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# Antibacterial and antibiofilm activities of polysaccharides, essential oil, and fatty oil extracted from *Laurus nobilis* growing in Lebanon

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ABSTRACT

**Objective:** To evaluate the antibacterial activity of the extracts of *Laurus nobilis* against three Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212 and *Staphylococcus epidermidis* CIP 444) and two Gram-negative bacteria (*Escherichia coli* ATCC 35218 and *Pseudomonas aeruginosa* ATCC 27853). Also, the antibiofilm activity has been investigated against the biofilm produced by *Staphylococcus epidermidis* CIP 444.

**Materials:** The polysaccharides, essential oil, and fatty oils extracted from the plant were used in broth microdilution methods to study the minimal inhibitory concentration, and then the minimal bactericidal concentration was determined.

**Results:** The results showed that alginate, fucoidan, fatty oils and essential oil have good antibacterial activities against the 5 bacterial strains, and a negligible biofilm eradication activity of fucoidan, laminaran, fatty oil, and essential oil was observed, but a promising biofilm eradication activity was obtained with alginate, which showed a reduced biofilm mass even at low concentration.

**Conclusions:** The extracts obtained have promising antibacterial capacities which need further investigation for them to be incorporated in medical or nutritional applications.

## 1. Introduction

For every disease, there is a cure given by the nature through one way or another[1]. Over the past few years, medicinal plants has been developed extensively[2]. They are the richest biological resources of drugs of folk medicines, traditional medicinal systems, modern medicines, nutraceuticals, food supplements, pharmaceuticals, intermediate and chemical entities for synthetic drugs[1].

The discovery of pharmaceutical compounds and medicines can be traced using plants which are good sources. Humans as well as livestock species can use the natural products as potential drugs. The analogues of these products can act as intermediates for synthesis of useful drugs[3]. Extraction of medicinally active portions of the plant tissues can be done using selective solvents following standard procedures. Metabolites of relatively complex structures constitute the products so different components can be obtained in liquid, semisolid state or, after removing the solvent, in dry powder form. These products are intended for oral or external use[4].

Drug resistance is a consequence of the worldwide use of antibiotics, and the acute challenge for health care is to find measures to efficiently combat resistant organisms[5]. Seventy years after the first report on microbial biofilms[6], drug resistance is still a concern in a broad range of areas, and specifically in the biomedical, food and environmental fields[7-10]. It is natural for microorganisms to attach to wet surfaces, to multiply and to embed themselves in a slimy matrix composed of extracellular polymeric

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substances where they produce and form a biofilm. In this physiological state, they become difficult to be detected and to be eradicated. Currently, a renewed interest in natural substances has focused attention on plant products or extracts that are rich in bioactive compounds and well known for their antimicrobial and antibiofilm properties[11].

*Laurus nobilis* (*L. nobilis*), also known as sweet bay, bay laurel, Grecian laurel, true bay, and bay tree, is either an evergreen shrub or small tree, it belongs to the family Lauraceae which comprises numerous aromatic and medicinal plants[12]. Laurel usually grows to a height from 20 to 30 feet. It is grown in many warm regions of the world, particularly in Southern Europe and around the shores of the Mediterranean Sea which includes Lebanon[13]. It has been in this country since ancient times. *Laurus* essential oil has shown antibacterial and antifungal properties[14]. The essential oil exhibited strong antibacterial activity against a wide panel of tested foodborne spoilage and pathogenic bacteria[15].

Given the scarcity of studies on laurel antimicrobial activities especially local ones, the aim of the current study was to extract the polysaccharides, essential oil and fatty oil from *L. nobilis* and investigate its antibacterial and antibiofilm activities.

## 2. Materials and methods

### 2.1. Plant extracts

The samples of *Laurus* leaves and fruits were collected from Lebanon, in year 2013. The samples were air-dried at room temperature in the shade for some weeks. They had a final moisture content of 10.0%. Before using them, the dried samples were milled in a blender. At the end of the milling process, the particle sizes were in the range of 0.8 mm-0.9 mm.

### 2.2. Volatile oil extraction

The volatile oils of *Laurus* leaves or fruits were obtained by the process of hydrodistillation in the Clevenger apparatus. A total of 100 g *Laurus* (leaves or fruits) were placed in a flask (2.5 L) and hydrodistilled for 2.5 h. The oil samples were dried over anhydrous sodium sulphate and stored at 4 °C in the dark.

