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ORIGINAL PAPER

BIOCIDAL ACTIVITIES OF A TRITERPENOID SAPONIN AND FLAVONOID EXTRACTS FROM THE *ERICA MANIPULIFLORA* SALISB. AGAINST MICROFOULING BACTERIA

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Abstract

Investigations on the development of environmentally friendly, non-toxic products in the antifouling paint technology are becoming more and more widespread. The main purpose of this study was to investigate biocidal activities of fractions belonging to n-BuOH which is one of the polar extracts derived from the plant Erica manipuliflora Salisb. against marine biofilm bacteria, and to carry out a basic research in order to use the extracts determined to have high activity as antifouling agents. Of the fractions belonging to the n-BuOH extract, EBA-EBB-EBC (flavonoids, phenylethanoid glycosides) and EBD-EBE (triterpenoid saponins) were tested against marine biofilm bacteria (Pseudoalteromonas, Alteromonas, Exiguobacterium, Vibrio species) at varying concentrations. Inhibition concentrations of the fractions against marine biofilm bacteria were analyzed with the MIC test and disk diffusion method. As a result of the screening of inhibition concentrations of the n-BuOH fractions obtained from Erica manipuliflora Salisb., the species Alteromonas and Exiguobacterium were determined as the most sensitive bacteria. The fractions EBA, EBD and EBB were more effective. At the end of this study, it was assessed that n-BuOH fractions rich in flavonoids and triterpenoid saponins exhibited activity against some marine biofilm bacteria. It was also determined that it would be worth doing further studies in which pure substances to be obtained could be used as an antifouling additive in industrial areas to prevent marine biofilms.

Key words: Erica manipuliflora Salisb., biocidal activity, marine biofilm bacteria, flavonoids, triterpenoid saponins.

INTRODUCTION

The community formed by the organisms which develop by adhering to the surfaces of marine organisms, natural structures and man-made tools and devices (ships' hulls, water pipes, waste water discharge pipes, buoys, electricity cables, nets used in fishing, oil pipelines, etc.) in the sea is called fouling organisms and the development process of these organisms is called fouling (Railkin, 2004). During the biological fouling process, first bacteria adhere to a surface and form a slime biofilm structure by producing extracellular polymeric substances (EPS). The next process is the irreversible adhesion of macrofouling organisms to this slime layer (Chambers et al., 2006). Even a very small amount of a biofilm can affect the frictional resistance of a ship (A 1-mm thick biofilm can increase the friction of a ship hull by 80 per cent) (Yebra et al., 2006). To compensate this, more fuel is needed. A 40% increase in fuel consumption increases shipping costs up to 77%. Among the other negative effects are increases in the frequency of dry-docking operations, loss of time and production of other toxic wastes (Schultz and Swain, 2000).

To prevent fouling, marine paints containing heavy metals and biocides are used worldwide. However, efforts to prohibit the use of copper which has replaced organotin and is widely used are increasing because it is toxic to some marine organisms (e.g. a dose of 5-25 g L-1 is lethal to invertebrates) and accumulates in shellfish. The problem faced in the release of biocides is the development of strains resistant to antimicrobials and some heavy metals in nature, and therefore there is a need to develop new solutions. Investigations on the development of environmentally friendly, non-toxic products in the antifouling paint technology are becoming more and more widespread. Although works on natural antifouling products have been continuing during the last 20 years, research on the use of these products against biofouling is still very new. No matter how difficult to develop biocide-free antifouling paints is, the importance of their development cannot be denied when the damage biocide-containing antifouling paints cause to the environment is taken into account (Chambers et al., 2006).

