 and 72.5 ± 2.9 mg TE/g DM, respectively. The root extract from *E. acoroides* displayed a lower ORAC value than the extract from the leaves (45.1 ± 3.2 mg TE/g DM).

LC-MS analysis of secondary metabolites

E. acoroides, *H. ovalis* and *H. pinifolia* show different compositions of secondary metabolites (Fig. 2). The identification of individual compounds in the methanolic extracts was done via the comparison of MS/MS spectra with database entries. The three seagrass species showed different profiles of secondary metabolites, in this case mainly flavonoids and phenolic acids (Table 2). In *E. acoroides* leaves, three flavonoles based on kaempferol were found. In addition, two flavones (apigenin and luteolin), one phenolic acid (benzoic acid) and the saturated dicarboxylic acid azelaic acid were identified. The root extract of *E. acoroides* also contained two kaempferol-based flavonoles and luteolin and also a procyanidin and a flavanole (epicatechin). In *H. ovalis* three flavonoids and one phenolic acid was found. *H. pinifolia* contained several flavonoles, either based on kaempferol or quercetin and also epicatechin.

Seagrass extracts are not used as carbon and energy source by *C. albicans* and *E. coli* and do not affect their planktonic growth

C. albicans and *E. coli* planktonic cells grown only in the presence of medium supplemented with glucose were used as the positive control of the experiment (Fig. 3). Note that the mineral medium supplemented with the highest concentration of tested plant extracts did not promote the growth of the selected microorganisms.

The response of the planktonic growth of the selected microorganisms in the presence of the seagrass extracts at the highest concentrations (10 and 100 mg/l) is reported in Figs. 4 and 5. *C. albicans* and *E. coli* growth rates (table in Figs. 4 and 5) showed that there are no statistically significant differences between the presence and the absence of the extracts obtained from every plant portion at any tested concentration. Therefore, concentrations ≤100 mg/l plant extract were used in the subsequent studies.

E. acoroides leaf extract inhibits cell adhesion on a hydrophobic surface

The percentage reduction of the number of adhered cells of *E. coli* and *C. albicans* on hydrophilic and hydrophobic surface in presence of non-lethal concentrations of seagrass extracts is showed in Fig. 6. The results revealed that *E. acoroides* and *H. ovalis* were the most promising extracts for *C. albicans*, with excellent anti-adhesion activity, reducing fungal coverage up to 73.89 ± 1.01% and 68.37 ± 2.49% at 0.01 and 1 mg/l, respectively. For *E. coli*, 10 mg/l of *E. acoroides* leaf extract was found to be the concentration with the highest reduction in cell adhesion (reduction

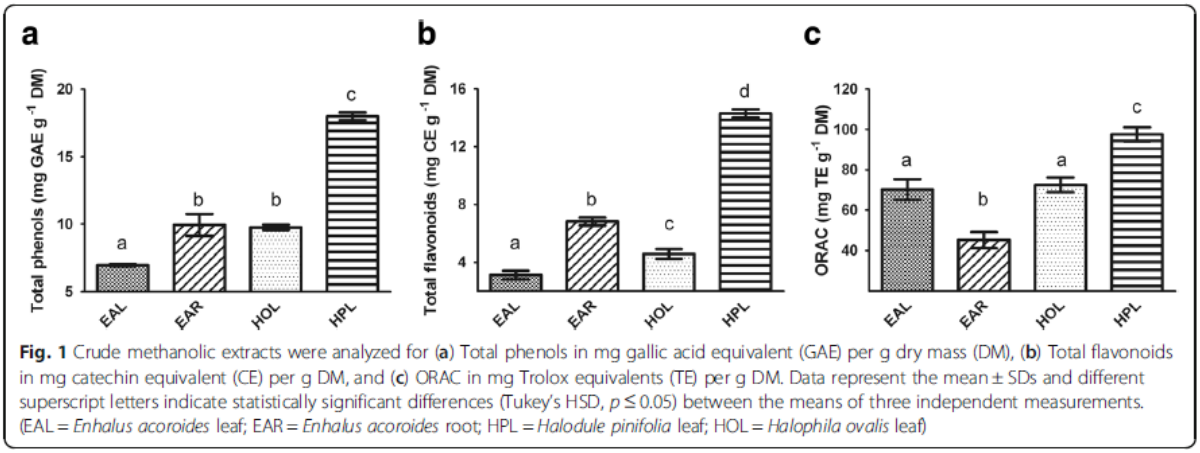


Fig. 1 Crude methanolic extracts were analyzed for (**a**) Total phenols in mg gallic acid equivalent (GAE) per g dry mass (DM), (**b**) Total flavonoids in mg catechin equivalent (CE) per g DM, and (**c**) ORAC in mg Trolox equivalents (TE) per g DM. Data represent the mean ± SDs and different superscript letters indicate statistically significant differences (Tukey's HSD, $p \leq 0.05$) between the means of three independent measurements. (EAL = *Enhalus acoroides* leaf; EAR = *Enhalus acoroides* root; HPL = *Halodule pinifolia* leaf; HOL = *Halophila ovalis* leaf)

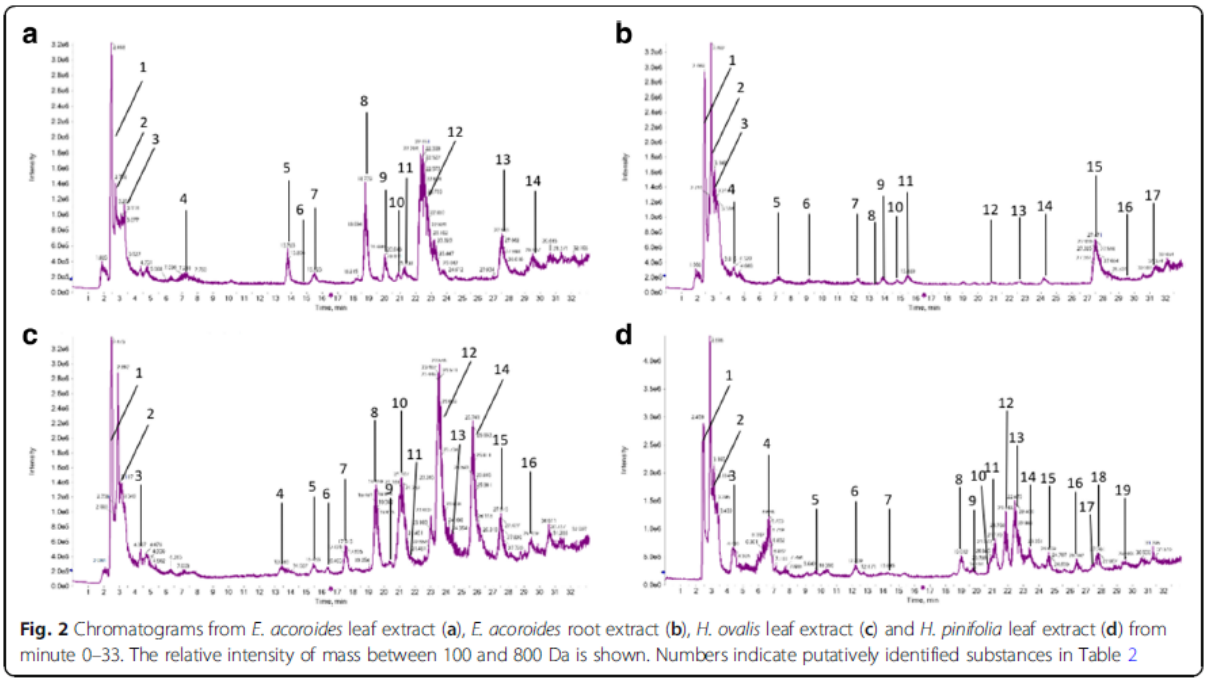


Fig. 2 Chromatograms from *E. acoroides* leaf extract (**a**), *E. acoroides* root extract (**b**), *H. ovalis* leaf extract (**c**) and *H. pinifolia* leaf extract (**d**) from minute 0–33. The relative intensity of mass between 100 and 800 Da is shown. Numbers indicate putatively identified substances in Table 2

of bacterial coverage by 60.86 ± 8.85%). Therefore, 0.01 mg/l and 10 mg/l *E. acoroides* leaf extract were chosen as the best non-biocidal concentrations for *C. albicans* and *E. coli* respectively, and were used in the subsequent studies.

E. acoroides leaf extract does not impact on biofilm growth curves, but does induce biofilm dispersion in *C. albicans* and interfere with AI2

A CDC reactor was used as the laboratory scale model system to grow a complex and mature *C. albicans* biofilm in the absence and presence of 0.01 mg/l *E. acoroides* leaf extract, the most effective concentration obtained from the adhesion assay.

Results in Fig. 7a indicated a significant reduction in the number of viable cells adhered on coupon surfaces treated with the extract, compared to the untreated ones, after 24 h (reduction of fungal coverage up to 26.77 ± 9.01%). Coupons collected after 48 and 72 h showed no significant differences between the treated biofilm and the control.

A significant increase in the number of dispersed cells in the treated biofilm (70 ± 6.83%) was observed (Fig. 7b).

A colony biofilm assay was used to grow a complex and mature *E. coli* biofilm in the presence and absence of 10 mg/l *E. acoroides*. Results in Fig. 8 showed no significant reduction in the number of viable cells during biofilm formation on the membrane treated with the extract, compared to the untreated, after 18 h in all the experimental conditions. Treatment 3 showed a growth rate slowdown in the interval 6–8, in which *E. coli* cells were in contact with the extract during both overnight growth and biofilm formation (reduction of cellular growth, compared to the control, up to 48.64 ± 4.02%). This growth curve was characterized by two exponential phases separated distinctly by an intermediate phase where the growth rate is very low. After that, at 16 h the number of viable cells was similar to the other treatments.

The effects of 10 mg/l of *E. acoroides* leaf extract on the cellular communication of *V. harveyi* were reported in Fig. 9. The results highlighted a significant increase in the relative luminescence emitted at time 8 h compared to the control (25.75 ± 7.49%).

Discussion

Biofilm resistance to antimicrobial agents is a major worldwide health care issue. Therefore, a successful reduction of surface colonization can be a potential strategy for the management of unwanted biofilms, especially on medical devices and work surfaces.

In this context, the use of plant-derived extracts to modulate biofilm genesis and dispersion may be a viable alternative. The present study is the first report describing the antibiofilm efficacy of non-lethal concentrations of *E. acoroides*, *H. pinifolia* and *H. ovalis* methanol extracts in counteracting microbial biofilms, highlighting the possibility that the selected seagrass species act as an extracellular signal mediating their biofilm activities.

E. coli and *C. albicans* were chosen as model systems for bacterial and fungal infections, respectively. *E. coli*

Table 2 Individual compounds identified by comparison of MS/MS spectra with database entries in *Enhalus acoroides* leaf extract (A), *E. acoroides* root extract (B), *Halophila ovalis* leaf extract (C) and *Halodule pinifolia* leaf extract (D)

No	RT	Mass	MS/MS	Name	Accession	Source
A - *E. acoroides* leaf extract						
1	2.5	343.03	201.02, 157.03, 59.01	n. i.	–	–
2	2.7	312.12	179.05, 132.06, 89.02	n. i.	–	–
3	3.3	367.1	277.07, 187.04, 157.03	n. i.	–	–
4	7.2	134.04	107.03, 92.02	Adenine	PT200393	ReSpect
5	13.7	637.1	461.07, 285.04	Kaempferol-3-glucuronide, mod.	PT209240	ReSpect
6	14.8	275.15	233.12, 119.05	n. i.	–	–
7	15.2	121.03	92.02, 77.03	Benzoic acid	KO000321	MassBank
8	18.6	527.02	285.04, 241.00, 96.96	n. i.	–	–
9	20.1	511.05	269.04, 241.00, 96.96	n. i.	–	–
10	20.8	187.09	169.08, 125.09, 97.06	Azelaic acid	KO000124	MassBank
11	21.3	447.09	285.04	Kaempferol-3-O-glucoside	PS042209	ReSpect
12	22.5	461.07	285.04	Kaempferol-3-glucuronide	PS092408	ReSpect
13	27.5	285.04	151.00, 133.03	Luteolin	PS040410	ReSpect
14	29.5	269.04	225.05, 151.00, 117.03	Apigenin	PT203930	ReSpect
B - *E. acoroides* root extract						
1	2.4	343.03	201.02, 157.03, 59.01	n. i.	–	–
2	2.7	312.12	179.05, 132.06, 89.02	n. i.	–	–
3	2.9	377.08	341.11, 179.05, 119.03, 89.02	Galactinol dihydrate, mod.	PT211910	ReSpect
4	4.3	216.98	173.02, 156.98, 136.94, 59.01	n. i.	–	–
5	7.2	134.04	107.03, 92.02	Adenine	PT200393	ReSpect
6	9.6	577.12	451.10, 425.08, 407.07, 289.07, 125.02	Procyanidin B2	PT204580	ReSpect
7	12.3	289.07	245.07, 203.07, 151.04, 109.03	+(−) Epicatechin	PT204560	ReSpect
8	13.8	637.1	461.07, 285.04	Kaempferol-3-glucuronide, mod.	PT209240	ReSpect
9	14.0	469.08	275.02, 193.05, 178.02, 149.06, 96.96	n. i.	–	–
10	14.8	275.15	233.12, 119.05	n. i.	–	–
11	15.3	121.03	92.02, 77.03	Benzoic acid	KO000321	MassBank
12	20.8	187.09	169.08, 125.09, 97.06	Azelaic acid	KO000124	MassBank
13	22.6	461.07	285.04	Kaempferol-3-glucuronide	PS092408	ReSpect
14	24.1	299.05	284.03, 256.03, 133.03	Kaempferide	PT204030	ReSpect
15	27.5	285.04	151.00, 133.03	Luteolin	PS040410	ReSpect
16	29.5	269.04	225.05, 151.00, 117.03	Apigenin	PT203930	ReSpect
17	31.2	329.23	229.14, 211.13, 171.10	n. i.	–	–
C - *H. ovalis* leaf extract						
1	2.4	343.03	201.02, 157.03, 59.01	n. i.	–	–
2	2.9	377.08	341.11, 179.05, 119.03, 89.02	Galactinol dihydrate, mod.	PT211910	ReSpect
3	4.3	216.98	173.02, 156.98, 136.94, 59.01	n. i.	–	–
4	13.3	261.04	217.05, 189.05, 133.02	n. i.	–	–
5	15.5	121.03	92.02, 77.03	Benzoic acid	KO000321	MassBank
6	16.3	306.17	288.16	n. i.	–	–
7	17.5	479.08	316,02	Myricetin-3-galactoside	PS092809	ReSpect
8	19.5	463.09	301,03	Quercetin-3-O-beta-D-galactoside	PS046509	ReSpect
9	20.8	187.09	169.08, 125.09, 97.06	Azelaic acid	KO000124	MassBank

Table 2 Individual compounds identified by comparison of MS/MS spectra with database entries in *Enhalus acoroides* leaf extract (A), *E. acoroides* root extract (B), *Halophila ovalis* leaf extract (C) and *Halodule pinifolia* leaf extract (D) *(Continued)*

No	RT	Mass	MS/MS	Name	Accession	Source
10	21.1	317.02	271.02, 149.02	n.i.	–	–
11	21.3	447.09	285.04	Kaempferol-3-O-glucoside	PS042209	ReSpect
12	23.5	301.03	255.03, 165.02, 133.03	n.i.	–	–
13	24.1	299.05	284.03, 256.03, 133.03	Kaempferide	PS040309	ReSpect
14	25.7	285.04	239.03, 185.06, 143.05, 117.03	Kaempferol	PR040027	MassBank
15	27.5	285.04	285.04, 151.00,133.02	Luteolin	PT204043	ReSpect
16	29.4	269.04	225.05, 151.00, 117.03	Apigenin	PT203930	ReSpect
D - *H. pinifolia* leaf extract						
1	2.4	343.03	201.02, 157.03, 59.01	n. i.	–	–
2	2.9	377.08	341.11, 179.05, 119.03, 89.02	Galactinol dihydrate, mod.	PT211910	ReSpect
3	4.3	216.98	173.02, 156.98, 136.94, 93.03, 59.01	n. i.	–	–
4	6.6	473.07	311.04, 293.03, 179.03, 149.01	n. i.	–	–
5	9.6	577.12	451.10, 425.08, 407.07, 289.07, 125.02	Procyanidin B2	PT204580	ReSpect
6	12.1	289.07	245.07, 203.07, 151.04, 109.03	+(−) Epicatechin	PT204560	ReSpect
7	14.0	469.08	275.02, 193.05, 178.02, 149.06, 96.96	n. i.	–	–
8	19.1	641.17	473.13, 311.07, 167.03	n. i.	–	–
9	19.7	549.09	505.10, 463.09, 300.02, 271.02, 255.02	Quercetin-3-(6-malonyl)-glucoside	PT209340	ReSpect
10	20.8	187.09	169.08, 125.09, 97.06	Azelaic acid	KO000124	MassBank
11	21.1	505.09	463.08, 300.02, 271.02	Quercetin-3-O-beta-D-galactoside, mod.	PT204650	ReSpect
12	21.8	463.08	300.03, 271.02	Quercetin-3-O-beta-D-galactoside	PT204650	ReSpect
13	22.4	433.07	300.02, 271.02, 255.03, 179.00	Quercetin-3-arabinoside	PT209320	ReSpect
14	23.4	447.09	284.03, 255.03, 227.03	Kaempferol-3-glucoside	PT209270	ReSpect
15	24.6	417.08	284.03, 255.03, 227.03	Kaempferol-3-O-alpha-L-arabinoside	PT209220	ReSpect
16	26.3	301.03	178.99, 151.00, 121.03, 107.01	Quercetin	PT204090	ReSpect
17	27.4	285.04	199.03, 175.04, 151.00, 133.02	Luteolin	PT204043	ReSpect
18	27.7	315.05	300.02, 271.02, 255.03	Isorhamnetin	PM007432	ReSpect
19	29.5	269.04	225.05, 151.00, 117.03	Apigenin	PT203930	ReSpect

No = number of peak in Fig. 9, RT = retention time, Mass = mass of precursor ion, MS/MS = fragment spectra obtained at −30 eV, Accession = accession number in database, Source = database used, n. i. = not identified, mod. = modified

biofilms are found to be the major causative agent of many intestinal infections, for recurrent urinary tract infections, and it also responsible for indwelling medical device-related infectivity [43]. *C. albicans* is one of the very few fungal species causing disease in humans. These infections range from superficial mucosal and dermal infections, such as thrush, vaginal yeast infections, and diaper rash, to vascular catheters and dental implants infections [44].

