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Table Of Contents

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Vol. 10 No. 2 (2025): December
DOI: 10.21070/acopen.10.2025.13088

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Antibacterial Efficacy of Myrtus communis Nanoemulsion Against Multidrug-Resistant, ESBL-Producing Pseudomonas aeruginosa and Its Association with β-Lactam Resistance Profiles

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Abstract

General Background: Pseudomonas aeruginosa is a major opportunistic pathogen responsible for severe hospital-acquired infections and is increasingly associated with multidrug resistance mediated by extended-spectrum and metallo-β-lactamases. **Specific Background:** The rapid dissemination of ESBL- and MBL-producing P. aeruginosa has compromised the efficacy of third-generation cephalosporins and carbapenems, creating an urgent need for alternative antimicrobial strategies. **Knowledge Gap:** Evidence remains limited regarding the effectiveness of phytochemical nanoemulsions against genetically characterized MDR P. aeruginosa isolates. **Aims:** This study evaluated the antibacterial activity of a Myrtus communis essential oil nanoemulsion against clinical ESBL-producing P. aeruginosa and examined its association with β-lactam resistance profiles. **Results:** Among 350 clinical samples, 40 P. aeruginosa isolates showed high resistance to cephalosporins and carbapenems, with prevalent VIM, IMP, CTX-M, and SHV genes. The formulated nanoemulsion exhibited a mean droplet size of 96.15 nm and demonstrated strong antibacterial activity, with a minimum inhibitory concentration of 0.07 mg/µL and a minimum bactericidal concentration of 0.2 mg/µL, effectively inhibiting most MDR isolates. **Novelty:** This study integrates molecular resistance profiling with nanoemulsion-based phytotherapy against MDR P. aeruginosa. **Implications:** Myrtle nanoemulsion represents a promising adjunct or alternative antimicrobial approach for managing life-threatening MDR infections, particularly in settings with limited therapeutic options.

Highlight :

- ESBL and MBL genes were highly prevalent in multidrug-resistant P. aeruginosa.
- Myrtus communis nanoemulsion showed stable nanoscale properties.
- Low MIC and MBC values confirmed strong antibacterial activity...

Keywords : Pseudomonas aeruginosa, Myrtus communis nanoemulsion, Antibiotic resistance, ESBL and MBL genes, Antibacterial activity

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Introduction

The Pseudomonas genus encompasses over 220 species, the majority of which are saprophytic, while more than 25 species are recognized as opportunistic pathogens(1) .Pseudomonas species are broadly divided into two groups: fluorescent (e.g., P. aeruginosa, P. fluorescens, and P. putida) and non-fluorescent (e.g., P. stutzeri and P. mendocina) (2). P. aeruginosa has emerged as a significant Gram-negative pathogen, particularly among immunocompromised individuals. It is frequently isolated from patients hospitalized for more than one week and is a common cause of nosocomial infections (3). Infections caused by P. aeruginosa can be severe and life-threatening, affecting multiple organ systems. Clinical manifestations include respiratory tract infections (e.g., pneumonia), bloodstream infections (bacteremia), endocarditis, central nervous system infections (e.g., meningitis, brain abscess), ear infections (otitis externa and media), ocular infections (e.g., bacterial keratitis, endophthalmitis), osteomyelitis, gastrointestinal disorders (e.g., diarrhea, enteritis, enterocolitis), urinary tract infections, and characteristic skin lesions such as ecthyma gangrenosum (4). P. aeruginosa is classified among the ESKAPE pathogens (Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, P. aeruginosa, and Enterobacter spp.), which are commonly associated with multidrug-resistant, life-threatening hospital-acquired infections in critically ill and immunocompromised patients (5). The emergence of P. aeruginosa strains producing extended-spectrum β-lactamases (ESBLs) represents a significant public health concern. Clinical isolates frequently exhibit resistance to multiple antimicrobial classes, including β-lactams, aminoglycosides, and fluoroquinolones. A worrying global trend involves the increasing prevalence of strains resistant to third- and fourth-generation cephalosporins and carbapenems (6). β-lactamases can break down these medicines. They are found in penicillins, oxyimino-cephalosporins (third and fourth generation), and aztreonam. There is no way for β-lactamase to do its job when clavulanic acid, sulbactam, or tazobactam are present. They also often make these medicines not work. It's getting easier for P. aeruginosa to spread, and many drugs can't kill it. To stop this, we will need strong infection control, close monitoring, and antibiotic care. That's why we need to find new drugs and other ways to heal people quickly. Too few new drugs are being made to keep up with the rise in drug tolerance. These days, microparticles (NPs) are a good pick. Nodes, or NPs, are very little things that are just one to one hundred nanometers across. Because of how they are made and how chemicals respond with them, they kill germs very well. They hurt the walls of bacteria, stop biofilm from forming, mess up cell signals, and lead to oxidative stress, among other things. Furthermore, NPs can be functionalized to selectively target specific bacterial strains, thereby enhancing their antimicrobial precision and efficacy(9). Essential oils also offer natural antimicrobial potential, though their hydrophobicity, volatility, and strong organoleptic properties limit their practical application(10). Encapsulation in oil-in-water emulsion systems particularly nanoemulsions with nanoscale droplet sizes can overcome these limitations by improving solubility, stability, and bioavailability(11) . Due to their high surface area-to-volume ratio, nanoemulsions enhance the antimicrobial activity of essential oils compared to conventional emulsions (12). Accordingly, the specific objective of the present study was to evaluate the antibacterial activity of myrtle (Myrtus communis) essential oil formulated as a nanoemulsion against clinical isolates of ESBL-producing P. aeruginosa

