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ORIGINAL RESEARCH

Rosmarinus officinalis Might be Exploited as a Natural Antifouling Agent: A Potentially Promising Strategy for Curbing Membrane Biofouling

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ABSTRACT
Membrane biofouling is the coverage of membrane surfaces due to undesirable development of biofilms causing a decrease and subsequent loss of productivity in water treatment settings. Continuous use of synthetic chemicals against biofouling is inept as it leads to the emergence of multi-antibiotic resistance. Application of natural products such as plants can be apt in curbing biofouling while checking the resistance challenge. This study aimedto evaluate the potential of *Rosmarinus officinalis* in the control of membrane biofouling. Bacteria from biofouling environments were subjected to a biofilm confirmation test and identified at cultural, morphological, biochemical and molecular levels. Leaves of *R.officinalis* were extracted in solvents of varying polarity and activities. These extracts were evaluated against bacterial biofilm formation via minimum biofilm inhibitory concentration (MBIC), minimum biofilm eradication concentration (MBEC) and mesocosm bioassays. Biofilm formation was confirmed in 68% of the isolates identified as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Staphylococcus aureus*. The methanol and ethyl acetate extracts of *R.officinalis* indicated the least MICs (0.313mg/L and 1.25mg/L) against *Pseudomonas aeruginosa* and *Staphylococcus aureus,* respectively. Both extracts recorded the highest MBIC (50.00%) against *Pseudomonas aeruginosa*. The peak MBEC (57.88%) was obtained from the methanol extract against *Staphylococcus aureus* and this same extract inhibited 56.23% density of bacterial biofilms on glass slides. The methanol and ethyl acetate crude extracts of *R. officinalis* appreciably reduced bacterial biofilms; hence, this plant can be exploited as a natural antifouling agent, with reduced toxicity and low risk of resistance.

KEYWORDS: Bacterial biofilms, natural product, quorum sensing, rosemary, water treatment.

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1. Introduction

Membrane Bioreactor (MBR) is an efficient, state-of-the-art, high-quality water treatment technology that consists of bioreactors modified with membrane filtration units for biomass retention (Meng et al., 2017; Waheed et al., 2017). Recent technological innovations and significant footprint reduction further made MBR an established water treatment system (Oh and Lee, 2018). Despite its advantages, MBR is characterized with challenges such as: pre-treatment huddles, lack of long-term performance and, to a larger extent, membrane biofouling. Membrane biofouling

is the coverage of membrane surfaces due to undesirable development of biofilms (sessile slimy multicellular microbial communities) (Whiteley et al., 2017). This phenomenon hinders the effective use of MBR in water treatment settings (Biofilms, 2019).

Strategies employed to control membrane biofouling mostly spin around physical cleansing of biofilms, modification of the membranes and incorporation of antimicrobial substances such as peptides and nitrofurazones (Hook et al., 2012). In addition to being pricy, the use of these chemicals is associated with resistance, environmental pollution and non-specificity (Lade et al., 2014; Alghamdi and Quijada, 2019). Addressing biofoulingusing natural products (such as plants) as an alternative can be apt since the life of the bacteria must not be the primary target but their ability to form and express biofilms (Paluch et al., 2020). Thus, it would be imperative to develop a strategy for mitigating membrane biofouling using natural products; which can be safe, efficient, readily available, cost-effective and eco-friendly. In this study, the leaves (commonly used part) (Meziane-Assane et al., 2013) of rosemary (*Rosmarinus officinalis*) were exploited based on its perceived ethno medicinal advantages and wide applications as herb and spice (Kalamartzis et al., 2020). The objectives of the research were to: (1) Isolate and purify bacteria from visibly biofouled environments, (2) screen and confirm the ability of the isolates to form biofilms and identify the biofilm-forming bacteria (3), extract the leaves of *R.officinalis*using organic solvents of different polarity and (4) investigate the antifouling activities of the extracts against the biofilm-forming bacteria.