The bioactive properties of the seagrass species selected in this work are well known, and have been reported in detail by several authors [45–47]. However, until now attention has mainly focused on the antimicrobial activity of seagrass extracts, which, through disk diffusion assays, were investigated not in their capacity as biofilm-forming microorganisms but in their planktonic state. Using lethal concentrations, Umamaheshwari et al. [46] reported the antibacterial activity of *H. ovalis* and *H. pinifolia* extracts, obtained using different solvents, against different microbial strains, recording maximum antibacterial activity by the ethanol extract of *H. pinifolia*. Instead, Choi et al. [48] reported the antimicrobial properties of *Zostera marina* methanol extract and its organic solvent fractions on three human skin pathogens (*Staphylococcus aureus*, *S. epidermidis* and *C. albicans*), and Natrah et al. [47] reported the antibacterial properties of methanol extracts of *E. acoroides* and other seagrass and seaweed species on different aquaculture pathogens (*Aeromonas hydrophila*, *Vibrio alginolyticus*, *V. parahaemolyticus*, *V. anguillarum* and others).

In contrast, to the best of our knowledge, no papers have investigated the antibiofilm activity of *Enhalus acoroides*, *Halodule pinifolia* and *Halophila ovalis* at non-lethal concentrations against bacterial (*E. coli*) and

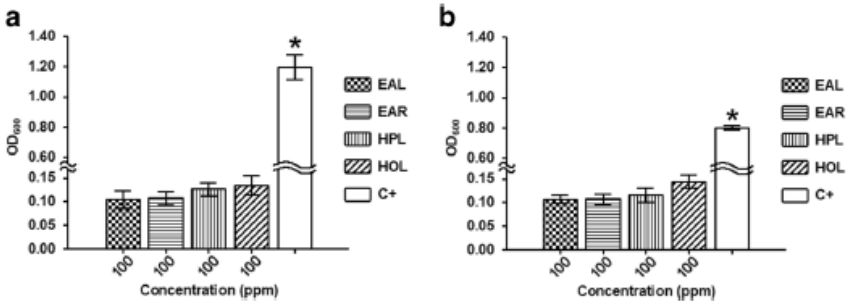
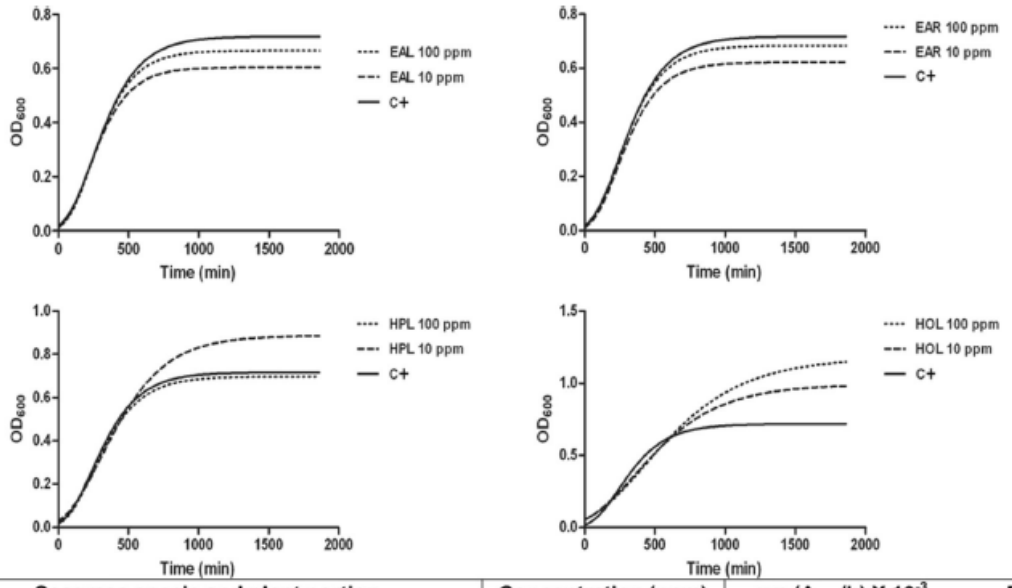
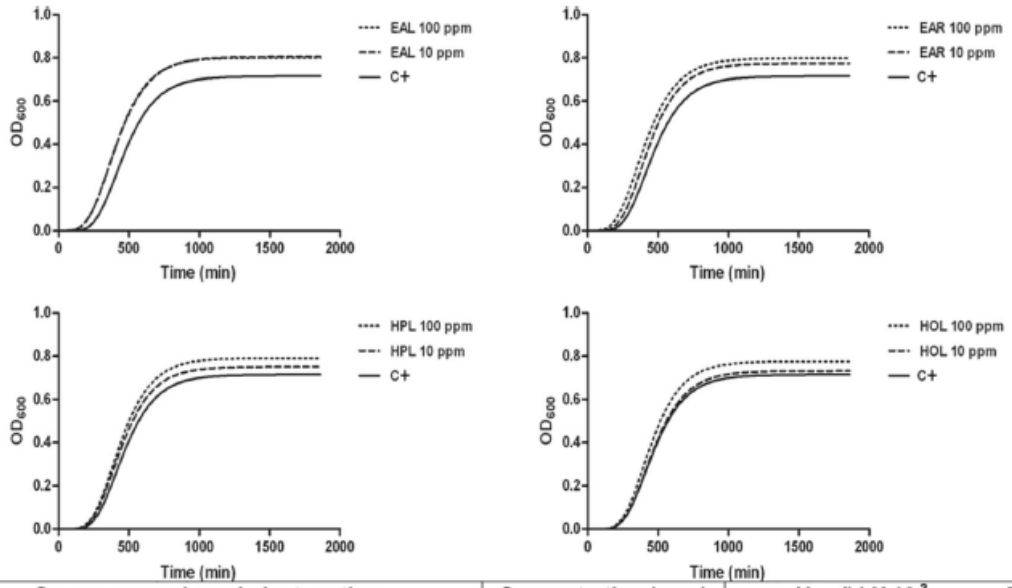


Fig. 3 *E. coli* (**a**) and *C. albicans* (**b**) planktonic growth without (positive control) and with each seagrass extract at 100 ppm. The positive control was set up with mineral medium supplemented with glucose at 5 g/l. Stars indicate statistically significant differences (Tukey's HSD, $p \leq 0.05$) between the means of three independent replicates. (EAL = *Enhalus acoroides* leaf; EAR = *Enhalus acoroides* root; HPL = *Halodule pinifolia* leaf; HOL = *Halophila ovalis* leaf; C + = Positive control)



Seagrass specie and plant portion	Concentration (ppm)	μm (A_{600}/h) X 10^{-3}	R^2
Control (LB)	0	1.42±0.04	0.9945
Enhalus acoroides leaf	100	1.47±0.03	0.9959
	10	1.40±0.04	0.9946
Enhalus acoroides root	100	1.40±0.06	0.9953
	10	1.37±0.03	0.9948
Halodule pinifolia leaf	100	1.37±0.02	0.9949
	10	1.38±0.06	0.9937
Halophila ovalis leaf	100	1.42±0.01	0.9881
	10	1.45±0.09	0.9919

Fig. 4 OD-based growth curves of *C. albicans* in absence (positive control) and in presence of each seagrass extract at 10 and 100 ppm. Maximum specific growth rate (μ_m) and the goodness of fit (R^2) obtained by the Gompertz model. Data represent the mean ± SDs of three independent measurements. Means reported showed no statistically significant differences between the positive control and treated samples (Tukey's HSD, $p \geq 0.05$). (EAL = *Enhalus acoroides* leaf; EAR = *Enhalus acoroides* root; HPL = *Halodule pinifolia* leaf; HOL = *Halophila ovalis* leaf; C + = Positive control)



Seagrass specie and plant portion	Concentration (ppm)	μm (A_{600}/h) X 10^{-3}	R^2
Control (YNBG)	0	1.95±0.03	0.9943
Enhalus acoroides leaf	100	1.97±0.04	0.9930
	10	1.93±0.02	0.9936
Enhalus acoroides root	100	1.91±0.05	0.9919
	10	1.96±0.03	0.9921
Halodule pinifolia leaf	100	1.95±0.04	0.9938
	10	1.84±0.08	0.9929
Halophila ovalis leaf	100	1.93±0.05	0.9924
	10	1.96±0.05	0.9940

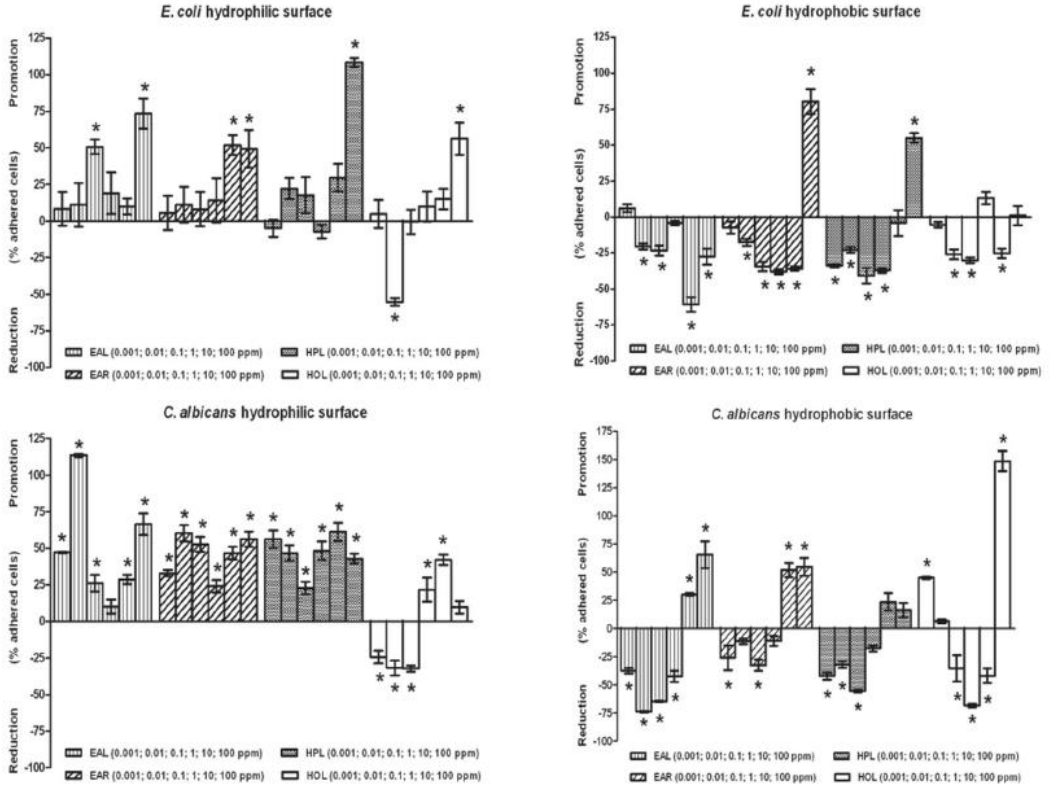
Fig. 5 OD-based growth curves of *E. coli* in absence (positive control) and in presence of each seagrass extract at 10 and 100 ppm. Maximum specific growth rate (μ_m) and the goodness of fit (R^2) obtained by the Gompertz model. Data represent the mean ± SDs of three independent measurements. Means reported showed no statistically significant differences between the positive control and treated samples (Tukey's HSD, $p \geq 0.05$). (EAL = *Enhalus acoroides* leaf; EAR = *Enhalus acoroides* root; HPL = *Halodule pinifolia* leaf; HOL = *Halophila ovalis* leaf; C + = Positive control)

fungal (*C. albicans*) biofilms. To this end, methanol extracts, obtained from different organs of three seagrass species (namely, *Enhalus acoroides* leaves and roots, *Halophila ovalis* leaves and *Halodule pinifolia* leaves) were screened for their ability to modulate biofilm genesis without killing cells. Methanol was used as the extraction solvent, having been previously reported as the most effective solvent to obtain high concentrations of bioactive compounds with antibacterial activity from seagrasses, compared to other extraction solvents [45, 49, 50].

Before evaluating the antibiofilm activity, the extracts, at concentrations of 100 mg/l, were first proved to not act as a carbon and energy source nor to affect the cellular growth of *C. albicans* and *E. coli*. Therefore, concentrations ≤100 mg/l plant extract were used in the subsequent studies.

With the aim of investigating the effects of seagrass extracts on cell adhesion to surfaces, the first step of biofilm formation, microtiter based assays were performed. The results revealed excellent anti-adhesion activity for *E. acoroides* leaf extract, reducing fungal coverage up to 74% and bacterial coverage up to 61% at 0.01 and 10 mg/l, respectively. Therefore, 0.01 mg/l and 10 mg/l *E. acoroides* leaf extract were chosen as the best non-biocidal concentrations for *C. albicans* and *E. coli* respectively, and were used in the subsequent studies. These concentrations significantly decreased the number of adhered cells on a hydrophobic surface, more so than on the hydrophilic one. Previous studies had highlighted the preference for hydrophobic surfaces, these reporting a decreased adhesion on the hydrophobic surface compared to the hydrophilic [51, 52]. This is probably due to the hydrophobic nature of the aerial surfaces of plants [53].

In the present study the anti-adhesion activity of the seagrass extracts was dose-dependent, but the highest concentrations did not correspond to those with the



		0.001 ppm	0.01 ppm	0.1 ppm	1 ppm	10 ppm	100 ppm
E. coli hydrophilic surface	EAL	8.25±24.71%	11.07±25.61%	50.71±8.63%	19±28.76%	9.93±11.13%	73.51±17.95%
	EAR	5.51±20.27%	11.15±21.19%	8.11±20.25%	0.03±12.43%	51.92±13.64%	49.35±22.32%
	HPL	-4.89±10.31%	22.16±14.26%	17.65±24.88%	-7.24±8.25%	29.54±18.86%	108.34± 5.51%
	HOL	4.77±16.49%	-55.37±4.64%	-0.54±16.59%	15.34±17.96%	15.00±12.16%	56.36±22.18%
E. coli hydrophobic surface	EAL	6.05±4.59%	-20.60±3.72%	-23.35±6.07%	-4.36±3.09%	-60.76±8.86%	-27.66±9.95%
	EAR	-7.41±8.43%	-17.65±5.19%	-34.45±5.91%	-38.04±2.93%	-36.02±2.44%	80.29±14.74%
	HPL	-33.95±2.07%	-22.98±3.83%	-40.82±9.47%	-37.03±2.74%	-4.43±15.65%	54.92±5.95%
	HOL	-5.37±3.45%	-26.03±5.82%	-30.22±3.10%	13.14±8.63%	-25.31±6.81%	1.01±11.48%
C. albicans hydrophilic surface	EAL	47.13±12.91%	113.65±2.12%	26.07±9.83%	10.15±8.43%	28.65±5.79%	66.40±12.83%
	EAR	32.97±3.35%	60.28±9.53%	52.67±8.83%	24.03±7.27%	46.72±7.50%	56.25±8.98%
	HPL	56.21±10.47%	46.75±9.32%	22.97±7.16%	48.29±10.86%	61.23±12.34%	42.87±5.81%
	HOL	-24.32±7.48%	-31.76±8.75%	-32.23±4.20%	21.71±13.99%	42.03±6.00%	9.63±7.53%
C. albicans hydrophobic surface	EAL	-37.50±4.03%	-73.89±1.01%	-64.74±0.69%	-42.57±8.69%	29.90±2.07%	65.32±20.58%
	EAR	-25.81±19.29%	-11.14±4.99%	-32.69±8.81%	-10.91±7.64%	51.68±12.59%	54.69±13.88%
	HPL	-42.33±5.15%	-32.18±4.95%	-55.39±2.09%	-17.54±4.82%	23.59±13.40%	16.17±11.43%
	HOL	44.86±1.47%	6.23±3.02%	-35.67±20.22%	-68.36±2.99%	-41.95±10.92%	148.60±15.39%

Fig. 6 Microplate-based biofilm assay. Percentage reduction of the number of adhered cells of *E. coli* and *C. albicans* on hydrophilic and hydrophobic surface in presence of non-lethal concentrations of seagrass extracts. According to post hoc analysis (Tukey's HSD, $p \leq 0.05$), stars indicate statistically significant differences between the means of three independent replicates. In addition, the mean ± SDs of the percentage reduction of the number of adhered cells with seagrass extracts at non-lethal concentrations on hydrophilic and hydrophobic surface are reported in the table. The higher anti-adhesion effect for each microorganism was highlighted. (EAL = *Enhalus acoroides* leaf; EAR = *Enhalus acoroides* root; HPL = *Halodule pinifolia* leaf; HOL = *Halophila ovalis* leaf)

best performance. Indeed, several studies have reported a weak activity of the compounds at low and high concentrations, and excellent activity at intermediate concentrations [54]. Such a response, widely known in literature, is defined as hormesis, an adaptive behavior of microorganisms to provide resistance to environmental stress and improve the allocation of resources to ensure cell stability [19, 55].

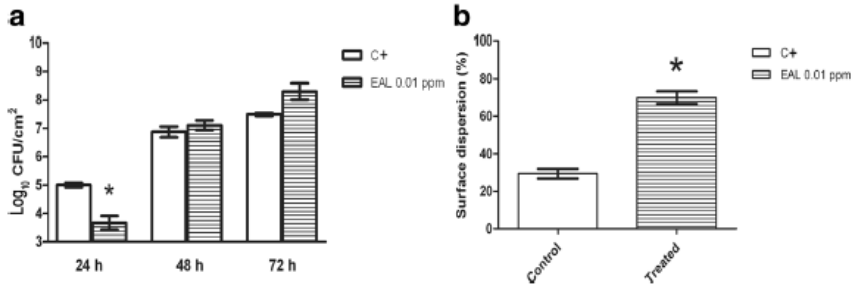


Fig. 7 CDC biofilm growth on polycarbonate coupons (**a**) and biofilm dispersion rate (**b**) of *C. albicans* in absence (positive control) and in presence (treated) of 0.01 ppm of *Enhalus acoroides* leaf extract. Stars indicate statistically significant differences (Tukey's HSD, $p \leq 0.05$) between the means of three independent replicates. (C + = Positive control; EAL = *Enhalus acoroides* leaf)

To further explore the effect of the most promising seagrass extract on biofilm development and detachment, CDC reactors were employed to reproduce biofilm at the solid/liquid interface, while for the assessment of the anti-biofilm effect in the adhesion phase microplate-based biofilm assays are the most suitable [41, 56, 57]. In this study, a significant reduction in fungal coverage (up to 26.77 ± 9.01%) after 24 h (static adhesion phase) was observed in presence of 0.01 mg/l *E. acoroides* leaf extract. This result confirms the anti-adhesion activity observed in microtiter assays. Coupons collected after 48 and 72 h showed no significant differences between treated and control samples.

