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Miraculous Antibacterial Activity With A Synergy Between Salicylic Acid And *Rosmarinus Officinalis* Essential Oil Against The Multidrug Resistant Pathogen *Pseudomonas Aeruginosa.* In Vitro And In Silico Studies.

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Abstract

Pseudomonas aeruginosa occupies a central position in the current problematic of nosocomial infections. Such infections are becoming an everyday occurrence. This dire scenario requires stringent adherence to antimicrobial stewardship and isolation of colonized or infected patients at all times. Unfortunately, some secondary metabolites from plants, such as essential oils, phenolic compounds, and alkaloids, were shown to be ineffective against *Pseudomonas aeruginosa*, allowing us to test the interaction of an antibiofilm with an antibacterial drug and evaluate how effective it was.

The purpose of this research is to see how effective *Rosmarinus officinalis L* essential oil alone and combined with salicylic acid is against *Pseudomonas aeruginosa*, on the other hand, to evaluate in silico, the inhibition of Las B elastase and Las A alkaline by the major compounds of rosemary essential oil and involved interactions for the first time between them.

Rosemary essential oil was ineffective against hospital strains of *Pseudomonas*, although *Pseudomonas aeruginosa* ATCC7853 was sensitive to the pure concentration. The docking of Las B elastase and Las A alkaline protease with these parameters is achieved for the first time in this study, and no other investigations in this context have been uncovered.

The effects of key components in *Rosmarinus officinalis* essential oil on the Las B elastase and Las A alkaline protease responsible for *Pseudomonas aeruginosa*'s multidrug resistance capacity were demonstrated in this study.

We propose association of salicylic acid with Eucalyptol, camphor, alpha pinene and borneol as a new treatment against multidrug resistant *Pseudomonas aeruginosa.*

Key words: Autodock vina., salicylic acid., *Rosmarinus officinalis* essential oil., Las B elastase., Las A protease

Abbreviations

CF, cerebrospinal fluid., CFU, colony forming unit., DMSO, diméthylsulfoxyde., ESBL, Extended Spectrum Beta-Lactamase., MBC, minimum bactericidal concentration., MIC, minimal inhibitory concentration., MF, mc farland unit., QS, Quorum sensing

1- Introduction

Because of the versatility of the various virulence factors that it contains, *Pseudomonas areruginosa* is the causal agent of a wide range of infections in the hospital setting. This bacteria, in particular, has the potential to live a biofilm lifestyle on tissues and medical device surfaces, which provides various benefits, including the ability to evade host immunity, which can lead to infection chronicity. One of the *Pseudomonas* tactics for contributing to antibacterial therapy failure is the creation of the bioflm **(Rasamiravaka et al., 2015)** because Antibiotics can only target free cells and are unable to penetrate the biofilm matrix **(Brindhadevia et al., 2020).**

Indeed, *P.aeruginosa* is an opportunistic pathogen that has been extensively studied in human, plant, and animal infections for its ability to produce virulence factors. Tumors, plastic surgery, nosocomial infection, immunocompromised patients all have an impact on bacterial infection induced by clinical pathogens. Quorum sensing (QS) and QS-mediated biofilm development and release of virulence agents such as LasB elastase, pyocyanin, LasA protease, pyoverdin, rhamnolipids, and aliginate are the main reasons for *P. aeruginosa* pathogenicity **(Muzamil al.,2021; Pearson et al.,2018; Bjarnsholt et al., 2010; Storey et al.,1998).**

For this reason, and to combat pathogenic bacteria that can form biofilm, three strategies have been proposed: avoiding microbial attachment to a surface, disrupting biofilm development and/or affecting biofilm architecture to improve antimicrobial penetration and affecting biofilm maturation and/or inducing its dispersion and degradation **(Masak et al., 2014; Blackledge et al., 2013; L. Yang et al., 2012).**

In order to develop new antibacterial chemicals, researchers are focused on herbal medicine. Indeed, the wide variety of medicinal plants and the large number of natural chemicals extracted explains the high number of natural chemicals extracted. Furthermore, secondary metabolites of medicinal plants are known to have antibacterial activities in traditional medicine **(Borges et al., 2016; Heinrich et al., 2012).**

As a result, the focus of this study is on the antibacterial activity of one of *Rosmarinus officinalis* L's extracts, which is used as an antiseptic in phytomedicine.

R. officinalis L., often known as rosemary, is a Lamiaceae-family plant that originates in the Mediterranean region. It might, however, be found all over the planet. It is a perennial and aromatic plant with green leaves that radiate a distinctive fragrance. It is shrub-shaped with branches full of foliage, growing up to two meters tall with green leaves that exude a distinctive fragrance. it is a culinary spice, an ornamental and medicinal plant **(Rafael et al., 2019).**

Indeed; Antimicrobial, antioxidant, anti-diabetic, and anti-tumorigenic properties of *Rosmarinus officinalis* have long been known. It is also known to impact the cell's adhesion characteristics, self-aggregation, and protein release, which could be beneficial in the treatment of cardiovascular disorders including thrombosis **(Abdul sattar et al., 2017).**

Concerning biofilm-inhibiting chemicals, and because of its antithrombotic, anti-inflammatory, and analgesic effects, aspirin is used by a large percentage of the world's population **(Gargiulo et al., 2016**). SAL is the primary metabolite produced in vivo by aspirin deacetylation **(Laudy et al., 2016),** it reduced platelet antibacterial activity **(Hannachi et al., 2020)**, and numerous effects of SAL on bacterial pathogenicity and biofilm formation have been documented **(Cristian et al., 2021).**

Using essential oils and their constituents in combination with antibiotics can reduce bacterial resistance. **(Faleiro et al., 2013)**. Unfortunately, several plant secondary metabolites are insufficient on their own as traditional medicine to have been proven effective against *Pseudomonas aeruginosa*. It is for this reason that the new approach of this study is to create a synergy with a biofilm inhibitor rather than with another antibiotic to clarify the efficacy and antibacterial activity of natural extracts alone and to find new antibiotic molecules, so it is time to turn back to traditional medicine to combat nosocomial infections.