From ancients, medicinal plants have been known to possess diverse biological activity as antimicrobial, pain-killer, anticancer, and antihypertensive activity and an important source of many biological active compounds (Inatain

et al., 1996; Alma et al., 2003; Andrade et al., 2007; Webster et al., 2008). Traditional medicinal plants have been used extensively by a large proportion of the world for their health care and cure of diseases during the 2000 years (Singh et al., 2008). Plants have developed advanced defense mechanisms to survive in their ecosystems, and hence are plentiful sources of pharmaceutical compounds (Zhao et al., 2005; Li and Vederas, 2009). Phytotherapy is based on the use of biological active components contained in plants (Garza et al., 2007). The most interesting area of practice for medicinal plant extracts is the inhibition of growth and reduction in numbers of the pathogens (Okolo et al., 1995; Kuete et al., 2007; Kotzekidou et al., 2008). Recent studies have been focused on growing interest in plants as a significant source of new pharmaceuticals (Rates, 2001; Kang et al., 2011; Lee et al., 2013). Of the 500,000 plant species found globally, only 1% has been phytochemically investigated, mainly with respect to antimicrobial activity (Cowan, 1999; Palombo, 2009). Several plant extract libraries have been examined regarding the control of pathogenic biofilms including 54 plants and common food products (Rasmussen et al., 2005) and six Florida plant extracts (Adonizio et al., 2008) against *Pseudomonas aeruginosa*, 168 plant extracts against *Staphylococcus aureus* (Quave et al., 2008), 13,000 fractions of 167 plant genera (Ren et al., 2005), six herbal plants (Wojnicz et al., 2012), and several grapefruit-derived flavonoids against *E. coli* strains (Vikram et al., 2010a; Vikram et al., 2010b).

The genus *Erica* L. (Ericaceae) is represented by more than 700 species in the world, *Erica manipuliflora* Salisb. are widespread species common in the coastal sides in Turkey (Stevens, 1978). Herbal teas are prepared from aerials parts of *Erica arborea* and *Erica manipuliflora* have been popularly used as diuretic, astringent and treatment of urinary infections in Turkey (Baytop, 1999; Tuzlacı and Eryasar-Aymaz, 2001).

A literature survey showed that there are no reports on the antimicrobial activity of this plant. Therefore, the aim of this study was to evaluate the antimicrobial activity of *Erica manipuliflora* Salisb. fractions which are obtained from the most active n-BuOH Extract against marine biofilm bacteria.

MATERIALS AND METHODS

Plant material and extraction

The plant material was collected from Datça, Turkey in March, 2010. Dried airel parts of *Erica manipuliflora* (5 kg) was grinded. 1.55 kg of powdered plant was extracted with n-hexane, CH_2Cl_2 ve MeOH respectively using Soxhlet apparatus. The extracts were concentrated with using rotary evaporator. The MeOH extract was dissolved in H_2O (500 mL), and partitioned against H_2O -saturated n-BuOH (7 x 300 mL) yielding a butanol fraction EB (33.83 g) and a water fraction EW (28.24 g). 5g of n-BuOH extract (EB) was submitted to VLC-RP eluted with H_2O: MeOH gradient, yielding 5 fractions EBA-EBE. The storax composition was determined with using TLC method. TLC was carried out on precoated Kieselgel 60 F254 (Merck 5554) plates. The solvent system was used: $CHCl_3$-MeOH-H_2O (61:32:7) and the spray reagent was used the vanillin H_2SO_4 reagent (a solution of 1% vanillin in EtOH) for the detection of compounds (Wagner and Bladt, 2009).

EBA: A fraction obtained from VLC-RPcolumn of Erica munipluiflora n-BuOH extract
EBB: B fraction obtained from VLC-RPcolumn of Erica munipluiflora n-BuOH extract
EBC: C fraction obtained from VLC-RPcolumn of Erica munipluiflora n-BuOH extract
EBD: D fraction obtained from VLC-RPcolumn of Erica munipluiflora n-BuOH extract
EBE: E fraction obtained from VLC-RPcolumn of Erica munipluiflora n-BuOH extract

Test microorganisms

The n-BuOH extracts were tested against marine biofilm bacteria. *Pseudoalteromonas marina* FJ200642, *P. haloplanktis* FJ040186, *P. elyakovii* FJ200650, *P. porphyrae* FJ200651, *P. agarivorans* FJ040188, *A. genoviensis* FJ200641, *V. lentus* FJ200649, *Exiguobacterium homiense* FJ200653 were used as biofilm forming seawater bacteria (Kacar et al., 2009).

