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Original Research

GC/MS Analysis, Antimicrobial, Fungicidal Activities and Toxicity of *Rosmarinus Officinalis* Essential Oil Plant from Algeria

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Abstract

Rosmarinus officinalis (family Lamiaceae), a spontaneous aromatic plant widespread in Algeria and widely used in folk medicine, is also known as "Eklil". Our work focuses on the study of the biological activities of the essential oil of *R. officinalis*. Its chemical composition was studied by GC/MS. Twenty one compounds were identified: The major compounds were: 1.8cineole (32.58%), Sabinene (15.92%), and Camphene (14.41%). The results show that the essential oil of *R. officinalis* has strong antimicrobial activity against strains tested, either bacterial (*Klebsiella pneumoniae, Staphylococcus aureus,* and *Streptococcus pneumoniae*) or fungal (*Penicillium expansum* and *Alternaria alternata*). In addition, we evaluated the toxicity induced by the essential oil of *R. officinalis* in a model of *Drosophila melanogaster*. Exposure of adult flies to the essential oil via a fumigation method resulted in a mortality-concentration relationship. The result of this study indicated that the values LC25, LC50, and LC90 of the EO of leaves obtained after 24 h were 50.91, 81.32, and 207.42 µL/Lair, respectively. In light of our findings, attention is drawn to the medicinal use of the plant. In addition, there is potential bio-insecticidal activity.

Keywords: *Rosmarinus officinalis*, Essential oil, Antibacterial activity, Antifungal activity, *Drosophila melanogaster*

Introduction

A number of issues are currently being raised regarding the efficacy and safety of chemicals used in medicine or the agri-food industry [1, 2]. Indeed, the development of resistance in microorganisms to various antibiotics is of concern to specialists [3]. On the other hand, the use of additives such as antioxidants is suspected to have negative effects on the health of the consumer [4]. The development of new therapeutic agents is essential to combating the phenomena of bacterial resistance and the oxidation of food [5]. Thus, essential oils are beginning to have a lot of interest as a potential source of bioactive natural molecules [6, 7] and are being studied for their possible use as an alternative for the treatment of infectious diseases [8] and for the protection of food against oxidation [9]. They are important raw materials for perfumery, cosmetics, and the flavoring industry [10].

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These substances are also used in the pharmaceutical industry, both as sources of active substances and for the flavoring of various products [11, 12]. Aromatic plant sources of essential oils are widespread in nature. Algeria is home to a host of important and varied species and thus testifies to an undeniable floristic wealth [13]**.** *R. officinalis* L. (rosemary) belongs to the Lamiaceae family, which represents one of the most important families of medicinal plants in Algeria [14], and grows spontaneously in the mountains of the Tebessa region (north-eastern Algeria) [15]. *R. officinalis* is the subject of recent research in the pharmaceutical, cosmetic, and food fields [16], appreciated for its aromatic, antioxidant, antimicrobial, antispasmodic, and anti-tumor properties, and widely used in traditional medicine [13]**.** Another objective of the study concerns the potential for insecticides in the essential oil of *R. officinalis.* Flies are considered insect pests, and among these species are *Drosophila melanogaster* (fruit flies), which threaten many economically important fruits [17]. In addition, fruit flies are carriers of pathogenic microbes posing risks of infection in humans [18], so it has become necessary to seek an environmentally friendly strategy to target these insect pests [6]. This study investigated the evaluation of the biological activity of essential oil from a spontaneous plant grown in the region of Tebessa (north-eastern Algeria). *R. officinalis*: In addition to the valuation of the antifungal and antibacterial effects, we also investigated the insecticidal activity of this oil against *D. melanogaster.*

Materials and Methods

Plant Materials and Extraction of Essential Oils

Fresh aerial parts of *R. officinalis* were harvested in March 2022 from the Boulhaf AL-Dairin Tebessa (south-eastern Algeria) (35°22′21.34 N, 8°14′7.45″ E, and 891m altitude). The plant was dried for two weeks in the dark. Once dried, the plant material was ground and then kept until its use. The dried plant material is subjected to hydrodistillation in a Clevenger (50 g of the plant with 500mL of distilled water). The oil was kept in well-sealed, opaque bottles and kept at a low temperature (4-5 Cº). The extraction process takes three hours from the onset of boiling [19]. The yield is calculated from the weight of the essential oil in relation to the dry weight of the vegetable mass used in the hydrodistillation, either: **Y = EOm/Dvm.100**

Y: EO yield (%)/EOm: essential oil mass/Dvm: dry vegetable mass

Gas Chromatography-Mass Spectrometry

The essential oil of *R. officinalis* obtained was analyzed by gas chromatography coupled with mass spectrometry (PERKIN Elmer/Turbo mass type GC) as previously described [20]. The essential oil is diluted by ethanol for chromatographic analysis at 10%. The temperature of the device was programmed to 60°C for 2 minutes, then it rose to a crescendo (3°C per minute) to 200°C, where it stabilized for 4 minutes. The scan is done for an hour to get all the compounds. The injection is made when the temperature of 200°C is reached by a "SPLIT" mode at a rate of 0.3 µL of diluted essential oil.