In order to assess the possibility of 0.01 mg/l *E. acoroides* leaf extract to promote *C. albicans* biofilm-detachment from the surface of coupons, a biofilm dispersion assay was performed. Results showed a significant increase in the number of dispersed cells in the treated biofilm, compared with the untreated (70 ± 6.83%), suggesting a further mechanism of action for the seagrass extract as biofilm dispersing agent. In fact, the phase of biofilm dispersion could be an interesting target for the development of new antibiofilm strategies, forcing the planktonic state and reestablishing the efficacy of traditional antimicrobial agents [4, 58]. Literature with information related to *C. albicans* biofilm dispersion is scarce. Farnesol and cis-2-decenoic acid showed dispersion-promotion of microcolonies of *C. albicans* biofilm [58, 59]. In addition, Villa et al. [60] reported that non-lethal concentrations of *Muscari comosum* ethanol bulb extract can modulate yeast adhesion and subsequent biofilm development on abiotic surfaces, and such concentrations could provide an extracellular signal responsible for biofilm dispersion.

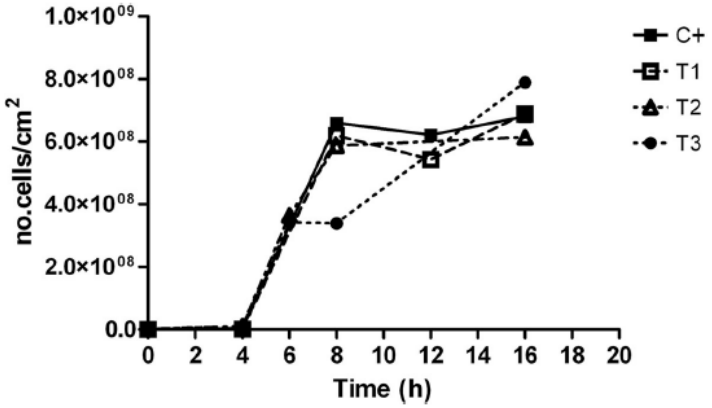


Fig. 8 Biofilm growth at the solid/air interface. *E. coli* biofilm grown on polycarbonate membrane under three experimental conditions: i) treatment 1: growth in contact with 1 ml of LB with 10 ppm of *E. acoroides* leaf extract; ii) treatment 2: overnight culture grown with 10 ppm of *E. acoroides* leaf extract and then growth in contact with 1 ml of LB; iii) treatment 3: overnight culture grown with 10 ppm of *E. acoroides* leaf extract and growth in contact with 1 ml of LB with 10 ppm of *E. acoroides* leaf extract. In the positive control, microorganisms grew in 1 ml LB inside a basolateral well without the extract. Data obtained were divided by the area of the membrane, and means were reported. The experiment was repeated three times. (T1 = treatment 1; T2 = treatment 2; T3 = treatment 3; C + = Positive control)

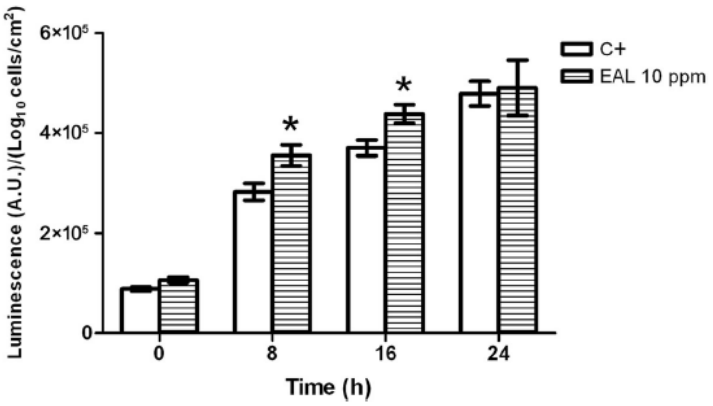


Fig. 9 Relative luminescence emitted by *Vibrio harveyi* in absence (positive control) and in presence of 10 ppm of *E. acoroides* leaf extract for 24 h. The relative luminescence has been calculated by normalizing luminescence by the number of adhered cells. Stars indicate statistically significant differences (Tukey's HSD, $p \leq 0.05$) between the means of three independent replicates. (C + = Positive control; EAL = *Enhalus acoroides* leaf)

For *E. coli*, the CDC reactor was not suitable to evaluate the possible effects of the extracts on the biofilm stages. Also other authors have reported the poor biofilm formation exhibited by *E. coli* K-12 strain under hydrodynamic conditions [61–63]. The effect of 10 mg/l of *E. acoroides* leaf extract on *E. coli* biofilm formation was then evaluated using a membrane-supporting biofilm reactor, which allowed the formation of a biofilm at the solid/air interface. This technique forced the cells to attach to a surface, a feature that allowed direct investigation of the effect of the selected extract on the development of the biofilm, whilst bypassing the effect on the adhesion phase.

No significant reduction in the number of viable cells during biofilm formation on the membrane treated with the extract, compared to the untreated, after 18 h in all the experimental conditions was observed. Treatment 3 showed a growth rate slowdown in the interval 6–8 h, in which *E. coli* cells were in contact with the extract during both overnight growth and biofilm formation (reduction of cellular growth, compared to the control, up to 48.64 ± 4.02%). Interestingly, treatment 3 showed a biphasic growth curve compared with the growth curves of the other treatments, a trend that could be explained by the bioluminescence produced by *V. harvey*. As signaling molecules play an important role in biofilm development and detachment, the effects of 10 mg/l of *E. acoroides* leaf extract were investigated using *V. harveyi*, suggesting other possible antibiofilm mechanisms of action of compounds in the chosen seagrass extract. The results revealed that at time 8 h, the samples treated with the leaf extract showed a significant increase in the relative luminescence emitted, compared to the control (25.75 ± 7.49). Villa et al. [64] reported an increase of autoinducer-2 (AI-2) activity and a reduction in biofilm formation in *E. coli* cells treated with zosteric acid, a phenolic compound occurring in the seagrass *Zostera marina*. In fact, it has been hypothesized that the accumulation of AI-2 above a threshold level leads to reduced biofilm formation due to the induction of a hypermotile phenotype that is unable to adhere to the surface [64]. Huber et al. [65] demonstrated that some polyphenolic compounds containing a gallic acid residue commonly produced by some plant species inhibited intercellular communication in bacteria. Truchado et al. [66] reported the ability of some phytochemical compounds (cinnamaldehyde, ellagic acid, resveratrol, rutin and pomegranate extract) to interfere with the quorum sensing system of *Yersinia enterocolitica* and *Erwinia cartovora*.

It has been well known that the antibiofilm activity of plant extracts is closely linked with the content of secondary metabolites, such as phenols and/or flavonoids, which represent the total amount of phenolic compounds in a plant extract [13]. The phenolic compound content is also deeply associated with the antioxidant activity of plant extracts [67]. Therefore, we determined the total phenolic acid (TPC) and flavonoid (TFC) content and the antioxidant activity (ORAC) of methanolic extracts in order to highlight features of the most promising antibiofilm extract, the *E. acaroides* leaf extract. Results show that *E. acaroides* leaf extract presents the lower TPC and TFC values compared to other seagrasse extracts. Although the low content of phenolic compounds, the *E. acaroides* leaf extract displays a higher ORAC value compared to the root extract. This indicates the abundance of other, non-phenolic compounds with antioxidant capacity in the leaves of *E. acoroides*. Cattò et al. [39] suggested the importance of antioxidant compounds in hindering biofilm formation. The researcher discovered that the mechanism of action behind the antibiofilm performance

of zosteric acid, a secondary metabolite of the seagrass *Zostera marina*, is related to the antioxidant activity of the molecule, and its interaction with the WrbA protein responsible maintaining cellular homeostasis and defense against oxidative stress.

To gain more insight into possible antibiofilm compounds in the seagrass extracts, individual substances in the methanolic extract were analyzed by LC-MS. Preliminary analysis shows that the phytochemical profile of the *E. acaroides* leaf extract is mainly characterized by the presence of the flavones apigenin and luteolin, three kaempferol derivates and the carboxylic acids benzoic and azelaic acid. This unique quantitative and qualitative chemical composition confers antibiofilm properties to the *E. acaroides* leaf extract.

Some of these compounds have shown to exhibit antibiofilm properties at non-lethal concentrations. Kaempferol, apigenin and luteolin from red wine reduced biofilm formation of methicillin-sensitive *S. aureus* significantly [68]. Sánchez et colleagues [69] reported that sub-lethal concentrations of plant extracts inhibit *E. coli* and *S. aureus* biofilms. The antibiofilm properties of the extracts were associated to the presence of flavonoids, such as kaempferol and apigenin, which modulate bacterial cell-cell communication by suppressing the activity of the autoinducer-2 [70]. However, we should keep in mind that the antibiofilm effects of plant extracts could be the result of interactions among different components of the extract at specific concentrations, and not only due to the effects of a single, predominant compound [4, 71].

Conclusions

In conclusion, the *E. acoroides* leaf extract proved to be the most promising extract among those tested. Indeed, the selected non-lethal concentrations of *E. acoroides* leaf extract were found to exert an antibiofilm effect on *C. albicans* and *E. coli* biofilm in the first phase of biofilm genesis, opening up the possibility of developing preventive strategies to hinder the adhesion of microbial cells to surfaces. The leaf extract also affected the dispersion and maturation steps in *C. albicans* and *E. coli* respectively, suggesting an important role in cell signaling processes. These effects could be explained by the presence of active compounds like kaempferol and apigenin at specific concentrations in the extracts of *E. acoroides*, which are known to possess biofilm inhibiting properties. Furthermore, there could be a synergistic action of these flavonoids with other compounds occurring in the plant, enhancing the global antibiofilm effect. Currently, the leaf extract is being investigated with the objective of testing fractions for identifying the active compounds and to better understand the mechanisms of action of this seagrass species.

Acknowledgments
We would like to thank Dr. Vy Xuan Nguyen for the identification and collection of the seagrass samples in Vietnam used in this work.

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Availability of data and materials
The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Authors' contributions
DVL performed the biological experiments and wrote the manuscript. GY extracted and analyzed the crude extracts from the plants by MS. CC participated in the design of the study and provided technical advices and lab supports. VF conceived, designed and coordinated the study. VF contributed substantially to the writing and revising of the manuscript. CF and PJ participated in the design of the study, in discussions and reviewed the manuscript. All authors read and approved the final manuscript.

Competing interest
The authors declare that they have no competing interests.

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Chapter 5

Glasenapp, Y., Lucas, C., Wöltje, T., Fohrer, J., Papenbrock, J. (2019): Anti-adhesion activity of tannins isolated from the mangrove *Laguncularia racemosa*. Chemistry & Biodiversity 16: e1800632. doi.org/10.1002/cbdv.201800632

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FULL PAPER

CHEMISTRY & BIODIVERSITY

Anti-Adhesion Activity of Tannins Isolated from the Mangrove *Laguncularia racemosa*

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In the search of new compounds with biofilm-inhibiting properties, mangroves with their richness of secondary metabolites can be a valuable resource. Crude methanolic leaf extracts from the mangrove *Laguncularia racemosa* enriched in phenolic substances cause a reduction in initial cell adhesion of *Candida glabrata* and *Candida albicans*, but not on *Escherichia coli*. LC/MS-guided fractionation of the phenolic compounds resulted in 19 fractions, of which ten were analyzed for their bioactivity against cell adhesion. Effects on cell adhesion and planktonic growth of *Escherichia coli*, *Candida glabrata* and *Candida albicans* were measured in 96-well microtiter plates in the presence of 0.2 mg ml^{-1} of the isolated fractions. Two fractions caused a reduction of cell adhesion of *Candida albicans*. These fractions containing bioactive compounds were analyzed by LC/MS and NMR spectroscopy. Casuarinin and digalloyl-hexahydroxydiphenoyl-glucose were identified in the active fractions, in addition to three signals of ellagitannins. These results indicate a specific mode of action of hydrolysable tannins against cell adhesion of *Candida albicans*, which needs to be further analyzed.

Keywords: *Laguncularia racemosa*, bioactive compounds, biological activity, mangrove, phenolic compounds, tannins, phytochemistry.

Introduction

A major part of microbial life takes place not in a planktonic, but in a sessile lifestyle known as biofilm.[1] Bacteria and fungi, which attach to a surface, create a matrix around them consisting of extracellular polymeric substances (EPS).[2] These EPS can include polysaccharides, proteins, lipids and extracellular DNA. Once a biofilm is established, it provides protection for the microorganisms from physical impacts and increases the resistance against antibiotics and antifungal agents.[3] The awareness of biofilms as a cause of persistent infections and complications, especially in hospital environments, has increased recently.[4,5] Existing antimicrobials may have to be used in higher concentrations to cure the infection, which can have severe side effects on the patients.[6] In addition, biofilms can cause clogging in tubes of industrial systems or form layers on ship hulls. Removal of these structures requires mechanical effort or, in the case of ships, often the use of toxic chemicals.[7] These factors lead to a demand in new active compounds in the treatment and prevention of fungal and microbial biofilms, with a good tolerability. Natural compounds isolated from plants have a long tradition in medical applications and present a possible source of new active anti-biofilm compounds. Phenolic and polyphenolic substances with this activity have been reported, like zosteric acid from *Zostera marina* or epigallocatechin gallate from green tea.[8,9] Mangroves are an interesting group of plants, which can sustain in a

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highly challenging ecosystem with biotic and abiotic stress factors like salinity and flooding. Part of their adaptation mechanism is the production of phenolic compounds in the leaves, as their antioxidant capacities protect the plant cells from oxidative stress.[10] *Laguncularia racemosa* (L.) C.F.GAERTN. belongs to the family of Combretaceae and is distributed in the Neotropics and West Africa.[11] Plant leaves and bark contain a number of phenolic compounds including tannins, triterpenes and flavonoids and are used in traditional medicine.[12–14]

In the present study, phenolic compounds from *L. racemosa* were extracted and tested for their activity against cell adhesion of the Gram-negative bacterium *Escherichia coli* and the infectious yeasts *Candida albicans* and *Candida glabrata*. *Escherichia coli* was selected as model organism for bacteria, whereas *C. albicans* and *C. glabrata* are two common species in fungal infections.[15] The aim is to identify and describe bioactive compounds with a possible application in the prevention of biofilm formation.

Results and Discussion

Methanolic extracts prepared from *L. racemosa* leaves and further purified by solid phase extraction (SPE) lead to a reduction on *E. coli*, *C. glabrata* and *C. albicans* cell adhesion in the first phase of biofilm formation (*Figure 1*). Tested on *E. coli*, the highest concentrations of 0.2 and 0.4 mg ml^{-1} showed a reduced cell adhesion, which is not statistically significant. The different SPE extract concentrations did not have any considerable effect on the growth of planktonic *E. coli* cells except for 0.4 mg ml^{-1}, which reduced the planktonic cell density. In the case of 0.01, 0.02 and 0.05 mg ml^{-1}, the increased amount of adhered cells correlates with a reduced amount of planktonic cells. This indicates a redistribution of cells towards the sessile form. At a concentration of 0.4 mg ml^{-1}, both adhered and planktonic cell growth is reduced. *Candida glabrata* cell adhesion was negatively influenced by the extracts at concentrations of 0.01, 0.1, 0.2 and 0.4 mg ml^{-1}. At the highest concentrations of 0.2 and 0.4 mg ml^{-1}, planktonic cell density was substantially reduced. A concentration of 0.1 mg ml^{-1} caused a reduced cell adhesion, but elevated the presence of planktonic cells. Here, the reduced amount of sessile cells favors the presence of planktonic cells. On *C. albicans*, 0.05, 0.2 and 0.4 mg ml^{-1} lead to a reduced cell adhesion. Planktonic cell density was not negatively influenced.

These results indicated the presence of adhesion-inhibiting substances, which belong to the group of phenolic compounds, as other chemical compound groups were eliminated by SPE purification. In order to identify the active substances, the SPE-purified *L. racemosa* methanolic extract was subjected to LC/MS-guided fractionation. Peaks where only one main mass occurred were selected, which lead to a total number of 19 fractions (*Figure 2*). Times of collection were set accordingly, with at least 0.3 min collection time and a maximum of 0.8 min, depending on the peak width.