2. MATERIALS AND METHODS

2.1. Description of Sampling Locations

The sample sites (biofouled environments) were selected based on their likelihood to harbor biofilm-forming bacteria. These environments include parts of a biofouling model, locally fabricated at the Department of Environmental Science, Kaduna Polytechnic, Kaduna, Nigeria (10° 29' 20.79''N, 07° 25' 21.35''E); which consist of membrane filter (BMm), glass (BMg) and plastic (BMp) substrates. Other sampling points include surfaces of solid objects (metals, plastics and wooden materials) from River Kaduna (RK), Kaduna, Nigeria (10° 29' 47.13''N, 07° 25' 19.95''E) as well as walls and floors of water reservoirs and chambers (IB) in Kaduna State Water Treatment Plant, Malali, Kaduna, Nigeria (10° 33' 26.80''N, 07° 29' 01.11''E).

2.2 Sample Collection and Transportation

A total of 117 slimy scrapings were collected using a simple random sampling technique using a sterile scoop over the duration of 6 months (January-June, 2020). Each sample was immediately transferred to a tightly capped Bijou bottle containing 10mL of peptone water (Digel et al., 2018). The samples were transported in a cold condition to the microbiology laboratory, Department of Environmental Science, Kaduna Polytechnic, Kaduna, Nigeria for microbiological analyses.

2.3 Isolation of Bacteria from the Biofouled Environments

The bacterial isolation was conducted using standard plate technique as described by Wilson et al. (2018). The biofouled scrapings in peptone water were vortexed (Digel et al., 2018) and serially diluted to 10^{-6} using sterile normal saline as the diluent. A volume (100µL) from each dilution was inoculated (using spread plate technique) onto correspondingly labeled Nutrient Agar (gL^{-1} of peptone: 5.0, NaCl: 5.0, beef extract: 1.5, yeast extract: 1.5 and agar 15; pH: 7.2) as well as MacConkey agar (gL^{-1} of peptone: 17, proteose: 3g, lactose monohydrate: 10g, bile salt: 1.5, NaCl: 5, neutral red: 0.03, crystal violate: 0.001 and agar: 13.5; pH: 7.1) plates (Aryal, 2019; Julistiono et al., 2018). The inoculated Petri dishes were incubated at 28ºC for 24 hours, following when randomly selected isolates were sub-cultured and purified using the same media, under similar culture conditions.

2.4 Confirmation of Bacterial Biofilm Formation

Ability of the bacterial isolates to form biofilms was verified using a tube method (TM) as described by Kırmusaoğlu (2019). Freshly-grown bacterial culture was inoculated into replicate test tubes, each of which contained 5mL of prepared Tryptic Soy Broth (TSB) (gL^{-1} of tryptone: 17.0, soytone: 3.0, glucose: 2.5, NaCl: 5.0 and dipotassium phosphate: 2.5; pH 7.3 ± 0.2) and incubated for 24 hours at 37°C. Following this incubation, the tubes were carefully emptied and the planktonic cells were discharged by rinsing twice with phosphate-buffered saline (PBS, pH 7.2). The sessile isolates of biofilms formed on the test tubes were stained with safranin for 1 hour. The safranin-stained tubes were rinsed twice with PBS to discharge the excess stain. After air drying, appearance of a visible film lining the walls and bottom of the tubes indicated biofilm production. The same volume (5mL) of a sterile uninoculated TSB was used as control under similar culture conditions.

2.5 Identification of the Biofilm-Forming Bacteria

The biofilm producing bacterial isolates were characterized based on their morphological properties (Gram's stain reaction) and subjected to a series of biochemical (oxidase, catalase, indole, methyl red, Voges-Proskauer, triple sugar iron, citrate, urease, motility as well as H_2S production) tests (Farinde et al., 2014; Gohet al., 2014; UK Standards for Microbiology Investigations, 2014; Cappuccino and Sherman, 2013; MacFaddin, 2000). Identities of the various bacterial species were confirmed using 16S rRNA gene sequencing (Julistiono et al., 2018) via Genomic DNA Extraction, Polymerase Chain Reaction, Gel Electrophoresis and Visualization of the PCR Products, DNA Band Cutting, Gel Extraction, Purification of the PCR Fragments and Sequencing (Wang et al., 2011).