### 2.3. Fatty oil extraction

Fruits oil is extracted by using petroleum ether in ultrasonic bath 15 min up for twice. Then it is filtered and evaporated by using a rotary evaporator at 50 °C. Then the fatty oil were dried and stored in the dark at room temperature until use.

### 2.4. Isolation of the polysaccharides from Laurus

Fifty grams of dry leaves were extracted twice by ethanol (96%) for 3 h at 40 °C (*Laurus*: ethanol 1:0.8 w/w) to remove low molecular weight compounds. The supernatant was separated by centrifugation (3 000 r/min, 20 min). Then the leaves were dried and extracted for 3 h, 2 times by 150 mL HCl (pH 2.0-2.3) at 60 °C to separate the supernatant "1" (used for the extraction of fucoidan and laminaran) from the pellet "1" (used for the extraction of alginate). The pellet "1" was extracted successively with aqueous solutions (100 mL) of $Na_2CO_3$ 3% and 1.5% for 8 h at 60 °C and rinsed by water. The extracts were dialyzed for 24 h using a membrane with a retention limit of 100 KDa, and then precipitated using absolute ethanol. The precipitate was dissolved in water and the pH was adjusted to 2 by HCl (12%). The obtained precipitate of alginic acid was then dissolved in a minor amount of water with addition of NaOH to pH 8.6. The final solution was lyophilized to obtain the alginate powder. The supernatant "1" was neutralized with $NaHCO_3$ (3%) (pH 5.7 to 6.1), then lyophilized, laminaran will be separated from fucoidan by adding HCl as previously described by Imbs *et al*[16].

### 2.5. $H^1$NMR and $C^{13}$NMR spectra

NMR spectra of alginate and fucoidan were recorded using Bruker UltraShield 300 spectrometer at room temperature, with a frequency of 300 MHz, an acquisition time of 5.29 seconds and duration of impulse of 11 microseconds. Three milligrams of the sample was dissolved in 0.5 mL of 99% (deuterium oxide) D2O. Tetramethylsilane was used as internal standard.

### 2.6. Fourier transform infrared spectroscopy (FT-IR) analysis

The infrared spectra were recorded on a JASCO FT/IR6300 spectrometer. The resolution was 4 $cm^{-1}$. Data was collected in the range of 4 000 $cm^{-1}$-400 $cm^{-1}$. All the alginate samples were prepared for measurement in the form of KBr pellets.

### 2.7. Bacterial strains, media and reagents

Three Gram-positive bacteria [*Staphylococcus epidermidis* CIP 444 (*S. epidermidis*), *Staphylococcus aureus* ATCC 25923 (*S. aureus*) and *Enterococcus faecalis* ATCC 29212 (*E. faecalis*)] and two Gram-negative strains [*Escherichia coli* (*E. coli*)

ATCC 35218 and *Pseudomonas aeruginosa* ATCC 27853 (*P. aeruginosa*)] were used in this study. CIP 444 is a clinical strain which was isolated from a patient with infected implanted device, hospitalized in the Mignot Hospital of Versailles, France[17]. This strain was identified and characterized for many features in the previous studies and deposited to be enclosed within the microorganisms of the collection of Institute Pasteur in 2007[17-20]. The other strains are ATCC. The strains were stored at −80 °C in glycerol stocks and used as required. Brain heart infusion, brain heart agar, and Mueller-Hinton broth were purchased from Himedia (Mumbai, India), prepared and then autoclaved as indicated by the manufacturer.

### 2.8. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) assays

MICs and MBCs were determined by a microtiter broth dilution method as recommended by the Clinical and Laboratory Standards Institute[21]. The MIC was defined as the lowest extract concentration that yielded no visible growth.

Serial two-fold dilutions of alginate, fucoidan, essential oil and fatty oil in Mueller-Hinton broth were prepared in a 96-well plate (100 μL per well). Wells with no plant extract added were used as a positive growth control. A diluted bacterial suspension was added to each well to give a final concentration of $5\times10^5$ CFU/mL, confirmed by viable counts. Wells without bacteria added were used as a negative growth control. The plates were incubated for 24 h at 37 °C. The contents of the wells showing no visible growth were placed on brain heart agar and the number of colonies was counted after overnight incubation at 37 °C. The MBC was defined as the lowest concentration of the product reducing the initial inoculum by ≥99.9%. The MIC and MBC were determined for all strains. For each strain, at least three independent determinations were done and the modal value was taken.