All fractions obtained with the fractionation were analyzed by LC/MS. High resolution MS and MS/MS spectra were used for database searches. Precursor and corresponding fragment masses were compared to entries in MassBank, ReSpect and to the literature (*Table S1*, *Supporting Information*).[16–18] The obtained MS signals were compared to the signals present in an extract of the same leaf material prepared in 80% acetone, to identify possible byproducts due to the extraction and storage in methanol. For fractions 1 to 4, none of the masses previously detected in the chromatogram could be found. In fractions 5 and 6, different tannins were present, namely casuarinin, digalloyl-hexahydroxydiphenoyl (HHDP)-glucose and three ellagitannins of unknown structure.[19]

Fractions 7 to 9 contained methyl gallate, which does not occur in plants naturally and can be a result of methanolysis of gallic acid from the tannins in the presence of methanol.[20] Methyl gallate was not present in an extract of the same leaf material prepared with 80% acetone (*Figure S1*). A small peak of pentagalloylglucose was identified in fraction 11. A number of flavonols were identified in fractions 11, 12 to 15, 18 and 19. These were mainly myricetin and its glycosides. One phenolic acid glucoside was found in fraction 18. Six fractions (8, 10, 11, 16, 17, 19) contained masses that could not be identified with the databases used or the literature. The presence of phenolic compounds, including flavonoids and phenolic glycosides, has been described in the literature for *L. racemosa* twigs and bark.[12] There is little information available about detailed analysis of phenolic compounds in *L. racemosa* leaves, but the Combretaceae family is known to contain tannins, flavonols and their derivatives.[21,22]

All fractions, of which the amounts were sufficient to be used in three replicates in the adhesion assay and at least one replicate in the antimicrobial susceptibility test, were analyzed for their bioactivity at a concentration of 0.2 mg ml^{-1} (*Figure 3*). In addition to *C. albicans*, the activity on *E. coli* as a model organism

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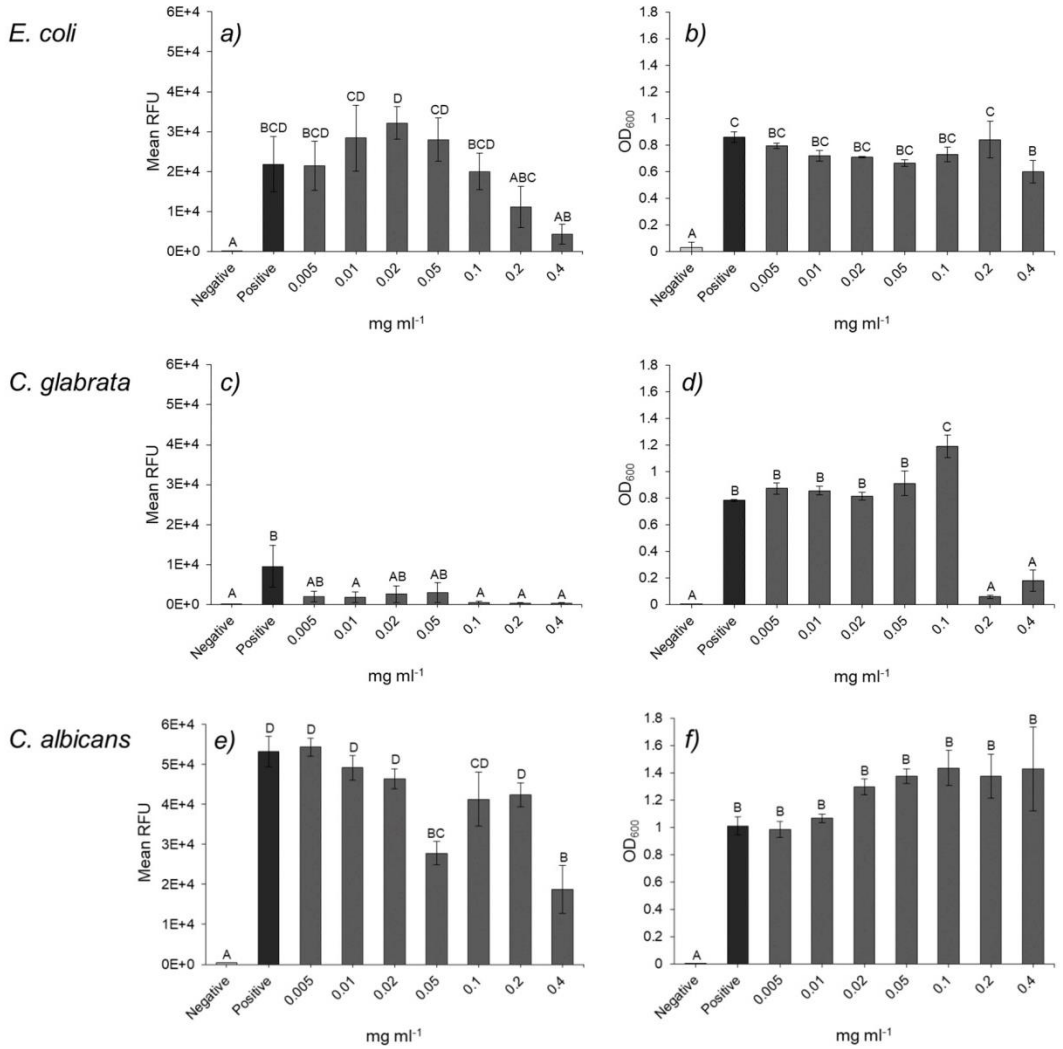

Figure 1. a), c), e) Cell adhesion ($n = 3$) and b), d), f) antimicrobial susceptibility test ($n = 3$) with SPE-purified *L. racemosa* methanolic extract on *E. coli*, *C. glabrata* and *C. albicans*. Negative control = growth medium, positive control = growth medium plus *E. coli* or *Candida* cells and 80 % MeOH. Data represent the mean values of three experiments (± SD). Different letters (A–D) above the bars indicate statistically significant differences with $p < 0.05$ tested with a one-way ANOVA with Tukey's test. RFU, relative fluorescence units; OD, optical density.

for Gram-negative bacteria and another yeast species, *C. glabrata*, were selected. Tested on *E. coli*, the adhesion of cells was not considerably influenced, only fraction 6 promoted the cell adhesion. The test for antimicrobial effects on the growth rate showed a reduction of the maximum OD_{600} for fractions 6 and 15, but due to the limited amount of fraction material no conclusions regarding the significance can be drawn. Since more cells adhered in the presence of fraction 6, the reduced planktonic cell concentration is likely to be due to a shift towards the sessile growth state. Cell adhesion of the yeast *C. glabrata* was promoted by fractions 2, 4 to 7 and 9, whereas fractions 10 and 14 showed no influence. The antimicrobial susceptibility assay showed a strong reduction by compounds in fraction 2, the other fractions showed a maximum OD_{600} close to the positive control at 0.8. The adhesion of *C. albicans* cells was substantially reduced by fractions 5 and 6 with 9208.2 ± 3553.5 relative fluorescence units (RFU) and

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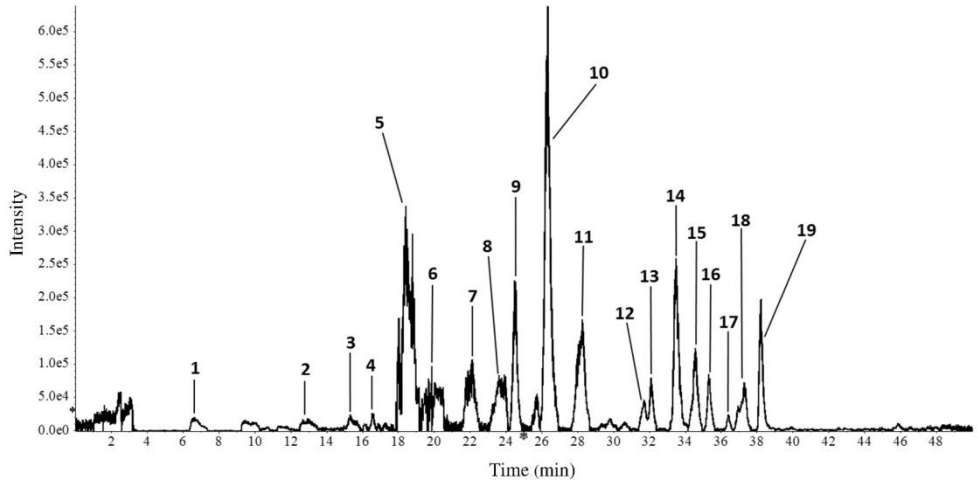

Figure 2. Total ion current (TIC) chromatogram from *L. racemosa* methanolic leaf extract after SPE purification in negative electrospray ionization LC/MS. Numbers indicate peaks described in *Table S1* (*Supporting Information*).

10,682.8 ± 3980.7 RFU compared to the positive control with 21.865.9 ± 3273.2 RFU. The antimicrobial susceptibility test was repeated three times for fractions 2, 4 to 7, 9, 10 and 14. Fraction 5 showed a considerable increase of the maximum OD_{600} with 1.38 compared to the positive control with 0.99. Here, the reduction of cell adhesion leads to an increase in the concentration of planktonic cells.

Interestingly, the fractions showed no inhibitory effect on cell adhesion on *C. glabrata*, even though the SPE-purified leaf extract reduced the adhesion. A possible explanation is a synergistic effect of two or more compounds present in the whole extract, which are separated after fractionation and therefore their combined activity is lost. Another factor influencing the compound composition might be the drying step of the fractions in the rotary evaporator. Here, the fractions are exposed to a temperature of 60 °C, which could lead to a degradation process or structural changes of the compounds. A cell adhesion test was carried out with the highest concentration of SPE extract, 0.4 mg ml^{-1}, which was previously heated to 60 °C for 2 h (data not shown). The results showed a loss of adhesion reducing activity on *E. coli*, but not on *C. glabrata* and *C. albicans*.

The different activity of the fractions on the two yeasts *C. albicans* and *C. glabrata* can be explained by their different biofilm formation mechanisms. They possess diverse adhesins, which are a possible target for anti-biofilm compounds, as they modulate the attachment of yeast cells to a surface.[23,24] In the progress of biofilm development, other processes could be influenced by biofilm inhibiting reagents. *C. albicans* biofilms are composed of yeast cells, hyphae and pseudohyphae, whereas *C. glabrata* forms a multilayer of yeast cells.[25] As the active fractions did not inhibit general cell growth, their activity is more likely connected to the disruption of processes in *C. albicans* adhesion or matrix development.

To gain more insight into the concentration dependency of the cell adhesion inhibition on *C. albicans*, dilution series of fractions 5 and 6 were analyzed. Fractions 5 and 6 were tested in concentrations of 0.05, 0.1, 0.2 and 0.3 mg ml^{-1} on *C. albicans* (*Figure 4*). The diluted fractions showed a linear relationship between concentration and cell adhesion, with 0.3 mg ml^{-1} showing the highest reduction. For fraction 5 at 0.3 mg ml^{-1}, this effect was statistically significant compared to the positive control. The planktonic cell density of *C. albicans* increased at concentrations of 0.1 mg ml^{-1} for both fractions and 0.2 and 0.3 mg ml^{-1} for fraction 6. The two highest concentrations of fraction 5 could not be tested more than one time. A possible reason for the promotion of growth in the antimicrobial susceptibility test can be the shift between the planktonic and adhered cell subpopulations towards the planktonic state.

As fractions 5 and 6 showed the highest reduction rate on *C. albicans*, their chemical structures were elucidated by MS/MS and NMR spectroscopy in the case of fraction 5. LC/MS of fraction 5 revealed the presence of one main peak at *m/z* 935.08 $[M-H]^-$ and

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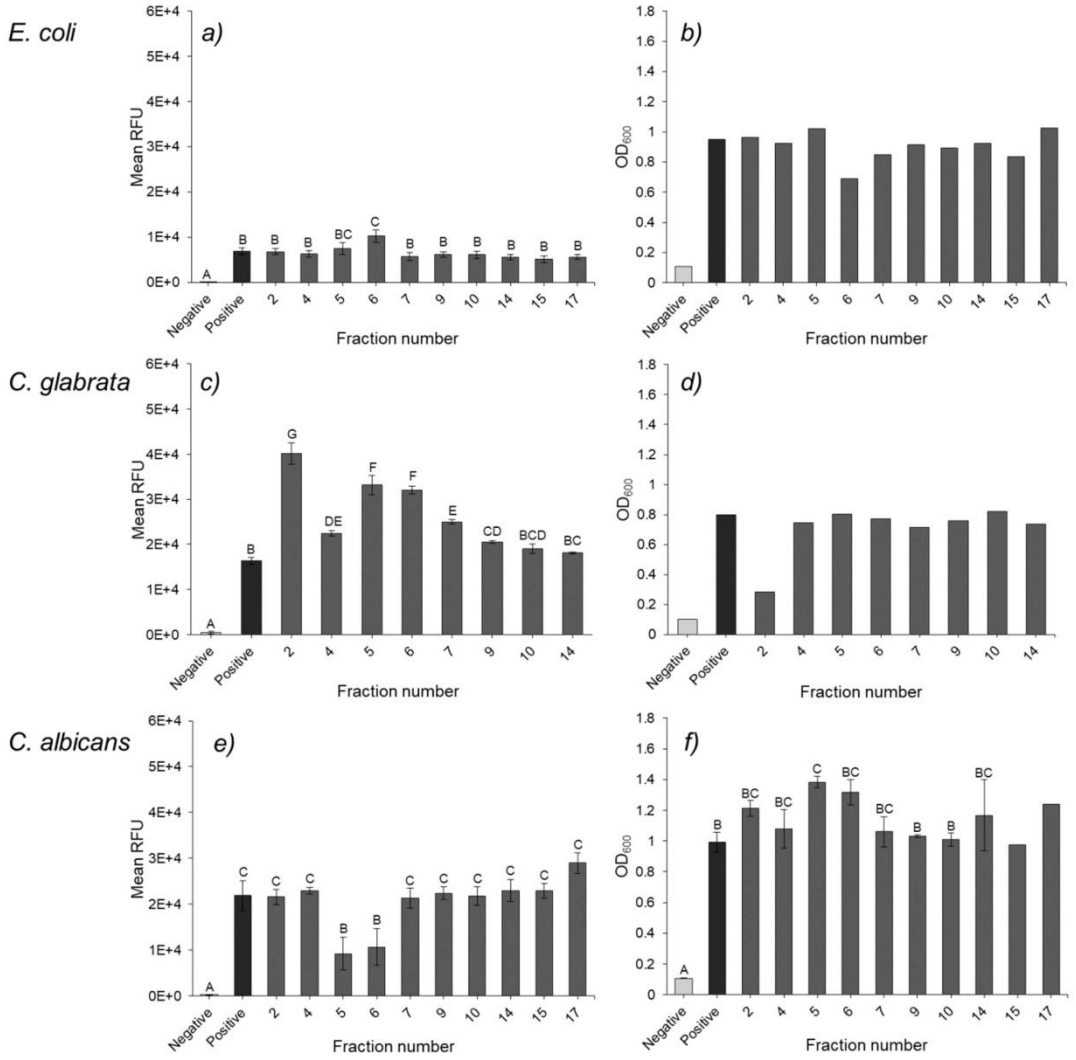

Figure 3. a), c), e) Cell adhesion ($n=3$) and b), d), f) antimicrobial susceptibility test (b), d): $n=1$, f): $n=3$, fraction 15: $n=1$ and 17: $n=2$) with fractions of *L. racemosa* methanolic extract after SPE purification on *E. coli*, *C. glabrata* and *C. albicans*. Negative control = growth medium, positive control = growth medium plus *E. coli* or *Candida* cells and 80% MeOH. Data represent the mean values of three experiments (±SD). Different letters (A–G) above the bars indicate statistically significant differences with $p<0.05$ tested with a one-way ANOVA with Tukey's test. RFU, relative fluorescence units; OD, optical density.

467.03 $[M-2H]^{2-}$ (*Table 1*). The fragmentation pattern showed *m/z* of 169 and 125, representing gallic acid and the cleavage of its CO_2. The presence of fragments with 301, 275 and 249 *m/z* corresponds to the presence of a HHDP group. The signal at 633 *m/z* represents the loss of one HHDP group. The mass of 935 $[M-H]^-$, the high abundance of its double charged species $[M-2H]^{2-}$ and their MS/MS fragments indicate a galloyl-bis-HHDP-D-glucopyranose.[26] In addition, three minor compounds presented *m/z* values of 939.11, 953.12 and 965.09 $[M-H]^-$ in the negative electrospray ionization and were identified as ellagitannins with unknown structure. The fragments of *m/z* 301, 275 and 249 typical for the HHDP group were also present in these three tannins. One of them presented a precursor ion of *m/z* 939.11 $[M-H]^-$ and fragments of 787, 769 and 431. In a relative comparison of the peak areas of all peaks present in fraction 5, the signal of 935.08 contributes to 44%, 939.11 to 23%, 953.12 to 9% and 965.09 to 24% of the peak

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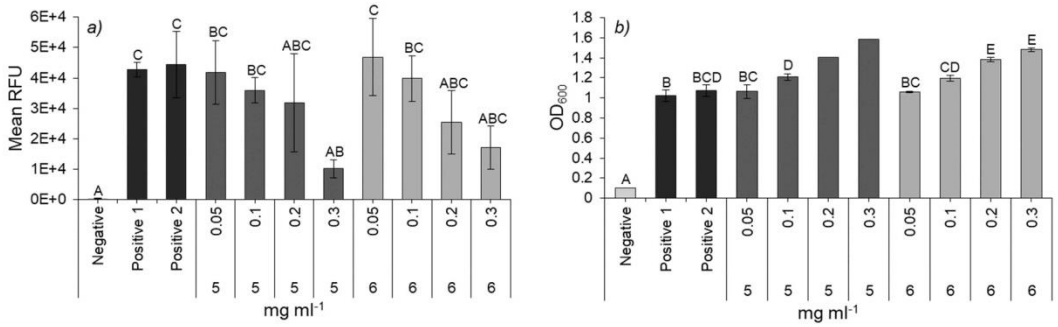

Figure 4. a) Cell adhesion and b) antimicrobial susceptibility test with dilutions of chromatographic fractions 5 and 6 from SPE purified methanolic *L. racemosa* extract on *C. albicans*. Negative control = growth medium, positive control = growth medium plus *C. albicans* cells and 80 % MeOH. Data represent the mean values of three experiments (± SD). Different letters (A–E) above the bars indicate a statistical difference with $p < 0.05$ tested with a one-way ANOVA with Tukey's test of three independent biological replicates ($n = 3$). Due to a limited sample amount, only one biological replicate of fraction 5 at 0.2 and 0.3 mg ml^{-1} was used in the antimicrobial susceptibility test ($n = 1$). RFU, relative fluorescence units; OD, optical density.

Table 1. MS^1 and MS^2 data in *m/z* for compounds present in fractions 5 and 6. MS^1 was recorded for $[M-H]^-$ and $[M-2H]^{2-}$, the latter is shown in brackets.