2.6 Collection and Identification of the Plant Sample

Apparently healthy whole plant of *R.officinalis* was identified using standard keys and descriptions (Myers, 2019) and harvested from its cultivated site in Malali

Plant Gardens, Kaduna, Nigeria (10°32'8.7"N, 09°27'37.2" E) in May, 2020. A taxonomist authenticated the plant's identity at the herbarium section of the Department of Plant Biology, Bayero University, Kano, Nigeria. Voucher specimens of the authenticated plant was pressed in-between clean sheets of paper, dried (Bulugahapitiya, 2013) and deposited at the herbarium for reference purpose.

2.7 Preparation of Plant Material

Leaves of the freshly collected *R.officinalis* were detached, pre-washed with clean water to remove extraneous materials (Lohaet al., 2019), rinsed with distilled water and distributed evenly to air-dry at room temperature (Gahlot et al., 2018). The dried leaves were excised and pulverized to fine powder using laboratory mortar and pestle. The ground powder was sieved through a 0.5mm mesh gauze to standardize the particle size (Teresa-May, 2018) and stored at room temperature in an air-tight dry container until needed for analyses (Ibrahim et al., 2017).

2.8 Extraction of the Plant Material

The leaf powder was extracted using cold maceration method (De Oliveira et al., 2019) in n-hexane, acetone, ethyl acetate, methanol and distilled water, based on the phases of non, less, medium, high and comparably high polar solvents, respectively (Bulugahapitiya, 2013). According to a procedure described by Sagbo et al. (2020), 100g of the leaf powder was macerated in 1000mL of the solvent and the set-up was allowed to stand for 72 hours at room temperature with intermittent agitation (De Oliveira et al., 2019; Loha et al., 2019). The damp plant material was passed through a cheese cloth, allowed to settle and re-filtered via a Whatman grade 1 filter paper (11μm) (Loha et al., 2019). The residue (marc) was re-extracted in similar solvent to recover as much occluded solution as possible. The extracts were concentrated using rotary evaporator at 40ºC *in vacuo* and air dried in a fume hood (Teresa-May, 2018). The dried fractions of the crude extracts were stored in air tight glass containers at 4°C under refrigeration until required for further analyses (Sagbo et al., 2020).

2.9 Preparation of the Plant Extracts

Exactly 0.1g of the dried crude extract was dissolved in 10mL of 1% Dimethyl Sulfoxide (DMSO) to achieve 10mg/mL (Famuyide et al., 2019). This (stock) was diluted serially to prepare subsequent test concentrations (5.000, 2.500, 1.250, 0.625, 0.313, 0.156 and 0.078mg/mL). Sterility of the extracts was verified by inoculation (via streaking) on freshly prepared NA, which was incubated at 37°C for 24 hours.

2.10 Minimum Inhibitory Concentration (MIC) Assay

Five milliliters (5mL) of standardized (0.5 OD_{595nm}) bacterial inoculums (Lade et al., 2014) from an overnight culture was inoculated into 95mL of freshly prepared LB broth (Lade et al., 2014) and incubated at 37°C for 48 hours (Julistiono et al., 2018). Wells of a flat bottom polystyrene microtiter plate were conditioned by introducing 200µL of (plain) LB broth to

each and allowed to stand for 1 hour at room temperature (Lade et al., 2014). The wells were emptied and 20µL of the activated culture, followed by 180µL of the plant extract (0.078–10.000mg/mL)were added to each (Taufik et al., 2018). The plate was covered and incubated under static condition at 28°C for 48 hours (Biswa and Doble, 2013). Plain (uninoculated) LB broth was used as negative control while ciprofloxacin (5µg/mL) was applied as positive control. Bacterial MIC was determined with the use of a micro plate reader (AMR-100, China) at OD_{595} as the minimum concentration where absorbance of the treatment was less or equal to that of the negative control (Da Rosa et al., 2016).

