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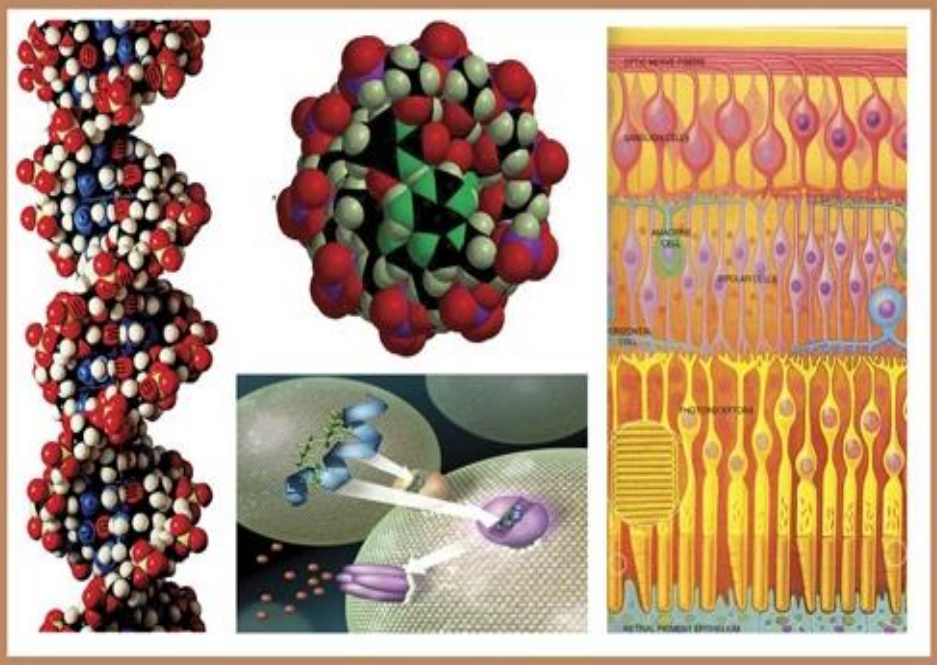

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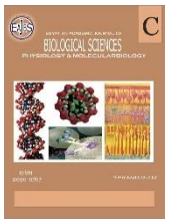

Contribution To the Evaluation of The Antimicrobial Activity of Essential Oils Extracted from *Mentha Pulegium* and *Laurus Nobilis*

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ABSTRACT

The extensive use of antibacterial agents in human medication has led to the appearance of resistant microbial strains, hence the importance of directing research towards new molecules, where a good part of scientific research is currently directed towards the way of the use of active biological extracts of medicinal plants, in particular towards essential oils.

In this context, the objective of this work is to evaluate the antibacterial activity of essential oils extracted from *Mentha pulegium* and *Laurus Nobilis* on some pathogenic bacterial species such as *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*. The extraction of essential oils from aromatic plants: *Mentha pulegium*, *Laurus Nobilis* is carried out by the hydrodistillation technique.

The results reveal that *Mentha pulegium* and *Laurus Nobilis* show a yield of 2.7% and 0.79% respectively. Our results indicate that the two essential oils *Mentha pulegium* and *Laurus Nobilis* have very low activity against *Escherichia coli*, *Staphylococci aureus*, *Pseudomonas aeruginosa*, which represent inhibition diameters of 6 mm; 4mm and 2mm respectively. The bacterium *Pseudomonas aeruginosa*is the most resistant to the two essential oils extracted from *Mentha pulegium*, *Laurus Nobilis*.

INTRODUCTION

Algeria, by its geographical location, offers rich and diverse vegetation. A large number of aromatic plants grow there spontaneously. The main species listed are thyme, mentha, lavender, germander, Laurus and rosemary. Interest in these aromatic and medicinal plants has continued to grow in recent years. The essential oils and extracts of these plants have found their place in the preservation of food and the protection of stored foodstuffs. Their use is linked to their broad spectra of recognized biological activities (Meriga*et al.*, 2012; Pandini *et al.*, 2015; Aghraz *et al.*, 2017; Ben Othman *et al.*, 2017; SritiEljazi *et al.*, 2018).In addition, the extensive use of antibacterial agents in human medication has led to the appearance of resistant microbial strains, hence the importance of directing research toward new molecules, where a good part of scientific research is focused on. Currently directed toward the way of the use of active biological extracts of aromatic and medicinal plants, in particular towards essential oils (Essawi T, &Srour M, 2000).