Fraction	Compound	t_R [min]	MS^1 [m/z]	MS^2 [m/z]
5	Casuarinin	16.6	935.0816 (467.0375)	633.0749, 300.9987, 275.0202, 249.0403, 169.0140, 125.0244
	Ellagitannin	17.3	939.1123 (469.0546)	603.0614, 335.0397, 300.9987, 275.0193, 249.0396, 169.0133
	Ellagitannin	18.6	953.1268 (476.0599)	921.0950, 773.0173, 619.0882, 300.9954, 275.0168, 249.0376, 169.0115
	Ellagitannin	18.2	965.0917 (482.0425)	933.0569, 903.0835, 573.0461, 300.9954, 275.0165, 249.0373, 169.0123
6	Digalloyl-HHDP-glucose	17.9	785.0847 (392.0381)	633.0750, 615.0633, 483.0779, 463.0490, 300.9991, 275.0199, 249.0407
	Ellagitannin	17.3	939.1150 (469.0535)	603.0612, 335.0399, 300.9962, 275.0222, 249.0371
	Ellagitannin	18.6	953.1274 (476.0600)	921.1006, 773.0176, 619.0923, 300.9969, 275.0181, 249.0393
	Ellagitannin	18.1	965.0930 (482.0428)	933.0679, 903.0983, 573.0550, 300.9977, 275.0207, 249.0434

area sum. The precursor mass of 965.09 $[M-H]^-$ could not be assigned to a specific tannin, and has previously been described as an unknown ellagitannin by Dias et al.[19] The fragment detected at *m/z* 481 represents a HHDP-hexose. The mass difference of 32 Da can be caused by a methanol adduct or the addition of oxygen. The main compound of fraction 6 was observed at *m/z* 785.08 $[M-H]^-$ and 392.03 $[M-2H]^{2-}$ (*Table 1*) and identified as digalloyl-HHDP-glucose. Fragment masses of 615, 483 and 301 represent the losses of gallic acid, the HHDP group and digalloylglucose.[18–20] The fragments of *m/z* 301, 275 and 249 indicate the presence of a HHDP group.[20,27] This peak makes out 32 % of the complete peak area sum. In addition, three other compounds with *m/z* 939.11, 953.12 and 965.12 were detected, which were assigned as ellagitannins with unknown structure as for fraction 5. The peak of 939.11 contributes to 23 %, 953.12 to 27 % and 965.12 to 18 % of the complete peak area sum of fraction 6.

Since fractions 5 and 6 both showed a similar inhibitory effect on *C. albicans* cell adhesion, the main active substance could not be identified. The compounds present in both fractions belong to the plant secondary metabolite group of ellagitannins, which are a subgroup of hydrolysable tannins. All hydro-

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lysable tannins share common structural elements, as the aromatic groups and the hexose, and might present similar activities.[21]

For structural elucidation and confirmation of the suggested galloyl-bis-HHDP-D-glucopyranose, NMR spectrum was recorded on fraction 5 (*Table 2*, *Figures S2–S6*). The chemical shifts of one major compound in the ^{1}H and ^{13}C spectra at position C-1 to C-6 indicate an open chain sugar comparable to casuarinin (*Table S2*, *Supporting Information*).[28] NMR signals of the minor compounds were too low to be assigned to one substance individually. Similar to the major compound, the NMR data indicates an open chain structure of the sugar. Based on MS/MS and NMR data, the structure of the main compound of fraction 5 was determined as casuarinin (*Figure 5*).

Table 2. NMR chemical shifts ^{1}H, ^{13}C in (D_6)acetone of compounds present in fraction 5.

Position	Casuarinin		Minor compounds	
	δ(C), type	δ(H)	δ(C), type	δ(H)
1	68.2, CH	5.50	67.0, CH	5.63
2	72.0, CH	5.76	75.7, CH	4.74
3	72.7, CH	5.54	69.1, CH	5.39
4	62.4, CH	5.42	73.5, CH	5.43
5	78.1, CH	4.98	70.2, CH	5.35
6	64.0, CH_2	4.97, 3.96	63.8, CH_2	4.82, 4.10

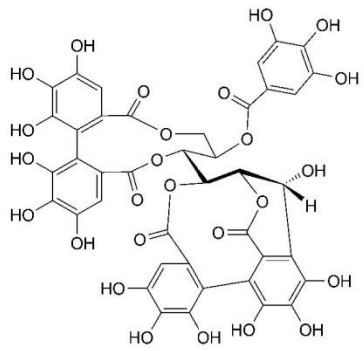

Figure 5. Structure of casuarinin, one compound present in bioactive fraction 5 of SPE-purified *L. racemosa* leaf extract.

Ellagitannins are complex plant secondary metabolites, which are important for the defense of plants against herbivores.[29] All ellagitannins are derived from pentagalloylglucose, from which two or more galloyl groups are linked by oxidative coupling.[30] In the majority of ellagitannins, the coupled groups form a HHDP group.[31] The occurrence of ellagitannins, including casuarinin, has been reported for the plant family Combretaceae.[32] In the related species *Lumnitzera racemosa*, the occurrence of eleven tannins has been demonstrated, including castalagin and 1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyranose.[33]

To gain further insight into the mode of action of the ellagitannins, two smaller aromatic compounds have been tested for their ability to inhibit *C. albicans* cell adhesion. Gallic acid is a moiety of the casuarinin molecule which can be cleaved by hydrolysis. In addition, pyrogallol, which is derived from gallic acid by decarboxylation, was tested (*Figure 6*). The results show no inhibitory effect of gallic acid at concentrations between 0.0125 and 1.2 mg ml^{-1}. Similarly, pyrogallol did not influence the adhesion in a significant way, only at high concentrations of 0.8 and 1.2 mg ml^{-1} there seems to be redistribution towards the adhesion phase, as the density of planktonic cells decreases. The gallic acid moiety alone is not responsible of the inhibitory effect of the ellagitannins present in the active fractions. Further tests with ellagic acid and other breakdown products as well as the whole ellagitannins need to be carried out to identify which exact molecular structure leads to the inhibition of cell adhesion.

A possible mode of action in the inhibition of cell adhesion on *C. albicans* can be the precipitation of proteins involved in biofilm formation. The biofilm matrix of *C. albicans* is mainly composed of proteins (55%) and to a lesser amount of carbohydrates (25%), lipids (15%) and nucleic acids.[34] The precipitation of matrix proteins would drastically decrease the stability of the developing biofilm matrix. Lim at al. (2006) have observed alterations in cell wall morphology in *C. albicans* cells exposed to hydrolysable tannins extracted from the mangrove *Rhizophora apiculata* at concentrations of 100 mg ml^{-1}.[35] During the time course of 36 h, cells first appeared as sticky and clustered, then showing cavities and finally cell walls collapsed. Pomegranate extracts rich in hydrolysable tannins have also been found to change *Candida* cell morphology in a study by Anibal et al.[36] Cell membranes and hyphae formed irregularly, indicating an effect of the present metabolites on the cell wall structure. This could be caused by interactions with structural elements such as polysaccharides and proteins in the cell wall.[37] The exact mode of action and the suitability for a practical application in biofilm prevention need to be further analyzed.

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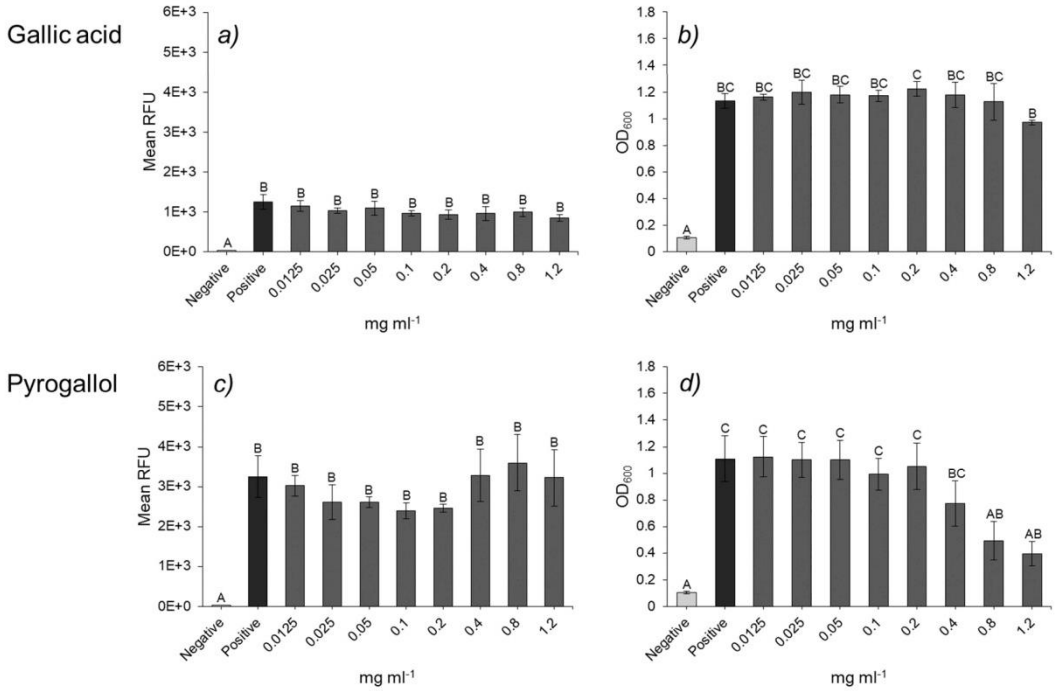

Figure 6. a), c) Cell adhesion ($n=3$) and b), d) antimicrobial susceptibility test ($n=3$) with different concentrations of gallic acid and pyrogallol on *C. albicans*. Negative control = growth medium, positive control = growth medium plus *C. albicans* cells and 80% EtOH. Data represent the mean values of three experiments (±SD). Different letters (A–C) above the bars indicate statistically significant differences with $p<0.05$ tested with a one-way ANOVA with Tukey's test. RFU, relative fluorescence units; OD, optical density.

Conclusions

In this study, the potential occurrence of bioactive compounds of the mangrove *Laguncularia racemosa* against initial biofilm cell adhesion was evaluated. Crude methanolic extracts enriched in phenolic compounds have shown activity against *Candida albicans*, *Candida glabrata* and *Escherichia coli* cell adhesion in the first 24 h of biofilm growth, but the fractions analyzed showed only activity against *C. albicans*. The compounds showing activity against *C. glabrata* and *E. coli* seem to be present in the fractions which could not be tested, or the activity is based on synergistic effects of several compounds. The fractions inhibiting *C. albicans* adhesion have been found to contain a mixture of tannins. These polyphenolic compounds have been reported in the literature to interact with *Candida* cell wall structure. To further analyze the bioactivity of tannins from *L. racemosa*, a more sensitive fractionation method needs to be applied. In addition, testing single compounds in the form of chemical standards would give more insight, but only a small number of tannins are commercially available. The activity of the ellagitannins could also be based on breakdown products, since they all share structural elements like gallic acid and ellagic acid. Overall, *L. racemosa* was found to contain ellagitannins with inhibitory activity against *C. albicans* cell adhesion, which gives the fundament of further research on anti-biofilm compounds from natural sources.

Experimental Section

General

TOF-MS and MS/MS spectra were either obtained on a TripleTOF 4600 or 6600 quadrupole time of flight (qTOF) mass spectrometer (AB Sciex, Canby, USA) with negative electrospray ionization (ESI) coupled to a Shimadzu high performance liquid chromatography (HPLC; Shimadzu, Kyoto, Japan) or an Agilent 1260 HPLC (Agilent, Santa Clara, USA). NMR spectra were acquired on a Bruker 500 MHz UltraShield with Avance-IIIHD console and Cryo TCI $^{1}H,^{13}C/^{15}N$ probe

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(Bruker BioSpin GmbH, Rheinstetten, Germany) at 500 MHz for ^{1}H and 125 MHz for ^{13}C at 25 °C. (D_6) acetone was used as solvent. Chemical shifts are reported in parts per million (δ). In addition to 1D experiments, 2D spectra including COSY, TOCSY, HSQC, HMBC and NOESY were recorded. All chemicals used were of analytical grade. Methanol used for extraction and LC/MS was purchased from Honeywell (Morris Plains, USA), (D_6)acetone for NMR analysis from Deutero GmbH (Kastellaun, Germany).

Plant Material

Leaves of an adult plant of *L. racemosa* grown in the greenhouse of the Institute of Botany, Hannover, Germany, were harvested in March 2017 and directly frozen in liquid nitrogen. The plant was originally identified by Prof. Schnetterer and was further analyzed by DNA barcoding by Glasenapp et al.[38] A voucher specimen of the plant is available in the Herbarium of the Institute of Botany, Leibniz University Hannover, under the registration number 'Mangrove 100'.

Extraction

The frozen leaf material was ground to a fine powder using liquid nitrogen. For extraction, 140 ml of methanol (MeOH) were added to seven grams of leaf powder and stirred at room temperature. After 1 h, the extract was filtered into a round bottom flask and the plant material was extracted again by using the same volume of MeOH for two times. In the last step, the extraction occurred overnight. The extraction solvent was evaporated with a rotary evaporator (Büchi, Flawil, Switzerland). The extract was dissolved in MeOH to a concentration of 20 mg ml^{-1} and stored at 4 °C in the dark. After a dilution of 1:5 with water, the extract was purified on solid phase extraction (SPE) columns (C18 U, Phenomenex, Torrance, USA). The resulting eluate was collected in reaction tubes and the solvent was evaporated in a speedvac (Eppendorf, Hamburg, Germany) and then dissolved in 80% MeOH to a final concentration of 10 mg ml^{-1}. Acetone extracts were prepared by weighing 50 mg of frozen leaf material into a reaction tube and adding 800 µl aqueous acetone (80%). After 10 min of extraction at room temperature, the tubes were centrifuged for 5 min at 18,000 *g* and the supernatant was transferred into a new tube. This process was repeated three times with 400 µl aqueous acetone.

LC/MS Guided Fractionation

SPE-purified *L. racemosa* leaf extract was fractionated on a semi-preparative HPLC column (250×8 mm, ProntoSIL 120–5 C18H, Knauer, Berlin, Germany) in a HPLC (Shimadzu) coupled to a mass spectrometer (TripleTOF 4600, AB Sciex). Fifty microliter of the purified extract were injected, water (A) and MeOH (B) were used as solvents and were both supplemented with 2 mM ammonium acetate and 0.01% acetic acid. The flow rate was 1.4 ml min^{-1} and the run time was 50 min. A gradient was applied with 90% A at the start, changing linear to 90% B over 45 min, followed by 2 min at 90% B and changing to 90% A in 1 min and 2 min at 90% A. Peaks of extracted compounds were monitored by mass spectrometry in negative ionization mode, with a nebulizer temperature of 0 °C, voltage −4.500 eV and curtain gas 15 psi. Times for collection were set according to the most prominent peaks. Solvent flow was partitioned with a 20:1 flow splitter (ASI, Richmond, USA) and fractions collected by a fraction collector (Shimadzu) in glass vials. In each run, 50 µl of SPE purified extract at a concentration of 10 mg ml^{-1} was injected, the total number of runs was 60. The collected fractions were dried in a rotary evaporator (Büchi), dissolved in 80% MeOH, transferred in reaction tubes and evaporated in a speedvac (Eppendorf), weighed and dissolved to a concentration of 5 mg ml^{-1}.

MS/MS Analysis

Compounds isolated from the purified *L. racemosa* leaf extract by LC/MS fractionation were identified by comparison of mass and MS/MS data with entries in publicly available databases (ReSpect, MassBank). Each isolated fraction was analyzed by LC/MS with the same equipment as described above. The runtime was shortened to 40 min, the injection volume was 10 µl. Ionization was achieved in negative ionization mode at a nebulizer temperature of 600 °C, voltage −4500 eV and curtain gas 35 psi. MS/MS fragmentation occurred in information dependent acquisition (IDA) mode at a collision energy of −30 eV with a collision energy spread of ±15. Fractions 5 and 6 were further analyzed on an Agilent 1260 HPLC coupled to a Hybrid Quadrupole-TOF LC/MS/MS Mass Spectrometer (TripleTOF 6600, AB Sciex). Water (A) and Methanol (B) were used as solvents, both supplemented with 0.1% formic acid. Separation was realized on a Phenomenex prodigy column (150×4.6 mm, phenyl-3, 5 µm, Phenomenex). The total run time was 54 min,

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with the following gradient setting: 95% A from 0–5 min, 70% A at 11 min, 100% B at 23 min, 100% B at 37 min, 95% A at 39 min until 54 min. 15 µl were injected, the flow rate was 0.55 ml min^{-1}. MS acquisition was achieved in negative ionization mode at a nebulizer temperature of 550 °C, voltage −4500 eV and curtain gas 30 psi. MS/MS fragmentation occurred in information dependent acquisition (IDA) mode at a collision energy of 0 eV with a collision energy spread of ±15, and masses between 40 and 1200 Da were recorded. The plant leaf extract prepared with 80% acetone was analyzed on a Shimadzu HPLC coupled to a Hybrid Quadrupole-TOF LC/MS/MS Mass Spectrometer (TripleTOF 6600, AB Sciex). Water (A) and methanol (B) were used as solvents, both supplemented with 0.1% formic acid. A fluorophenyl column (100× 2.1 mm, 3 µm, Restek, Bellefonte, USA) was used for separation, the total run time was 35 min. The gradient was set as following: 90% A at min 1, 98% B at 25 min, 98% B at 32 min, 90% A at 32.1 min until 35 min. 2.5 µl of the sample were injected, the flow rate was 0.55 ml min^{-1}. MS data was acquired in negative ionization mode at a nebulizer temperature of 550 °C, voltage −4500 eV and curtain gas 30 psi. MS/MS fragments were recorded at a collision energy of −10 eV, for masses between 70 and 1200 Da.

Standards

Gallic acid and pyrogallol were purchased from Sigma-Aldrich and dissolved in 80% ethanol. Dilutions of 0.0125, 0.025, 0.05, 0.1, 0.2, 0.4, 0.8 and 1.2 mg ml^{-1} were prepared in 80% ethanol and stored at −20 °C prior to use.

Strains and Media

Escherichia coli ATCC 25404, *Candida albicans* ATCC MYA-2876 and *Candida glabrata* ATCC 90030 were used as model organisms to test for effects of the plant extract and fractions on adhesion and planktonic growth. The *E. coli* and the *C. albicans* strains are both reported as good biofilm formers.[39,40] *C. glabrata* shows weaker biofilm formation, but was chosen as a model for other clinical important *Candida* species.[39] Stock cultures with 50% glycerol and 50% cell culture were stored at −80 °C. *Escherichia coli* was cultivated in Luria Bertani broth (LB), *C. albicans* in amino acid-free yeast nitrogen base (YNB, Sigma-Aldrich, St. Louis, USA) supplemented with 0.5% glucose and *C. glabrata* in YM (Medium 186, DSMZ, Germany). The same media were used for antimicrobial and anti-adhesion assays and cells were grown at 37 °C in case of *E. coli* or 30 °C for yeasts.