2.11 Minimum Biofilm Inhibitory Concentration (MBIC) Assay

Inhibition of the bacterial biofilm formation was determined using a crystal violet method in microtiter plate (Julistiono et al., 2018). Equal volumes (100µL each) of the activated culture and the extract (0.078–1.250mg/mL) were introduced to each well following conditioning. The plate was incubated in static condition at 28ºC for 48 hours. Supernatants from the wells were carefully aspirated out without disrupting the biofilms on the base and the wells were washed thrice with PBS to remove any unattached bacterial cells. The plate was incubated at 37°C for 15minutes. Formed biofilms were fixed with 200µL of 99% methanol for 20 minutes and stained with 100µL of 0.2% (w/v) crystal violet solution for 15 minutes at room temperature. Excess stain was removed from the wells by rinsing four times with PBS, after which 100µL of 95% ethanol was introduced to extract the crystal violet in solution from the biofilms (Hossain et al., 2017). Absorbance of the dissolved crystal violet (which corresponds to a measure of bacterial cells that formed the biofilms) was determined at 595nm (Taufik et al., 2018) using the micro plate Reader. Inoculated LB broth (without the extract) was used as control. The MBIC was determined as the minimum concentration where absorbance of the treatments was less than or equal to that of the control (Da Rosa et al., 2016). Percentage inhibition of biofilm was calculated using the equation below (Julistiono et al., 2018):

$$\%\ \text{biofilm inhibition (595nm)} = \frac{\text{OD of control} - \text{OD of test}}{\text{OD of control}} \times 100$$

2.12 Minimum Biofilm Eradicating Concentration (MBEC) Assay

Following conditioning of the micro plate wells, 100µL of the activated culture was inoculated into each well. The set-up was incubated under static condition at 28ºC for 48 hours (Taufik et al., 2018). The liquid culture was aspirated out and the wells were washed with PBS. Wells with successfully induced biofilms were filled with 200µL of the extracts at 0.078–1.250mg/land the plates were incubated for 6 hours at 37°C. The biofilms were fixed with 200µL of 99% methanol for 20 minutes and stained with 100µL of 0.2% (w/v) crystal violet solution for 15 minutes at room temperature. Excess stains were

removed by rinsing four times with PBS, which preceded the addition of 100µL of 95% ethanol (to each well) to extract the crystal violet in solution from the biofilm (Hossain et al., 2017). Absorbance (595nm) of the dissolved crystal violet was determined with the use of micro plate reader as a measure of cells that formed the biofilms (Taufik et al., 2018). Inoculated LB broth (without the extract) was used as control. The MBEC was calculated as the minimum concentration where absorbance of treatment was at least 50% less than that of the control, indicating up to 50% eradication of the formed biofilms (Da Rosa et al., 2016).

2.13 Mesocosm Experiment

To further confirm their antifouling activities, the crude plant extracts were subjected to mesocosm experiment in accordance with a procedure described by Dobretsov et al. (2011). Specifically, 2 sets of 2L capacity transparent plastic containers were filled with 1L of the compound solution. This solution was prepared by dissolving the extract in standard unfiltered seawater, sampled from the Eleko beach, Lagos, Nigeria (06º 26' 17.23"N, 03º 51' 06.51"E) using a standard procedure (Cheesbrough, 2006). Final concentrations of 0.313mg/mL and 0.156mg/mL extract in seawater were used. Replicates of sterile microscope glass slides (25 × 75mm) were immersed horizontally into each container. Microscope slides were also dipped into a similar container filled with an equal volume of the unfiltered seawater (control). Containers with the slides were kept under illumination at room temperature (25°C±2°C) for 5 days (Wilson et al., 2018). The slides were brought out and fouling was fixed with 1% formaldehyde in seawater. These slides were then stained with DNA-binding fluorochrome 4,6-diamidino-2-phenylindole solution (0.5µgmL^{-1}) and air dried. Bacteria in 10 randomly selected fields of view were enumerated under an epifluorescence microscope (Wild M20, Switzerland) using direct count at a total magnification of ×2000.