According to the World Health Organization (WHO), 75 to 95% of rural populations (particularly in developing countries) use traditional medicine made largely from plants (WHO, 2003). It has been proven that approximately 20% of plant species in the world have therapeutic or cosmetic virtues since they contain molecules or active principles with different biological properties, which find their application in various fields (medicine, pharmacy, cosmetology and agriculture, etc.) (Suffredini, 2004)

Algeria is one of the countries of the Mediterranean basin richest in phylogenetic resources of aromatic and medicinal interest, given the diversity of its bioclimatic stages. There are more than 300 species for therapeutic or aromatic use among the 3,150 plant species in our country (Morales. 2002). Currently, aromatic and medicinal plants have a considerable advantage thanks to the progressive discovery of the applications of their essential oils and their extracts in medicine and in other fields of economic interest. Essential oils for example, also called volatile odoriferous oils, are aromatic oily liquids extracted from different parts of plants; leaves, bark, flowers, buds, seeds, etc. (Tongnuanchan P, &Benjakul S. 2014.). These natural substances rich in antimicrobial compounds and antioxidants can be used to solve different problems.

Pennyroyal, known by the vernacular Arabic name “fliyou”, is widely used in folk medicine in many cultures [Agnihotri, 2005]. The flowering aerial parts of this plant are traditionally used for their antimicrobial, expectorant, carminative and antispasmodic properties in the treatment of colds, bronchitis, tuberculosis, sinusitis, cholera, food poisoning, flatulence and intestinal colic (Zargari,1990).

Bay laurel (*Laurus Nobilis* L., Lauraceae) is an endemic species of the Mediterranean region, which is grown in many countries with a temperate subtropical climate. From the lauraceae family, *Laurus Nobilis* is one of the spontaneous plants of the Mediterranean region, it is found in the form of a tree or a rustic shrub with evergreen foliage. It is covered with small cream flowers in spring. It is found in rich and moist but well-drained soils, in a sunny orientation and protected from the wind. Coastal regions suit it very well such as the North or the Eastern regions of Europe. It is mainly cultivated for its condiment and medicinal characteristics (HaddouchiF. et Benmansour A, 2008). The main objective of this study is to evaluate the antibacterial activity of essential oils of *Mentha pulegium* and *Laurus Nobilis* via pathogenic bacteria such as *Escherichia coli*. *Staphylococcus aureus*. *Pseudomonas aeruginosa*.

MATERIALS AND METHODS

Biological Materials:

This present work is focused on two aromatic medicinal plants which are *Mentha pulegium* and *Laurus Nobilis*. *Collection of plant material: the sample of pennyroyal was collected in the region of Saïda in the month of April, and the laurel was collected in the region of Dhaya in SBA in the same period. Workplace: nutrition laboratory at the Faculty of Nature and Life Sciences of the University of Djilali Liabes Sidi Bel Abbes.

Table 1: Presentation of plants used for the extraction of essential oils (EOS).

Botanical name	Common name	Family	Organs used
Mentha pulegium L. (MP)	Arabe: Fliou. Frensh : *Menthe Pouliot*.	Lamiaceae	aerial parts (leaves, flowers and stems)
Laurus Nobilis L.	Arabe : Rende. Frensh: Laurier.	Lauracee.	leaves

Method of Obtaining EOS:

The extraction of essential oils from these plants is carried out by the technique of hydro-distillation under well-defined conditions.

EOS Analysis:

Quantitative Analysis:

Yield: The yield of essential oil EO corresponds to the ratio between the mass of EOS (m EOS) obtained and the mass of plant material (m MV) used for the extraction (AFNOR, 2000)**.**

YEO (%) =m/m0 X 100 (Chama*et al.*, 2020).

YEO: Essential oil yield in %.

m: Mass of essential oil in grams.

m0: Mass of fresh plant material used in grams.

Qualitative Analyses:

Organoleptic Control:

*Consists of controlling the organoleptic characteristics of the EO obtained: smell; color; and appearance. These indications make it possible to assess the quality of the oil studied (Vahid *et al.*, 2013).

Study of Physico-Chemical Properties:

In order to determine the quality of our EO, we have determined a certain number of Physicochemical characteristics.

The Relative Density:

The density of an oil is the ratio of the mass of a certain volume of oil at 20°C (Chama *et al.*, 2020). D= m /m0

Where: m: the mass in grams of the oil, m0: the mass in grams of distilled water

The Refractive Index:

Using the Abbe refractometer, the refractive index of EO is measured at a temperature of 21°C (indicated by the built-in thermometer of the device). To do this, place a drop of EO on the prism of the refractometer, then make the adjustment using the micro screw and read the value. The refractive index of the essential oil is given by the following formula

[n]°D = nDt' + 0,00045(t'-t).