We are prevented from evaluating the effectiveness of *Rosmarinus oficinalis'* essential oil using an antibiofilm rather than an antibiotic because it is one of the medicinal plants that have been used since Antiquity and has maintained a place in the inventory of remnants traditional healers from all over the Mediterranean basin.

This is why our research was divided into two stages: first, the activity of the essential oil of *Rosmarinus officinalis* alone to determine its antibacterial potency against *Pseudomonas*, and subsequently, the synergy with a biofilm-inhibiting drug, salicylic acid.

With the in vitro model of LasB elastase, LasA protease, molecular docking was done to make clear the inhibitory mechanism and interaction types, using autodock vina with different parameters disclosed here for the first time. The discovery studio visualizer was used to treat the results.

2- Materials and methods

2-1 Compilation of plants

The rosemary harvest took place in the Ouedmoura region in March 2021. At an elevation of 1303 meters, it is 91 kilometers from the wilaya of Laghouat (Algeria) (3417824°N. 2, 30016°E).
Mr. Yousfi M collected the plant and compared it to Rosmarius references from the Mediterranean basin to identify it. He recognized the specimens, and voucher specimens were deposited in the herbarium of Amar Thelidji University in Laghouat, Algeria. Pl Algéria 1852: no444. 1852[in sched].

2-2 Extraction and Chemical composition of essential oil by GC-MS

The research was done at Laghouat University's research laboratory. Hydrodistillation using the Clevenger is the procedure for extracting essential oils. The essential oil is separated by a density differential. To eliminate all traces of water, anhydrous sodium sulphate is added to the essential oil before it is collected in opaque bottles.
Chemical composition of essential oil is determined by GC-MS, using a SHIMADZU GCMSQP2020 Instruments, equipped with a fused Rxi®-5ms capillary column (Phase: Crossbond® 5% diphenyl/ 95% dimethyl polysiloxane). By comparing calculated (LRI) values to those found in the literature, components were identified.

2-3 Bacterial strains collection

The work was done in the main hospital at Laghouat's central laboratory from March to June 2021.
We selected four *Pseudomonas aeruginosa* bacterial strains positive from 784 samples supplied to the lab by various medical departments, each from a different type of sample: urine, pus, cerebrospinal fluid, and ascetic fluid.
Pseudomonas aeruginosa ATCC 7853 is from Abou Bekr Belkaid Tlemcen University.

2-4 Bacterial strain classification

Diagnosis of Pseudomonas *aeruginosa* as ESBLs is more difficult due to a link with other resistance mechanisms, such as hyperproduction of cephalosporinases. The antibiotics employed include Ticarcillin + Clavulanic acid 75/10 g (TCC), 30 mm of a disk of Ceftazidim30 g, and Aztreonam 30 g, according to the antibiogram's usual circumstances **(Magiorakos et al., 2012; Barguigua et al., 2011).**
The development of ESBLs will be detected if there is an image of synergy in the form of a champagne cork. A technique called the twin disc technique can be used to identify ESBLs if the diameter around the 3rd generation cephalosporin discs decreases **(CLSI., 2011).**
Different antibiotics were used to detect many forms of *Pseudomonas aeruginosa* and classify them according to their sensitivity.
The antibiotics used are Ticarcillin + Clavulanic acid 75/10 µg (TCC) 30 mm of a disk of Ceftazidime (CAZ) 30µg, Cefotaxime (CTX) 30 µg, Imipenem(IPM), Piperacillin (PRL)100 µg.

2-5 Testing of essential oil against different type of *pseudomonas aeruginosa*

The antibacterial activity of *Rosmarinus officinalis L* essential oil was tested using the disk diffusion method on four bacterial isolates and the ATCC 7853 strain.

2-6 Salicylic acid synergies with essential oil evaluation against *Pseudomonas aeruginosa*

The stock solution was made by dissolving 250 mg of salicylic acid in 10 mL of distilled water. Standard solutions were created by diluting stock solutions to achieve final concentrations
The disk diffusion method was used to test the antibacterial activity of *Rosmarinus officinalis L* oil linked with salicylic acid on the four bacterial isolates and the ATCC 7853.
The inoculum was made with a brush to remove bacterial colonies more easily after an 18 to 24 hour pure culture (storage medium) on nutritional agar media.

The bacteria were cultured in petri dishes poured with MH medium with a thickness of 4 mm associated with salicylic acid solution with varied dosages ranging from (1-25 mg / ml).
After discharging the swab into 5 to 10 ml of sterile 0.9 percent physiological saline at 0.5 MF (or an optical density of 0.08 to 0.1 read at 625nm).
The use of sterile 6 mm diameter discs (FIORONI S.A) gorged with 15 l of pure essential oil at an initial concentration of 365.8 mg/ml, as well as a series of dilutions in 10% DMSO, 1/2 and 1/10. DMSO is used as a negative control **(Nabti et al., 2020).**

2-7 MIC and MBC determination

The minimal inhibitory concentrations were determined using 96-well microtiter plates (MIC). The initial concentration of Rosmarinus essential oil is 365.8 mg/ml.
In Müller-Hinton broth, a dilution series of essential oil was created by adding 10% DMSO; the positive control is the bacterial inoculum without extract; and the negative control is DMSO.
The bacterial suspension was made by diluting a pure culture of 48H to a density of 0.5 MF in 10 ml of physiological water or medium MH liquid.
The 0.5MF opacity suspension is diluted a second time 1/10 to distribute 5 * 105 CFU / ml of germ in each well. Purity was censured by isolation on nutrient agar**.**
The volume of the inoculum deposited in the wells is 5 µl, 70 µl of liquid MH, and 25 µl of extract.
For 24 hours at 37 degrees Celsius, the microplate was incubated. the MIC is determined by pouring 40 µl of 0.2 mg/ml iodonitrotetrazolium chloride into all wells of the microplate, incubating for 30 minutes at 37 ° C, and observing a pink purple color if bacterial growth is present; thus, the MIC is determined by the lowest concentration, which corresponds to the first clear cup without color change **(Ibrahim et al., 2021).**
The MBC was measured by culturing the extract from the wells from the MIC to the first concentration. After an 18-hour incubation period at 35° C, the colonies of each reculture were counted and compared to a control that represented a 0.01 percent dilution of the beginning inoculum. MBC is the lowest essential oil concentration at which the number of bacterial colonies is less than or equal to the number of colonies present on the initial inoculums' dilution **(Denis et al., 2011).**