Antimicrobial Susceptibility Testing

Effects of the SPE-purified plant extract, fractions and standards on the growth rate of *E. coli*, *C. albicans* and *C. glabrata* were analyzed in 96-well plates. Cells were grown overnight in the respective medium and temperature. The cell density was determined with a hemocytometer and dilutions of 10^8 cells ml^{-1} prepared. Plant extracts and fractions were added to the cell solution and appropriate medium to a defined concentration in mg ml^{-1} for SPE extracts and fractions. Cells mixed with medium and 80% MeOH instead of extract or fraction were used as a positive control, and the respective medium as a negative control. From each sample, 200 µl were placed in a well of a 96-well clear microtiter plate with three replicates per sample. The plate was incubated at 37 °C for *E. coli* and 30 °C for the yeasts and the OD_{600} was recorded every 20 min. Growth curves of the samples with plant extract were compared to the positive control to detect possible negative effects on the growth rate and final OD_{600}.

Anti-Adhesion Activity Testing

Cell adhesion was analyzed in black 96-well microtiter plates (96F untreated, Thermo Scientific, Waltham, USA). Culturing, cell dilution and sample preparation were carried out in the same way as described for the antimicrobial susceptibility test. The plates were incubated statically for 24 h at 37 °C for *E. coli* and at 30 °C for yeasts. Afterwards, the liquid was removed, the adhered cells were dried for 20 min and then stained with 4′,6-diamidino-2-phenylindole (DAPI, 0.01 mg ml^{-1}) in the case of *E. coli* and fluorescent brightener 28 (FB28, 0.01 mg ml^{-1}) for yeasts. The staining solution, incubated for 20 min, was thereafter removed and the plate was dried again. Next, the wells were washed two times with phosphate buffered saline (PBS) and finally dried. The fluorescent signal was measured in a microplate reader (Biotek, Winooski, USA) at 355/460 nm for DAPI and 365/435 nm for FB28.

Statistics

Statistically significant differences between samples were evaluated with an analysis of variance (ANOVA) and Tukey Alpha multiple comparison test in InfoStat

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version 2016e. Significant differences were characterized with $p < 0.05$.

Acknowledgements

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Author Contribution Statement

Y. G. and *J. P.* conceived and designed the experiments. *Y. G.* and *C. L.* performed the LC/MS experiments and analyzed the data. *Y. G.* and *T. W.* performed the microbial assays. *J. F.* contributed the NMR analysis and analyzed the data. *Y. G.* and *J. P.* wrote the article.

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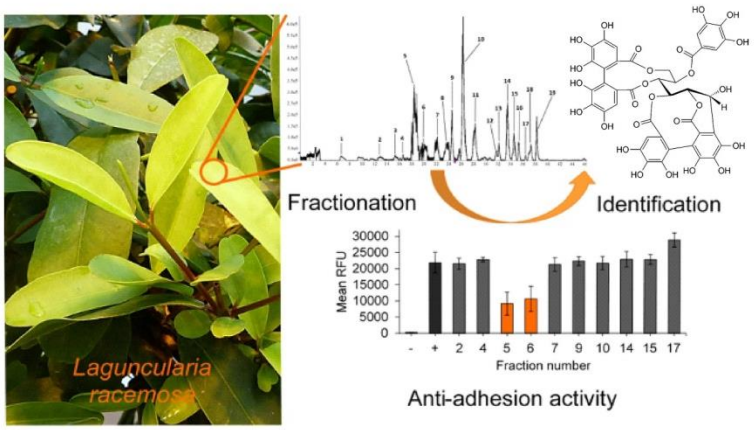

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Anti-Adhesion Activity of Tannins Isolated from the Mangrove *Laguncularia racemosa*

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Supporting Information

Anti-adhesion Activity of Tannins Isolated from the Mangrove *Laguncularia racemosa*

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Table S1. Compounds annotated in *Laguncularia racemosa* leaf extracts by database comparison of MS/MS fragment masses. MS^1= mass of precursor ion, MS^2= fragment masses obtained at -30 eV collision energy, n. i. = not identified, mod. = modified, italic = only present in the methanolic extract, *= ReSpect accession, #= MassBank accession.

Fraction	MS^1 [m/z]	MS^2 [m/z]	Putative Compound Annotation	Source
1	-	-	No Peak	-
2	-	-	No Peak	-
3	-	-	No Peak	-
4	-	-	No Peak	-
5	935.0816 (467.0375)	633.0749, 300.9987, 275.0202, 249.0403, 169.0140, 125.0244	Casuarinin	This study
	939.1123 (469.0546)	603.0614, 335.0397, 300.9987, 275.0193, 249.0396, 169.0133	Ellagitannin	This study
	953.1268 (476.0599)	921.0950, 773.0173, 619.0882, 300.9954, 275.0168, 249.0376, 169.0115	Ellagitannin	This study
	965.0917 (482.0425)	933.0569, 903.0835, 573.0461, 300.9954, 275.0165, 249.0373, 169.0123	Ellagitannin	This study
6	785.0847 (392.0381)	633.0750, 615.0633, 483.0779, 463.0490, 300.9991, 275.0199, 249.0407	Digalloyl-HHDP-glucose	Dias et al. 2016
	939.1150 (469.0535)	603.0612, 335.0399, 300.9962, 275.0222, 249.0371	Ellagitannin	This study
	953.1274 (476.0600)	921.1006, 773.0176, 619.0923, 300.9969, 275.0181, 249.0393	Ellagitannin	This study
	965.0930 (482.0428)	933.0679, 903.0983, 573.0550, 300.9977, 275.0207, 249.0434	Ellagitannin	This study
7	*183.0306*	168.0056, 124.0166, 78.0118	Methyl gallate	PM012531*
8	*183.0303*	168.0067, 124.0173, 78.0125	Methyl gallate	PM012531*
	457.0773	305.0664, 169.0153, 125.0255	Ellagitannin	-
	639.0902	319.0461, 301.0349, 257.0454, 193.0149, 175.0047, 151.0043, 125.0254	n. i.	-
9	*183.0307*	168.0066, 124.0173, 78.0122	Methyl gallate	PM012531*
10	639.0979	319.0464, 301.0348, 257.0446, 193.0147, 175.0042, 151.0024, 125.0246	n. i.	-
11	639.0956	319.0468, 301.0348, 193.0150, 175.0039, 151.0039, 125.0251	n. i.	-
	303.0907	96.9606	n. i.	-
	445.1982	385.1894, 223.1344, 205.1238, 161.0452, 153.0924, 89.0252	n. i.	-
	939.1034 (469.05)	769.0921, 617.0779, 447.0581, 169.0148, 125.0234	Pentagalloylglucose	Boulekbache-Makhlouf et al. 2010
	463.0887	316.0240, 271.0262, 179.0014	Myricitrin	PT204270*
	317.0322	289.0339, 271.0253, 178.9990, 151.0039, 137.0247	n. i.	-
12	303.0517	285.0401, 275.0564, 241.0500, 217.0493, 199.0395, 177.0191, 151.0408, 125.0259	Taxifolin	PS069609*
	479.0842	316.0224, 271.0248, 178.9977	Myricetin-3-galactoside	PR100972#
	449.0730	316.0234, 271.0244, 178.9984, 151.0031	Myricetin-3-xyloside	PT209300*
13	449.0745	316.0237, 271.0262, 178.9998, 151.0048	Myricetin-3-xyloside	PT209300*
	463.0899	316.0238, 271.0251, 178.9998, 151.0048	Myricitrin	PT204270*
14	463.0882	316.0229, 271.0242, 178.9987, 151.0025	Myricitrin	PT204270*

16	408.9885	329.0328, 314.0095, 298.9858, 270.9897	n. i.	-
17	433.0780	300.0275, 271.0254, 255.0302, 179.0001	Quercetin-3-arabinoside	PT209320*
18	431.2302	371.2084, 161.0462, 101.0252, 59.0157	Syringin (Eleutheroside B)	TY000098#
	623.1639	315.0532	Isorhamnetin-3-rutinoside (Narcissin)	PS091210*
19	423.0033	343.0468, 328.0236, 313.0002, 297.9764	n. i.	-

Table S2. NMR chemical shifts ^{1}H, ^{13}C of the hydrolysable tannin casuarinin. Chemical shifts of ^{13}C as reported by Hatano et al. (1988) in acetone-d_6 and ^{1}H by Souza-Moreira et al. (2013) in DMSO-d_6.

Casuarinin		
Position	**δ_C, type (Hatano et al. 1988)**	**δ_H (Souza-Moreira et al. 2013)**
1	67.6, CH	5.39
2	76.7, CH	4.56
3	69.8, CH	5.21
4	74.2, CH	5.26
5	71.2, CH	5.17
6	64.6, CH_2	4.53, 4.0

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T. Hatano, T. Yoshida, T. Shingu, T. Okuda, '^{13}C Nuclear magnetic resonance spectra of hydrolysable tannins. III. Tannins having $^{2}C_4$ glucose and C-glucosidic linkage'. *Chem. Pharm. Bull.* **1988**, *36*, 3849-3856.

T. M. Souza-Moreira, J. A. Severi, K. Lee, K. Preechasuth, E. Santos, N. A. R. Gow, C. A. Munro, W. Vilegas, R. C. L. R. Pietro, 'Anti-*Candida* targets and cytotoxicity of casuarinin isolated from *Plinia cauliflora* leaves in a bioactivity-guided study', *Molecules* **2013**, *18*, 8095-8108.

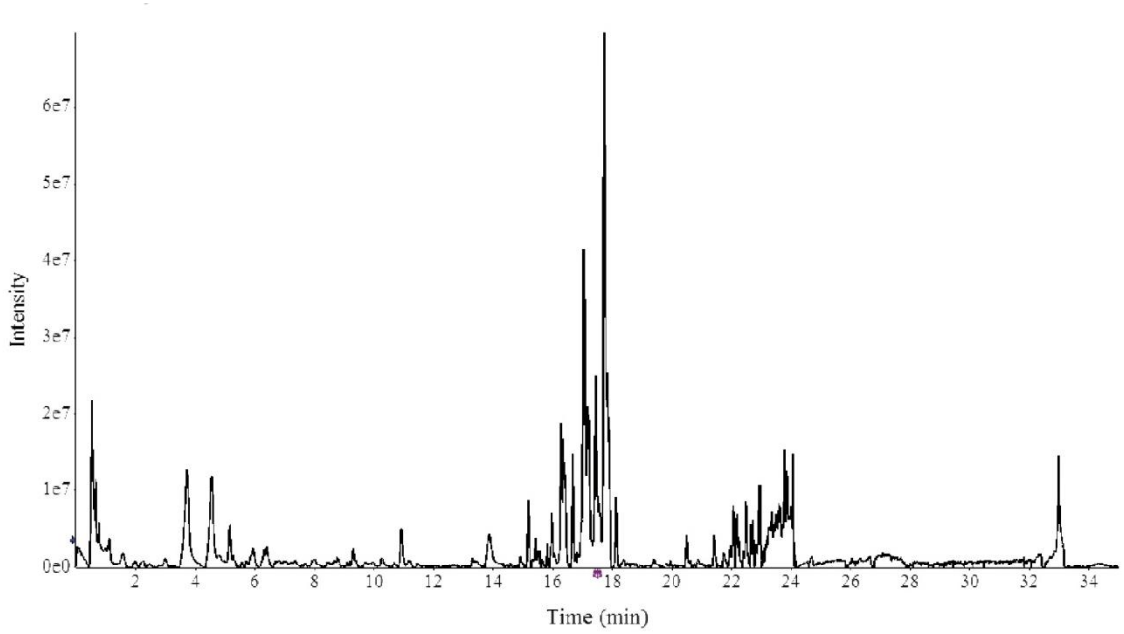

Figure S1. TOF-MS spectrum of *Laguncularia racemosa* leaf extract prepared in 80% acetone.

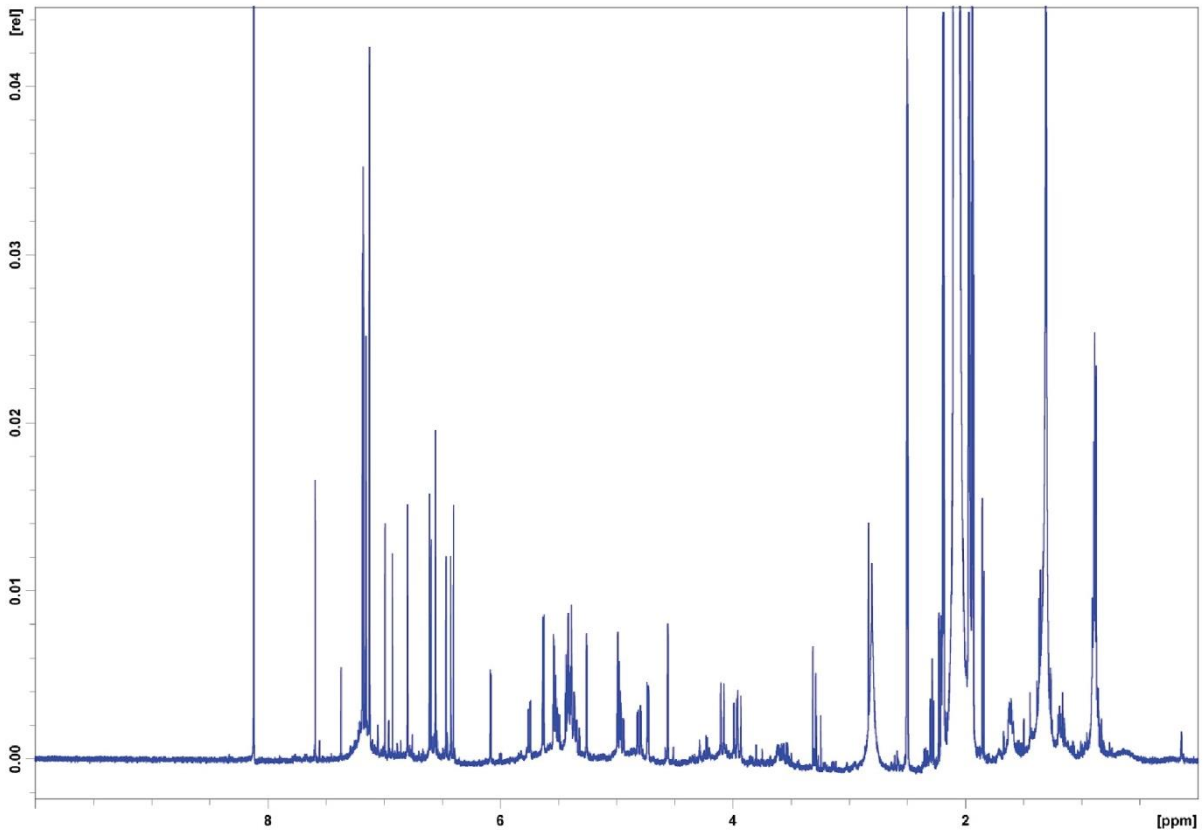

Figure S2. 1H-1D NMR spectrum of fraction 5 in acetone-d_6 from *Laguncularia racemosa* leaf extracts enriched for phenolic substances by solid phase extraction (SPE).

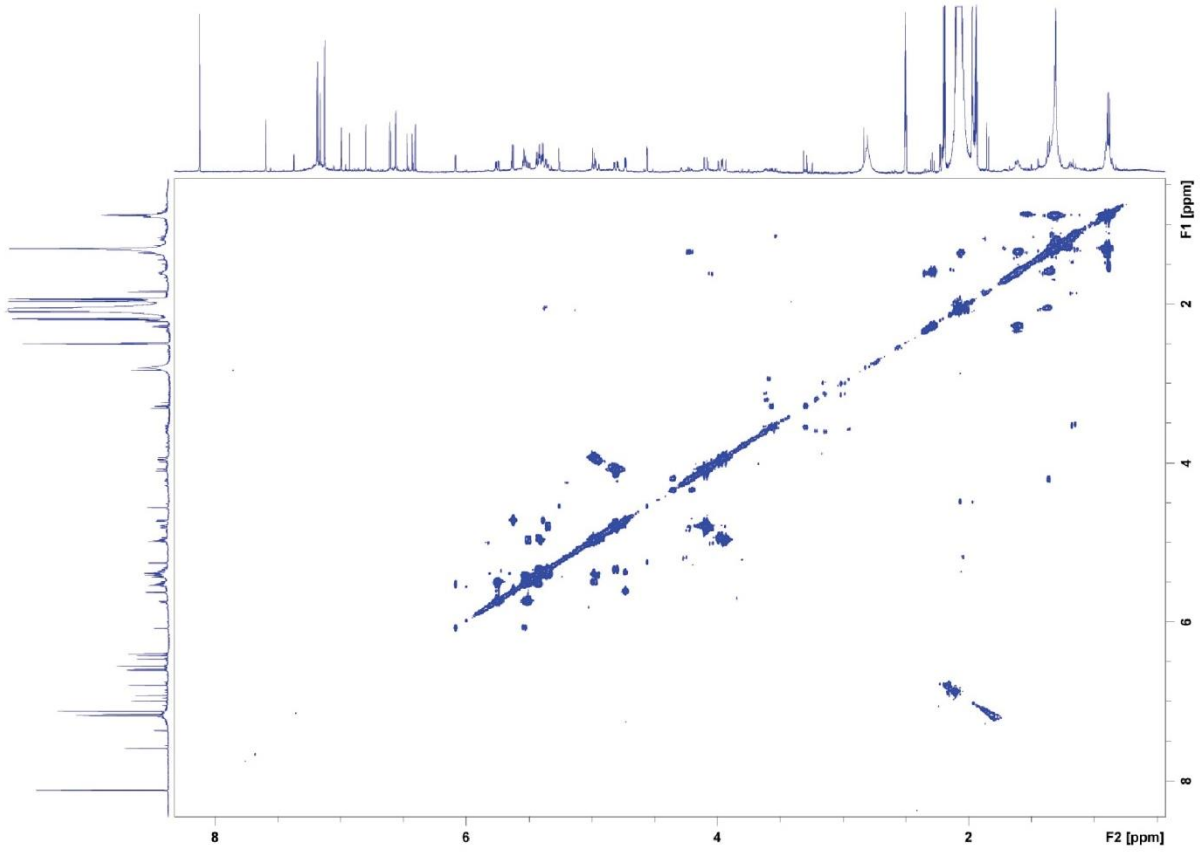

Figure S3. COSY NMR spectrum of fraction 5 in acetone-d_6 from *Laguncularia racemosa* leaf extracts enriched for phenolic substances by solid phase extraction (SPE).