With:

nDt': Measured refractive index.

t: Reference temperature which is 20°C.

t': Temperature at the time of measurement.

Methods for the Detection Of Bacterial Strains:

***Escherichia coli*:**

The search for *Escherichia coli* was based on direct invasive methods, requiring cytobacteriological examination of urine.

The collection of a urinary quantity was carried out in hospitalized patients contaminated because of infections associated with "nosocomial" care.

The urine of the patients is collected in sterile pots and passed through microscopic examination in the urology department

Isolation:

Isolation is performed on Mac Conkey medium. This medium (selective) is used for the culture of *Enterobacteriaceae*, using a Pasteur pipette inoculated with 2 drops of urine in this medium. Incubation is carried out at 37°C for 18 to 24 hours. After a morphological reading, isolated colonies are obtained on MacConkey medium.

***Staphylococcus aureus*:**

The search for *Staphylococcus aureus* was in infected skin regions, especially in newborns The study was carried out by taking a sample of this strain from the infected part of the patient Sampling is carried out at the level of the skin by a skin biopsy examination in the dermatology department at the hospital.

Isolation:

Isolation is performed on Chapman medium. This medium (selective) is used for the culture of Gram-positive cocci to have pure cultures of Staphylococcus, using a Pasteur pipette inoculated with 2 drops of the sample in this medium. Incubation is carried out at 37°C for 18 to 24 hours. After a morphological reading, isolated colonies are obtained on the Chapman medium.

Pseudomonas aeruginosa:

The search for *Pseudomonas aeroginosa* was based on a direct method consisting of taking an eye sample from the eye of a patient with a corneal ulcer. The sample is taken by scraping the edges of the corneal ulceration with a vaccinostyle after

local anesthesia. In this case, there are standardized sampling kits including all the tools necessary for scraping and sowing. If wearing lenses, we also analyze the lenses as well as the case and the preservation liquid. Patients who have undergone this sampling are at the level of the ophthalmology department and the ophthalmologist who will take the sample and inoculate the culture media.

Isolation:

Isolation is performed on the ordinary medium (BCP). This medium (selective) is used for the culture of Pseudomonas to have pure cultures, using a Pasteur pipette inoculated with 2 drops of the sample in this medium. Incubation is carried out at 30°C for 18 to 24 hours. After a morphological reading, isolated colonies are obtained on the BCP medium.

Identification of Bacterial Strains:

Identification is based on the determination of morphological (macroscopic, microscopic) and biochemical characters.

Macroscopic Examination: This examination aims to determine the color, shape and appearance of the colonies.

Microscopic Examination: It can be performed without staining the sample by direct observation (examination in the fresh state), or after staining the sample (Gram stain).

Biochemical tests, Catalase research, Catalase is an enzyme found in most strict aerobic and optional anaerobic bacteria (chama *et al.*, 2021 urine), (chama *et al.*, thymus,2020). A colony is suspended with a drop of hydrogen peroxide on a clean slide. The appearance of bubbles which corresponds to the released oxygen indicates the presence of catalase.

Search For Oxidase: The enzyme sought is phenylene-diamine-oxidase (chama *et al.*, 2021 urine), The search for this enzyme consists in depositing in a hemolysis tube, an "Ox" disc and soaking it with a drop of distilled water. Then take part of the colony to be studied and spread it on the disk. After about 10 minutes a dark purple coloration appears on the disc and then turns black: oxidase test + (chama *et al.*, 2021 honey)

Evaluation of the Antibacterial Activity of Eos:

Aromatogram Method: The disc diffusion method, also called Vincent's method or the aromatogram technique developed by Schroeder and Messing in 1949. This examination is done in the same way as an antibiogram where the antibiotics are replaced by aromatic essences, previously selected and recognized (Bachiri,2016). The antibacterial activity is evaluated by the aromatogram method, which makes it possible to determine the sensitivity of the different bacterial species to the essential oils used (chama *et al.*,2022). In this method, sterilized 6 mm filter paper discs are saturated with a filtered sterilized plant extract of the desired concentration. The impregnated discs are then placed on the surface of a suitable solid agar medium such as Mueller Hinton. Media were pre-inoculated with test organisms. The standard inoculum size is 1x108 CFU/ml of bacteria for the inoculation diffusion plates which is equal to the McFarland turbidity standard of 0.5.

Preparation of the Different Concentrations of EOS:

It is a question of preparing a stock solution of pure tween 80 to make the different essential oils. The recommended technique is to dilute pure tween 80 in 90ml of distilled water; this solution will be sterilized at 120°C for 15 min. In a volume of 9 ml of this solution, we aseptically add 1 ml of HE, then after vortexing the contents, in order to obtain a well-homogenized solution, we will carry out successive dilutions, ranging from -1 to -6. We do the same for the second EO.