Materials and Methods

A total of 350 clinical samples were collected from patients who referred to the medical and emergency centers of Tehran hospitals. Sample were cultured culture medium, and then biochemical tests were done to diagnosis of bacteria. Diagnostic tests were: Oxidase, Catalase, Cultivation environment TSI Triple Sugar Iron Agar, Indole production, Methyl red MR, VP, Citrate consumption, SIM (Sulfide Indole Motility Medium) and urease tests.

Determination of antibiotic Susceptibility Patterns

Antimicrobial susceptibility testing was performed using the Kirby–Bauer disc diffusion method (13) on Mueller–Hinton agar (Merck, Germany), in accordance with the guidelines established by the Clinical and Laboratory Standards Institute (CLSI, 2023). A pure culture of each *P. aeruginosa* isolate, recovered from clinical specimens, was used to assess susceptibility to a panel of antimicrobial agents, including amikacin, imipenem, meropenem, cefepime, piperacillin, ciprofloxacin, gentamicin, ceftazidime, tobramycin, ceftriaxone, and cefotaxime. Bug samples were changed to a 0.5 McFarland standard so that everyone could use them and take the same tests. The new CLSI breakpoints made it clear that the results were either weak, average, or strong.

Molecular Identification by PCR

Karmania Pars Gene (Iran) made the DNA extraction kit. It was possible to get genetic DNA from different kinds of bacteria by following their steps. Different types of P. aeruginosa have genes that are only found in those types. These genes helped us figure out what kind of bacteria it was. You can see the primer sequences, the genes they work on, and the temperature cycle conditions in Table 1 (14, 15). Amplified PCR products were separated by electrophoresis on a 1% (w/v) agarose gel containing DNA-safe stain (a non-toxic alternative to ethidium bromide). A DNA molecular weight ladder was included in each run to verify the expected amplicon sizes. Gels were visualized under ultraviolet (UV) transillumination, and the presence of bands at the anticipated molecular weights was used to confirm successful amplification and isolate identity

Table 1. Primer sequences, thermal cycling conditions, and expected amplicon sizes for detection of ESBL-encoding genes in ***P. aeruginosa***.

Gene	Thermal Cycling Conditions	Primer sequences (5'→3')	Product Size (bp)
TEM	95°C / 5 min; (95°C / 1 min, 56°C /30 sec, 72°C / 45 sec) ×30; 72°C /5 min	F: 5' ATGAGTATTCAACATTTCCG 3' R: 5' GACAGTTACCAATGCTTAATCA 3'	402
SHV	95°C / 5 min; (95°C / 1 min, 58°C /45 sec, 72°C / 45 sec) ×35; 72°C /5 min	F: CCTGACCACCATCGTCGCCACAAC R: CAGTTCCGTTTCCCCAGCGGT	471
CTX	95°C / 5 min; (95°C / 1 min, 57°C / 1 min, 72°C / 45 sec) ×30; 72°C /5 min	F: CACACGAATTGAATGTTTCAG R: TCATCCACATGGTGAGT	540
VIM	95°C / 5 min; (95°C / 1 min, 59°C / 45 sec, 72°C / 1 min) ×30; 72°C /5 min	F: 5' AGTGGTGAGTATCCGACAG 3' R: 5' ATGAAAGTGCGTGGAGAC 3'	390
IMP	95°C / 7 min; (95°C / 1 min, 55°C /1	F: 5' ACCGCAGCAGAGTCTTTGCC 3'	587

