Impact of Antibacterial Activity of Physical Storage Extracts on Pathogenic Bacteria

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Abstract

The overuse of antibiotics can lead to resistance among pathogenic bacteria. A new antibiotic that is effective against new and resistant bacterial strains is needed. Plants and marine organisms may offer such novel treatments. In this study, extracts of the seaweed *U. lactuca*, and the plant seeds *N. sativa* were tested against strains of Gram-positive cocci and Gram-negative bacilli *S. aureus*, and *P. aeruginosa*. The results of the bacterial inhibitor showed high activity in both extracts with inhibition of *S. aureus* growth up to 30 mm and 20 mm and *P. aeruginosa* growth inhibition was up to 12 mm and 15 mm, after the treated with 100 μl *U. lactuca* and *N. sativa* extracts, respectively. The MICs and MBCs were reflected with the growth inhibitor with values of 2 μl, 8 μl and 4 μl, 8 μl for *S. aureus* and *P. aeruginosa* after treated with *N. sativa* respectively. Kill-time increases as concentrations of *U. lactuca* and *N. sativa* extracts increase. Moreover, extracts stored in the transparent bottle decreased in effectiveness after one month of storage with percentage of 58.85%. After three months, heating the extracts of *U. lactuca* and *N. sativa* to 90°C increased their antibacterial activity.

Keywords

Resistant Bacteria, *S. aureus*, *P. aeruginosa*, *U. lactuca*, *N. sativa*, Kill-Time

1. Introduction

Bacterial infections are usually treated with antibiotics. However, these drugs can be expensive. The continued use of antibiotics can also lead to bacterial resistance and thus decreased efficiency. Antibiotics can also cause...
adverse effects, such as hypersensitivity and depletion of beneficial microbes in the gut [1]. For these reasons, the WHO has suggested the need for alternative treatments [2]. The increased demand for biodiversity in drug development has led to the identification of several compounds that can inhibit pathogenic bacterial growth and may provide new antibiotic medicines.

Marine organisms, especially seaweeds, have a broad range of antibacterial, antifungal, antitumor, and antioxidant behavior. The chemical structures of seaweed include sterols, isoprenoids, amino acids, terpenoids, phlorotannins, steroids, phenolic compounds, fatty acids, and acrylic acid [3]-[5]. Seaweed-based products include alginate, carrageenan, and agar as phycocolloids. These products are abundantly available, renewable, and have been used for decades in medicine and pharmacy [6]. Seaweeds are also known to produce certain bioactive molecules [7]-[10], which interact with other organisms in the environment to inhibit bacterial or fungal growth [6] [11]. Seaweeds antimicrobial activity may also include the ability to synthesize bioactive secondary metabolites [12]-[14]. The extracts and active constituents of various marine seaweeds have been shown to have antibacterial activity against Gram-positive and Gram-negative bacteria [15]-[17].

The use of plant extracts in the treatment of diseases dates back to ancient times, and medicinal plants are widely used in contemporary medicine. Medicinal plants contain active compounds and essential oils. Si et al. [18] studied 66 essential oils and found that nine demonstrated particularly high efficacy against S. typhimurium DT104, E. coli O157: H7, and E. coli with K88 pili and significantly inhibited E. coli and coliform bacteria in the digestive system, with little effect on beneficial lactobacilli and anaerobic bacteria.

The coastlines of Jeddah in Saudi Arabia are abundant resources of many varieties of seaweeds and plant materials used in traditional medicine. The aim of the present study was to screen the effect of heat and storage on the antibacterial activity of Ulva lactuca and Phoenix dactylifera against Staphylococcus aureus, Pseudomonas aeruginosa.

2. Materials and Methods

2.1. Study Bacteria

Bacteria were isolated and identified at King Abdulaziz University Hospital in Jeddah, Saudi Arabia as Staphylococcus aureus, Pseudomonas aeruginosa, plates were prepared by inoculating 100 μl −1 of each sample (1 × 10^5 colony-forming units) onto Mueller-Hinton agar (OXOID CM 337) was as follows (g/L): beef, 300.00; casein acid hydrolysate 17.50; starch 1.50; agar 17.00. The final pH was 7.3 ± 0.1 at 25°C.