Biological Materials

The microbial support consists of *Staphylococcus aureus* ATTC 25923 (Gram+), *Klebsiella pneumoniae* ATTC13883 (Gram-), and *Streptococcus pneumoniae* ATTC 49619 (Gram+). The fungal strains tested came from the microbiology laboratory of Tebessa University, Algeria. (*Penicillium expansum* and *Alternaria alternate)* isolated from apple rotare are identified by a microbiologist Dr.Fenghour H, from Tebessa University (Table 1).

Determination of the Antibacterial Activity of the Essential Oil

Aromatogram Test (Agar Diffusion Method)

A 0.5 McFarland bacterial suspension is prepared from a pure and young culture of 18 hours. It should be noted on the one hand that the adjusted suspension should contain 10^8 UFC/mL (units forming colony/mL) and on the other hand that the inoculum thus prepared should not be used beyond 15 minutes. Seeding is done by swabbing from the freshly prepared inoculum. 2 boxes for each dilution plus 2 boxes for positive contains neither pure essential oil nor DMSO) and negative controls (contains DMSO). 6mm diameter chromatographic paper discs,

Table 1. Origin of the different microbial strains tested

Microbial strains		Family	Nature of the levies
GRAM$^+$ bacteria	*Staphylococcus aureus*	Staphylococcaceae	pulmonary
GRAM$^-$ bacteria	*Klebsiella pneumoniae*	Enterobacteriaceae	pulmonary
	Streptococcus pneumoniae	Sterptococcaceae	pulmonary
Fungi	*Penicillium expansum* (link 1809)	Trichocomaceae	Apple rot
	Alternaria alternata (Keissl,1912)	Pleosporaceae	Apple rot

previously sterilized, are deposited on the seeded Muller Hinton agar surface after being loaded with 15µL of essential oil diluted in DMSO (dimethylsulfoxide) at 1/2, 1/4, 1/8, and 1/16 (v/v). Other discs loaded with 15µL of DMSO are used as negative controls. Antibiograms are performed in parallel with aromatograms [21]. After 24 hours of incubation at 37°C, the inhibition diameter is measured.

Determination of Growth MICs

Based on the previous screening essential oil of *R. officinallis* was identified as having potent antibacterial activity, and its Minimum Inhibitory Concentration (MIC) was determined. The agar dilution method recommended by the National Committee for Clinical Laboratory Standards [21] was used with the following modification: a final concentration of 0.5% (v/v) Tween-20 (Sigma) was incorporated into the agar medium to enhance oil solubility. A series of two-fold dilutions of each oil, ranging from 0.2 to 25.6 mg/ml, was prepared in Muellur Hinton agar at 48°C. Plates were dried at room temperature for 30 min prior to spot inoculation with 3µL aliquots of culture containing approximately 10^5 CFU/mL of each bacteria. Inoculated plates were incubated at 37°C for 18 h. and the MIC was determined. Experiments were carried out in triplicate. Inhibition of bacterial growth in the plates containing test oil was judged by comparison with growth in blank control plates. The MICs were determined as the lowest concentration of oil inhibiting the visible growth of each organism on the agar plate [22].

Minimum Bactericidal Concentration (BMC*)*

The Bactericidal Minimum Concentration (BMC) corresponds to the lowest essential oil concentration capable of killing more than 99.9% of the initial bacterial inoculum (less than 0.01% of survivors). The lowest concentration leaves only 0.01% of the surviving bacterial strains after 24 hours of oil exposure corresponds to BMC [23]**.** The nutrient agar poured in Petri dishes is seeded in streaks by 100µL of the contents of the wells having the minimum inhibitory concentration and then having a concentration higher than the IJC. BMC is determined after 24-hour incubation at 37°C.