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	min, 72°C / 50 sec) ×35; 72°C /10 min	R: 5' ACAACCAGTTTTGCCTTACC3'	

Biosynthesis of Myrtle Nanoemulsion

The nanoemulsion was prepared using a two-step emulsification process(16). The aqueous phase was prepared by dissolving Tween 20 (polyoxyethylene sorbitan monolaurate) in Milli-Q ultrapure water at a concentration of 3% (w/w) under ambient temperature (~25 °C) with continuous stirring for 20 min. The oil phase consisted of a mixture of 25% soybean oil and 75% *Myrtus communis* L. (myrtle) extract (w/w). The final oil-to-water ratio was set at 10% (w/w). A coarse pre-emulsion was first generated by homogenizing the oil and aqueous phases using a rotor–stator homogenizer (Ultra-Turrax T25, IKA, Germany) at 10,000 rpm for 5 min. This pre-emulsion was subsequently processed through a high-pressure homogenizer (Nano DeBee, BEE International, USA) at 100 MPa for five cycles to achieve a stable nanoemulsion with reduced droplet size. The droplet size distribution and polydispersity index (PDI) of the resulting nanoemulsion were determined by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments, UK). All formulations were stored in sealed glass vials at 4 °C until further use.

Characterization of Biosynthesized Nanoparticles

The morphology and surface topography of the biosynthesized nanoparticles were examined using scanning electron microscopy (SEM; Hitachi SU3500, Japan). To make them, drops of nanoparticle solutions were put on clean glass slides. After that, they were left outside to dry. On top of it was a gold-palladium metal layer that was 10–15 nm thick. They became more electrical as a result, and charging stopped going wrong. A 15 kV voltage and a zoom range of 5,000x to 50,000x were used to scan it. For the backscatter study (173°), a Zetasizer Nano ZS from Malvern Instruments in the UK was utilized. It was 25°C hot. There were many paths of light (DLS). The PDI and the group of particle sizes could be found. Three of each number were made. The functional groups that help make nanoparticles and keep them steady were found using FTIR (PerkinElmer Spectrum Two, USA). It was then put in the freezer and ground into a very fine powder. After that, they were looked at as KBr pellets in the 400–4000 cm^{-3} range, with a 4 cm^{-3} accuracy.

Antibacterial Activity of Myrtle Nanoemulsion Against ***P. aeruginosa*** Isolates

They did a test to see if a nanoemulsion of myrtle (Myrtus communis L.) could get rid of P. aeruginosa samples from patients. For 15 hours, one cell of each strain grew in 5 mL of Luria–Bertani (LB) broth at 37°C and 180 rpm. In this way, a society was made that would last right away. Then, ten times as much clear, clean water was added to make a standard inoculum with 10^7 CFU/mL. First, 100µL of the bacterial solution was mixed with either 900µL of clean water or myrtle nanoemulsion. To be sure, this was done. They didn't stop until the number got to 0.03, 0.05, 0.07, 0.1, 0.2, and 0.3 mg/µL. For 24 hours, the hot plates didn't move, and they stayed at 37°C. Before they were used, ten times as much clean water was put into the tanks. After that, two 100-µL plates with the right amount of bacteria were put on top of the Mueller–Hinton agar. The plates were checked to see how many CFU/mL of bacteria were still on them after 24 hours at 37°C. The number of CFU/mL went down because the medicine worked better than the poison.

Results

In about 11.42 percent of the 350 clinical cases of P. aeruginosa, this was found by itself. Twelve species were found in pee, and nine (75.0%) were P. aeruginosa. Most of the time, it took place here. In the same way, P. aeruginosa was found in 16 of the 21 samples from burn wounds, which is 76.19%. It was only found in two of seven (28.5%) samples from wound infections in general, though. A lot of people have found P. aeruginosa in their burns, pee, and wound infections, but at least one drug wasn't able to get rid of them all.