Disc diffusion method

The biocidal activity of all extracts was determined by using the paper disk diffusion method. The biofilm bacteria strains were inoculated on Zobell Broth (Oxoid) and incubated for 24 h at 26 ^{0}C. The counts of bacteria strains were adjusted to yield approximately 10^7- 10^8 cfu ml-1, using the Standard McFarland method. The concentration of the fractions obtained was 20 mg/ml and dilutions series were prepared as 1/2-1/1024 (10mg/ml-19.5µg/ml). The all fractions were filter-sterilized using a 0.22 µm membrane filter. The test organisms (100µl) were inoculated on the surface of appropriate solid medium Mueller Hinton Agar in plates. The agar plates inoculated with the test organisms were incubated for 1 h before placing the fraction impregnated paper disks on the plates. Sterile paper disk of 6 mm diameter were impregnated these fractions (50 µl) and plates were incubated at 26 ^{0}C for 24 h. After incubation, all plates were observed for zones of growth inhibition, and the diameters of these zones were measured in millimeters. All tests were performed under sterile conditions and disks imbued with 50 µl of pure MeOH were used as a negative control (Bradshaw, 1992; Kim et al., 1995; NCCLS, 1999). Additionally, Vancomycin and Tobramycin (Becton Dickinson Gmbh, Heidelberg) were used as the standard antibacterial agents.

Microdilution method

Determination of the minimum inhibitory concentration (MIC) was carried out according to the method described by Zgoda and Porter (2001), with some modifications. A dilution series of the fraction, ranging from 1/2-1/1024, were prepared and then transferred to the broth in 96–well microtitre plates. Before inoculation of the test organisms, the bacterial strains were adjusted to Standard McFarland method. The plates were incubated at 26 °C for 24 h. The MIC values of the fractions were defined as the lowest concentration that was not showed growth. The results were enhanced by the use of 15 µL of Triphenyl Tetrazolium Chloride 1% (TTC) (Sigma USA), which may be reduced when there is bacterial growth, forming the triphenyl formazan with red color.

RESULTS AND DISCUSSION

The results of the present study revealed that n-BuOH fractions obtained from *Erica manipuliflora* Salisb. were rich in flavonoids, phenylethanoid glycosides (EBA-EBC) and triterpenoid saponins (EBD-EBE) (Figure 1). The MICs and disc diffusion analysis were given in Tables 1 and 2. The results showed in the table which were based on the principle, indicating that at which concentration the highest zone diameter is determined. Effective concentrations and zone diameters varied from one species to another. According to the results of the disc diffusion method (Table 1), the results of the screening of inhibition concentrations of n-BuOH fractions against marine biofilm bacteria indicated that *Alteromonas genoviensis* and *Exiguobacterium homiense* were the most sensitive biofilm species.