### 2.9. Biofilm formation

Assay of biofilm formation in polystyrene was performed essentially according to a standard procedure with some modifications[17]. Actually, *S. epidermidis* CIP 444 was grown in trypticase soy broth medium overnight at 37 °C. Then the overnight cultured *S. epidermidis* CIP 444 suspension with defined volume at concentration of $4.16\times10^5$ CFU/mL (confirmed by viable count) was added to a trypticase soy broth medium supplemented with 0.25% glucose. A total of 120 μL of this bacterial suspension was inoculated into each well of a sterile 96-well flat-bottom polystyrene tissue culture-treated microtiter plate (Corning®Costar® 3598, Corning, NY 14831, USA) except for column 12 which was used as a negative control and filled only with the sterile medium, and then the plates were incubated for 24 h at 37 °C. The next step included discarding the biomass and washing the microtiter plates with saline (0.9% NaCl) to remove any non-adherent bacteria, then drying the plates at room temperature for several minutes. After that, the remaining biofilm attached to the wall and the bottom of the wells was fixed by heating at 90 °C for 50 min, thus the plates were ready for treatment with the plant extracts.

### 2.10. Antibiofilm activity of the plant extracts

After the fixation of the formed biofilm as previously described, each well of the microtiter plate was filled with 120 μL of sterile physiologic water to be used as a diluent for the serial dilution of our plant extracts. A serial 1/2 dilution was then made with equal volume of the extract in the saline water in the wells except for column 11 which was used a positive untreated control, then the microtiter plates are incubated at 37 °C for 18 h. Tests were performed in quadruple.

The wells were then washed 2 times by saline water, filled with 100 μL 0.1% crystal violet and left at room temperature for 10 min. The stain was then discarded and the wells were washed by saline water for 3 times. They were finally filled with 100 μL of physiologic water and the $OD_{490\ nm}$ was measured. The results were expressed as mean±SEM.

## 3. Results

### 3.1. Isolation of the polysaccharides from Laurus

According to the method of Imbs *et al.*[16], the amount of alginate obtained from the extraction of 50 g of *Laurus* leaves was 0.26 g with respective yield of 0.52%, while the fucoidan amount was about 0.63 g and the laminaran amount was 0.21 g.

### 3.2. $H^1$NMR spectroscopy of alginates

$H^1$NMR spectroscopy is considered to be the most reliable method to determine the composition and the detailed structure of alginates, which are typically described by their mannuronic/guluronic ratio (M/G)[22]. Usually the M/G is calculated using the method proposed by Grasdalen *et al*[23].

The result from $H^1$NMR spectra of alginate isolated from *Laurus* showed:

FG=IA/(IB+IC)=4.7623/9.1057=0.52

FM=1−FG =1−0.52=0.48

M/G=FM/FG=0.48/0.52=0.92

This ratio was between 0.2 and 1.4, so the alginate isolated from *Laurus* was a good elastic gelling agent.

### 3.3. FT-IR spectroscopy analysis of alginate

The FT-IR spectrum of alginate isolated from *Laurus* is presented in Figure 1[24]. In the region between 3615.6 $cm^{-1}$ -1 600.63 $cm^{-1}$, there are three bands appeared: a broad band centred at 3438.46 $cm^{-1}$ assigned to hydrogen bounded O-H stretching vibrations, the weak signal at 2920.66 $cm^{-1}$ is due to CH stretching vibrations, and the asymmetric stretching vibration of O-C-O is centered at 1600.63 $cm^{-1}$. The band at 1423.21 $cm^{-1}$ may be due to the C-OH bending vibration with contribution of carboxylate group O-C-O[25,26], the weak bands at 1097.3 $cm^{-1}$ may be assigned to O-C-H deformation, the band at 1022.09 $cm^{-1}$ may be also due to C-O stretching vibration. Moreover, the band at 703.89 $cm^{-1}$, which is a weak band assigned to the Cl-H deformation vibration of α-L-guluronic acid[27]. While the bands 538.042 $cm^{-1}$ may be due to C-H alkenes.