Antibiotic Susceptibility Profile

Scientists used the disk diffusion method to test 40 types of P. aeruginosa to see how well drugs worked against them. A lot of them didn't get better with more than one drug (Figure 1). The best protection was seen with ipenem (50%), then meropenem (45%), and finally amikacin (60%). That is, aminoglycosides and carbapenems may still be useful in some medical situations. Piperacillin (40%) and ciprofime (42.5%) both worked most of the time. It was okay to use fluoroquinolones and some β-lactams, though. Gentamicin and tobramycin both worked on 30% of the samples, while ciprofloxacin only worked on 22.5% of them. Third-generation cephalosporins had very high rates of resistance: 87.5% for ceftazidime (10%), 97.5% for cefotaxime (2.5% resistance), and 72.5% for ceftriaxone (27.5% resistance). It is clear from these data that most types of P. aeruginosa are not sensitive to many drugs. Long-spectrum cephalosporins and fluoroquinolones have a hard time killing them because of this. Resistance rates between 40% and 50% in these classes show that there is a new threat. Most of the time, amikacin and carbapenems work better. Antibiotics don't work as well against P. aeruginosa attacks because of this. Antibiotics need to be taken care of, resistance needs to be watched for, and new drugs like nanoemulsion-based mixtures need to be made.

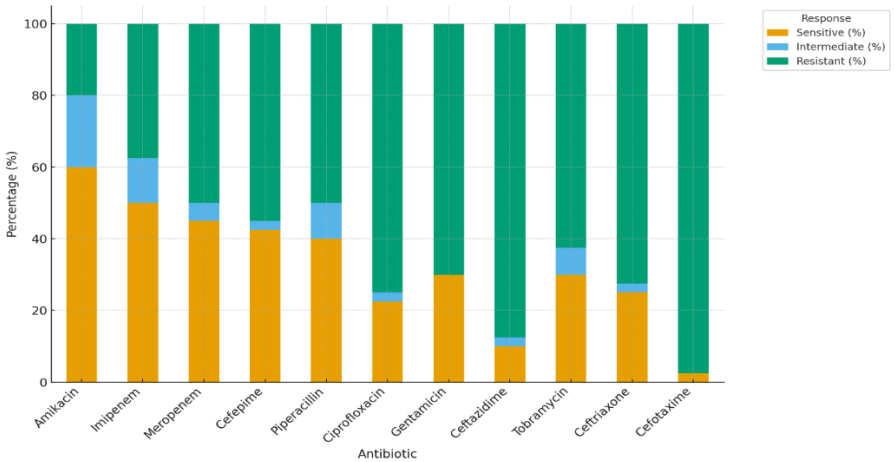

Figure 1: Antibiotic resistance pattern of *P. aeruginosa* isolates

Academia Open
Vol. 10 No. 2 (2025): December
DOI: 10.21070/acopen.10.2025.13088

With PCR, we looked at 40 kinds of P. aeruginosa. These strains had many genes that coded for β-lactamase, but the ones that coded for metallo-β-lactamase (MBL) were the most common (Figures 2, 3, 4, and 5). 55% of the isolates, or 22, had the VIM gene. Six of the isolates, or 15%, had SHV. These are enzymes, such as TEM, SHV, and CTX-M. It was found that some of the strains have genes that make them. These genes help germs stay safe from ceftazidime and penicillins, which was proven. Remember that MBL genes (VIM and IMP) were found a lot of the time. Eight of the samples, or about 20%, had IMP in them. In other words, these kinds can break down carbapenems. Most of the time, carbapenems are only used to treat P. aeruginosa when nothing else has worked. Also, 14 isolates (35.0%) had more than one resistance gene present at the same time, like VIM + SHV or IMP + CTX-M. This lets us see genes that are tough to change. ESBL and MBL are made by P. aeruginosa. Researchers from all over the world say that it is growing in hospitals, which are places where drugs are used all the time. These genes are also taken up and stay there. There was a high level of safety, as shown by behavior reaction studies. It turned out that a group of genes was to blame. These "superbugs" are very dangerous because many drugs can't kill them.