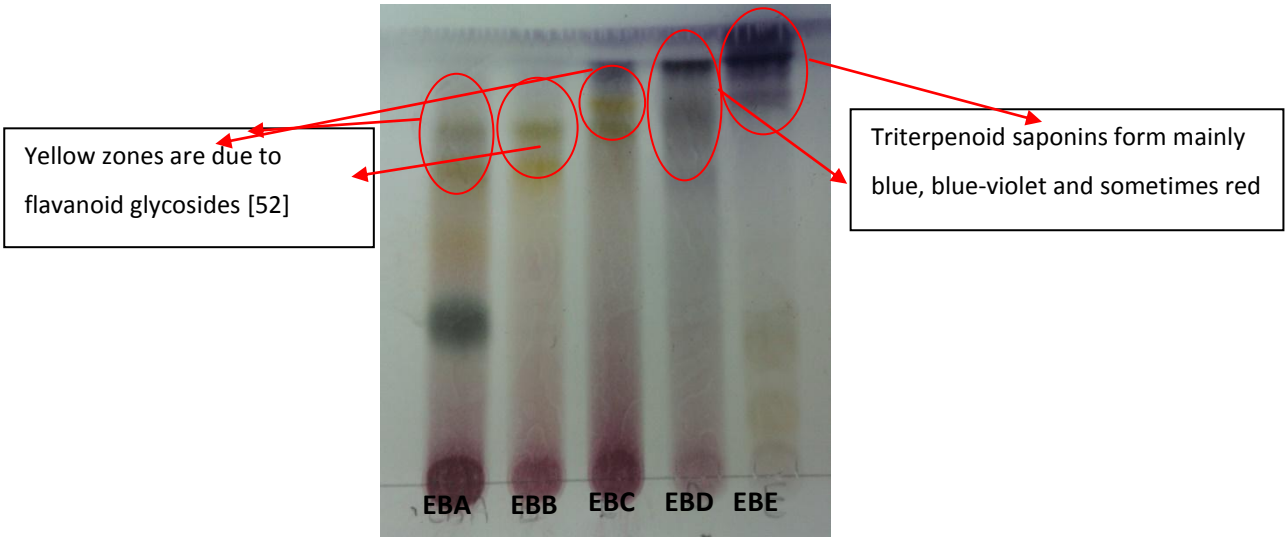

Figure 1. The TLC picture of fractions Solvent System: CH_2Cl_2:MeOH:H_2O:61:32:7, after sypraying with 1% vanillin H_2SO_4 and heating

The most effective fraction was EBB, and the inhibition zone diameter was determined to be 24 mm. in the species A. genoviensis and 16 mm. in the species E. homiense. Zone diameters were 10 to 15 mm for the EBA fraction, 10-20 mm for the EBC fraction, 8-15 mm for the EBD fraction and 10-20 mm for the EBE fraction. Pseudoalteromonas elyakovii and P. marina were the most resistant species to all fractions. Furthermore, EBA and EBD were determined as the most effective fractions in the minimum inhibition concentration analysis (Table 2). Growth was not detected even in 1/1024 (19.5µg/ml) dilution of these fractions. Alteromonas genoviensis, E. homiense and P. agarivorans were the most sensitive biofilm bacteria species to the MIC concentrations of the fractions. Although all the biofilm species investigated in the present study were in general resistant to both antibiotics (Vancomycin and Tobramycin), E. homiense and A. genoviensis were determined to be more sensitive.

Table 1. Disc Diffusion activity of *Erica manipuliflora* Salisb. extracts against biofilm bacteria

Isolates	Extracts					Antibiotics	
	Disc Diffusion Zone Diameter (mm) – Dilution					Zone Diameter (mm)	
	EBA	EBB	EBC	EBD	EBE	Vanc*.	Tobr.**
P. marina	10 - (1/2)	12 - (1/2)	10 - (1/32)	8 - (1/16)	10 - (1/2)	7	9
P. haloplanktis	12 - (1/16)	12 - (1/512)	10 - (1/8)	10 - (1/256)	12 - (1/16)	-	9
P. elyakovii	10 - (1/512)	12 - (1/512)	10 -(1/1024)	10 - (1/256)	10 - (1/64)	-	10
P. porphyrae	13 - (1/2)	14 - (1/4)	13 - (1/8)	11 - (1/4)	12 - (1/2)	7	10
P. agarivorans	12 - (1/256)	13 - (1/16)	10 - (1/128)	12 - (1/16)	12 - (1/64)	-	8
A. genoviensis	15 - (1/4)	24 - (1/2)	20 - (1/64)	15 - (1/4)	20 - (1/2)	8	30
V. lentus	12 - (1/16)	13 - (1/16)	12 - (1/16)	12 - (1/128)	10 - (1/2)	8	14
E. homiense	12 - (1/256)	16 - (1/16)	12 - (1//32)	12 -(1/1024)	12 - (1/32)	21	20

* Vancomycin, ** Tobramycin

Table 2. Minimum inhibition concentrations (µg/ml) of *Erica manipuliflora* Salisb. extracts against biofilm bacteria