2-8 In silico: Molecular docking of the investigated compounds

2-8-1 Selection and preparation of ligands and enzymes

To gain a better understanding of the interactions and inhibition mechanisms between ligands and enzymes, we should select a target protein from the protein data bank (PDB) based on a set of criteria, namely its 3D active site, which should interact with the inhibitor. Following PDB research (protein data bank) **(Berman et al., 2000).**
The major chemical composition of *Rosmarinus officinalis*, represented by Eucalyptol, Camphor, Borneol and Alpha pinene, was compiled from public databases and published research publications and downloaded in SDF format as ligands from the PubChem database. https://pubchem.ncbi.nlm.nih.gov **(Kim et al., 2019).**
As the enzymes evaluated in silico, we chose LasB elastase and LasA protease because they represented the virulence agents of Quorum sensing (QS) and QS-and the key causes for *P. aeruginosa* pathogenicity.
"Molecular docking is a computational procedure that attempts to predict noncovalent binding of macromolecules or, more commonly, a macromolecule (receptor) and a small molecule (ligand) efficiently, starting from unbound structures, structures obtained from molecular dynamics simulations, or homology modeling," according to the autodock vina program for molecular docking and virtual screening. The objective is to forecast bound conformations and binding affinity" **(Trott et al., 2010).**
First, all superfluous ligands such as water molecules, heteroatoms, and any cocrystallized solvent are removed. The structures of the 3D inhibitors were discovered.
Then, the AutoDock Tools (ADT) was used to accomplish intermediate tasks such as creating pdbqt files for protein and ligand preparation and creating grid boxes. The protein was given polar hydrogens, Kollman charges, solvation parameters, and fragmental volumes via ADT. The prepared file was saved in PDBQT format by AutoDock.
AutoGrid was used to create a grid map using a grid box. The grid size was set to 40 40 40 xyz points, and the grid center was set to 60.536, 59.659, 26.220 (x, y, and z) for Las A alkaline protease, and 27.066, 28.592, -8.851 for Las B elastase.

The protein and ligand information, as well as grid box attributes in the configuration file, were used for docking with AutoDock/Vina. Iterative local search global optimizer is used by AutoDock/Vina (**Syed et al., 2013).**
In this study, the specific docking type was chosen, and the lowest interatomic distance modes in were chosen for analysis. Finally, the docking findings were imported into the discovery studio visualizer v 4.0 applications.

3- Results and discussion

Some secondary metabolites from plants, such as essential oils, phenolic compounds, and alkaloids, were shown to be ineffective against *Pseudomonas aeruginosa*, allowing us to test the interaction of an antibiofilm with an antibacterial drug and evaluate how effective it was. As a result, the purpose of this research is to see how effective *Rosmarinus officinalis L* essential oil alone and combined with an antibiofilm represented by salicylic acid is against *Pseudomonas aeruginosa.*
First, we used *Pseudomonas aeruginosa* isolates and ATCC to evaluate a variety of drugs. The antibiogram indicates a diverse array of multidrug-resistant bacteria. *Pseudomonas aeruginosa* from hospital and ATCC have a high resistance to third-generation cephalosporins.
Pseudomonas aeruginosa ATCC 7853 is more sensitive than hospital strains isolated from LCR, the positive ESBL and the negative ESBL to antibiotics such as Imipenem**. (figure1)**

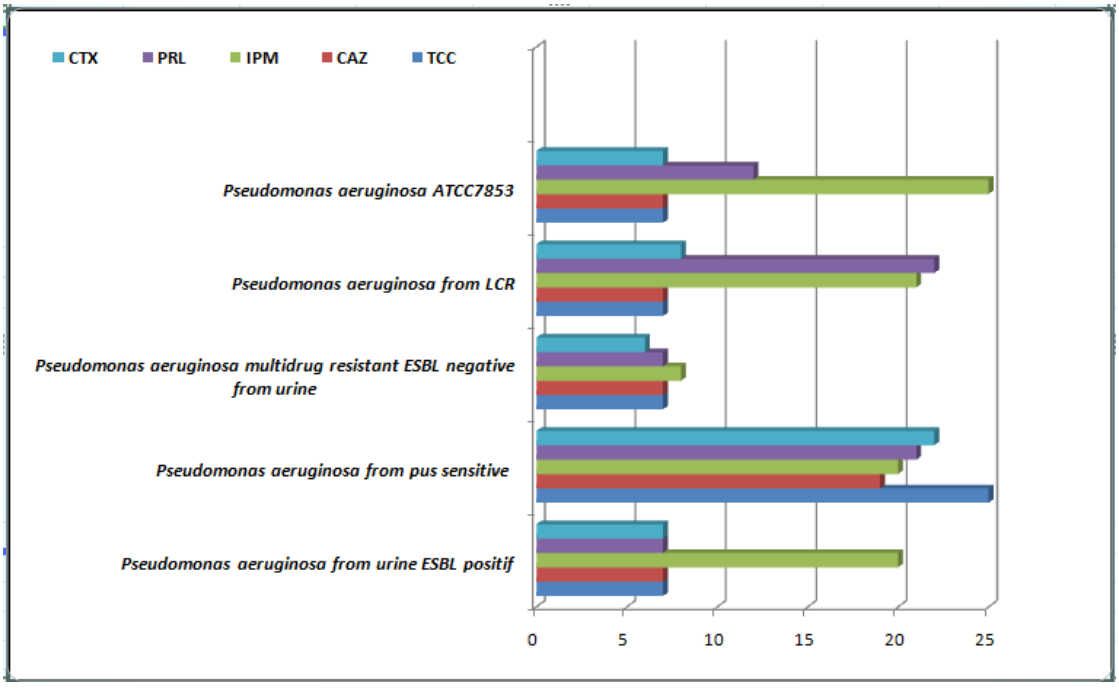


Figure 1: Resistance of bacterial isolate and ATCC against betalactamine, 3rd generation cephalosporins, imipenem

3-1 Results without and with salicylic acid

Unfortunately, rosemary essential oil was ineffective against hospital strains of *Pseudomonas*, although *Pseudomonas aeruginosa* ATCC7853 was sensitive to the pure concentration of the essential oil. **(figure2, table1)**
However, when the essential oil is combined with salicylic acid, a biofilm stabilizer, the isolates become more sensitive to the essential oil of rosemary, which is surprising**. (figure3A)**
(**Table 1**) shows the diameters of inhibitions with and without salicylic acids, where all *Pseudomonas aeruginosa* isolates became sensitive to the essential oil without and with the 1/2 dilution.
Furthermore, when salicylic acid was added to the essential oil, the zone of inhibition grew from 10 to 12 mm in width with reference strain *Pseudomonas aeruginosa* ATCC7853.