Preparation of the Inoculum:

It was prepared from a young 24-hour culture. For this, bacterial suspensions were made by taking 3 to 5 well-isolated colonies, which were deposited in 10 ml of distilled water. Then, we ensured a good agitation. Then incubated at 37°C for 24 hours. After incubation, centrifugation of

this bacterial suspension was performed (chama *et al.*, 2022 hindi)

Technical: Dip a sterile swab into the supernatant of the incubated bacterial suspension. Rub the swab over the entire M-H agar surface. *Finish inoculation by passing the swab over the agar peripheral. Let dry at room temperature for 15min. *Squeeze each sterile HE disk with sterile forceps on the medium and do not move the disks after application. The incubation was done at 37°C for 24 or 48.

Reading results: The measurement of the diameter of the zones of inhibition is transcribed in different activity symbols (Ponce *et al.*, 2003).

Table 2: Transcript of inhibition diameters of permeate discs.

Inhibition zone diameters (mm)	Transcription	Germ sensitivity
<8	-	Resistant.
9 – 14	+	Sensible.
15 – 19	++	Very sensitive.
>20	+++	Extremely sensitive.

RESULTS

Quantitative Analysis:

Calculation of EO Yield:

The EO yield of *Mentha pulegium* is 2.7%

The EO yield of *Laurus Nobilis* is 0.79%.

Qualitative Analyzes:

Organoleptic Control: The results of the Organoleptic characteristics of EOs are presented in the following Table 3.

Table 3: Organolepticcharacteristics of EOs

Essentielle oil	Odour	Aspect	color
Mentha pulegium	Gives off a strong characteristic mint odor.	Liqudcrystalal clear.	Pale yellow
Laurus Nobilis	Strong spicy.	. Moving liquid.	Light yellow

Study of Physico-Chemicalproperties:

Determination of Relative Density:

The relative density of *Mentha pulegium* is 0.0053 g/ml.

The relative density of *Laurus Nobilis* is 0.0013 g/ml.

Determination of the Refractive Index:

1-*Mentha pulegium.*

t: Temperature at the time of measurement: 21°C.

[n] °D: Refractive index: 1.465

2- *Laurus Nobilis*

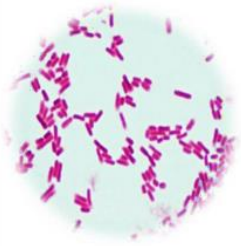
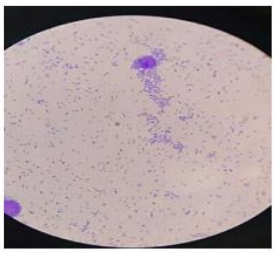
t: Temperature at the time of measurement: 21°C

[n] °D: Refractive index: 1.4909.

Results of Identification of Bacterial Strains:

ϖ Macroscopic and microscopic Examination: The results of the microscopic and macroscopic aspects are presented in the following Table 4.

Table 4: Results of the micro and macroscopic appearance of the bacterial strains.

Bacteria	***E. coli***	***P. aeruginosa***	***S. aureus***
Macroscopic Appearance	Smooth round colonies.	Fine Green Colonies.	Colonies large round regular Domed smooth Brilliant surrounded by a yellow halo
Microscopic Appearance	Mobile, coccobacilli, Gram-. 	Very mobile, bacilli Gram-fine. 	Immobile cocci in aman, Gram+. 

Biochemical Tests:

The results of biochemical tests are presented in the following Table 5.

Table 5: Demonstration of the production of oxidase and catalase in the three strains.

Biochemical tests	***E. coli***	***P.aeruginosa***	***S.aureus***
Oxidase	-	+	-
Catalase	-	+	+

Aromatogram Results:

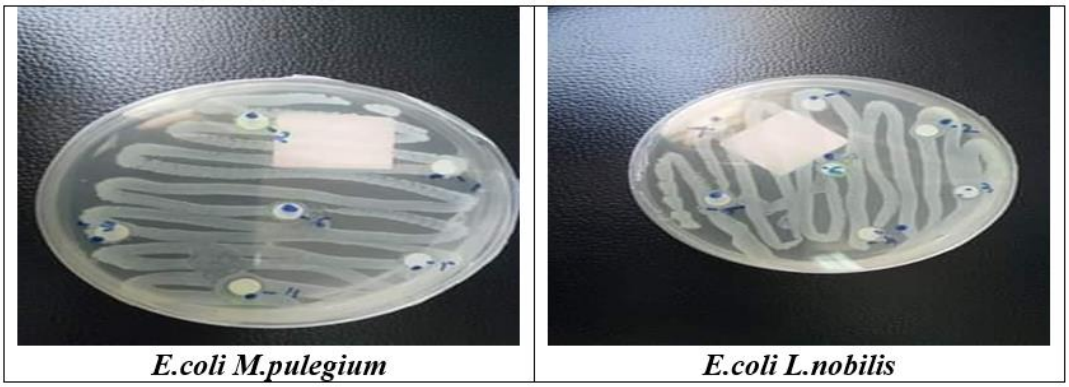


Fig. 1: Inhibitory effect of EOs on *E. coli.*

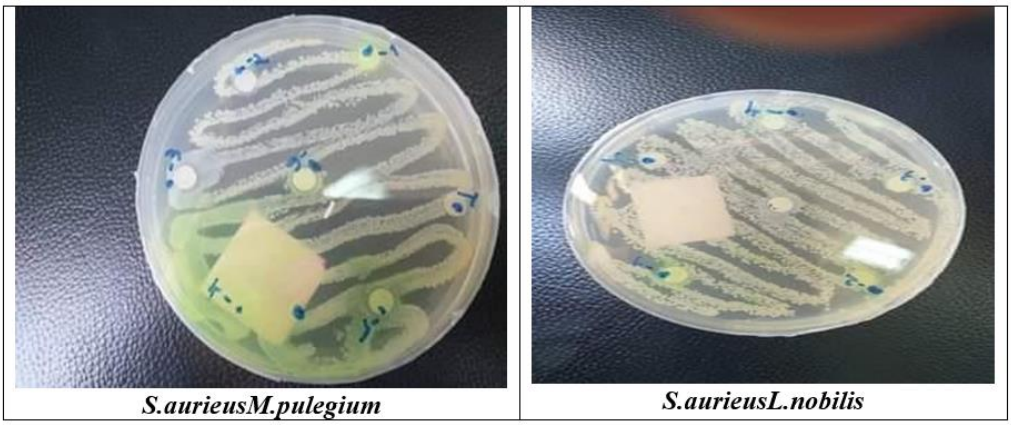


Fig. 2: Inhibitory effect of EOs on *S.aurieus*.

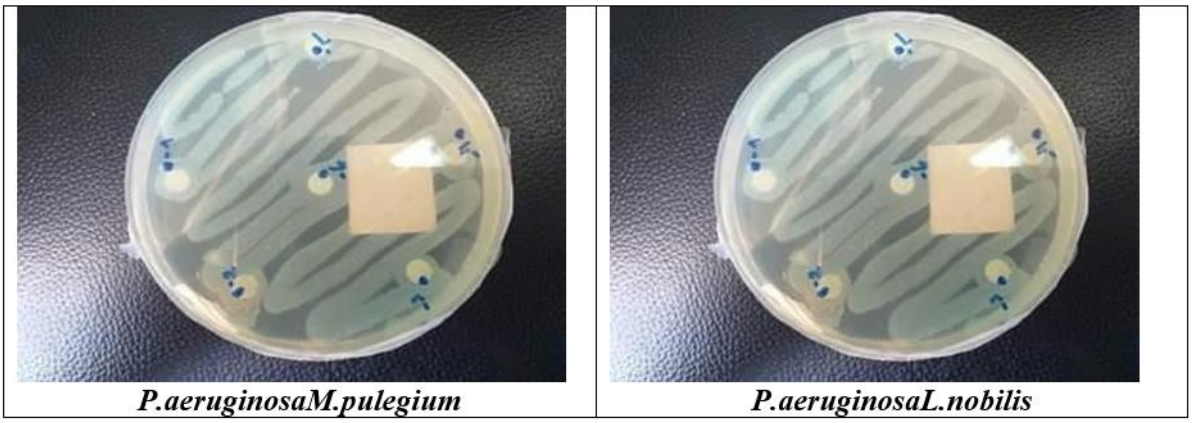


Fig. 3: Inhibitory effect of EOs on *P.aeruginosa*

Table 6: Antibacterial activity of *Mentha pulegium* on the 3 strains in the presence of different concentrations.