$$\text{Percentage biofilm inhibition} = \frac{\text{control count} - \text{test count}}{\text{control count}} \times 100$$

2.14 Statistical Analyses

Data were presented as mean ± standard deviation (SD) of replicate assays. The mean and standard deviation of bioassays were computed using Microsoft Excel (version 2016). Values of inhibitory activities were appraised by one-way analysis of variance (ANOVA) with the use of GraphPad Instat (version 3.10); in comparison with controls. All P-values <0.05 were regarded as statistically significant, which were illustrated with different superscript alphabets, while those >0.05 were considered insignificant and denoted by similar superscripts.

3. RESULTS AND DISCUSSION

In this study, biofilm production among bacteria isolated from the various biofouled environments was rated ‘strong’ where there was a visibly high biofilm adherence to both the wall and bottom of the test tubes. It was regarded as ‘moderate’ in case of less adherence to the tubes and ‘weak’ where

only a trace of biofilms manifests in the walls or bottom of the test tube(s). The result indicates that the trait of biofilm formation was confirmed in the majority (63.16%) of the screened isolates. This implies that most of the sampled environments harboured the typical biofilm-forming bacteria, which can easily be isolated using protocols adopted in this research. Among the biofilm formers, isolates from the membrane filter, plastic and glass substrates (M_1, M_2 and M_3, respectively) of the biofouling model excelled with up to 25% 'strong' formation. Since all the three isolates originated from the (controlled) biofouling model, this signifies that biofilm formation could depend strongly on environmental conditions such as availability of nutrients, moisture and temperature. The result of Zuberi and Nadeem (2017) corroborates the finding of this study as they similarly reported as high as 63.64% biofilm formation in bacteria isolated from contact lenses and their accessories in Karachi, Pakistan.

4.1 Identity of the Biofilm-Producing Bacteria

The isolates with confirmed biofilm formation ability were identified as *Staphylococcus aureus* RBSB2_C1, *Pseudomonas aeruginosa* NT 10038 and *Klebsiella pneumoniae* C2244. This confirmed the inherent biofilm formation property of these bacterial species. Awoke et al. (2019) support this finding as they similarly identified the capability of biofilm production in *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Klebsiella* sp. in Southwest Ethiopia. Although, *Staphylococcusaureus* itself has been reported (Paluch et al., 2020) to control biofilm production in some other bacteria.

4.2Minimum Inhibitory Concentration of the Plant Extracts

The MICs of the extracts were determined as 0.31 to 1.25mg/mL against *Pseudomonas aeruginosa*, *Klebsiella pneumonia* and *Staphylococcus aureus.* Majorly, lower MICs were recorded from the methanol and ethyl acetate extracts. This might owe to the fact that many phytochemicals are polar in nature; hence, significant biological activities concentrate mostly in the polar regions (Bogavaca et al., 2017). The least MIC (0.31mg/mL) was obtained from the methanol extract against *Pseudomonas aeruginosa*, followed by 0.63mg/mL from the same extract against *Klebsiella pneumonia*. This implies that the biofilm producing bacteria were most susceptible to the methanol extract. The study of Van-Vuuren (2008) corroborates this as MICs ≤1.25mg/mL of some South African plants were reported and regarded as strong values. In the result of Bogavaca et al. (2017), much higher (50mg/mL) MIC value of *R. officinalis* (but essential oil)was identified against *Pseudomonas aeruginosa*. Similar to our finding, Jarrar et al. (2010) recorded activities of the ethanol extract of *R. officinalis*, collected