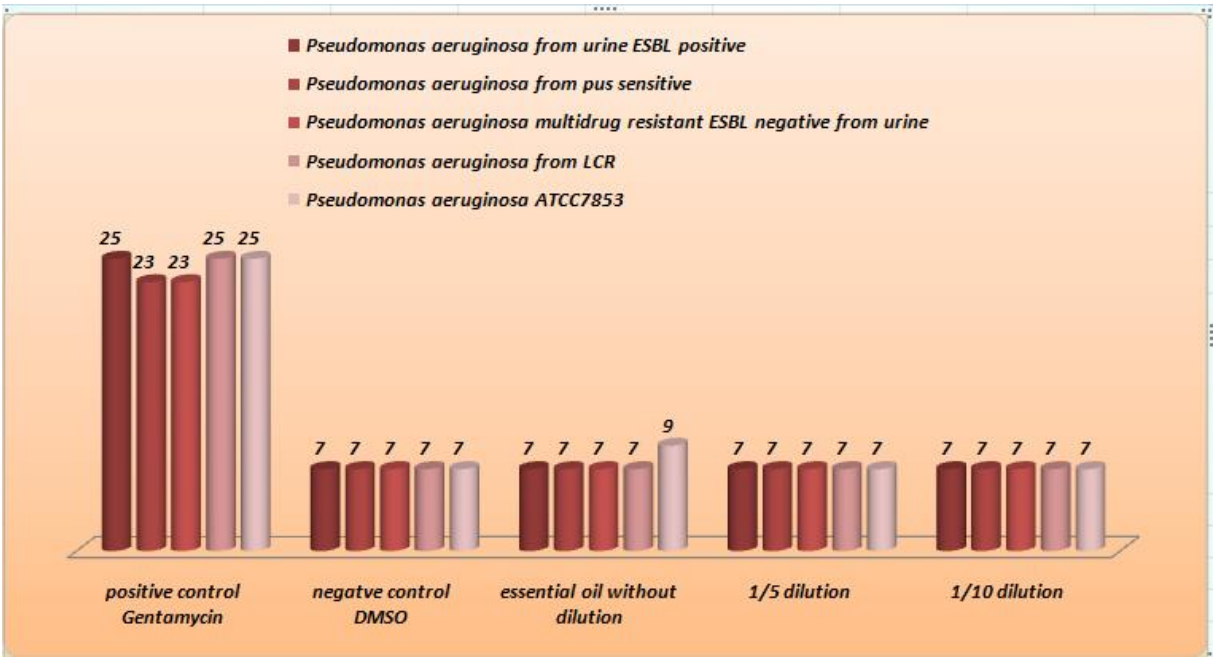


Figure2: Evaluation of the antibacterial activity of rosemary essential oil with 1/5 and 1/10 dilutions against the isolate of *Pseudomonas* and the ATCC.

According to research, salicylic acid stabilized the bioflms, possibly preventing bacterial cell spread. However, it may encourage the spread of infections locally, increasing bacterial persistence and ultimately leading to therapeutic failure.

By interfering with Agr expression, salicylic acid reduces bioflm spread, it inhibits the formation of protease and surfactant molecules, as well as bacterial cell autolysis. Furthermore, bioflm matrix demonstrated the absence of extracellular DNA as a result of salicylic acid treatment **(Cristian et al., 2021).**

As a result, we may conclude that utilizing salicylic acid to reduce biofilm spread permits active molecules from the essential oil of *Rosmarinus officinalis* to penetrate and inhibit *Pseudomonas aeruginosa* isolates.

Table1: Inhibition diameters of rosemary essential oil with and without salicylic acid with Pseudomonas isolates and ATCC

***Pseudomonas aeruginosa* types**	**inhibition zone without salicylic acid(mm)**	**Inhibition zone of *Rosmarinus officinalis* essential oil with salicylic acid (mm)**		
		Essential oil without dilution	**Essential oil dilution 1/2**	**Essential oil dilution 1/10**
***Pseudomonas aeruginosa* ESBL positive**	/	**11± 0,288**	**9± 0,152**	/
***Pseudomonas aeruginosa* sensitive**	/	**12± 0,251**	**10± 0,173**	/
***Pseudomonas aeruginosa* multidrug resistant ESBL negative**	/	**12± 0,288**	**9± 0,200**	/
***Pseudomonas aeruginosa* from LCR**	/	**10± 0,100**	**8± 0,115**	/
***Pseudomonas aeruginosa* ATCC 7853**	**8± 0,353**	**10± 0,115**	**8± 0,300**	/

3-2 MIC and MBC determination

The MIC and MBC values of different strains were fairly diverse; the MIC and MBC values of *Rosmarinus officinalis L* essential oil with salicylic acid against the isolates are listed in the table below**. (Table 2, figure3B)**

By comparing the result of the MIC and the MBC before and after the addition of salicylic acid for the strain ATCC7853, a MIC of 1143.1 µg/ml decreases to 142.8 µg/ml, and a MBC of 1143.1µg/ml. we may say that salicylic acid is a factor favoring the penetration of active molecules to inhibiting the bacteria.

Table2: Minimal inhibitory concentration and minimal bactericidal concentration of *Rosmarinus Officinalis L* essential oil with salicylic acid against different isolate of *Pseudomonas aeruginosa*.

