

Effects of Biochemical and Molecular Inhibitors of Plant Extracts on Pathogenic Bacteria

Ashwag Al-Zahrani¹, Effat Al-Judaibi², Hanan Omar^{2,3}, Awatif Al-Judaibi²

¹Department of Food Science, College of Sciences and Arts, Jeddah University, Al-Kamil, KSA

²Department of Biological Science, Science Faculty for Girls, King Abdulaziz University, Jeddah, KSA

³Department of Botany and Microbiology, Faculty of Science, Tanta University, Tanta, Egypt

Email: aamaljudaibi@kau.edu.sa

How to cite this paper: Al-Zahrani, A., Al-Judaibi, E., Omar, H. and Al-Judaibi, A. (2017) Effects of Biochemical and Molecular Inhibitors of Plant Extracts on Pathogenic Bacteria. *Journal of Biosciences and Medicines*, 5, 44-55.

<https://doi.org/10.4236/jbm.2017.55005>

Received: April 3, 2017

Accepted: May 23, 2017

Published: May 26, 2017

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Abstract

The study aimed to evaluate the effect of the green alga *Ulva lactuca* and medicinal plant *Nigella sativa* extract on the activity of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The bacteria were incubated with the crude extracts and extracellular free potassium and phosphorus ions were measured in the medium. The levels of potassium and phosphorous were the maximum in the medium of *S. aureus* treated with *N. sativa* extract. The medium of *P. aeruginosa* incubated with *U. lactuca* extract was found to have the lowest phosphorous and the greatest potassium levels. The highest activity against *P. aeruginosa* was noticed with *U. lactuca* extract, where it caused reduction in the dry weight and glucose consumption of bacteria estimated by 28.41% and 41.09%, respectively. The antibacterial activity of *N. sativa* extract was the greatest against *S. aureus* and recorded 32.59% and 39.96% reduction in the bacterial dry weight and glucose uptake, respectively. Scanning Electron Microscopy study showed morphological changes in the cell wall of treated bacteria. The treatment of bacteria with the tested extract induced gene mutations. The results assessed the possible application of *U. lactuca* and *N. sativa* as a source of pharmacological benefits.

Keywords

Natural Products, Antibacterial Activity, Biochemical Analysis, Molecular Analyses, Morphological Changes

1. Introduction

The use of antibiotics has been an effective treatment option for a variety of microbial infections. Antibiotic misuse or overuse has contributed to the creation of a new generation of antibiotic-resistant microorganisms. One important area

of study is testing the level of susceptibility and drug-resistance in microorganisms to specific antibiotics. The European Centre for Disease Prevention and Control (ECDC) and the Centers for Disease Control and Prevention (CDC) created terminology to define the various levels of the acquired antibiotic resistance profiles in microorganisms: multidrug resistant (MDR) was defined as “acquired non-susceptibility to at least one agent in three or more antibiotics”, extremely drug resistant (XDR) was defined as “non-susceptibility to at least one agent in all but two or fewer antimicrobial categories”, and pan drug resistant (PDR) was defined as “non-susceptibility to all agents in all antibiotics”. The study covered *Staphylococcus aureus*, *Enterococcus* spp., *Enterobacteriaceae*, *Pseudomonas aeruginosa* and *Acinetobacter* spp. [1]. Many bacteria strains were identified as MDR, XDR or PDR, including *P. aeruginosa*, *Klebsiella pneumoniae*, *S. aureus* and *Enterococcus* spp., *E. coli*, and *Acinetobacter* spp. [2] [3] [4].

The development of new antimicrobial agents required to address the new generation of drug-resistant microorganisms currently on the rise is vital. Microbiota found in malt and marine environments, shows great promise in being potential sources of antimicrobial active compounds.

Several medical research studies have shown some marine algae contain organic compounds that have a broad range of biological activities, including antibacterial, antifungal, antioxidant, antifouling, anti-inflammatory, cytotoxic, anticancer and antimutagenic properties [5] [6] [7] [8]. The macroalgae *Ulva lactuca* has been shown to exhibit antimicrobial activity against gram positive and gram negative bacteria such as *S. aureus*, *S. epidermidis*, *S. saprophyticus*, *Streptococcus agalactiae* (group B), *S. pyogenes*, *Enterococcus faecalis*, *Bacillus subtilis*, *Lactobacillus acidophilus*, *P. aeruginosa*, *E. coli*, *Enterobacter aerogenes*, *Stenotrophomonas maltophilia*, *Salmonella typhimurium*, *Shigella sonnei*, *Proteus vulgaris* and *P. mirabilis* [9] [10] [11].