Table 1: Confirmed Biofilm Production of Bacterial Isolates from Slimy Surfaces of the Membrane Filter, Plastic and Glass Substrates of the Biofouling Model, Water Reservoirs from Kaduna State Water Treatment Plant and Solid Objects from River Kaduna

Bacterial Isolates	I_1	I_2	I_3	I_4	I_5	I_6	I_7	M_1	M_2	M_3	M_4	M_5	R_1	R_2	R_3	R_4	R_5	R_6	R_7
Extent of Biofilm Production	-	++	++	+	-	+	++	+++	+++	+++	-	++	+	-	-	+	+	-	-

Percentage Biofilm Formation: N (%)	19(63.16)
Strong: n (%)	12(25.00)
Moderate: n (%)	12(33.33)
Weak: n (%)	12(41.67)

Key: +, ++ and +++ = Weak, moderate and strong biofilm production respectively; - = No visible biofilm production.
$M_{1\text{-}5}$ = Isolates from plastic, glass and membrane filter substrates of the biofouling model, $R_{1\text{-}7}$ = Isolates from biofouled substrates from River Kaduna and $I_{1\text{-}7}$ = Isolates from surfaces of water reservoirs from Kaduna state water treatment plant.

Table 2: Cultural, Morphological and Biochemical Identities of the Biofilm-Forming Bacteria

Colonial Characteristics on NA	Colonial Characteristics on MacConkey	Gram's Stain Reaction	Ox	Cat	Ind	MR	VP	Gl	Lc	Su	H_2S	Cit	Ur	Mot	Inference
Blue-green	Yellow-green	Gram - bacilli	++	+++	-	-	-	+	+	-	-	+	-	+	*Pseudomonas aeruginosa* **
Mucoid milky	Pink mucoid	Gram - bacilli	-	+	-	-	+	+	+	+	-	+	+	-	*Klebsiella*sp.
Large smooth circular	NG	Gram + cocci	-	+	-	+	+	+	+	+	-	+	+	-	*Staphylococcus aureus*

Key: - = negative, + = positive, Ox = oxidase, Cat = catalase, Ind = indole, MR = methyl red, VP = VogesProskauer, Gl = glucose, Lc = lactose, Su = sucrose, Cit = citrate (Simon's), Ur = urease, Mot = motility, ** = grown at an elevated temperature (42°C), NG = No growth.

Table 3: Minimum Inhibitory Concentration (mg/mL) of *Rosmarinus officinalis* Leaf Extracts against *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Staphylococcus aureus*

	Pseudomonas aeruginosa					*Klebsiella pneumoniae*					*Staphylococcus aureus*				
Extracts	Aq	M	E	A	H	Aq	M	E	A	H	Aq	M	E	A	H
R.officinalis	2.500	0.313*	1.250	-	-	-	0.625*	-	-	2.500	-	-	1.250	-	-
Ciprofloxacin	< 0.078					< 0.078					< 0.078				
Plain LB	-					-					-				

Key: Aq = Aqueous, M = Methanol, E = Ethyl acetate, A = Acetone and H = N-hexane. Results ≤1.250mg/mL (especially those indicated with*) were considered strong MIC values. - = MIC values >5.000mg/mL.Ciprofloxacin and Plain LB = positive and negative controls respectively.