Isolates	Extracts - Minimum Inhibition Concentration				
	EBA	EBB	EBC	EBD	EBE
P. marina	19.5	19.5	39.0	19.5	78.1
P. haloplanktis	19.5	78.1	78.1	19.5	78.1
P. elyakovii	19.5	78.1	78.1	19.5	78.1
P. porphyrae	19.5	78.1	78.1	19.5	78.1
P. agarivorans	19.5	19.5	19.5	19.5	78.1
A. genoviensis	19.5	19.5	19.5	19.5	78.1
V. lentus	19.5	78.1	78.1	19.5	78.1
E. homiense	19.5	19.5	19.5	19.5	78.1

A review of studies conducted on plant extracts showed that studies have mostly focused on the identification of antimicrobial activity against pathogenic microorganisms, and anti-biofilm activities of these pathogens which form biofilms. However, research on marine biofilm bacteria is limited. On the other hand, current marine paints used to prevent biofouling are toxic and cause damage to marine organisms, research is focusing on the screening of environmentally friendly, non-toxic compounds, determining which of these compounds are active and using them as active agents in paints.

Plants develop various defense mechanisms to compete in the ecosystem and become rich resources of antimicrobial agents and other pharmaceutical compounds (Cowan, 1999; Zhao et al., 2005; Li and Vederas, 2009). For instance, in their study conducted in 2009, Dulger and Aki investigated antimicrobial activity of *Stachys pseudopinardii R. Bhattacharjee* and *Hub.–Mor.* (Lamiaceae) which is endemic in Turkey against some pathogens with using the MIC and Disc diffusion methods. At the end of their study, it was determined that their inhibition zones ranged between 6 and 24 mm. and that they had a strong antimicrobial activity. Upon the completion of the micro-dilution analysis, minimum values were determined as 16 mg / mL for *Stachys pseudopinardii R. Bhattacharjee* and 32 µg / mL for *Hub.–Mor.* (Lamiaceae). Phytochemical analyses of Stachys species have confirmed the occurrence of diterpenes, phenyl ethanoid glycosides, flavanoids and saponines. Flavonoids may be responsible for their antibacterial activity. The results indicated that *S. pseudopinardii* possessed significant activity against both bacteria and yeast cultures. This activity may be indicative of the presence of metabolic toxins or the compounds which are stated above. Therefore, this plant extract should be analyzed further, as it might contain as yet unknown compound that is effective against pathogens (Dulger and Aki, 2009).

In another study conducted by Kang et al. in 2011, the methanol extract of 12 medical plants were tested on gram-positive and gram-negative bacteria. Based on their findings and the results of previous studies, they decided that because of hydrophilic outer membrane which had gram-negative bacteria, methanol extracts were not completely soluble in water and penetrate the outer membrane; thus, methanol extracts disrupted cell function, metabolism and cell integrity and led to deaths. While some researchers (Rajeshwar et al., 2005; Kuete et al., 2007) obtained similar results, some other researchers (Rabe and Staden, 1997; Rezende et al., 2006) obtained different results, which might be due to the composition of plant extracts and the extraction process (performed with either water or solvent). Antibacterial mechanisms of medical plants have been known not only to affect cell membrane permeability in other words depletion of energy (Conner, 1993), but also to suppress cell wall synthesis (Marcucci et al., 2001; Kang et al., 2011).

In their study, Rodrigues de Araujo et al. (2015) investigated pharmacological properties, and antibacterial, anti-biofilm and cytotoxic effects of ethanol extracts of Terminalia species (three fractions: AqF, HaF and WSF). Serial dilutions of the ethanol extracts were prepared in the MIC test, and concentrations were tested at 12.5 µg / ml to 400 µg / ml. Within the anti-biofilm activity, concentrations were tested by diluting them at the ratios of 1/2, 1/4 and 1/8. In