Strain tested	Stock solution	-1	-2	-3	-4	-5	-6
E.coli	6mm	5mm	4mm	3mm	2mm	1mm	1mm
S.aurieus	5mm	4mm	3mm	3mm	2mm	1mm	0mm
P.aeruginosa	3mm	2mm	2mm	1mm	1mm	0mm	0mm

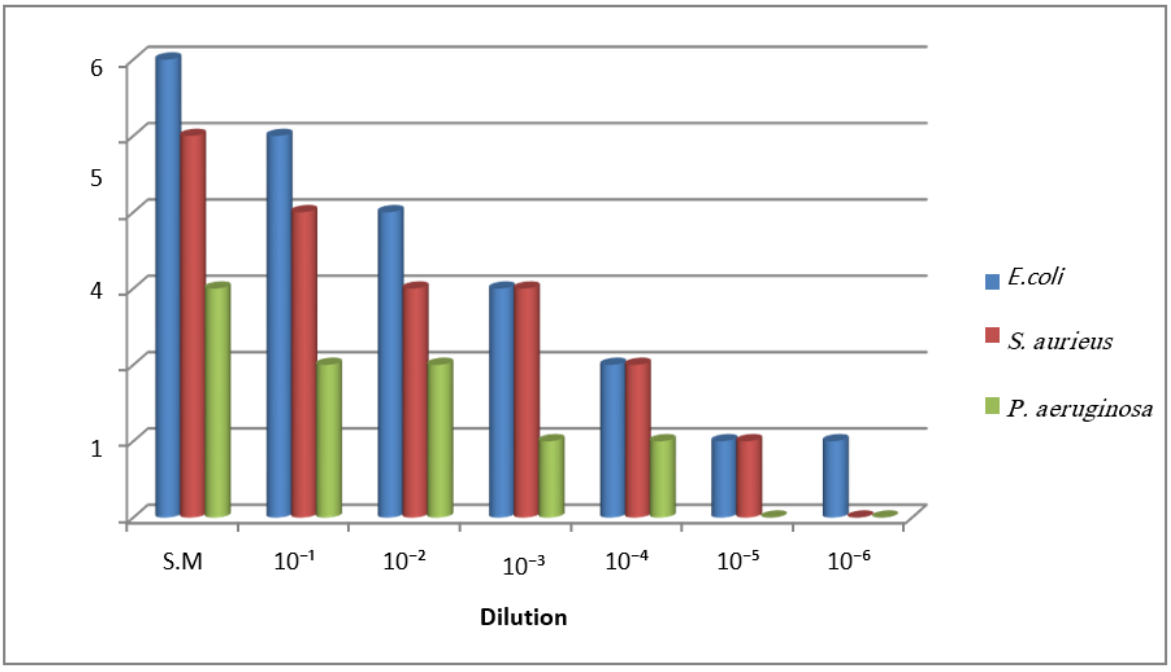


Fig. 4: Graph represents the antibacterial activity of *Mentha pulegium* on the 3 strains in the presence of different concentrations.

Table 7: Antibacterial activity of *Laurus Nobilis* on the 3 strains in the presence of different concentrations

Strain tested.	Stock solution	-1	-2	-3	-4	-5	-6
E. coli	4mm	4mm	3mm	2mm	1mm	1mm	0mm
S. aurieus	3mm	2mm	2mm	1mm	1mm	0mm	0mm
P. aeruginosa	2mm	1mm	1mm	1mm	0mm	0mm	0mm