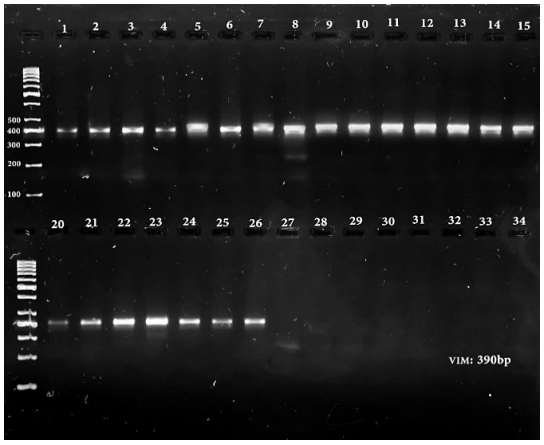

Figure 2: Electrophoresis of the PCR product for the *VIM* gene on a 1% agarose gel (The observed bands at the expected size (390 base pairs) confirm the presence of the *VIM* gene in *P. aeruginosa* isolates).

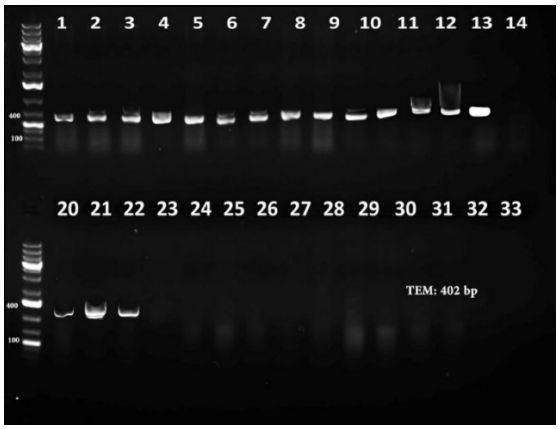

Figure 3: Electrophoresis of the PCR product for the *TEM* gene on a 1% agarose gel (The observed bands at the expected size (402 base pairs) confirm the presence of the *TEM* gene in *P. aeruginosa* isolates).

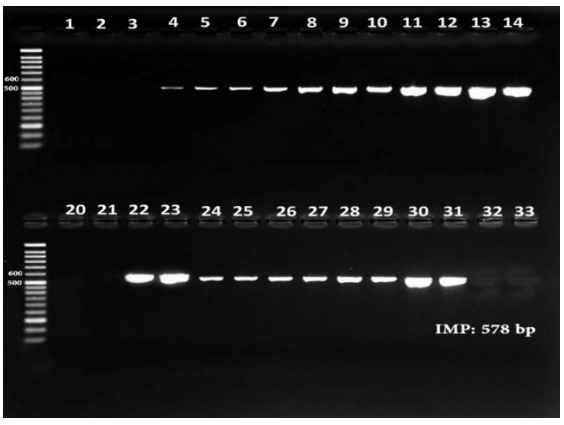

Figure 4: Electrophoresis of the PCR product for the *IMP* gene on a 1% agarose gel (The observed bands at the expected size (578 base pairs) confirm the presence of the *IMP* gene in *P. aeruginosa* isolates).

Academia Open
Vol. 10 No. 2 (2025): December
DOI: 10.21070/acopen.10.2025.13088

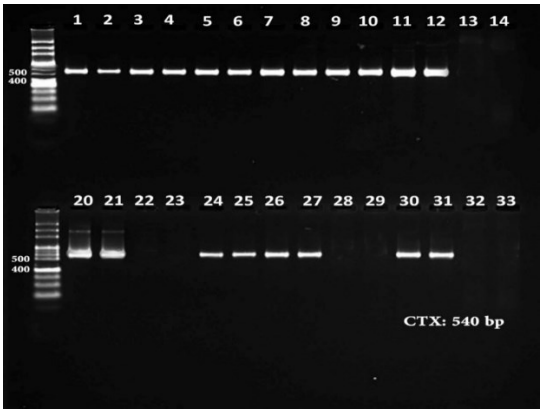

Figure 5: Electrophoresis of the PCR product for the *CTX* gene on a 1% agarose gel (The observed bands at the expected size (540 base pairs) confirm the presence of the *CTX* gene in *P. aeruginosa* isolates).