Table 4: Inhibitory Potential of the Methanol and Ethyl Acetate Leaf Extracts of *Rosmarinus officinalis* Against the Biofilms of *Pseudomonas aeruginosa*,*Klebsiella pneumonia* and *Staphylococcus aureus*

Inhibition of Biofilm Development (%)			
	Bacterial species		
Extracts (0.078–1.250mg/mL)	*Pseudomonas aeruginosa*	*Klebsiella pneumonia*	*Staphylococcus aureus*
Methanol	50.00±0.66(b)^{g}	43.10±3.84(c)^{i}	37.69±5.48(c)^{i}
Ethyl Acetate	NA	42.41±2.24(a)^{j}	48.8±1.00(b)^{j}
Ciprofloxacin (0.078mg/mL)	51.90±12.61^{h}	51.38±3.51^{f}	53.80±4.82^{k}

Values are mean (±SD) percentage biofilm inhibition.
Values with different superscripts across the same column are significantly different (P<0.05).
Key: NA = not active against biofilms at all tested concentrations.
a, b, c, d and e = 0.078, 0.156, 0.313, 0.625 and 1.250mg/mL respectively.

Table 5: Eradication Potential of *Rosmarinus officinalis*Leaf Extractsagainstthe Biofilms of *Pseudomonas aeruginosa*,*Klebsiella pneumonia* and *Staphylococcus aureus*

Eradication of formed Biofilms (%)			
	Bacterial species		
Extracts (0.078–1.250mg/mL)	*Pseudomonas aeruginosa*	*Klebsiella pneumoniae*	*Staphylococcus aureus*
Methanol	48.67±6.14(c)^{m}	**51.17±2.24(c)^{m}	**57.88±4.76(c)^{m}
Ethyl Acetate	**52.35±6.79(c)^{n}	41.92±4.09(d)^{n}	48.22±3.84(c)^{n}
Ciprofloxacin (0.078mg/mL)	**64.21±3.02^{p}	**61.55±3.38^{p}	**56.39±1.58^{p}

Values are mean (±SD) of percentage biofilm eradication.
Values with different superscripts across the same column are significantly different (P<0.05).
Key: - = no biofilm eradication recorded, ** = active eradication of biofilms (≥50%)
a, b, c, d and e = 0.078, 0.156, 0.313, 0.625 and 1.250mg/mL respectively.

Table 6: Density of Biofilm-Forming Bacteria on Glass Slides Exposed to the Extracts of *Rosmarinus officinalis*in Unfiltered Seawater

	Count (cells/mm^2)		
Plant Extracts	Control	0.156mg/mL	0.313mg/mL
Methanol	127.93±4.65^q	102.47±2.96^r (19.90%)	56.00±2.76^s (56.23%)
Ethyl Acetate	125.67±4.19^q	103.17±3.59^r (17.90%)	73.00±4.16^s (41.91%)

Values are mean (±SD) bacterial density with percentage inhibition in parenthesis.
Mean values with different superscripts across the same row are significantly different (P<0.05).

from the Northern Palestine against *Staphylococcus aureus,* at an MIC of 0.39mg/mL. It was established (Abkhoo et al., 2010) that ethanol extracts of *R. officinalis* from Tehran, Iran, significantly inhibited the growth of *Pseudomonas aeruginosa* at the MIC of 0.1mg/mL.

Generally, variation in MIC values was observed across the three (3) bacterial isolates in the present study. This can be due to the presence of different intrinsic levels of tolerance to the tested plant compounds, as similarly observed by Ahmad and Aqil (2007).