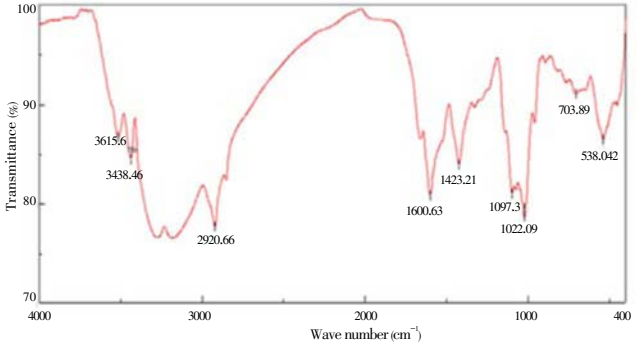


**Figure 1.** Infrared spectrum of alginate isolated from the leaves of *Laurus*.

### 3.4. $C^{13}$NMR study of fucoidan

The $C^{13}$NMR spectrum of fucoidan fractions was complex, with the main signals of L-fucans sulphates appearing at 93.78-107.00 ppm (C1) and 15.0-16.7 ppm (C6). The signals [18-20 ppm ($CH_3$), 170-179 ppm (C=O)] indicated the presence of acetyl groups and signal 16.75 ppm refer to α-L-fucan.

### 3.5. FT-IR spectroscopy analysis of fucoidan

The FT-IR spectrum of fucoidan isolated from *Laurus* is presented in Figure 2[24]. A broad band centred at 3305.39 $cm^{-1}$ is assigned to the hydrogen bonded O-H stretching vibration; a weak band at 2932.23 $cm^{-1}$ is assigned to a C-H stretching vibration. The band centred at 1608.34 $cm^{-1}$ is assigned to the carbonyl group C=O, the one at 1521.56 $cm^{-1}$ is assigned to the C-O bond of the carboxylate group. The band at 822.491 $cm^{-1}$ and 773.915 $cm^{-1}$ corresponds to the bond S-O-C group sulphate. The fingerprint in the region 674.963 $cm^{-1}$-466.082 $cm^{-1}$ is difficult to analyse, since neither previous work on fucoidan has been done nor the structure of it has been identified yet.

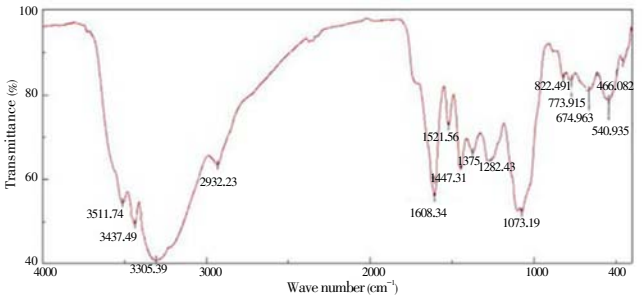


**Figure 2.** Infrared spectrum of fucoidan isolated from the leaves of *Laurus*.

### 3.6. Antibacterial activity of MIC and MBC

The extracts showed different inhibitory capabilities toward the tested bacterial strains, with both Gram-positive bacteria and Gram-negative bacteria being sensitive. Among Gram-negative bacteria, *P. aeruginosa* was globally the most sensitive; whereas *S. epidermidis* was globally the most sensitive among the Gram-positive bacterial strains. The MIC and MBC results are summarized in Table 1 and Table 2, respectively. Fatty oil was more efficient against *S. epidermidis*, *E. faecalis*, and *P. aeruginosa* than *S. aureus* and *E. coli* whereas *S. aureus* and *P. aeruginosa* seem to be more resistant to alginate than the other strains. Regarding fucoidan, *S. epidermidis* showed the highest sensitivity followed by *P. aeruginosa*, *E. faecalis*, *S. aureus* and *E. coli*. All the products have shown bactericidal activity against the tested bacterial strains with the highest efficiency obtained with fucoidan against *S. epidermidis*.

**Table 1**

Results of MIC of laurel extracts on the used bacteria.

| Bacterial type | Bacterial strain | Isolated compound | MIC (mg/mL) or % of the initial concentration |
|---|---|---|---|
| Gram-positive bacteria | *S. epidermidis* CIP 444 | Alginate | 61.32<MIC≤78.84 |
| | | Fucoidan | ≤60.97 |
| | | Essential oil | ≤90% |
| | | Fatty oil | ≤70% |
| | *E. faecalis* ATCC 29212 | Alginate | 61.32<MIC≤78.84 |
| | | Fucoidan | >78.39 |
| | | Essential oil | ≤90% |
| | | Fatty oil | ≤70% |
| | *S. aureus* ATCC 25923 | Alginate | >78.84 |
| | | Fucoidan | >78.39 |
| | | Essential oil | ≤90% |
| | | Fatty oil | 70%<MIC≤90% |
| Gram-negative bacteria | *E. coli* ATCC 35218 | Alginate | 61.32<MIC≤78.84 |
| | | Fucoidan | >78.39 |
| | | Essential oil | ≤90% |
| | | Fatty oil | 70%<MIC≤90% |
| | *P. aeruginosa* ATCC 27853 | Alginate | >78.84 |
| | | Fucoidan | 60.97<MIC≤78.39 |
| | | Essential oil | ≤90% |
| | | Fatty oil | ≤70% |