Many soil-grown plants, called medicinal plants, have been shown to express bioactive compounds that can act as antimicrobial agents. These secondary metabolites produced by medicinal are an important source for pharmaceutical drugs. In fact, bioactive products derived from medicinal plants are a principal source of pharmaceutical agents used in traditional medicine and work by acting as antimicrobial agents against pathogenic microorganisms [12] [13] [14].

Several plants have been defined as medicinal and have been used for years to treat infectious diseases, such as *Thymol* sp., *Ocimum* sp., *Oregano* sp. and *Nigella* sp. [15] [16] [17]. Seed extracts and essential oils of *Nigella sativa* have been used to treat a variety of diseases and are shown to have various pharmacological properties including antimicrobial actions [18] [19]. *N. sativa* has an inhibitory effect on MRSA, *S. aureus*, *S. epidermidis*, *E. coli*, *S. typhi*, *S. enteritidis*, *Klebsiella* sp. and *Enterobacter aerogenes* [20] [21].

The present investigation aims to study the influence of methanol extract of green alga *Ulva lactuca* and medicinal plant *Nigella sativa* on the activity and molecular genetics of pathogenic Gram positive cocci *Staphylococcus aureus* and the Gram negative bacilli *Pseudomonas aeruginosa*.

2. Materials and Methods

2.1. Extract Preparation

The green alga *Ulva lactuca* and the seeds of medicinal plant *Nigella sativa* were collected from Jeddah, Saudi Arabia. The samples were cleaned, washed with distilled water, dried at 40°C and powdered in a mixer grinder. The powdered samples were extracted by soaking in methanol (1:10, w/v) for several times at room temperature. The deposits were then used as crude extracts. The extracts were dried under vacuum pressure at 40°C [22] [23]. The crude extract was stored at -20°C until required.

2.2. Test Bacteria

The tested bacteria *Staphylococcus aureus* and *Pseudomonas aeruginosa* were isolated and identified at King Abdulaziz University Hospital, Jeddah, Saudi Arabia. The isolates were grown on Mueller-Hinton agar (OXOID CM 337) [24] [25] [26].

2.3. Biochemical Analyses

The pathogenic bacteria (1×10^5 CFU·ml⁻¹) were incubated with 100 µl crude extracts (100 mg·ml⁻¹) at different intervals (20, 40, 60 and 80 mins) in Mueller-Hinton broth at 37°C [27]. The extracellular potassium and phosphorus concentrations were estimated by a photometric procedure using EasyRA Medica and COBAS® INTEGRA 400 plus, respectively. The results were expressed as the value of extracellular free potassium and phosphorus ions in the medium (mmol·L⁻¹).

The effect of different concentrations (50 and 100 µl) of crude extract (100 mg·ml⁻¹) on glucose uptake and dry weight of bacteria was investigated according to [28]. The pathogenic bacteria (1×10^5 CFU·ml⁻¹) were inoculated into Mueller-Hinton broth and incubated with the different concentrations of extract on a shaker (180 rpm) at 37°C for 24 h. Samples were then centrifuged at 10,000 rpm for 10 mins. Glucose uptake was measured in the suspension solution by using COBAS® INTEGRA 400 plus. The pellets were washed triple with distilled water and centrifuged at 10,000 rpm for 10 mins and then dried at 80°C. Bacterial growth was measured as dry weight. Each treatment was performed in triplicate.

2.4. Molecular Analyses

The effect of *U. lactuca* and *N. sativa* extract on the genetic material of pathogenic bacteria was studied as recommended by [29]. The bacterial DNA was extracted with methanol using Qiagen DNA extraction kit (Molecular sequencing of the *mecA* gene in *S. aureus* and *acsA* gene in *P. aeruginosa*) and the following PCR primers:

S. aureus

MR1 TAGAAATGACTGAACGTCCG;

MR2 TTGCGATCAAATGTTACCGTAG;
 MR3 AAAATCGATGGTAAAGGTTGGC;
 MR4 AGTTCTGCAGTACCGGATTTTGC.

P. aeruginosa

acsA-F GCCACACCTACATCGTCTAT;
 acsA-R AGGTTGCCGAGGTTGTCCAC.