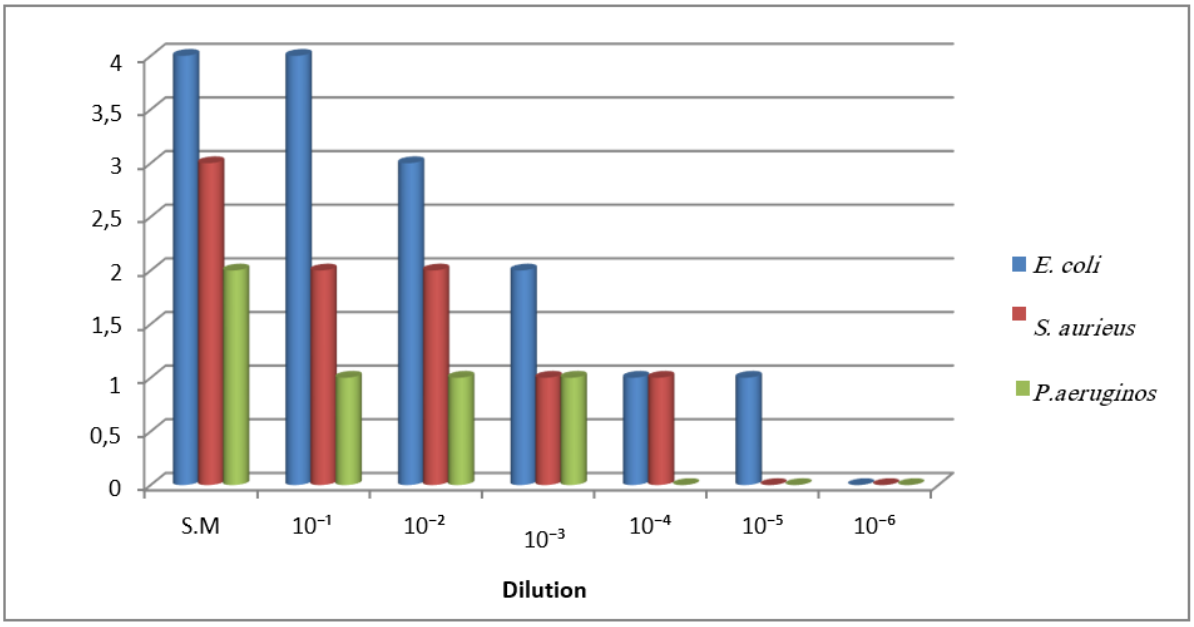


Fig. 5: Graph represents the antibacterial activity of *Laurus Nobilis* on the 3 strains in the presence of different concentrations.

Antibiogram Results:

Our results show that the three bacterial strains tested are resistant to EOs of *Mentha pulegium* L and *Laurus Nobilis*. Table 8.

Table 8: Transcription results of the inhibition diameters of the impregnated discs.

Essential Oil	*E. coli*	*S. aurieus*	*P. aeruginosa*
Mentha pulegium.	Resistant.	Resistant.	Resistant.
Laurus Nobilis.	Resistant.	Resistant.	Resistant.

DISCUSSION

The yield stated by the literature for *Laurus Nobilis* L. varies from 0.63 to 0.7% (Guedouari, 2012). By carrying out the experimental plan relating to this plant, we noticed that obtaining a yield which is 0.79% is possible, for the yield of *Mentha pulegium* varies from 1.2 to 2.7, our results reveal a yield which is 2.7% which shows the interest of this study. These results are different from those reported in other regions of Algeria. According to the results cited in the scientific literature, hydrodistillation remains the most coveted EO extraction method in current practice. (Goudjil *et al.*, 2015). The study of the physicochemical characteristics of our EOs made it possible to highlight their compliance with established standards, it is distinguished by a relative density, and a refractive index, globally comparable to those given by the French pharmacopoeia. It should however be noted that the chemical composition of the essential oils of a plant depends on several factors such as the geographical origin, the harvest period, the place of drying, the temperature and the duration of drying and the method of extraction (Goudjil, 2016°. Essential oils are usually liquid at room temperature and volatile, note that EOs are more or less colored and their density is generally lower than that of water. The refractive index of our essential oil is 1.4909 (*Laurus Nobilis*), and 1.465 for *Mentha pulegium*. They are normative according to French standards for essential oils. This index depends on the chemical composition which increases according to the lengths of the acid chains, their degrees of establishment and the temperature. The results show that the three strains are essential oils of *Mentha pulegium* L and *Laurus Nobilis*. We can deduce that

our extracts have low antibacterial power on the bacterial strains tested. Expressed by diameters of the inhibition zones between 0 and 6 mm and which do not agree with reports of the antibacterial activity of pennyroyal essential oil; Benabdallah (2008) describes diameters of inhibition of the essential oil of the Algerian pennyroyal between 10 and 22 mm, the largest diameter of which concerned *E. coli*. However, the antibacterial activity of essential oils rich in pulegone has been reported. Hajlaoui *et al.* (2009) showed that the oil essence of *M. pulegium* from Tunisia showed great antibacterial activity with inhibition diameters of 10 to 31 mm higher than our results. According to Ait-Ouazzou*et al.*, (2012), the essential oil generated diameters inhibition of 12.6 ± 0.5 mm against a range of bacteria, while *Pseudomonas aeruginosa* was resistant to it. Mahboubi M. &Haghi G., (2008) noted good antibacterial activity with inhibition diameters and values of the order of 8-21 mm for the EOS of *M. pulegium* L. from Iran with piperitone/piperitenone chemotype.

However, this oil has no activity on *Escherichia coli*. Regarding the antibacterial activity, it can be attributed to the high content of the essential oil in an oxygenated compound: piperitone. Indeed, this activity always concerns the major compounds of the essential oil, involving either a high concentration of piperitone and the synergistic effects of the other constituents (Mahboubi M. &Haghi G, 2008), or a high content of pulegone (Hajlaoui *et al.*, 2009). Whatever the case, in general, oxygenated monoterpenes, which are significantly more active than hydrocarbon monoterpenes (Carson C.F. & Riley T.V., 1995) are usually present in significant concentrations in the essential oils of M. pulegium L. According to the results obtained, it is not possible to conclude that the essential oil of M. pulegium has a spectrum of antibacterial activity because this antibacterial power is subject to several factors and conditions. Regarding the activity of the *Laurus Nobilis* plant essential oil, the oil reacted negatively to the microbial strains tested. There is a small difference in the diameters of the inhibition zones obtained, ranging from 1 to 6 mm.