2.2. Study Materials

The study materials were the green algae Ulva lactuca which was from Red Sea coastal areas around Jeddah, located between 21˚N and 39˚E, and the seeds of Nigella sativa and was collected from Jeddah markets. They were washed with distilled water several times, spread on plates and dried at 40°C. After drying, they were ground and solubilized in methanol at the concentration of 100 g 100 ml −1. The extracts were incubated on a 120 rpm shaker at 30°C for 24 h and then filtered by using Whatman No. 1, after then it had dried under the reduced pressure at 40°C, and the deposits were used as crude extracts [19].

2.3. Antibacterial Assays

Each crude extract was determined in vitro against the methicillin resistant S. aureus and P. aeruginosa. The activity of each extract was measured by the disc diffusion method following Clinical and Laboratory Standards Institute protocols [20]. Each extract was dissolved in dimethylsulfoxide (DMSO) with 3 μg·ml −1 and filtered by a 0.22 μm filter (Millipore, Billerica, MA). 100 μl of each solution were placed on 1 mm paper discs. Negative control was prepared with the solvent, and Augmentin XR (1 mg/ml) was used as the positive control. After inoculation, plates were incubated at 37°C for 24 h and the inhibition zones were measured. All tests were performed in triplicate.

The extracts that inhibited the bacterial growth were tested to determine the minimum inhibitory concentrations (MICs) using serially diluted with Mueller-Hinton broth in a 96-well microplate [21]. Bacteria were cultured overnight on Mueller-Hinton agar and then suspended in 1 ml −1 of Mueller-Hinton broth (OXOID CM 405) to give a final concentration of 5 × 10^5 colony-forming units ml −1. The microplate were inoculated with the bacteria and incubated at 37°C for 16 - 20 h, and were evaluated for the visible presence or absence of bacterial growth. MICs were determined as the lowest concentration of an extract for which there was no visible growth.
compared to the control [22].

The minimum bactericidal concentrations (MBCs) were determined by inoculating 0.1 ml of the negative growth wells in the MICs assays onto nutrient agar. Plates were incubated at 37°C for 24 h. MBCs were considered to be the concentration that showed no growth of the tested bacteria, negative control was a plate that contained only medium [23]-[25].

2.4. Kill-Time Determination

This experiment was assayed to estimate the rate of killing bacteria by the crude methanol extract. Using the method of [26], the extract was incorporated into 10 ml Mueller Hinton broth in McCartney bottles at 1/2 × MIC, 1 × MIC, and 2 × MIC. Two controls, a Mueller-Hinton broth without extract inoculated with test organisms and a Mueller-Hinton broth incorporated with the extract at the test concentrations without the test organisms, were included. Inoculums density, approximately 10^5 cfu/ml further verified by total viable count, was used to inoculate 10 ml volumes of both in the McCartney bottles and control bottles. The bottles were incubated at 37°C. A 100-μl aliquot was removed from the culture medium at 0, 2, 4, 8, 12, and 24 h to determine cfu/ml by using the Standard Plate Count technique [27]. After incubating at 37°C for 24 h, the visible colonies were read by using an Interscience scan of 500 colony counters. Bacterial colonies were counted as cfu/ml and compared with the control count [28] [29]. Each treatment was performed in triplicate.

2.5. Effect of Storage on U. lactuca and N. sativa Methanol Extracts

To investigate the effect of storage time and the type of storage material on antibacterial activity of U. lactuca and N. sativa, extracts were divided into two major groups. In the first group, the extracts were stored in sterilized dark bottles. In the second group, the extracts were stored in sterilized transparent bottles. Each group was further subdivided into three portions that were stored for 1, 2, and 3 months before laboratory analyses. The positive control was crude of U. lactuca and N. sativa at 4°C. Overnight nutrient broth cultures of S. aureus and P. aeruginosa. The cells were then suspended to the concentration of 5 × 10^5 colony-forming units ml^-1. By using holes in the agar, 100 μl of S. aureus or P. aeruginosa were inoculated in the plates, 100 μl of storage-treated extracts were added to the hole, and then the cultures were incubated at 30°C for 24 h. Three replicates of each treatment were done. Results were recorded by measuring the diameter of the inhibition zone [30].