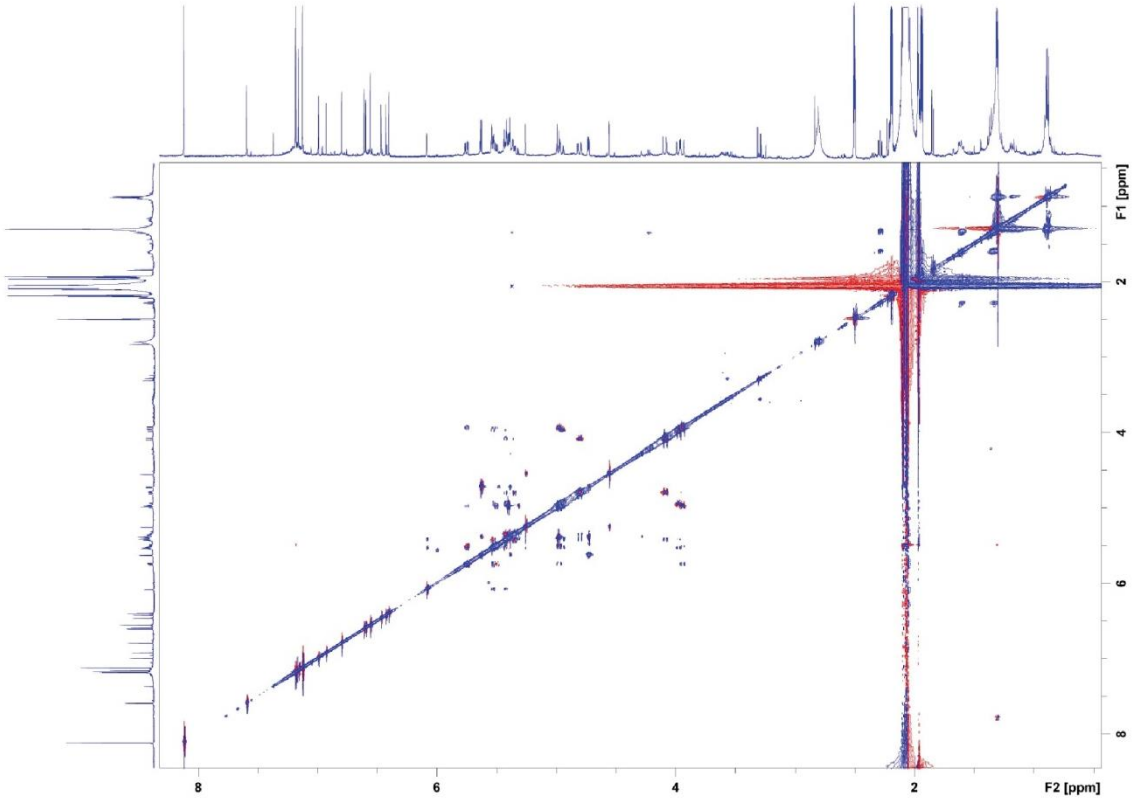

Figure S4. TOCSY NMR spectrum of fraction 5 in acetone-d_6 from *Laguncularia racemosa* leaf extracts enriched for phenolic substances by solid phase extraction (SPE).

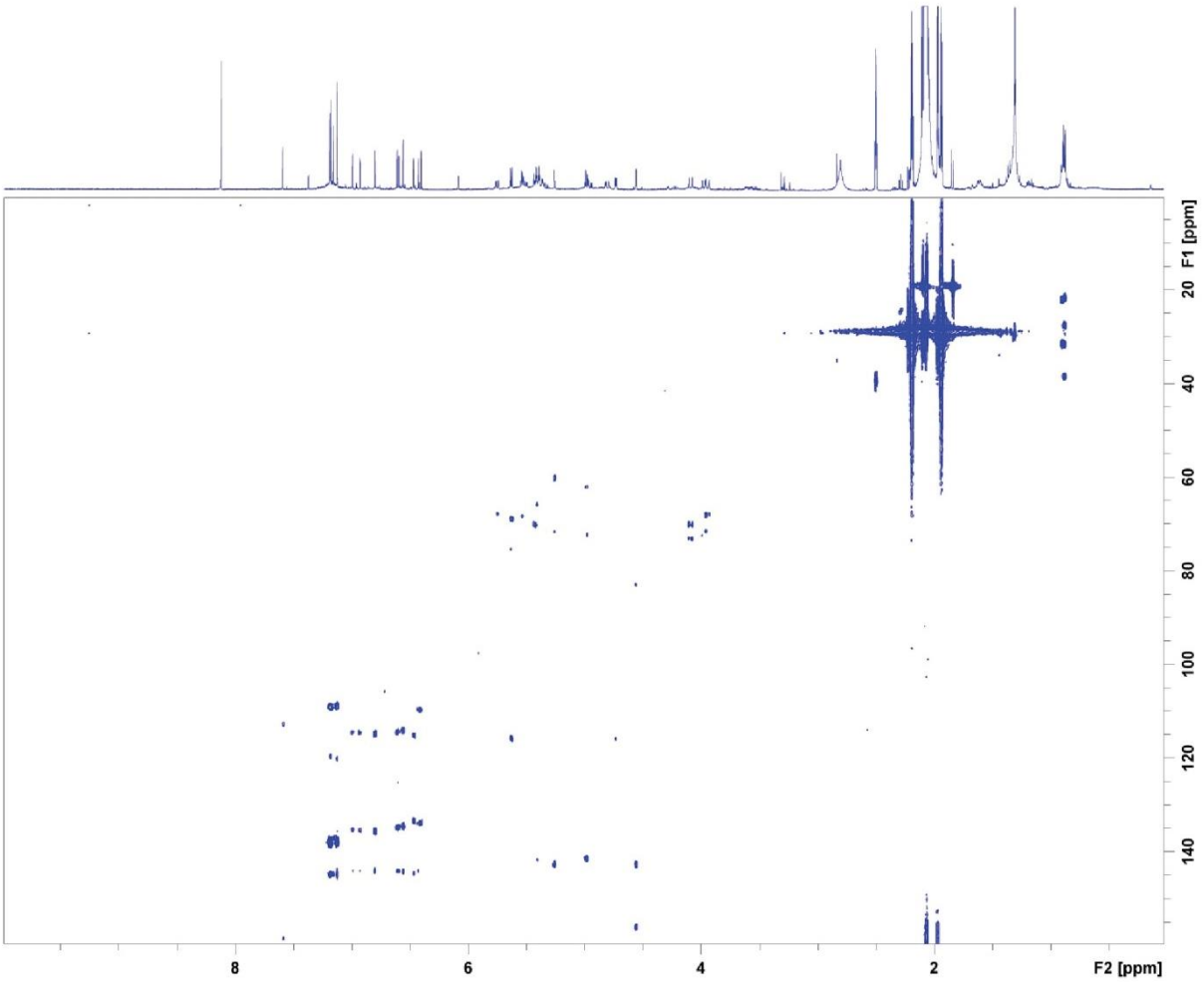

Figure S5. HMBC NMR spectrum of fraction 5 in acetone-d_6 from *Laguncularia racemosa* leaf extracts enriched for phenolic substances by solid phase extraction (SPE).

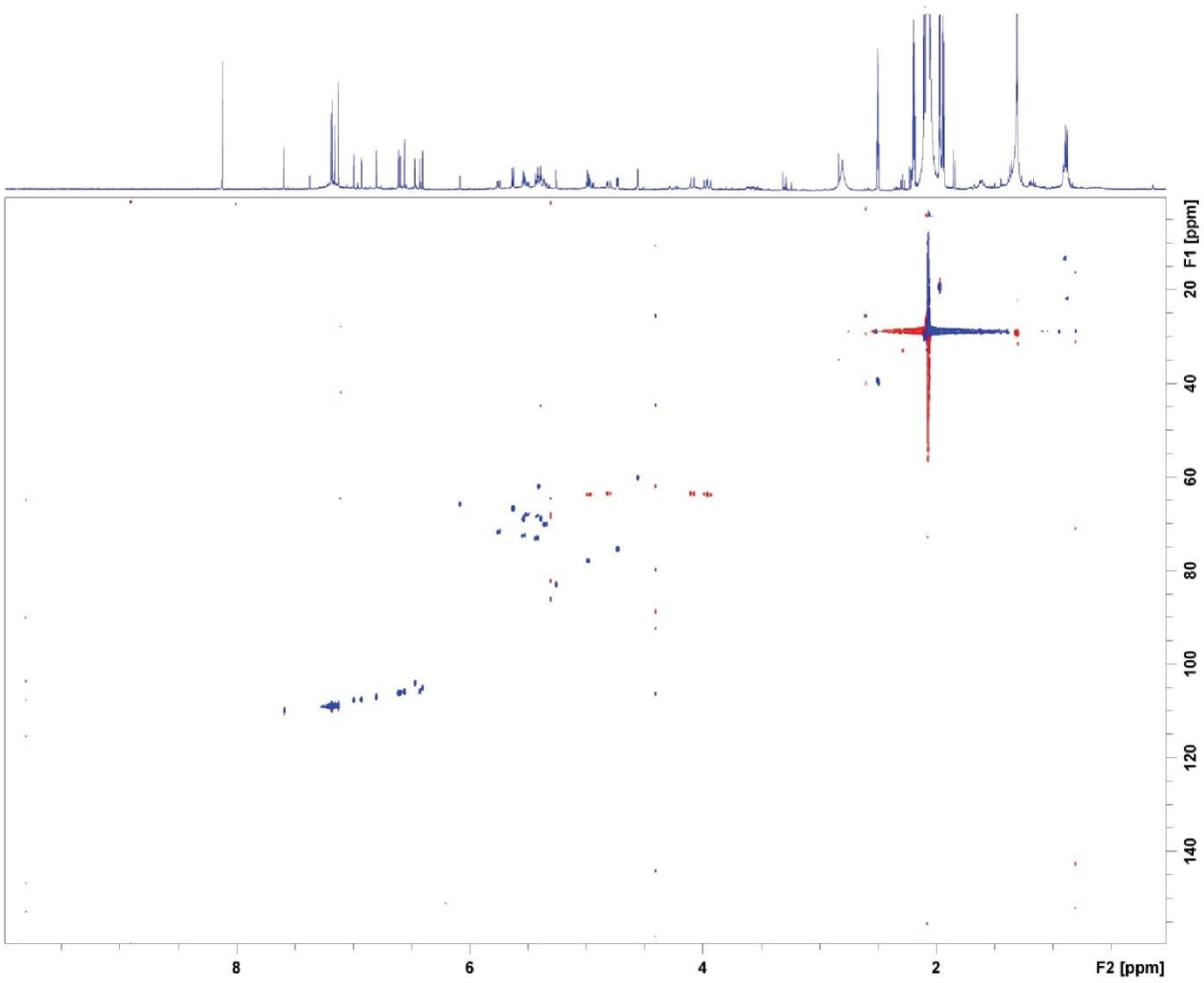

Figure S6. HSQC NMR spectrum of fraction 5 in acetone-d_6 from *Laguncularia racemosa* leaf extracts enriched for phenolic substances by solid phase extraction (SPE).

Chapter 6

Discussion

Plants as a source of bioactive substances

Plants are a valuable resource of bioactive compounds, which are described in ethnobotany as remedies against a big variety of conditions (Brusotti et al. 2014). The documented bioactivities cover a broad field of antibacterial, -fungal and –viral actions. These are of interest, when developing new pharmaceuticals from natural sources. Of 122 plant-derived drugs, the use of 80% is based on their application in ethnopharmacology (Fabricant & Farnsworth 2001). A big issue is the development of new antibiotic and antifungal substances, since many microorganisms have gained resistances against available products (Hall & Mah 2017). Antimicrobial activities, including anti-biofilm, have been reported for plant extracts and isolated compounds (Rabin et al. 2015). An advantage compared to synthetically designed chemicals is the already existing knowledge and experience regarding the toxicity and long-term effects of many plant metabolites. Many of these are used and known since centuries, and have become lead compounds in modern drug research (Dias et al. 2012).

Salt-tolerant halophytes: Mangroves and seagrasses

A common feature of seagrasses and mangroves is their tolerance to salt in the water and soil in their habitat. This tolerance is achieved by many specific morphological adaptations which reduce the salt uptake or mitigate the negative effects of the salt inside the plant (Flowers & Colmer 2015). Another factor contributing to their enhanced salt tolerance could be the synthesis of secondary metabolites. In other halophytes, elevated salt concentrations have shown to increase the total content of flavonoids and phenols, and therefore the overall antioxidant capacity (Boestfleisch et al. 2014). For the mangrove species *Avicennia germinans* and *Laguncularia racemosa*, the induction of secondary metabolite synthesis through elevated salt concentrations in greenhouse experiments could not be demonstrated (Glasenapp 2014, Loewner 2015). In their natural habitat, other biotic and abiotic stress inducers like herbivore feeding and UV radiation contribute to the synthesis of specific secondary compounds (Wink 2009). Secondary metabolites in seagrasses are mainly related to their defense against herbivores and growth of biofouling bacteria and algae (Harrison

1982, Harrison & Durance 1985). An induced production of secondary metabolites as a response to herbivore feeding in *Posidonia oceanica* was investigated by Vergés et al. (2008), but total contents did not elevate. In this stress situation, the synthesis of secondary metabolites is decreased in favor to the production of primary metabolites. Still, secondary metabolites in mangroves and seagrasses play an important role in their response to their challenging habitat. The individual effects of single stress factors on the secondary metabolite content is a possible field of future research. There is a high diversity of secondary metabolites in the halophytic plant species of mangroves and seagrasses, which can have possible prospects as bioactive compounds.

Mangroves as a potential source of bioactive compounds

In the search for new bioactive compounds, natural sources like plants and fungi are promising, as many available pharmaceuticals are derived from them (Newman & Cragg 2016). Numerous traditional remedies are based on medicinal plant parts, which contain secondary metabolites causing health promoting activities. To avoid taking plant material out of the wild, greenhouse-grown plants are an alternative. Mangroves contain numerous secondary metabolites as an adaptation mechanism to biotic and abiotic stress factors in their natural habitat.

Therefore, mangroves were investigated as a source of bioactive compounds, with a focus on biofilm inhibiting activity. Before conducting detailed analysis on the compounds of the plants, an unambiguous species identification is mandatory. DNA barcoding with internal transcribed spacer (ITS) as a molecular marker has provided good species resolution, for example in seagrasses (Nguyen et al. 2015) and plants of the iris family (Yao et al. 2010). Mangrove plants available in the greenhouse in Hannover and 30 samples of *Avicennia, Lumnitzera* and *Rhizophora* collected in the wild were compared to publicly available sequences in GenBank. Overall, ITS analysis provided a good species resolution (Chapter 2, Figure 1). According to the results, the local collectors misidentified four *Avicennia* and two *Rhizophora* samples. One plant from the greenhouse considered as *A. marina* proved to be *A. germinans* instead. These results underline the importance of careful species identification before any further analysis of plant samples, for example for secondary compounds.

Another considerable factor is the difference of plants grown in controlled greenhouse conditions to those exposed to more variable environmental conditions in the natural habitat. *A. germinans* plant leaves from the greenhouse share 70% of phenolic secondary metabolites identified in this work with samples collected from naturally grown plants in Guatemala. Phenolic acids are identical for two out of three detected in both sample groups. A number of ten flavonoids is present in both sample groups, but greenhouse-grown plants contain five additional flavonones. For both sample groups, a high variability between individual plant samples was observed and was exemplarily analyzed for naringenin and rutin (Chapter 2, Figure 5). In greenhouse grown plants, the differences between samples are lower compared to the plant samples from Guatemala, which can be explained by more consistent conditions in the greenhouse. Differences in the metabolite profile of greenhouse and outdoor growing plants are observed for other plant species as well. Zobel (1991) describes differences in the furanocoumarin concentration in *Ruta chalepensis* L. grown outdoors and in the greenhouse. The factors influencing the secondary metabolite composition, like e.g. UV exposure, can also be applied in a directed manner to increase the concentration of certain compounds. Controlled artificial light sources like LEDs can be used to induce an increased secondary metabolite production, as proposed by Ouzounis et al. (2015).

In summary, one has to keep in mind possible differences in metabolites present in samples collected in the wild to those cultivated in a greenhouse. A possible lower variability in their content due to more stable conditions can be an advantage. The regular fertilization in the greenhouse supplies the plants with all major and minor nutrients, which is reflected in the elemental composition of plant leaves. Compared to plant samples from Guatemala, contents in calcium, manganese, phosphorus and zinc are higher (Chapter 2, Figure 6). The content of phenolic acids and flavonoids should be monitored over a longer time period, to see if there is an advantageous higher stability in their concentration compared to outdoor grown plants.

Different biofilm test systems- advantages and disadvantages

To analyze potential bioactivities of natural products, a number of test systems is available. Each one has certain advantages and limitations, which have to be considered when choosing the appropriate method. In biofilm analysis, the time course of the developmental stages needs to be taken into account.

In the 96-well microtiter assay used in this study, the first phase of biofilm formation, the attachment, takes place. Then, after growth for 24 h, cells attached to the bottom of the wells are subsequently stained and measured (Chapters 4 and 5). It can be discussed, if this structure can already be considered as a biofilm. For *Escherichia coli*, biofilm formation has been reported to occur after 4 to 8 days (Reisner et al. 2003, Beloin et al. 2004). Structures observed after 24 h by Reisner et al. (2003) show further development after 36 and 42 h. The yeasts *Candida albicans* and *Candida glabrata* need 38-72 h and 48 h, respectively, to develop mature biofilms (Chandra et al. 2001, Kucharíková at al. 2011). This data indicates that the structures observed in the 24 h microtiter assay give information about the effect of the tested substances on the adhesion of microbial cells, but not on the development of a mature biofilm. To study the long-term effect and inhibitory activity of the analytes, other experimental systems can be applied. Bioreactors are a possible way to observe biofilm formation over longer time periods, as the biofilm is supplied with fresh medium constantly (William et al. 2011). This is applicable for yeasts, as described in Nailis et al. (2009) and shown by the results on *C. albicans* (Chapter 4, Figure 5). For *E. coli*, flow chambers and microfermenter-based continuous flow culture systems can be applied for an elongated biofilm growth and analysis (Beloin et al. 2004). Another advantage of these systems is the visibility of changes in the general structure when taking microscopic pictures.

For *E. coli*, specific changes like the production of amyloid curli fibers are also visible on agar plates. As described in Chapter 3, the macrocolonies are growing for six days and colony size as well as the intensity of curli fiber production is documented. The biofilm is growing on the solid-air interface, whereas in most other systems the biofilm grows on the solid-liquid interface. As most natural occurring biofilms of *E. coli* are growing on human tissues in a more liquid environment, the obtained results on biofilm inhibition from this assay should be surveyed with another solid-liquid assay.