The DNA was sequenced by MacroGene (<https://www.macrogenusa.com/>) and analyzed by BLAST software (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

2.5. Scanning Electron Microscopy (SEM) Analyses

A thin film of treated bacterial cells with 50 μl of *U. lactuca* and *N. sativa* extracts (100 $\text{mg}\cdot\text{ml}^{-1}$) were smeared on a silver stub for analysis by SEM [30] [31]. The samples were coated with gold by cathodic spraying (Polaron gold) and then dried under a mercury lamp for 5 mins. The morphology of *S. aureus* and *P. aeruginosa* were observed by using scanning electron microscope at Nano Center of KAU (JEOL, JSM-7600F 450).

2.6. Statistical Analysis

The results were analyzed by paired-samples *t*-test using the IBM SPSS 20 statistical software to compare the mean values of each treatment. The results are expressed as the means \pm SD. Probability levels of less than 0.01 were considered highly significant.

3. Results

3.1. Biochemical Analyses

The treatment of *S. aureus* and *P. aeruginosa* with 100 μl *U. lactuca* and *N. sativa* crude extract (100 $\text{mg}\cdot\text{ml}^{-1}$) showed an increase in the leakage of potassium and phosphorus ions with increasing time of incubation (20, 40, 60 and 80 minutes). The levels of potassium 68.54% and phosphorus 38.24% recorded highly significant increase ($P < 0.01$) in the bacterial medium of *S. aureus* treated with *N. sativa* extract (Figure 1). At the same time, the treatment of *P. aeruginosa* with *U. lactuca* extract (Figure 2) induced the presence of maximum level of potassium 36.05% and the minimum value of phosphorous 13.10% as compared with *N. sativa* extract 27.29% and 18.97%, respectively. *S. aureus* was the most sensitive to the extract of *N. sativa* with respect to the decrease in the ability to regulate cell permeability, whereas *U. lactuca* extract was more effective against *P. aeruginosa*.

Glucose uptake is reflected in the bacterial biomass that determined as dry weight in the untreated cells and directly contrasted with the biomass growth weight analyzed during the incubation period with *U. lactuca* and *N. sativa* extracts. The influence of 50 and 100 μl tested extract on the bacterial metabolism expressed as glucose uptake is shown in Table 1. As the concentration of *U. lactuca* and *N. sativa* treated extracts increased, glucose uptake was found to be decreased with *S. aureus* 53.00% and 39.96%, respectively and *P. aeruginosa*

41.09% and 44.74%, respectively. Therefore, a decrease in glucose uptake was reflected in the less dry weight of treated bacteria with tested extracts. The results in Table 2 clarified that the lowest dry weight of *S. aureus* 32.59% was observed when treated with *N. sativa* extract. However, *P. aeruginosa* showed the minimum dry weight 28.41% during the incubation with *U. lactuca* extract.

The results of an SEM study showed the changes in the bacterial morphology and structure in response to the methanol extracts of *U. lactuca* (Figure 3) and *N. sativa* (Figure 4). The treatment of bacteria with the extract of *U. lactuca* and *N. sativa* resulted in the formation of cavities in cells as well as shrinkage, aggregation, rupture, and partial deformation of the cell wall.

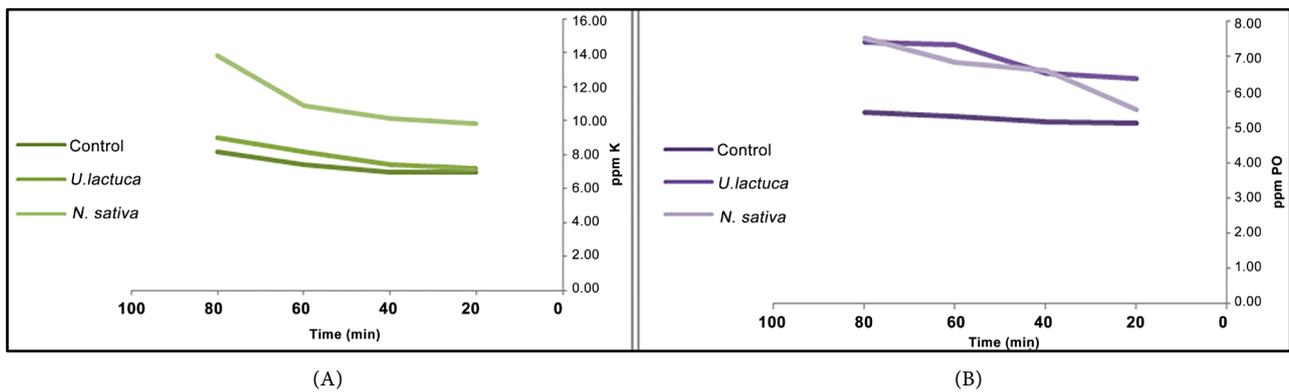


Figure 1. Potassium (A) and phosphorus (B) leakage of *S. aureus* treated with 100 µl of *U. lactuca* and *N. sativa* extract after 20, 40, 60 and 80 minutes of incubation.