2.6. Effect of Temperatures on U. lactuca and N. sativa Methanol Extracts

This study evaluates the effect of several temperature treatments on the antibacterial activity of the U. lactuca and N. sativa methanol extracts. Both U. lactuca and N. sativa were tested for their antibacterial activity after treated with −80°C, 5°C, 22°C, 50°C, and 90°C. Overnight nutrient broth cultures of S. aureus and P. aeruginosa were centrifuged, and the cells were washed three times with sterilized deionized water. The cells were then suspended in a concentration of 5 × 10^3 colony-forming units ml^-1. Using holes in the agar, 100 μl of S. aureus or P. aeruginosa were inoculated in the plates, 100 μl of temperature-treated extracts were added to the hole, and then were incubated at 30°C for 24 h. Three replicates of each treatment were done. Results were recorded by measuring the diameter of the inhibition zone [30].

2.7. Statistical Analysis

Results were analyzed by paired-samples t-test using the IBM SPSS 20 statistical software to compare the mean values of each treatment, and they are expressed as means ± SE. Probability levels of less than 0.01 were considered highly significant.

3. Results

In this study, the effect of the ethanol extracts of U. lactuca and N. sativa was estimated by the inhibition of the bacterial growth and the determination of MICs and MBCs. The MICs concentrations of the algae and plant extracts were then assayed to determine the ability of the extracts activity after storage and temperatures treatments. The results in Table 1 showed the effect of U. lactuca and N. sativa ethanol extracts on the growth inhibition with the concentrations of 30, 50, 100, 150 and 200 mg/ml, S. aureus was sensitive to all the concentrations and
the 100 μl were the best concentration with inhibition zone of 30 mm and 20 mm after the treated with the extract of *U. lactuca* and *N. sativa* respectively. Increasing the concentrations of the extracts to 150 mg/ml and 200 mg/ml resulted approximate effect with the concentration 100 mg/ml with inhibitions of 30 mm, 32 mm by the treated with *U. lactuca*, while the extract of *N. sativa* showed a different result in decreased of inhibition effect to 14 mm and 16 mm after treated with 150 mg/ml and 200 mg/ml respectively.

On the other hand, *P. aeruginosa* showed more resistance than *S. aureus*, the concentration 30 mg/ml has no inhibitory effect. While the inhibition at the concentrations of 50, 100, 150 and 200 mg/ml were approximate with the average of 12 mm and 15 mm after the treated with *U. lactuca* and *N. sativa* respectively.

The growth inhibition reflected on the MICs and MBCs and the results shows in Table 2. The extract of *N. sativa* was more effective on the tested bacteria with MIC and MBC of 2 μl, 8 μl and 4 μl, 8 μl for *S. aureus* and *P. aeruginosa* respectively.

Table 3 shows the antibacterial activity of 1/2, 1, and 2 MIC concentrations of *U. lactuca* and *N. sativa* extracts on bacterial vitality during incubation. The cells were counted after 0, 2, 4, 8, 12, and 24 h. The results of *P. aeruginosa* showed different cell decreases after incubation with *U. lactuca* and *N. sativa*, with percentage of cells vitalities up to 48.04%, 74.02%, 9.22%, and 66.75% after incubation with 1×MIC of *U. lactuca* and *N. sativa* extracts for 2 and 4 h, respectively. The 2 × MIC concentration decreased cell vitality to 44.32% and 72.78% after incubation with *U. lactuca* extract for 2 and 4 h. Incubation with *N. sativa* extract decreased cell vitality to 31.07% after 2 h. The number of vital cells of *S. aureus* decreased at the concentration of 1/2 × MIC to 2.10%, 31.36%, and 65.30%, respectively, after 2, 4, and 8 h of incubation with *U. lactuca* extract. The decreasing percentages of cells incubated with *N. sativa* were 1.89%, 21.79%, and 76.33%, respectively.

The results in Table 4 and Figure 1 show the effect of storage conditions; a transparent and dark storage bottle, on the activity of *U. lactuca* and *N. sativa* extracts against *S. aureus* and *P. aeruginosa*. Extracts stored in the transparent bottle decreased in effectiveness after one month of storage with percentage of 58.85%. After three months, the inhibition of the bacterial growth decreased to 38.90%. Further, after one month of storage, growth inhibition decreased after treated with *N. sativa* to 33.33%.

Extracts stored in the dark bottles performed better. *S. aureus* growth was inhibited to 53.70%, after stored for one month. While after three months, the growth inhibitor decreased to the percentage 46.30% by *U. lactuca* treatment.

As shown in Table 5, heating the extracts of *U. lactuca* and *N. sativa* to 90°C increased their antibacterial activity. *U. lactuca* and *N. sativa* extracts stored at −08°C decreased the growth of *S. aureus* and *P. aeruginosa.* *U. lactuca* and *N. sativa* extracts stored in transparent bottles for three months decreased the growth of *S. aureus*. Dark bottles reduced the percentage inhibitory of stored extracts for less than 10% in the *U. lactuca* extract and 20% in the *N. sativa* extract.