	Pseudomonas aeruginosa **ESBL positive**	***Pseudomonas aeruginosa*** **sensitive**	***Pseudomonas aeruginosa*** **multidrug resistant ESBL negative**	***Pseudomonas aeruginosa*** **from LCR**	***Pseudomonas aeruginosa*** **ATCC 7853**	
					Without salicylic acid	**With salicylic acid**
MICµg/ml	**142.8**	**71.4**	**142.8**	**142.8**	**1143.1**	**142.8**
MBCµg/ml	**571.5**	**285.7**	**571.5**	**1143.1**	**/**	**1143.1**

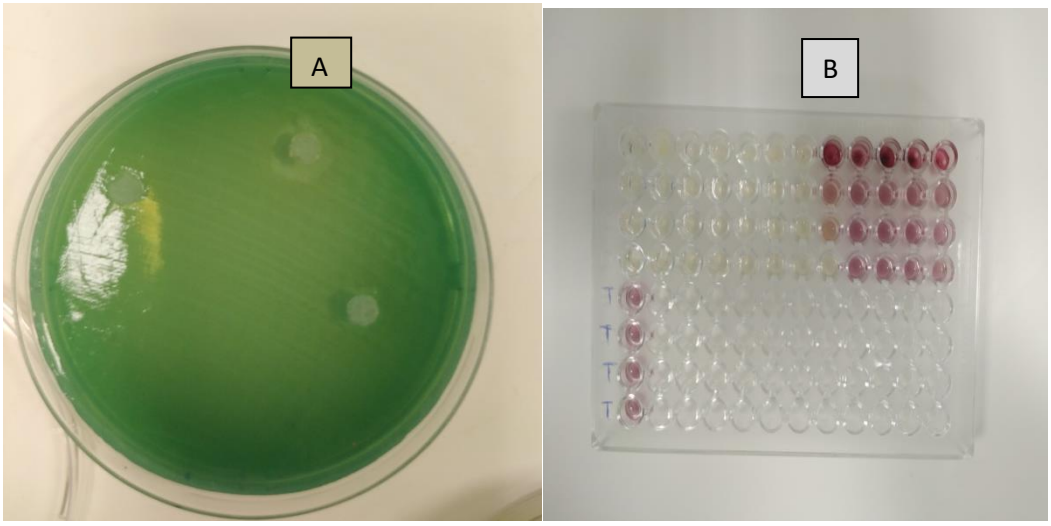


Figure3: A: zones of inhibition for rosemary oil associated with salicylic acid against *Pseudomonas* **B:** 96-well microplate with minimum inhibitory concentrations using dinitrotetrazolium as growth indicator.

3-3 *Rosmarinus officinalis* essential oil Chemical composition by GC-MS

The main chemical composition is Eucalyptol 40.09 percent, Camphor 24.59 percent, according to gas chromatography coupled with mass spectroscopy. **(figure4)**

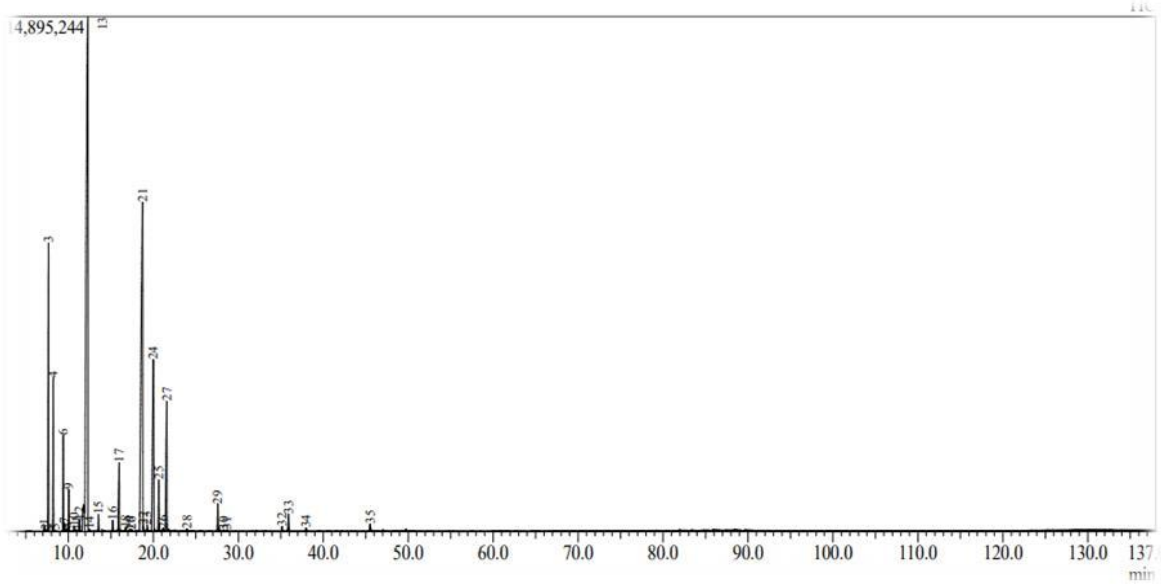


Figure 4: *Rosmarinus officinalis L* essential oil's gas chromatography coupled with mass spectrometry (GC–MS) spectrum.

Borneol 9.12 percent, Alpha pinene 7.07 percent, Terpineol (aplha); cyclohex-3-ene-1-meta 5.36 percent, Camphene 3.12 percent, linalool 2.41 percent, Pinene beta; bicyclo(3.1.1)haptane6,6 2.07 percent, and Terpinen-4-ol 1.41 percent are among the chemicals discovered with a percentage of 1 to 10%. The other ingredients with minor percentages are shown in the table below**. (table3)**

Some other works have been done on the components of rosemary essential oil, with the antibacterial activity being attributed to 1, 8 cineole, camphor, alpha pinene, verbenone, beta caryophyllene, and myrcene **(Djenane et al., 2011).**

Unlike earlier research, our findings show that *Rosmarinus officinalis L* essential oil from the Laghouat (Algeria) region has significantly stronger antibacterial action against gram-negative bacteria. The principal constituents of *Rosmarinus officinalis L* essential oil from the region of Laghouat (Algeria), such as Eucalyptol, Camphor, Borneol, and Alpha pinene, account for the highest percentage of the essential oil.