The antibacterial action of our oil can be attributed to its richness in three main compounds (Davanone, Camphor and Thujone) which have been reported for their antibacterial power against several bacterial strains tested. The combined action (synergy) of different compounds at the origin of this extract can explain the variation in results between the same species from different regions of the world. According to Oussou, Kanko, these molecules act most often by a synergistic action, either alone or with minor compounds that can contribute significantly to the activity of essential oils. (Denis F., Poly M.C. 2007). It is known in the literature that Gram-positive bacteria are more sensitive to essential oils and plant extracts than Gram-negative bacteria (Karaman I, 2003). This is due to structural differences in their outer membranes (Inouye S, 2001), These chemical compounds exert their antimicrobial activity on microorganisms by disrupting membrane integrity. (Knobloch *et al.*, 1989). The penetration of active compounds present in EOS is therefore different (Kheyer et al., 2014). In Gram-bacteria, the outer membrane constitutes an effective permeability barrier, rich in lipopolysaccharides whose negative surface charges prevent the diffusion of hydrophobic molecules (Nikaido H., 2003), however, some low molecular weight phenolic compounds can adhere to these bacteria by binding to membrane proteins and lipopolysaccharides using their functional groups and sneaking up to the more vulnerable inner membrane (Dorman HJD. et Deans SG., 2000). In other words, hydrophobic compounds are able to disrupt the plasma membrane and the outer membrane of Gram-bacteria by inducing its permeability and cell death (Wang *et al.*,2012). Gram-positive bacteria are less protected against antibacterial agents since

the peptidoglycan only hinders the diffusion of molecules larger than 50 kDa (Nikaido H. &Vaara M., 1985). Interest in the scientific study of the therapeutic power of medicinal plants has steadily increased in recent years with the aim of seeking alternatives to chemical substances, which pose risks to human health and the environment. In this context, we tried to evaluate in vitro the antibacterial activity of essential oils extracted from the *Mentha pulegium* L plant and the *Laurus Nobilis* plant used in traditional medicine in Algeria.

CONCLUSION

Interest in the scientific study of the therapeutic power of medicinal plants has steadily increased in recent years with the aim of seeking alternatives to chemical substances, which pose risks to human health and the environment. In this context, we tried to evaluate in vitro the antibacterial activity of essential oils extracted from the *Mentha pulegium* L plant and the *Laurus Nobilis* plant used in traditional medicine in Algeria. The oil is extracted by the hydrodistillation method. Quantitative analyzes of these oils showed a yield of 2.7% for *Mentha pulegium* and 0.79% for *Laurus Nobilis*. The study of the physicochemical characteristics of our EOs made it possible to highlight their compliance with established standards, it is distinguished by a relative density, and a refractive index, globally comparable to those given by the French pharmacopoeia. Regarding the antibacterial potency by the disc diffusion method of the EOs of *Mentha pulegium* L and *Laurus Nobilis*, our results show that the three bacterial strains tested are resistant to EOs of *Mentha pulegium* L and *Laurus Nobilis*. We can deduce that our extracts have a very low antibacterial power on the bacterial strains tested. Expressed by diameters of the inhibition zones between 0 and 6 mm. These results remain preliminary and require in-depth complementary studies at different levels of the approach through a fine and thorough characterization of these essential oils by other techniques such as GC/SM or HPLC/SM in order to establish a structure-activity relationship. The antimicrobial activity must be evaluated in other in vitro systems (cellular and enzymatic) as well as in vivo in order to better understand the molecular interactions of these oils with respect to their targets. However, further research is needed to assess the effectiveness of the extracts studied:

-Evaluate and test the different molecules isolated in vivo on different biological models with a view to using them for therapeutic purposes and for the preservation of products intended for consumption.

-The isolation and characterization of active compounds in EOs in order to identify the different molecules responsible for the different biological activities of these plants,

-Develop plant-based products that can be an alternative to the use of synthetic products to fight against pathogens.

-Exploit the antimicrobial power in the pharmaceutical industry to enrich the therapeutic arsenal. In the end, the results obtained as well as the perspectives proposed will make it possible to open new paths in the therapeutic field.

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