### 4. Discussion

The overuse of prescribed antibiotics can lead to resistance among these pathogenic bacteria. Thus, new antibiotics that are effective against new and resistant bacterial strains are needed. Plants and marine organisms may

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**Table 1. Inhibition of bacterial growth (mm) after 24 hours of treated with 100 μl *U. lactuca* and *N. sativa* methanol extracts (Mean ± SD).**

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>U. lactuca</th>
<th>N. sativa</th>
<th>Augmentin XR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 mg/ml</td>
<td>50 mg/ml</td>
<td>100 mg/ml</td>
</tr>
<tr>
<td>S. aureus</td>
<td>25 ± 1.527</td>
<td>28 ± 1.000</td>
<td>30 ± 1.155</td>
</tr>
<tr>
<td></td>
<td>30 ± 1.000</td>
<td></td>
<td>14 ± 2.517</td>
</tr>
<tr>
<td></td>
<td>14 ± 1.000</td>
<td></td>
<td>20 ± 1.000</td>
</tr>
<tr>
<td></td>
<td>20 ± 0.000</td>
<td></td>
<td>14 ± 1.732</td>
</tr>
<tr>
<td></td>
<td>16 ± 3000</td>
<td></td>
<td>12 ± 0.135</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>0</td>
<td>9 ± 1.527</td>
<td>12 ± 1.000</td>
</tr>
<tr>
<td></td>
<td>12 ± 1.000</td>
<td></td>
<td>13 ± 4.000</td>
</tr>
<tr>
<td></td>
<td>14 ± 1.000</td>
<td></td>
<td>15 ± 0.577</td>
</tr>
<tr>
<td></td>
<td>14 ± 2.000</td>
<td></td>
<td>15 ± 1.528</td>
</tr>
<tr>
<td></td>
<td>17 ± 0.017</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*P ≤ 0.01.

**Table 2. MIC and MBC (μl/m) of bacterial growth after incubation with *U. lactuca* and *N. sativa* methanol extracts.**

<table>
<thead>
<tr>
<th>MIC</th>
<th>U. lactuca</th>
<th>N. sativa</th>
<th>MBC</th>
<th>U. lactuca</th>
<th>N. sativa</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus</td>
<td>4</td>
<td>2</td>
<td>&gt;32</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>2</td>
<td>4</td>
<td>32</td>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>
Table 3. Kill-time determination of *S. aureus* and *P. aeruginosa* by cell vitality (cfu) after treatment with *U. lactuca* or *N. sativa* extracts for serial times and incubated for 24 h. Mean ± SD.

<table>
<thead>
<tr>
<th>Tested bacteria</th>
<th>Control</th>
<th>1/2 × MIC</th>
<th>1 × MIC</th>
<th>2 × MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 h</td>
<td>4 h</td>
<td>8 h</td>
<td>12 h</td>
</tr>
<tr>
<td><em>U. lactuca</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>3.89</td>
<td>3.81 ± 0.020**&lt;sup&gt;**&lt;/sup&gt;</td>
<td>2.67 ± 0.020**&lt;sup&gt;**&lt;/sup&gt;</td>
<td>1.35 ± 0.105**&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>4.85</td>
<td>2.54 ± 0.020**&lt;sup&gt;**&lt;/sup&gt;</td>
<td>2.44 ± 0.020**&lt;sup&gt;**&lt;/sup&gt;</td>
<td>1.30 ± 0.110**&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>N. sativa</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>5.28</td>
<td>5.18 ± 0.020**&lt;sup&gt;**&lt;/sup&gt;</td>
<td>4.12</td>
<td>1.25</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>4.12</td>
<td>3.89 ± 0.020**&lt;sup&gt;**&lt;/sup&gt;</td>
<td>2.72 ± 0.020**&lt;sup&gt;**&lt;/sup&gt;</td>
<td>1.37 ± 0.020**&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>**P < 0.01</sup>
Table 4. Inhibition of bacterial growth (mm) after 24 h of incubation with 100 μl of *U. lactuca* or *N. sativa* extract stored for one, two and three months in two types of storage bottles Mean ± SD.