Table3: *Rosmarinus officinalis L* essential oil chemical composition, including areas, percentages, retention times, and names of each metabolite

peak	Retention time	Area%	Retention index	Name
1	7.188	0.10	921	Tricyclene
2	7.356	0.03	925	Thyujene alpha
3	**7.673**	**7.07**	**933**	**Alpha pinene**
4	**8.228**	**3.12**	**947**	**Camphene**
5	8.439	0.01	952	Verbenene
6	**9.407**	**2.07**	**975**	**Pinene beta; bicyclo(3.1.1)haptane6,6**
7	9.521	0.17	978	1 octen-3-ol
8	9.802	0.01	985	3-octanone
9	10.050	0.91	991	Myrcene;octa1,6 diene
10	10.679	0.12	1005	Alpha phellandrene
11	10.981	0.03	1010	Delta-3- carene
12	11.309	0.33	1016	Terpinene (alpha)
13	**12.294**	**40.09**	**1034**	**Eucalyptol**
14	12.471	0.09	1038	Trans beta ocimene
15	13.548	0.36	1057	Gamma terpinene
16	15.209	0.29	1088	Terpinolene
17	**15.982**	**2.41**	**1102**	**Linalool**
18	16.752	0.10	1114	Fenchol
19	17.199	0.03	1121	p-menth-2-en-l-ol
20	17.433	0.04	1125	Aplhacampholenal
21	**18.740**	**24.59**	**1146**	**Camphor**
22	18.841	0.11	1147	Terpineol (trans-beta)
23	19.274	0.15	1154	Isopulegol-1
24	**20.002**	**9.12**	**1166**	**Borneol**
25	**20.643**	**1.41**	**1176**	**Terpinen-4-ol**
26	21.164	0.09	1185	Cymen-8-ol (para)
27	**21.588**	**5.36**	**1192**	**Terpineol (aplha); cyclohex-3-ene-1-meta**
28	23.973	0.05	1229	Citronellol
29	27.589	0.76	1285	Bornyl acetate
30	28.228	0.02	1296	Thymol
31	28.670	0.03	1302	Carvacrol
32	35.167	0.12	1405	Methyleugenol
33	35.905	0.50	1417	Caryophyllene
34	37.971	0.07	1451	Alpha- Humulene
35	45.529	0.22	1579	Caryophyllene oxide

3-4 In silico: Molecular docking of the investigated compounds

Las B elastase: Elastase of *Pseudomonas aeruginosa* is an enzyme classified among the hydrolases, with a total Structure Weight: 33.82 kDa, unique protein chains and with 2570 atom, sequence length 301 **(Bitto., 2004). (figure5)**

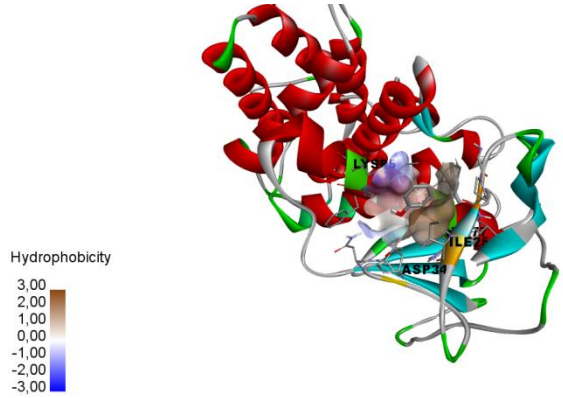


Figure 5: The 3D structure of Las B Elastase colored by structure with the active site of enzyme complexed with ligand: Camphor, docked by discovery Studio visualizer 2021.

Alkaline protease from *Pseudomonas aeruginosa* is an enzyme classified among the metalloproteinase with Total Structure Weight: 49.92 kDa , unique protein chain with 3845 atom, sequence lengh is 470**(Miyatake et al., 1995). (Figure 6)**

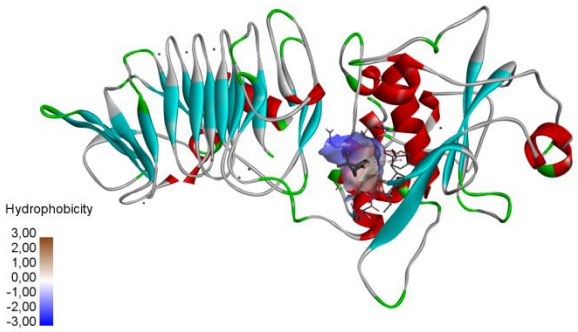
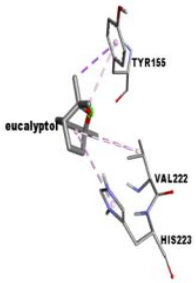
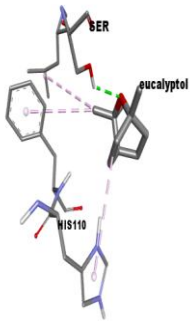
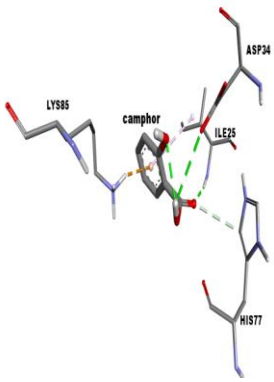
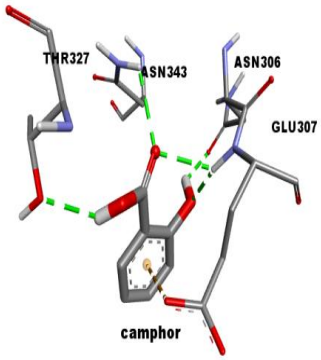
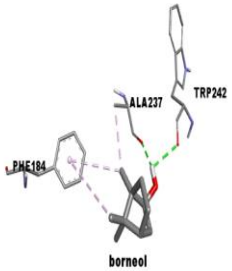
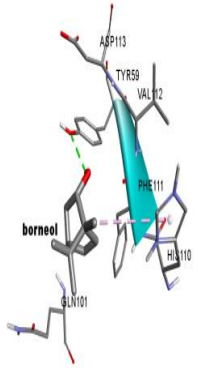


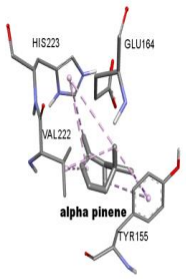
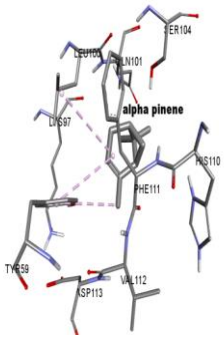
Figure 6: The 3D structure of Las A alkaline protease colored by structure with the active site of enzyme complexed with ligand: Alpha pinene docked by discovery studio visualizer 2021.