4.3 Biofilm Inhibitory Potential of the Plant Extracts

Almost all the extracts inhibited the formation and development of preformed biofilms at appreciable limits. The extracts were able to hinder the development of biofilms formed by *Pseudomonas aeruginosa*, *Klebsiella pneumonia* and *Staphylococcus aureus* at the range of 24.89 to 50.00%. The methanol extract displayed the highest (50.00%) potential of biofilm inhibition against *Pseudomonas aeruginosa* at 0.16mg/mL. This unveils the puissance of *R. officinalis* as a potential antifouling agent. The probable mechanism of this biofilm inhibition may be reduction in the production of extra polymeric substances (EPS), which is an important component of bacterial biofilms, crucial to the maintenance of the spatial structure of the consortium, as established by Paluchet al. (2020). Exactly 50.00% of biofilm formed by *Pseudomonas aeruginosa* was similarly inhibited but at 7.80mg/mL of *R. officinalis* in the study of Yazdeliet al. (2021). Ziemichód and Skotarczak (2017) further established the capability of plant products to inhibit both formed and preformed biofilms of *Staphylococcus aureus.* Studies conducted by Endo et al. (2018) were in agreement with ours as they equally revealed as high as 50.00% activity against the preformed biofilm of *Staphylococcus aureus* and *Pseudomonas aeruginosa* at 30.00 to 250.00μg/mL of the leaf extracts of *R. officinalis.* Likewise, Ceylan et al. (2014) recorded 39.49% and 51.30% inhibition capacity of the preformed biofilms of *Pseudomonas aeruginosa* and *Staphylococcus aureus,* respectively using *R. officinalis* essential oil at 10.00 to

0.08µg/mL. Up to 57.00% of biofilm formed by *Staphylococcus Epidermidis* was inhibited by the essential oil of *R. officinalis obtained from Tunisia, as reported* in the work of Jardak et al. (2017).

4.4 Biofilm Eradication Potential of the Plant Extracts

Results of the eradication assay revealed that biofilms formed by *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Staphylococcus aureus* were appreciably reduced by the extracts, indicating an activity range of 33.95 to 57.88%. The highest (57.88%) activity was obtained from the methanol extract of *R. officinalis* at 0.31mg/mL against the biofilm formed by *Staphylococcus aureus.* Jardak et al. (2017) also established that the biofilm formed by *Staphylococcus epidermidis* was eradicated by up to 67.53% when exposed to *R. officinalis* essential oil at the concentration of 50.00mg/mL. In this study, *R. officinalis* might have reduced bacterial biofilms by producing and releasing compounds capable of affecting bacterial molecular signals and inhibiting behaviors under the control of quorum sensing (QS) (Yazdeli et al., 2021).

4.5 Effect of the Extracts on the Density of Biofilm-Forming Bacteria

From the mesocosm experiment, both methanol and ethyl acetate extracts of *R. officinalis* inhibited the formation of microbial communities on the glass slides at both tested concentrations. The methanol extract significantly ($P<0.05$) decreased bacterial densities (56.23% and 19.90%) at 0.313mg/mL and 0.156mg/mL respectively; in relation to the control. Activities recorded from this extract (especially at 0.33mg/mL) might indicate probable inhibition of QS among the exposed bacteria. This might have led to low attachment, hindering the subsequent biofilm formation and development. This idea was supported by Dobretsovet al. (2007), who established that QS inhibitors affect microbial composition and densities. According to Kjelleberg et al. (2001), anti-quorum sensing (AQS) agents may alter bacterial composition in biofilm formation, leading to a shift in the microbial communities from being dominated by Gram-negative to Gram-positive bacteria. However, this does not exclude the possibility that the biofilm inhibition (recorded in this study) might have also arisen from the obstruction (by the extracts) of other regulatory cascades that may govern the process of biofilm formation, as highlighted in the study of Dobretsovet al. (2011).

5. CONCLUSION

The biofilm formation trait was confirmed in the majority of the bacteria isolated from the biofouled environments. These bacteria were identified as *Staphylococcus aureus* RBSB2_C1, *Pseudomonas aeruginosa* NT10038 and *Klebsiella pneumoniae*C2244. Evaluation of the antibiofilm activities of *R. officinalis* revealed that this plant (especially its methanol extract) possessed some features of inhibitory activities against bacterial biofilms. This indicates that the methanol, followed by ethyl acetate extracts of this plant, might contain many phytoconstituents at varying concentrations,

which can be responsible for their antifouling effects. Therefore, this study has identified the potential of *R. officinalis* as, a source of active antifouling compounds that can be cheap, eco-friendly and readily available.

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Data Availability statements: The data presented in this study are available on request from the corresponding author.

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