<table>
<thead>
<tr>
<th>Storage periods</th>
<th>One month</th>
<th>Two months</th>
<th>Three months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of bottles</td>
<td>Transparent bottles</td>
<td>Dark bottles</td>
<td>Transparent bottles</td>
</tr>
<tr>
<td><em>U. lactuca</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. aureus</td>
<td>28 ± 0.096**</td>
<td>29 ± 0.096**</td>
<td>27 ± 0.096**</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>28 ± 0.096**</td>
<td>30 ± 0.096**</td>
<td>32 ± 0.096**</td>
</tr>
<tr>
<td><em>N. sativa</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. aureus</td>
<td>18 ± 0.115**</td>
<td>19 ± 0.096**</td>
<td>13 ± 0.096**</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>23 ± 0.096**</td>
<td>25 ± 0.096**</td>
<td>27 ± 0.096**</td>
</tr>
</tbody>
</table>

**P ≤ 0.01.

Figure 1. Bacterial inhibition growth after 24 h of incubation with *U. lactuca* or *N. sativa* extracts and stored for three months in transparent storage bottles.

*P. aeruginosa* treated with *U. lactuca* extract and stored for three month in transparent bottles

*S. aureus* treated with *U. lactuca* extract and stored for three month in transparent bottles

offer such novel treatments. To that end, extracts of the seaweed *U. lactuca*, and the plant *P. dectylefera* were tested against strains of Gram-positive cocci and Gram-negative bacilli *S. aureus*, and *P. aeruginosa*. Seaweed metabolism is influenced by extreme marine environmental conditions, including high pressure, temperature, and osmolarity, as found in our study sites, Red Sea coastal areas in Jeddah. It is also affected by the presence of
organic compounds, such as isonitrile, dichloramine, isocyanate, and halogenated compounds [31]. Bioactive compounds in antibacterial agents inhibit bacterial growth. These compounds include secondary metabolism components, such as alkaloids, peptides, terpenes, pigments, and sterols [32].

A study of antimicrobial activity of seaweeds *U. reticulata* and *U. lactuca* extracts showed promising antimicrobial activity against bacterial and fungal human pathogens [33]. In addition, *P. mirabilis* and *E. coli* were sensitive to the methanolic extract of *U. lactuca*, and *S. aureus* and *P. aeruginosa* were resistant to *U. lactuca* [34]. Selvin and Lipton [35] reported that the green alga *U. fasciata* exhibited broad spectrum antibacterial activity, these results agree with our results on *U. lactuca*. *U. fasciata* showed a broad spectrum of antibacterial activity, inhibiting the growth of both Gram-positive and Gram-negative tested organisms.

The effect of the bacterial inhibition growth reflected on the concentrations of both extracts’ MICs and MBCs. MICs and MBCs were detected from methanol extract of *Stachytarpheta indica*, *Ligaria cuneifolia* and *Jodina rhombifolia*, and the MICs and MBCs were comparable to the results in this study [36] [37].

Kill-time is the time it takes for cells to recover after incubation with an antimicrobial agent. Kill-time increases as concentrations of *U. lactuca* and *N. sativa* extracts increase. This is likely due to the increase in bioactive compounds that exist in the extracts.

The ideal antibiotic should remain stable during extended storage periods and temperature fluctuations. *U. lactuca* and *N. sativa* extracts maintained high antibacterial activity against *S. aureus* and *P. aeruginosa* after three months of storage at 90°C. This robustness may due to the concentrated and stability of its bioactive compounds. Other study results are consistent with ours [38]-[41]. Moreover, using dark bottles to store the extracts resulted in higher antibacterial activity, compared with the transparent bottles, this may due to biochemical light oxidation in the transparent bottles [42] [43].

### 5. Conclusion

The overuse of antibiotics can lead to resistance of pathogenic bacteria. A new antibiotic that is effective against new and resistant bacterial strains is needed. Medicinal plants and marine algae may offer such novel treatments. In this study, extracts of *U. lactuca*, and *N. sativa* were tested against *S. aureus*, and *P. aeruginosa*. The results of the bacterial inhibitor showed high activity in both extracts against *S. aureus* and *P. aeruginosa*. The MICs and MBCs were reflected with the growth inhibitor. Kill-time increases as concentrations of *U. lactuca* and *N. sativa* extracts increase. Moreover, extracts stored in the transparent bottle decreased in effectiveness after three months, and heating the extracts of *U. lactuca* and *N. sativa* to 90°C increased their antibacterial activity.

### References