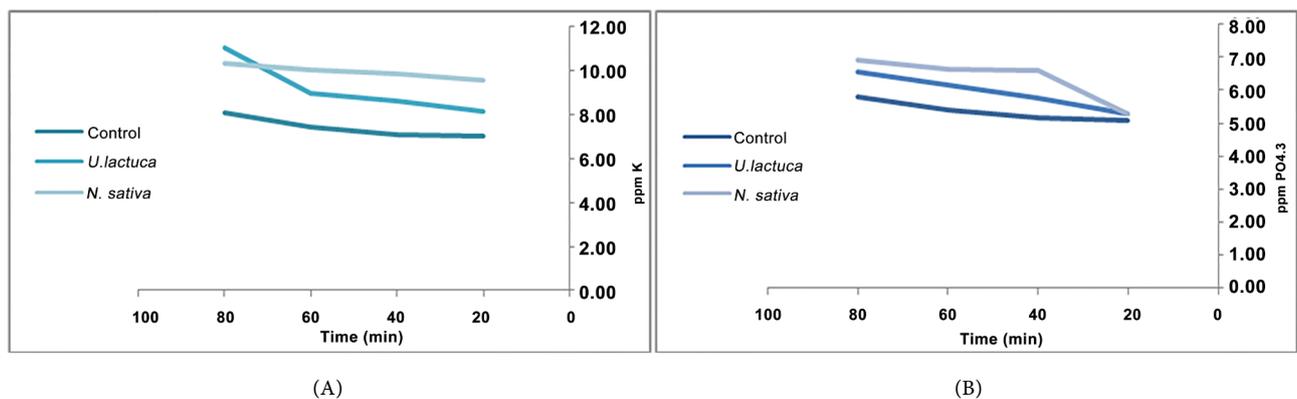


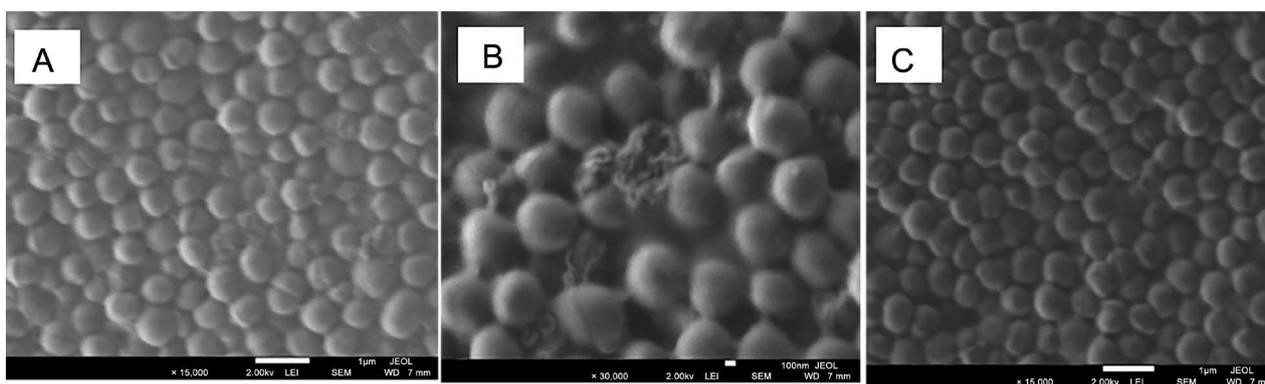
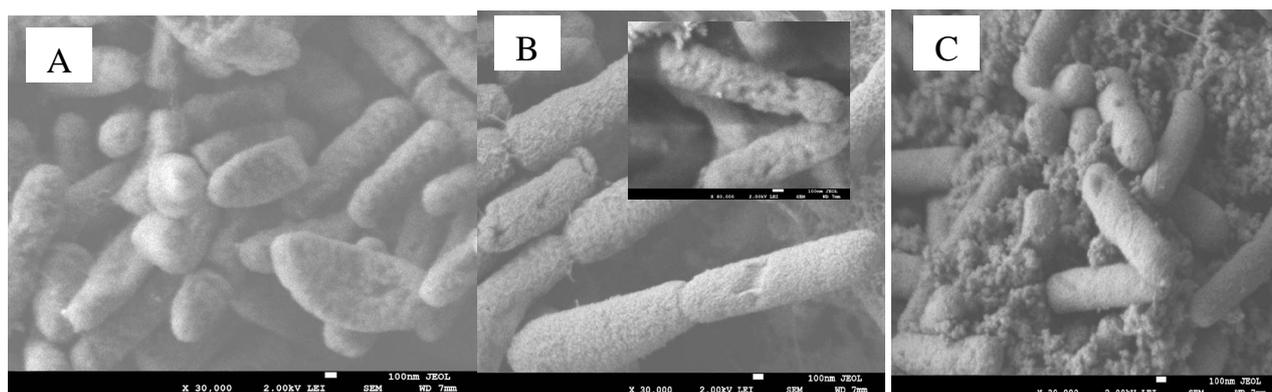
Figure 2. Potassium (A) and phosphorus (B) leakage of *P. aeruginosa* treated with 100 µl of *U. lactuca* and *N. sativa* extract after 20, 40, 60 and 80 minutes of incubation.