order for biofilm-forming bacteria to adhere surfaces, bacteria synthesize extracellular polysaccharides and release them. This structure not only helps adhesion but also acts like a defensive barrier. Between the organism and the material to which the organism will adhere, electrical charge and hydrophobicity of the surface and hydrophobic bonds such as Van der Waals interactions are important. EtE and the fractions such as AqF, HaF and WSF may have inhibited biofilm formation by leading to very little known metabolic changes. Among these possible changes are a decrease in the production and secretion of exopolysaccharides, and a change in electrical charge and/or hydrophobicity of the bacterial membrane. However, protocols which are more specific are needed to confirm the role of these fractions. Thus, results obtained indicated that the antibacterial activity of ethanol extracts and fractions was high and that biofilms formation was inhibited by 80% for some strains (Rodrigues de Araujo et al., 2015). In addition, it has been noted that plant-derived indole derivatives (Lee et al., 2011a) and flavonoids (Lee et al., 2011b) inhibited the development of biofilm of E. coli. Of the commonly used compounds, 11 trans-resveratrol and tannic acids significantly inhibited the biofilm formed by E. coli. In several previous studies, tannic acid has been reported to inhibit biofilm development (Hancock et al., 2010). In some other studies, auxin 3-indolylacetonitrile (Lee et al., 2011a) and flavonoid phloretin (Lee et al., 2011b) have been determined to inhibit biofilm. Shortly, tannic acid may be an important anti-biofilm compound, but as yet unknown compounds might have inhibited the biofilm process as well. Briefly, plants are the rich sources of bioactive molecules including antimicrobial compounds (Cowan, 1999; Palombo, 2009) and include inhibitors of biofilm formation (Lee et al., 2013).

Raut et al. (2013) were studied about terpenoids of plant origin were tested against biofilms formed by *C. albicans*. Of these terpenoids, linalool, nerol, isopulegol, menthol, carvone, α-thujone, and farnesol were determined to inhibit the biofilm-specific activity. Terpenoids are a large class of natural products exhibiting various anticancer, anti-parasitic, antiviral, anti-allergic, and antimicrobial biological activities (Gershenzon and Dudareva, 2007). Of the phenolic terpenoids, the most active ones are thymol, carvacrol, and eugenol, monocyclic ketone and β-ionone, followed by acyclic monoterpenes, citral, citronellal, citronellol, geraniol, linalool, and nerol. Their activities against *Candida* are considered to lead to damage on the cell membranes and to the inhibition of oxidative phosphorylation and the respiratory chain (Pauli, 2006). Another effect is the suppression of specific steps in the cell cycle (Zore et al., 2011), which suggests that changes in terpenoid-associated membrane permeability and viscosity could destroy the cell wall and cause problems in adhesion to a solid surface (Macros-Arias et al., 2011; Raut et al., 2013). In other study, MIC concentrations of flavonoid extracts obtained from *Moringa oleifera* were tested against *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms. At the beginning of the adhesion phase of the cell, it was tested whether flavonoid extracts inhibited adhesion. In vivo studies showed that flavonoids synthesized by plants exhibit antimicrobial potentials (Cushni and Lamb, 2005). Although studies in this field are not too much, some studies indicated that kaempferol and naringin present in citrus act like quorum sensing inhibitors by interfering with the interaction between acyl-homoserine lactones (AHLs, the signal molecules for Gram negative bacteria) and their receptors (Cushnie and Lamb, 2005; Vikram et al., 2010a). The present study also showed that the first adhesion could be prevented by flavonoids. NMR spectral analysis demonstrated that flavonoids had functional groups such as carbonyl and olefinic groups. In several studies, particularly flavonoids lacking hydroxyl groups at the B ring have been determined to be more active against microorganisms (Chabot et al., 1992). It is considered that the structure of active plant components of such a broad spectrum of the antimicrobials (not exhibiting mutagenic, cytotoxic effects) should be defined with further studies and that biofilm inhibition studies should be conducted more efficiently (Onsare and Arora, 2014).

CONCLUSIONS

This preliminary evaluation indicated that the n-BuOH extract of *Erica manipuliflora* Salisb has significant activity against the biofilm bacteria used. Further studies are necessary to identify the main active constituents and to obtain information about correlation between chemical composition and anti-biofilm activity of *Erica manipuliflora* Salisb. From these findings, we can suggest that n-BuOH fractions of plants which was used in this study may become the source for the discovery of novel anti-biofilm agents from plant sources. It is recommended that further studies in which pure substances to be obtained could be used as an antifouling additive in industrial areas to prevent marine biofilms should be performed.

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