**Table 2**

Results of MBC of laurel extracts on the used bacteria.

| Bacterial type | Bacterial strain | Isolated compound | MBC (mg/mL) or % of the initial concentration |
|---|---|---|---|
| Gram-positive bacteria | *S. epidermidis* CIP 444 | Alginate | >78.84 |
| | | Fucoidan | 60.97 |
| | | Essential oil | 90% |
| | | Fatty oil | ≤70% |
| | *E. faecalis* ATCC 29212 | Alginate | >78.84 |
| | | Fucoidan | >78.39 |
| | | Essential oil | 90% |
| | | Fatty oil | 75% |
| | *S. aureus* ATCC 25923 | Alginate | >78.84 |
| | | Fucoidan | >78.39 |
| | | Essential oil | 90% |
| | | Fatty oil | 90% |
| Gram-negative bacteria | *E. coli* ATCC 35218 | Alginate | >78.84 |
| | | Fucoidan | >78.39 |
| | | Essential oil | 90% |
| | | Fatty oil | 90% |
| | *P. aeruginosa* ATCC 27853 | Alginate | >78.84 |
| | | Fucoidan | >78.39 |
| | | Essential oil | 90% |
| | | Fatty oil | 70%<MBC≤90% |

### 3.7. Biofilm eradication activity

Our results showed that alginate had visually appreciable activity against established *S. epidermidis* CIP 444 biofilm, which had the highest eradication capacity of about 78.0% at the highest used concentration of 46.65 mg/mL. Its eradicating capability decreased in a proportional way with its concentration. It is about 78.0%, 67.3%, 52.7%, 44.3%, 34.6%, 13.5%, 25.3%, and 17.0% at 46.650 mg/mL, 23.325 mg/mL, 11.663 mg/mL, 5.831 mg/mL, 2.916 mg/mL, 1.458 mg/mL, 0.729 mg/mL, 0.364 mg/mL, 0.182 mg/mL and 0.091 mg/mL, respectively (Figure 3). Whereas the other extracts, fucoidan, laminaran, and essential oil showed relatively lower biofilm eradication capabilities than that of alginate. The biofilm eradication capacities of fucoidan, laminaran and essential oil were ranging between 3.7%-33.5%, 13.7%-33.0% and 8.5%-33.75%, respectively (Figures 4-6).

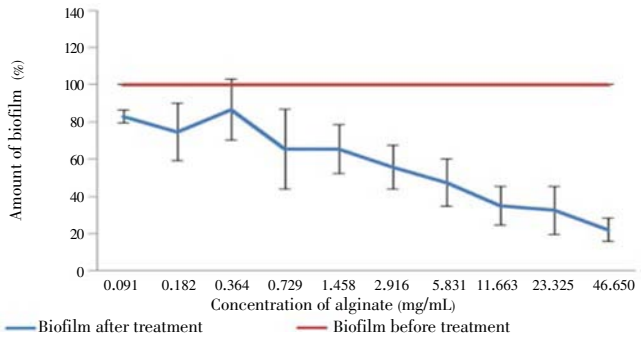


**Figure 3.** Eradication activity of alginate against established *S. epidermidis* biofilm at different concentrations.

The means±SD for at least 4 replicates are illustrated.

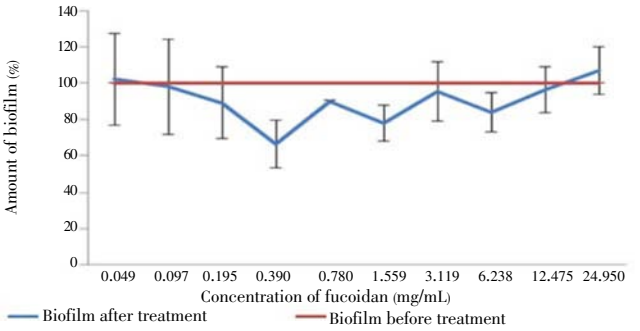


**Figure 4.** Eradication activity of fucoidan against established *S. epidermidis* biofilm at different concentrations.

The means±SD for at least 4 replicates are illustrated.