Between the ligand and the receptor, the shortest interatomic distance mode in A° were used.
Several amino acids, represented by hydrophobic bonds, played a critical role in the interaction between eucalyptol and the enzyme Las B elastase: HIS223 with a minimal distance of A°= 5.47, TYR155 with A°=3.88. The interactions between eucalyptol and the Las A alkaline enzyme entail the following bonds: The hydrogen type is represented by SER104 with A°=2.42, while the hydrophobic type is represented by LEU100 (A°=5.31), HIS110 (A°=4.91) and PHE111 (A°=5.15).
The interaction of camphor with Elastase is more specific, with bonds of various sorts portrayed as if joined: conventional hydrogen bond ILE25-HN with minimal distance A°=2.03, ASP34 (A°=3.07), carbon hydrogen bond HIS77 (A° =3.55), hydrogen bond electrostatic LYS85:H23 (A°=2.72), and a hydrophobic bond ILE25 with A°=4.46
In addition, the following is the association with alkaline protease:
Conventional hydrogen bond GLU307: HN with A°=2.37, GLU307 (A°=2.17), ASN343:HD21(A°=2.55), THR327/OG1(A°=2.37), ASN306/OD1(A°=2.28), and an electrostatic bond: GLU307:OE1(A°=4.07).
For the two enzymes, however, Borneol reacts with other amino acids, beginning with Elastase: two hydrophobic type bonds with PHE 184 at carbon levels 9 and 11, with minimal distance A°=4.67 and 5.44 respectly, and one with ALA237 (A°=3.88), two other hydrogen bonds ALA237 (A°=2.38), and TRP242 (A°=2.74).
A hydrogen bond with the amino acid TYR59 (A°= 2.51), and a hydrophobic bond with HIS110 with A°=5.36 for the protease.
Finally, with alpha pinene, where the bonds are all hydrophobic, for the Las B elastase enzyme, the amino acids introduced into the interaction with the ligand are as follows: TYR155 at carbon 7 and 8, VAL222 and HIS223 at carbon level 8 and 10.

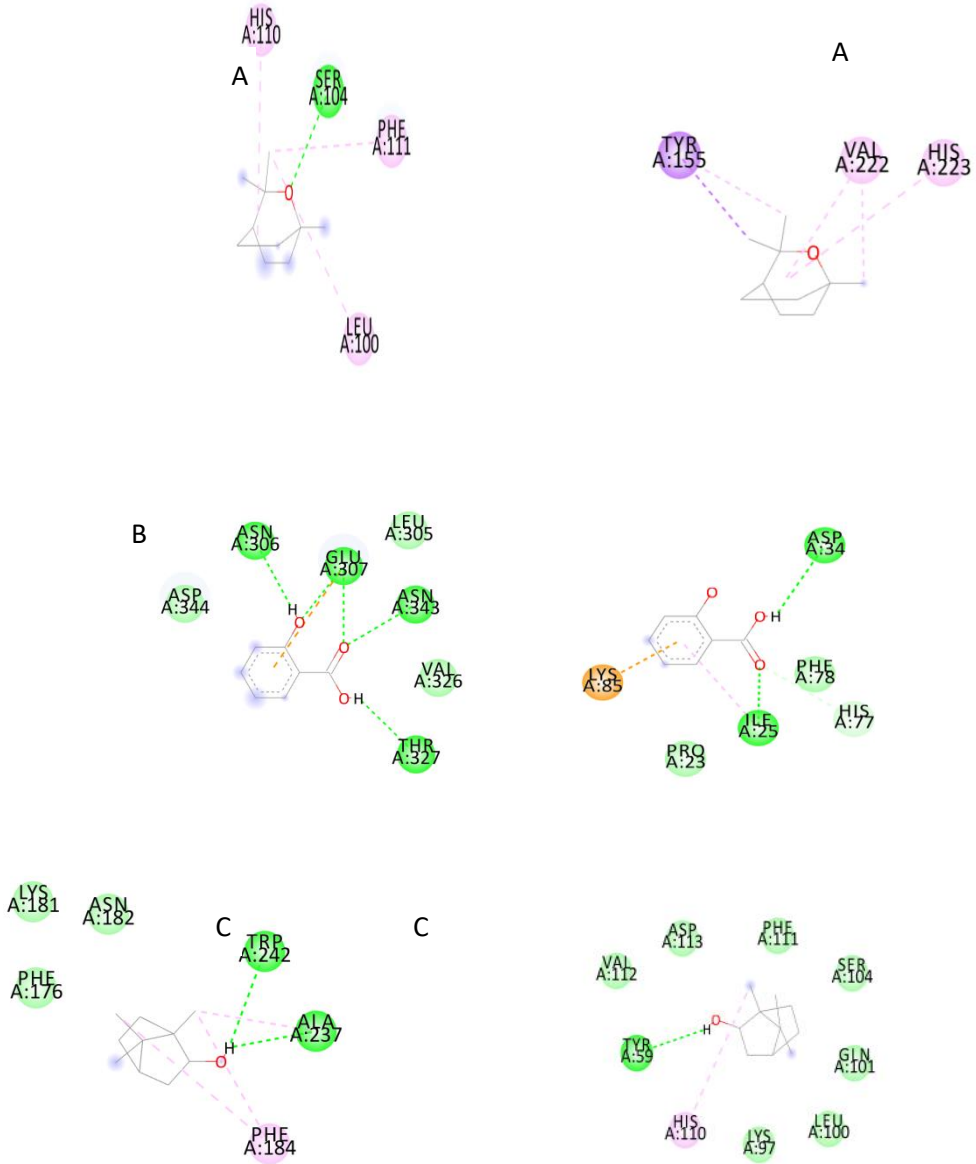
For Las A protease, the amino acids involved are: LEU100 with A°=5.32, TYR59 (A°=4.19) and TYR59 at carbon10 with A°=5.47. **(Table4)**

Table4: The docking pose with the inhibitors on the active site of Las B elastase and LasA alkaline protease for: eucalyptol, camphor, alpha pinene, borneol, Inhibitors: Presented in sticks and colored by default atom color.