Table 1. Glucose uptake ((mg DL-1) of *S. aureus* and *P. aeruginosa* treated with 50 and 100 µl *U. lactuca* and *N. sativa* extract after 24 hours of incubation (Mean ± SD).

Bacteria	Control untreated cells'	<i>U. lactuca</i>		<i>N. sativa</i>	
		50 µl	100 µl	50 µl	100 µl
<i>S. aureus</i>	4.83 ± 0.011	3.57 ± 0.006**	2.27 ± 0.021**	4.32 ± 0.010**	2.90 ± 0.006**
<i>P. aeruginosa</i>	4.94 ± 0.016	3.97 ± 0.006**	2.91 ± 0.045**	4.00 ± 0.285*	2.73 ± 0.006**

Table 2. Dry weight (mg) of *S. aureus* and *P. aeruginosa* treated with 50 and 100 μ l *U. lactuca* and *N. sativa* extract after 24 hours of incubation (Mean \pm SD).

Bacteria	Control untreated cells ^a	<i>U. lactuca</i>		<i>N. sativa</i>	
		50 μ l	100 μ l	50 μ l	100 μ l
<i>S. aureus</i>	31.30 \pm 1.54	26.30 \pm 0.46**	19.50 \pm 0.46**	22.50 \pm 1.00**	21.10 \pm 0.63**
<i>P. aeruginosa</i>	35.90 \pm 1.79	26.80 \pm 0.55**	25.70 \pm 0.05**	14.70 \pm 0.05**	14.60 \pm 0.41**

**Figure 3.** SEM showed the effect of *U. lactuca* (A) and *N. sativa* (B) extracts on the cell wall of *S. aureus*, compared to control (C).**Figure 4.** SEM showed the effect of *U. lactuca* (A) and *N. sativa* (B) extracts on the cell wall of *P. aeruginosa*, compared to control (C).

3.2. Molecular Analyses

The molecular investigation of the *S. aureus* antibiotics resistant gene *mecA*, and *P. aeruginosa* Acetyl coenzyme A synthetase gene *acsA*, are found in **Figure 5** and **Figure 6**. Bacteria treated with *U. lactuca* and *N. sativa* extract showed changes in the gene sequence of the selected microorganism, compared with the untreated sample.

As shown in **Figure 5** for *S. aureus*, five mismatches and two gaps were observed after treatment with *N. sativa*, and two mismatches and two gaps were noticed after treatment with *U. lactuca*. The primer used in this sequence was Mr3. Two gaps occurred in the treatment with the extract of *N. sativa* in the bases 26 and 432, whereas no gaps occurred in the gene after the treatment with *U. lactuca* extract for Mr4, there were 4 mismatches after treatment with *N. sa-*


```

Query 70952  GCGCCCACGCGATGAAGCCGGGCTCTGCAGCCAAGCCGTTCTTCGGCGTGGTACCGGCGC 97101
              |||
Sbjct 21     GCGCCCACGCGATGAAGCCGGGCTCTGCAGCCAAGCCGTTCTTCGGCGTGGTACCGGCAC 380

```

F. P. aeruginosa treated with *N. sativa*

```

Query 970604  GCCACAC-CTACATCGTCTATGGCCCGTTGGCCAACGGCGCCACCACCATTCTGTTTCGAG 97066
              |||
Sbjct 404     GCCACACNCTA-ATCGTCTATGGCCCGTTGGCCAACGGCGCCACCACCATTCTGTTTCGAG 346