Biofilm dispersal can be monitored as well. Extracts of *Enhalus acoroides* show an increasing detachment of cells from *C. albicans* biofilms (Chapter 4, Figure 5). This phase is another

target for anti-biofilm compounds. The advantage compared to biofilm-inhibiting compounds is the possible use against already formed mature biofilms. Interestingly, the extract of *E. acoroides* shows activity against adhesion as well as promoting the detachment of cells from existing biofilms.

In general, the transfer of successful applications in artificial biofilm test systems to a natural surrounding is critical. Multispecies biofilms are very common in infectious as well as other biofilms (Burmølle et al. 2006, Kolenbrander et al. 2010). The fusion of different species in one biofilm brings along synergistic and antagonistic effects, like an increased biomass and higher resistance to antimicrobial agents (Burmølle et al. 2014). To identify potential anti-biofilm agents, single-species biofilms are easier to manage and give a first evidence on inhibitory activities. In a later step, assays with biofilms consisting of several species can be performed, to see if the observed effects are still present in more complex biofilms. In the following steps towards a new anti-biofilm drug, animal models can give a confirmation if the agent is applicable in vivo, as e.g. against *C. albicans* biofilms on venous catheters (Nobile et al. 2006).

Activity of seagrass extracts against biofilms

Seagrasses are reported to contain secondary metabolites, which play an important role in their defense against other marine species feeding on them or growing on their leaves. Like mangroves, these marine halophytes are considered as a possible source of bioactive compounds. One sulphated phenolic acid, *p*-(sulphooxy)-cinnamic acid (zosteric acid) isolated from *Zostera marina* L., has been discovered to act inhibitory against bacterial biofilm (*E. coli*, *Bacillus cereus*) and fungi (*Aspergillus niger*, *Penicillium citrinum*) (Villa et al. 2010). These findings support the hypothesis of seagrasses as a source of bioactive compounds.

Three seagrass species, *E. acoroides*, *Halophila ovalis* and *Halodule pinifolia*, were analyzed for their potential activity on *E. coli* and *C. albicans* biofilm formation. All seagrass extracts are able to reduce cell adhesion in a concentration-specific manner. Interestingly, this dependency is not necessarily linear. In toxicology, non-linear reactions to a substance are known as hormesis (Calabrese & Baldwin 2003). The dose response can also be U-shaped, so that the highest effect is achieved at intermediate concentrations. This shape is also visible

in the results of the biofilm adhesion assay with the seagrass extracts tested on *E. coli* and *C. albicans* (Chapter 4, Figure 6).

Highest reduction is achieved with *E. acoroides* extract at a concentration of 0.01 mg l^{-1} for fungal and 10 mg l^{-1} for bacterial adhesion. Cell adhesion is reduced at a rate of 60.86 ± 8.85% and 73.89 ± 1.01% in *E. coli* and *C. albicans*, respectively, compared to the control. In addition, the concentration of 0.01 mg l^{-1} *E. acoroides* extract increases the dispersion of *C. albicans* biofilm cells up to 70 ± 6.83%.

The constituents of the three seagrass extracts have azelaic acid, luteolin and apigenin in common. *H. pinifolia* contains a variety of quercetin glycosides, whereas the other two species contain more or exclusively kaempferol derivatives. Flavones (apigenin, luteolin) and flavonols (kaempferol, quercetin) are associated with many physiological roles in plants and are distributed in primitive to higher plants (Zhang et al. 2013). In seagrasses, one main function of these metabolites is the defense against parasite feeding (Qi et al. 2008).

A compound occurring only in *E. acoroides* is kaempferol-3-glucuronide. It is the largest peak in the chromatogram and could possibly be the active component of the extract (Chapter 4, Figure 2). But also compounds found in lower amounts present possible active compounds, since they can be active at low concentrations. In *H. ovalis* and *H. pinifolia* kaempferol derivatives are present in very small amounts. In tests with kaempferol-3-glucuronide on *E. coli*, a concentration-dependent reduction of cell adhesion after 24 h of growth was observed (Glasenapp, data unpublished). As described by Dziri et al. (2012), an extract of *Allium roseum* L. var. *odoratissimum* containing kaempferol-3,7-di-O-rhamnoside and kaempferol-3-glucuronide as main components had weak antibacterial activity against *Staphylococcus aureus*, *Enterococcus feacium*, *Bacillus subtilis* and *E. coli*. Tested against periodontal pathogens, kaempferol-3-glucuronide showed no inhibitory action (Shahzad et al. 2015). Regarding the anti-biofilm activity of kaempferol and its glycosides, no data was found in the literature. In future experiments, fractions of *E. acoroides* leaf extract need to be tested to identify the active anti-biofilm compounds.

Bioactive compound isolation and analysis

In the search for new bioactive compounds, the extraction and isolation is a crucial step. Various techniques can be applied and may lead to a different outcome of isolated compounds. In this work, liquid chromatography-mass spectrometry was used for compound separation and identification. In the tests for bioactivity of seagrasses, crude extracts of milled leaf material extracted in methanol were used. Methanol is a common solvent for the extraction of phenolic compounds from plant material (Chan et al. 2007). Polar and slightly unpolar substances dissolve in it and therefore a wide range of compounds is isolated. The choice of methanol as extraction solvent excludes hydrophobic compounds like lipids and oils from the analysis for bioactivity. If these were of potential interest, other solvents like hexane or ethyl acetate have to be used. The results of the liquid chromatography-mass spectrometry (LC-MS) analysis of the seagrass extracts show that almost exclusively phenolic compounds were present and tested for bioactivity (Chapter 4, Table 2). LC-MS is a standard tool in the detection and identification of constituents of complex natural extracts (De Vos et al. 2007). High resolution time-of-flight (TOF)-MS provides information on the compound mass, which is complemented by information dependent acquisition (IDA) fragmentation. With this data, database comparison is undertaken to identify the substances present. A limiting factor in this method is the fact that databases are not complete for all possible metabolites. In that case, literature search is applied to find fragmentation patterns, which match the ones detected.

Another way to identify substances is nuclear magnetic resonance (NMR) spectroscopy. With this method, metabolites not present in databases can be identified since the molecular structure of the sample is analyzed (Kim et al. 2011). The disadvantages of this method are the limited availability of NMR spectrometers due to their high costs, the relatively large amounts necessary for analysis and the time needed for structure elucidation. Structural elucidation of casuarinin (Chapter 5, Table 2) provides valuable information about the open-chain formation of the glucose, which is not possible to detect by LC-MS only.

Crude extracts without further purification are very complex compound mixtures, therefore, the identification of individual active compounds is not possible. To overcome this limitation, mangrove extracts from *L. racemosa* were first purified by solid phase extraction (SPE), then only desired phenolic compounds are present. To test single compounds for their biofilm inhibiting activity, the purified extract was fractionated by preparative high performance

liquid chromatography (HPLC) fractionation. In the following LC-MS analysis of the fractions, in most of them more than one compound was present. This reflects the results of biofilm inhibition on *C. albicans*, as fractions five and six both presented a similar reducing activity with 57.8 and 51.1%, respectively (Chapter 5, Figure 3). Fraction five contains three compounds of the chemical group of tannins, from which two are also present in fraction six. These structurally very similar compounds elute at time points close to each other. Overlapping of the content of individual compounds in two or more fractions is also visible for other compounds. Myricitrin is present in three different fractions. These results reveal a suboptimal setting in the HPLC fractionation. Peaks in the chromatogram of the extract appear in short distances (Chapter 5, Figure 2). The fractionation program was set according to these times, but the practical outcome is not the same. To improve this, a better separation of peaks needs to be achieved. A longer run time can already lead to bigger distances between peaks and lower leaking of fractions into the neighboring ones. Another factor is the big number of runs; to gain enough material for the activity tests, 60 fractionation runs were conducted. Small shifts in the retention time are possible to occur in some of the runs. The HPLC column used for fractionation has a diameter of 8 mm. Preparative HPLC columns have a diameter between 20 and 50 mm (Knauer.net). With a bigger column, on which a bigger volume of extract is injected, the number of runs could be reduced.

SPE purified *L. racemosa* extracts are active against biofilm cell adhesion on all microorganisms tested, but fractions from this extract are only active on *C. albicans*. Here, technical aspects of the fractionation are also likely to play a role. The chromatogram of the SPE purified extract consists of 19 peaks, which were separated into individual fractions. From these 19 fractions, 10 resulted in at least 2.3 mg dry matter and could be tested in a sufficient amount of replicates. Since nine fractions were not tested, these might contain the compounds with the inhibiting activity on *E. coli* and *C. glabrata*. In addition, the fractions were exposed to conditions that favor a degradation process. To evaporate the solvent of each collected fraction, the water bath of the rotary evaporator heated up to 60°C. Some compounds might degrade or undergo structural changes in these conditions. Phenolic compounds in plant extracts stored in the dark at temperatures below 4°C are stable, so changes are not due to the storage time (Amendola et al. 2010).

Another possible explanation is a synergistic effect of two or more compounds present in the whole extract, which present no activity when separated from each other. Synergy of natural bioactive compounds has been reported in the literature. Curcumin shows an increased anti-biofilm activity on bacteria when combined with antibiotics (Kali et al. 2016). The biofilm inhibitory action of fluconazole on *C. albicans* biofilms can be elevated with the plant phenolics eugenol and cinnamaldehyde (Khan & Ahmad 2012).
Overall, the methods applied for compound isolation in this work show a need for improvement for a better separation of pure compounds in larger amounts.

Tannins as possible new anti-biofilm compounds

Two fractions from SPE purified *L. racemosa* leaf extract show a reducing activity on *C. albicans* biofilm cell adhesion (Chapter 5, Figure 3). These active fractions contain a variety of phenolic compounds. They were assigned to different compounds from the group of tannins by MS/MS fragmentation pattern comparison and NMR. Two compounds, pentagalloylglucose and a castalagin derivative, are present in both active fractions; casuarinin is only present in fraction five, whereas fraction six contains a digalloyl-hexahydroxydiphenoly (HHDP)-hexose and chebulagic acid. In plants, tannins act as defense compounds against herbivorous attacks (Salminen at al. 2001).
L. racemosa belongs to the family Combretaceae, which contains the five mangrove species *Conocarpus erectus*, *L. racemosa*, *Lumnitzera littorea*, *Lumnitzera racemosa* and *Lumnitzera rosea* (Wu et al. 2008). The occurrence of tannins in the mangrove family of Combretaceae has been described for *Lumnitzera racemosa*. Seven ellagitannins are present in leaves of this plant species collected in Taiwan, namely 2,3-(S)-HHDP-D-glucose, castalagin, chebulagic acid, chebulinic acid, corilagin, neochebulinic acid and punicalagin. In addition, the gallotannins 2,3-di-O-galloyl-D-glucopyranose, 1,2,3,6-tetra-O-galloyl-D-glucopyranose, 2,3,4,6-tetra-O-galloyl-D-glucopyranose and 1,2,3,4,6-penta-O-galloyl-b-D-glucopyranose were identified (Lin et al. 1993). For *L. racemosa*, no detailed analysis of tannins is available. Values for total contents of tannins are reported between 10 and 24% (Walsh, 1977) and around 0.2 mg gallic acid equivalents (GAE) mg^{-1} of gallotannins (McKee 1995).
The tannins identified in this work are in line with the results of Lin et al. (1993) except for casuarinin. Castalagin, casuarinin, chebulagic acid and digalloy-HHDP-hexose are

ellagitannins, since at least two galloyl groups are linked. Pentagalloylglucose is a gallotannin, and is the main precursor of all ellagitannins (Niemetz & Gross 2005). Consequently, the presence of pentagalloylglucose in a tannin containing plant sample is not unusual. The other ellagitannins differ in their combination of HHDP and galloyl groups, as well as in an open chain or closed sugar core. Which of these structures is responsible for the observed bioactivity against *C. albicans* cell adhesion remains to be analyzed. The identified ellagitannins and pentagalloylglucose need to be tested as single substances. Since not all of them are commercially available, they would need to be synthesized (Pouységu et al. 2011) or collected in sufficient amounts for several bioassays.

A possible mode of action is the protein precipitation activity of either microbial signaling molecules or structural biofilm proteins. Ellagitannins are considered less efficient in protein precipitation compared to gallotannins, but the activity is also dependent on the pH (Moilanen et al. 2013). In further studies, activity of the identified tannins in media with varying pH values should be compared. Bioactivities of ellagitannins and gallotannins have been reported in the literature. In a disk diffusion assay, chebulagic acid and corilagin showed strong inhibitory action against *C. albicans* (Fogliani et al. 2005). The authors related the antimicrobial effects observed to the enzyme inhibiting activity of tannins. In an experiment on the inhibition of the enzymatic activity of xanthine oxidase, they detected a moderate activity compared to quercetin as a reference inhibitor.

As shown in this work, tannins can be considered as promising new biofilm inhibiting compounds. In the next step, the applicability of these substances needs to be surveyed (Figure 1). Tannins are present in medical plants used in traditional medicine, but the tolerability of higher doses should be controlled (Haslam 1996). Factors as the stability of the substance and the solubility need to be taken into account. Improvement of water solubility could be improved through the complexation with e.g. cyclodextrins, as shown for gallic acid by Teodoro et al. (2017). Here, the activity of gallic acid against *C. albicans* biofilms was not reduced and the applicability of gallic acid was improved. In further studies, the usage of tannins as anti-biofilm compounds should be evaluated.

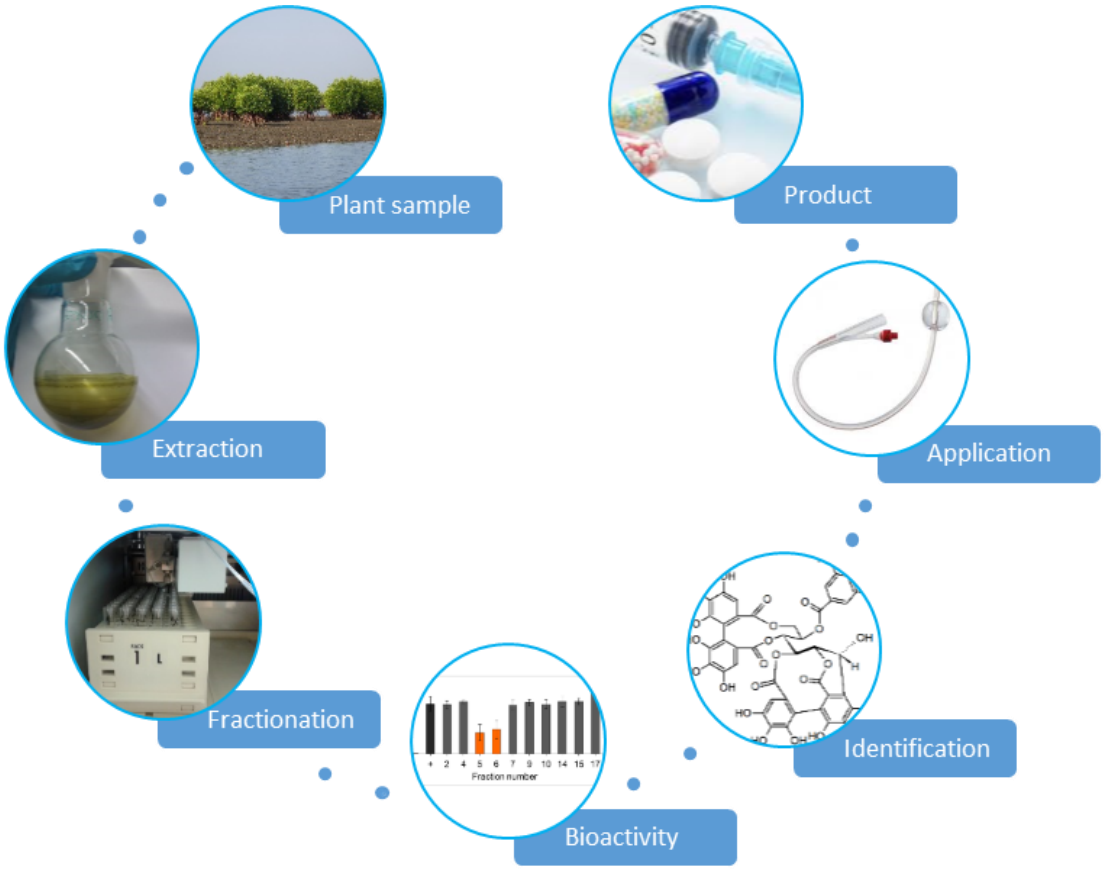

Figure 1. Flow chart of natural product development starting from plant material.

Conclusions

In this work, it was shown that species determination of mangroves can be achieved by DNA barcoding using ITS as a marker. This method can be considered in future works to support the results obtained with other markers and to verify the morphological identification.

The comparison of phenolic secondary metabolites present in greenhouse grown and naturally grown mangrove plants from Guatemala showed a comparable composition. For the use of mangroves in the search for bioactive compounds, this factor is important to utilize the full potential of these plant species. It remains to be analyzed, if the stable conditions in the greenhouse provide an advantage for the research on mangrove secondary metabolites due to less variable concentrations. Even though no induction of the secondary metabolite content by induced salt stress was possible, the factors influencing their concentration like e.g. UV-radiation are fields for future research.

Tea varieties and the flavonoid taxifolin have shown a reducing activity on *E. coli* macrocolony biofilm growth. The activity was not limited to the reduction of biofilm matrix components, but also influenced the general growth of bacterial cells. In future experiments, it needs to be analyzed if the observed effects on biofilm growth are due to an antibacterial or a specific anti-biofilm action.

Crude extracts from the seagrass species *Enhalus acoroides*, *Halophila ovalis* and *Halodule pinifolia* reduced biofilm growth on *E. coli* and *C. albicans*. The flavonoids identified in *E. acoroides* are promising candidates for future analysis. Fractions of the crude extracts should be tested to identify the active compounds present in the extract.

The extract of *L. racemosa* enriched for phenolic compounds inhibited cell adhesion in *E. coli*, *C. albicans* and *C. glabrata*. Two fractions of this extract inhibit *C. albicans* cell adhesion by 51 and 57%, and the compounds present in these fractions were identified as hydrolysable tannins with similar structural properties. They are promising candidates for further applications as biofilm inhibiting agents. First, the identified tannins need to be tested individually to identify the most active compound. The applicability of this compound for future use as an anti-biofilm agent can be analyzed in ongoing experiments.

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