Rosmarinus officinalis **essential oil major compounds**	**Las B Elastase of *Pseudomonas aeruginosa***	**Las A Alkaline protease of *Pseudomonas aeruginosa***
Eucalyptol		
Camphor		
Borneol		

Alpha pinene		

(Figure 7) depicts the bonds in detail as a 2D diagram, with the bonds between the active sites of the enzymes and the ligands plainly visible.



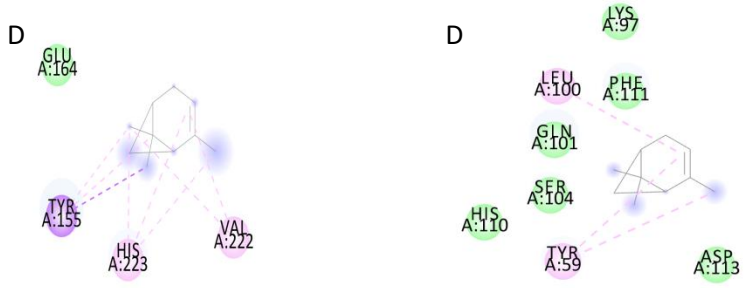


Figure7: A: 2D conformation of bonds between **eucalyptol** and the active site of LasB Elastase and Las A alkaline protease. Green dashed lines are drawn among atoms involved in hydrogen bond interactions, the pink involved in hydrophobic interactions, **B**: 2D conformation of bonds between **camphor** and the two enzymes,**C**: 2D conformation of bonds between **borneol** and the active site of LasB Elastase and Las A alkaline protease, D: 2Dconformation of bonds between **alpha pinene** and the two enzymes.

The docking of Las B elastase and Las A alkaline protease with these parameters is achieved for the first time in this study, and no other investigations in this context have been uncovered.

The effects of key components in *Rosmarinus officinalis* essential oil on the Las B elastase and Las A alkaline protease responsible for *Pseudomonas aeruginosa*'s multidrug resistance capacity were demonstrated in this study.

Hydrogen bonds are the most common directional intermolecular interactions in biological complexes, and they contribute significantly to the specificity of molecular recognition. Hydrophobic contacts are also the most common interactions in protein–ligand complexes, and they are the primary driving force in drug–receptor interactions **(Renato et al., 2017).**

In terms of ligand-enzyme interactions, the benzyl group can settle into a cavity lined with hydrophobic amino acid residues thanks to the molecules' binding mechanism.

HIS110, PHE111, PHE133, LEU100 from eucalyptol, PHE184 from borneol, and TYR 155, HIS 223, VAL 222 from alpha pinene make up this hydrophobic pocket.

About alkaline protease, the amino acid residues included are: TYR 155, VAL222, HIS223 from eucalyptol, HIS 110 from borneol, LEU100 and TYR 59 from alpha pinene.

This group has hydrophobic interactions with the various amino acids (face to edge and face to alkyl). The results show that eucalyptol, borneol, and alpha pinene are all competitive inhibitors.

Camphor reacts with the two enzymes via hydrogen type interactions, giving it the property of being a molecular recognizer.

4- Conclusion

In this work, We evaluated the most well-known essential oil in the Laghouat city, Algeria,is *Rosmarinus's* essential oil, for their ability to inhibit *Pseudomonas aeruginosa*, however it was only effective against *Pseudomonas aeruginosa* ATCC7853 and ineffective against hospital *Pseudomonas* strains. This allowed us to conclude, first and foremost, that the isolates are more resistant than the ATCC strain.

As a result, we used a biofilm inhibitor in combination with the essential oil as a novel idea to test the antibacterial activity against multidrug resistant *Pseudomonas* strains obtained from the hospital environment. The results were surprising, with multidrug resistant strain inhibition and increased zones of inhibition for ATCC7853.So, we can deduce that using salicylic acid to prevent biofilm spread allows active molecules from *Rosmarinus officinalis* essential oil to penetrate and inhibit *Pseudomonas aeruginosa* isolates.

On the other hand, GC–MS found that Eucalyptol, Camphor, Borneol, and Alpha pinene account for the highest percentage of *Rosmarinus officinalis L* essential oil from the region of Laghouat (Algeria). It allowed us to investigate the principal components of rosemary essential oil as inhibitors against the two enzymes responsible for *Pseudomonas aeruginosa* virulence, Las B elastase and Las A alkaline protease.

In silico study based on molecular docking using autodock vina program was carried out to study the Las B elastase and Las A alkaline inhibition mechanism and involved interactions for the first time. The results show

that Eucalyptol, Borneol and Alpha pinene gave an important and similar inhibition effect against both enzymes as competitive inhibitors, furthermore, Camphor being a molecular recognizer.

Therefore, we propose association of salicylic acid with one of the major compounds as a new treatment against multidrug resistant *Pseudomonas aeruginosa.*

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CONFLICT OF INTEREST

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

CREDIT AUTHOR STATEMENT

Fatima Zohra GUELLOUMA: Conceptualization, Methodology, Writing Original draft preparation. **Hadjer BOUSSOUSSA**: Supervision. **Ihsene KHACHBA**: Visualization. **Mohamed YOUSFI:** Validation, **Ibtissem ZIANE KHOUDJA**: Technical support, **Ibrahim BOURAHLA**: Provide assistance.

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