Characterization of Myrtle-Based Nanoemulsion

Two types of tools we used to study the live myrtle (Myrtus communis L.) nanoemulsion were FTIR (fourier transform infrared spectroscopy) and DLS (dynamic light scattering). FTIR tests showed that the nanoemulsion did a good job of keeping the phytochemicals inside. Between 700 and 950 cm^{-3}, there were bands of absorption. These were created when the aromatic rings changed shape and the carbon atoms moved around. Flavonoids and terpenoids in the plant juice caused this. Things 300 to 500 nm thick could be seen with a SEM. They were round, but not quite in the middle. Because they were round and didn't stick together much, they stayed put during green synthesis. This was possible because plants have chemicals that work on the outside. In Figure 4, there are some groups that are spread out and look like a mix of nanodroplets. The DLS data showed two big peaks. The first one was 84.06% bright and at 98.17 nm. The second one was at 623.99 nm and was 15.94% bright. There were 96.15 nm wide particles, and the PDI number was 0.08. There were a lot of pieces, but they didn't move. Phytochemicals can help us make a steady myrtle nanoemulsion, as this study shows. Figure 6 shows that it should be the right size and shape to kill germs.

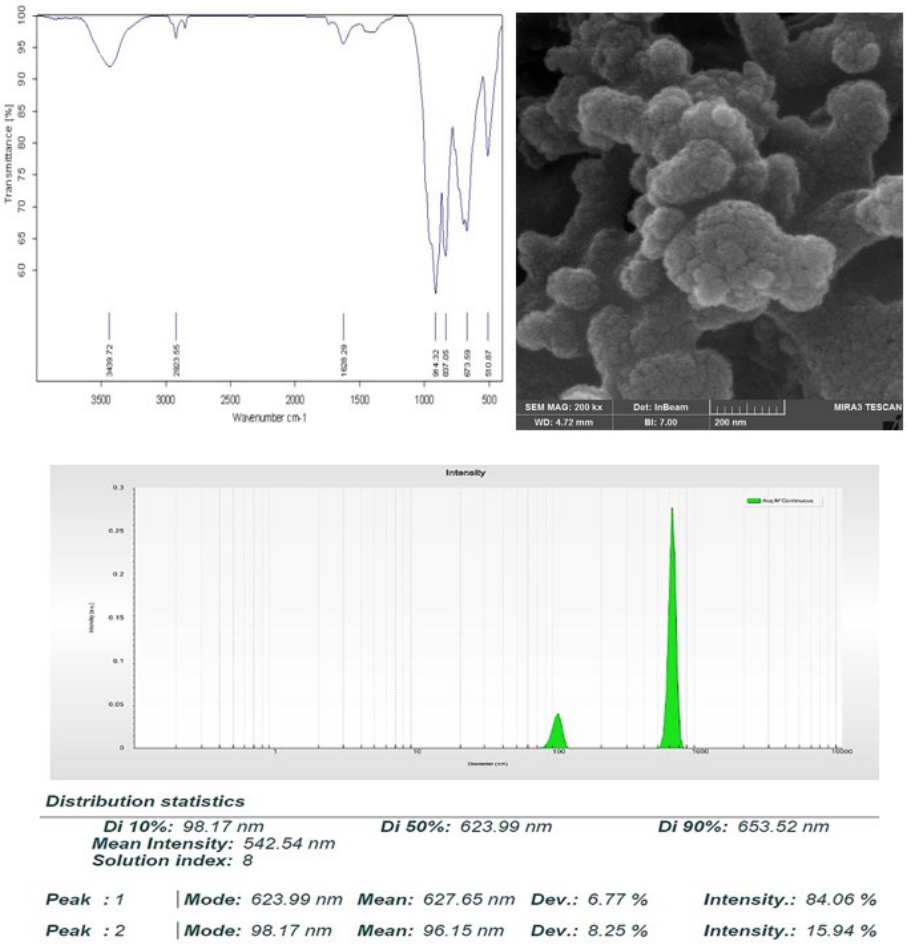

Figure 6. Characterization test of the nanoparticles

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Determination of MIC and MBC of Myrtle Nanoemulsion

Some types of P. aeruginosa that make ESBL were killed by the nanoemulsion made from myrtle (Myrtus communis L.). It mattered how much was used. The least amount that stopped all visible growth of bacteria was found to be 0.07 mg/µL. What else do you call this? What is the minimum limiting amount (MIC)? This level stopped germs from growing by 876.0% to 90.21 %, which is a huge amount. At 0.20 mg/µL, at least 99.9% of the active cells were killed, and bacteria could not live. The drug killed the bacteria because it stopped 92.87% to 95.87% of the flow and no colonies grew on subculture agar plates. At 0.10 mg/µL, more than 91% of the cells began to shrink. It's not even close to the MBC. This proves that it does kill germs. The myrtle nanoemulsion kills some P. aeruginosa and slows it down. In other words, it could be used instead of other drugs for types that don't get better with them.