```

R. P. aeruginosa treated with *N. sativa*

```

Query 970952  GCGCCCACGCGATGAAGCCGGGCTCTGCAGCCAAGCCGTTCTTCGGCGTGGTACCGGCGC 971011
              |||
321         GCGCCCACGCGATGAAGCCGGGCTCTGCAGCCAAGCCGTTCTTCGGCGTGGTACCGGCAC 380

Sbjct 971012  TGGTGGACAACCTCGGCAACCT 971033
              |||
381         TGGTGGACAACCTNGGCAACCT 402

```

F. P. aeruginosa treated with *U. lactuca*

```

Query 970604  GCCACAC -CTACATCGTCTATGGCCCGTTGGCCAACGGCGCCACCACCATTCTGTTTCGAG 970662
              |||
Sbjct 405     GCCACACTCTNNATCGTCTATGGCCCGTTGGCCAACGGCGCCACCACCATTCTGTTTCGAG 346

```

R. P. aeruginosa treated with *U. lactuca*

Figure 6. *P. aeruginosa* F and R in *ascA* gene detection after treatment with 100 µl of *U. lactuca* and *N. sativa* extracts.

Figure 6 showed the results of *P. aeruginosa* gene *acsA* sequence, which included one mismatch in the base 379 for the primer *acsA* F after treatment with *N. sativa* extract and 2 gaps in the bases 379 and 393 for the primer *acsA* R, compared with the untreated cells. The results show two mismatches in the bases 394 and 379 and two unread bases for bases 394 and 395 for the primer *acsA* F and one gap in the base 398 for the primer *acsA* R after treatment with the extract of *U. lactuca*.

4. Discussion

Antimicrobial agents can target several parts and processes of a microbe cell, including the cell wall, the cytoplasmic membrane, protein and enzyme production, DNA replication and the DNA genetic code [30] [31]. These alterations are caused by active compounds in the extracts of these medicinal plants, which have been shown to contain bioactive components that act as highly effective antimicrobial agents against microbial infections such as quinine, tannins, terpenoids, sterols, alkaloids and flavonoids [32] [33] [34].

Several studies that analyzed extracts of medicinal plants such as *Rhamnus globosa*, *Ocimum basilicum*, *Tecoma stans*, *Coleus forskohlii*, *Phoenix dactylifera*, *N. sativa*, *Elettaria cardamomum*, *Lawsonia inermis*, *Embelia ribes* and *Santalum album* have shown that they contain active compounds that can di-

rectly inhibit the growth activity of Gram negative and positive bacteria including *Bacillus subtilis*, *B. cereus*, *S. aureus*, *S. aureus* MRSA *Corynebacterium bovis*, *Pseudomonas aeruginosa*, *Pasteurella multocida* and *E. coli* [35] [36] [37].

It has been shown that *N. sativa* L. has several pharmacological effects that have been attributed to the active components in the seed extracts including thymoquinone, thymohydroquinone, dithymoquinone, thymol, carvacrol, nigellimine, nigellimine-x-oxide, nigellidine and alpha-hederin [38] [39]. Furthermore, the GC-MS analysis of *U. lactuca* extracts reveal the existence of phytochemical products such as phytol, hexadecanoic acid, ethyl ester and (E)-9-octadecenoic acid ethyl ester, thymoquinone, α -thujene, thymohydroquinone, p-cymene, dehydro-sabina ketone, carvacrol and longifolene [40]. The antimicrobial activity of *U. lactuca* has been verified by the studies with several pathogenic microorganisms including *K. pneumonia*, *E. coli*, *E. aerogens*, *P. aeruginosa*, *M. luteus*, *E. faecalis*, *S. aureus*, *S. aureus*, MRSA, *S. faecalis* and *B. subtilis* [41] [42] [43]. Moreover, the increase in potassium and phosphorus leakage returned to the effect of the extract on the permeability of the cytoplasmic membrane, as verified by SEM. In addition, the rate of gene mutation was shown to be higher in tested bacteria treated with *U. lactuca* and *N. sativa* extract [5] [27] [44] [45] [46].

5. Conclusion

The present study concluded that the extract of the green alga, *U. lactuca* and the seeds of medicinal plant, *N. sativa* can be used as a source for antibacterial agent. Further works should be done for isolation and characterization of the active compounds.

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