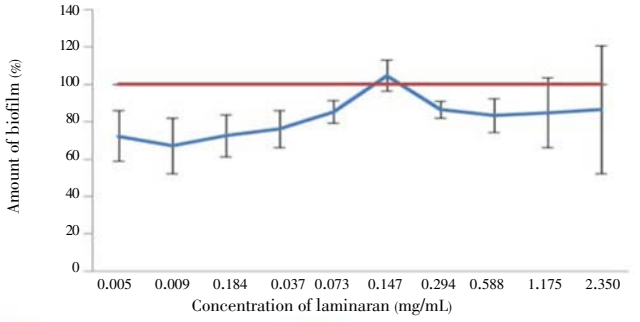


**Figure 5.** Eradication activity of laminaran against established *S. epidermidis* biofilm at different concentrations.

The means±SD for at least 4 replicates are illustrated.

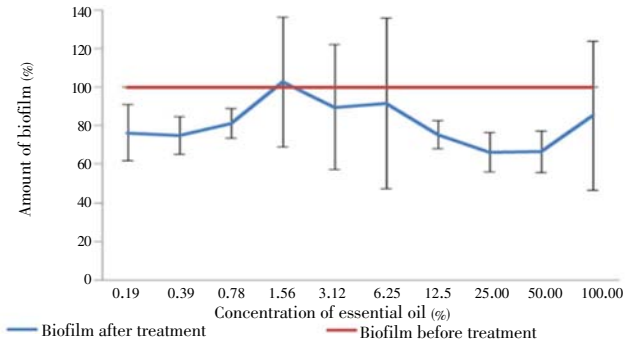


**Figure 6.** Eradication activity of essential oil against established *S. epidermidis* biofilm at different concentrations.

The means±SD for at least 4 replicates are illustrated.

## 4. Discussion

Interests in the medicinal plants investigation by scientists increased as the bacterial resistance toward the used chemicals increased alongside the transmittance of this resistance to several other bacteria. Furthermore, since using antibiotics and other synthetic compounds has adverse effects on human and animal health, interest in the fields of phytochemistry, phytopharmacology, phytomedicine and phytotherapy during the last decade has been generated[3]. On the other hand, it has been estimated that about 65% of human bacterial infections involve biofilms[28]. Biofilms have become the leading cause of infections related to indwelling

medical devices such as vascular catheters, prosthetic joints and artificial heart valves[29-31].

Likewise, biofilms are problematic in water treatment systems, and in particular food industry sectors such as brewing, dairy processing, fresh produce, poultry processing and red meat processing[32-35]. Bacteria in biofilms can be up to 1 000-fold more resistant to antibiotic treatment than the same organism growing planktonically[36-38]. The ability of biofilm-embedded bacteria to resist clearance by antimicrobial agents points to the importance of a continuous search for novel agents that are effective against both bacteria and their slime in the biofilm or the work in synergy with the currently available antimicrobial agents.

*S. epidermidis* was found as the most frequently isolated species in most studies, with this species being the most prevalent bacterium in human skin and mucosa[39]. In addition, *S. epidermidis* infections are commonly acquired in hospitals as a result of contamination of surgical cuts with microorganisms from the patients themselves or from the hospital personnel and the infection with *P. aeruginosa* is one of the most serious complications in burn patients, followed by infections with *E. coli*, *S. aureus* and other microorganisms[40]. Some of these species are also involved in foodborne and waterborne diseases.

Moreover, *S. epidermidis* CIP 444 is found to cause major problems regarding indwelling medical devices, also it has the capacity to develop persistent infections and shows an infection rate of 100% in *in vivo* experiments and this is due to its ability to form biofilm[20]. Therefore, our research was focused on these species specifically for the above reasons to test the effect of natural products such as laurel extracts against them.

Besides, our original results on the biofilm eradication activities at low, middle or high concentration depending on the extract, our results regarding the antibacterial activities of the extracts are in concordance with other findings that the essential oil of *L. nobilis* exhibited strong antibacterial activity against a wide panel of tested foodborne spoilage and pathogenic bacteria[15].

Our results hold a great promise for the medical community, due to the observed reasonable efficiency of alginate on the reduction of the bacterial biofilm, and the good antibacterial effect of most of the extracts of *L. nobilis*. Furthermore, it is the natural aspect of these products since we started with raw unmodified plant material. Therefore, the active substances present in this plant can be made available in the form of powder and it would play a non-negligible role in medicine, pharmacy, food and industry.

## Conflict of interest statement

We declare that we have no conflict of interest.

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