Discussion

A new study shows that MDR P. aeruginosa is getting worse in hospitals. Some types have genes for both ESBL and MBL, which makes this even more true. A huge number of isolates had MBL genes. Also, VIM genes were found in 55 percent of the isolates and IMP genes were found in 47. 5 percent. People from all over the world say that carbapenemases are being spread by Gram-negative bacteria (17, 18).

It's very scary that these genes are found in so many bacteria, since carbapenems are often the last line of defense against MDR P. aeruginosa (19). Genetics play a direct role in the resistance that is seen in drug susceptibility tests. Cefotaxime and other third-generation cephalosporins do not kill 97.5% of the germs in this case. Also, carbapenems don't kill all germs; they only kill about 50 to 55% of them. "Superbugs" that are immune to many drugs (XDR) or even all drugs are possible. This is because samples often contain more than one resistance factor, like VIM, IMP, SHV, and CTX-M. Burn units and critical care units all over the world are seeing this more and more often. This means that doctors often have to make plans that are risky, haven't been tried before, or don't work as well (20). Now it's important and good to come up with new ways to fight drugs. They're not just an idea anymore. We found that a nanoemulsion made from green chemistry and myrtle essential oil is very good at getting rid of these bad germs. The stable nanoemulsion could be made, as shown by a full physical study. It has small fluid particles (17.98–99.62 nm by DLS) that are all the same shape (300–500 nm by SEM). There are now more ways for bugs to use and connect with it(21). When the nanoemulsion was 0.07 mg/µL strong, it stopped about 90% of the growth. When it was 0.2 mg/µL strong, it killed more than 95% of the bacteria. As shown, it can kill P. aeruginosa that makes ESBL/MBL and not just slow it down. The nanoemulsion was used on samples that most common medicines didn't work on. It looks like it works in a way that is different from other drugs. It probably does this by hurting membranes, stopping biofilms, and causing reactionary stress. This works with efflux pumps and enzymes like β-lactamases that break down drugs. This seems like a good way to beat MDR(22). Plant-based nanoformulations can be used as drugs or instead of them. This is becoming clearer over time. But they need to be checked out many times to be sure they are safe. They also have to use tools that keep drugs safe and keep track of how well the body responds to them. Nanoemulsified essential oil of Myrtus communis kills P. aeruginosa bacteria very well, which is better than many drugs. Most of the time, the MIC values are between 0.05 mg/µL and 0.12 mg/µL (23). It's true after all. It was also found by Falleh et al. (2021) that Myrtus communis essential oil mixed as a nanoemulsion kills germs very well and stays stable in the body. The answers we got are very similar to theirs. The myrtle nanoemulsions were always well-made, and the drops were very small. Keep in mind that the poisons killed a lot of germs in both tests. Myrtle nanoemulsion kills bacteria the same way no matter what kind of bacteria it is or where it is tested. This means that the antibiotic is strong and works well. One study showed that nanoencapsulation not only makes bioactivity higher, but it also keeps it high over time. That makes it more likely that this green nanotechnology could be used in the real world to either keep food fresh or kill germs that are hard to kill (16). There will be many ways to deal with the MDR P. aeruginosa mess when it's all over. Doctors will be smart about how they use drugs to stop infections. Resistance will also be watched all the time, and new tools like phytochemical nanoemulsions will be created.

Conclusion

These results show that when P. aeruginosa is used in medicine, it usually has ESBL and MBL genes, especially VIM and IMP. Bacteria are hard to kill with carbapenems because of these genes. In other words, they can only be treated with certain kinds of medicines. With a MIC of 0.07 mg/µL and an MBC of 0.2 mg/µL, the myrtle (Myrtus communis) nanoemulsion made in this study did a great job of killing these bad bacteria. As you can see, nanoemulsion has a lot of promise as an extra or different antibiotic that can get around the problems that cause resistance. It is important to use these nano-phytotherapeutic ways along with strict drug care, infection control, and constant tracking to lower the risk that P. aeruginosa that makes ESBLs and MBLs is becoming.

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