Antifungal Activity of Essential Oils

Solid Medium Dilution Technique

This technique was applied to 9mm Petri dishes to determine growth rates and inhibition rates. 1000mL of Potato Dextrose Agar (PDA) have been prepared, sterilized, and preserved. All vials containing 50 mL of PDA, 1.5mL of tween 20, and different volumes of essential oil are added to prepare dilutions of 1, 0.5, 0.25, 0.05, and 0.01%. Each bottle is homogenized instantly by stirring, and then its contents are poured into Petri dishes. The mixture was thus poured and left to rest until cooling and solidification. Using a sterile platinum loop, 6mm diameter mycelial discs from the young (one-day) fungus culture were inoculated. The latter were deposited in the media previously prepared with dilutions of essential oils. Petri dishes (controls and tests) are incubated at 28°C for 7 days, respectively. On a daily basis, the growth of filaments on each can is recorded, and at the end of the appropriate incubation time, the diameters of different colonies of filamentous fungi are measured to calculate the inhibition rate (I%) [24].

I (%) =100 x (dC-dE)/dC

I(%) = Inhibition rate expressed as a percentage

dC = Colony diameter in "positive control" boxes

dE = Colony diameter in boxes containing the plant extract

Toxicity Assay

Essential oil was fumigation test of *R. officinalis* by shock and mortality methods in adults of *D. melanogaster* according to the method described by Grabsi et al., 2023 [25]. Several concentrations of 40 to 300µL of essential oil diluted in 1mL of ethanol and applied to Whatman paper 2 cm in diameter in glass vials. The lid was closed after 20 adult *D. melanogaster* flies were released for 1 hour and then transferred to vials without treatment. Mortality was recorded after 1 hour and 24 hours after exposure to the treatment, then corrected according to Abbott's formula (1925), which calculates the Sublethal and Lethal Concentrations (LC10, LC25, LC50, and LC90) with 95% confidence intervals (95% LC) using GRAPH PAD prism9 software. In parallel, Vials without essential oil were tested as negative controls (individuals do not undergo any treatment), and 1 mL of ethanol was used for positive controls adutes and received 1 mL of ethanol and are carried out in parallel. Each treatment were carried out five times. Flies were seen dead when they did not move in response to flask shaking.

Results

Yield and Chemical Composition of Essential Oils

The essential oil yield of the aerial part of *R. officinalis* is of the order of 1.42±0.02%, with a yellow color, a liquid appearance, and a pleasant odor. The results obtained by the chromatographic analysis (GC/MS) of rosemary obtained from the Boulhaf AL-Dair study station indicate that the essential oil of the sample contains 20 chemical components. Moreover, the results obtained by the chromatographic analysis (GC/MS) of rosemary from the Boulhaf AL-Dair study station indicate that the essential oil of the sample contains 21 chemical components. Moreover, it is important to note that the high-percentage chemotype of the essential oils studied is 1.8 cineole with 32.58%, followed by Sabinene (15.92%) and Camphene (14.41%) (Table 2).

Table 2. Chemical composition of essential oils of rosemary from the study station

N°	Compounds	RI	Percentage (%)
1	α-Pinene	932	3.37
2	Camphene	949	14.41
3	Sabinene	980	15.92
4	β-Pinene	987	0.11
5	p-Cymene	993	1.59
6	**1,8cineole**	**1032**	**32.58**
7	γ-Terpinene	1047	1.73
8	α-Terpinolene	1088	1.19
9	Linalol	1094	0.12
10	Camphre	1144	4.47
11	Pinocamphone	1154	6.19
12	Borneol	1169	9.69
13	Terpinene-4-ol	1178	3.67
14	1-Dodecene	1183	0.09
15	1-α-Terpineol	1188	3.34
16	Verbenone	1228	1.06
17	NI	1229	0.17
18	NI	1253	0.13
19	Bornylacetate	1287	0.34
20	α-Copaene	1378	0.07
21	Tetradecene	1386	0.17

RI: retention index (min); NI : unidentified

Antibacterial Activity of the Essential Oil of *R. Officinalis*

The antibacterial activity of EO is estimated in terms of the diameter of the inhibition zone around the discs containing the samples to be tested against three pathogenic germs, *K. pneumoniae, S. aureus*, and *S. pneumoniae*, after 24 hours of incubation at an adequate temperature of 37 C°. The bacterial strains studied are all sensitive to amoxicillin, with inhibition zones of 24.32± 0.44 and 23.30± 0.87mm for *K. pneumoniae* and *S. pneumoniae*, respectively. *S. aureus* is found to be highly sensitive to this antibiotic, with an inhibition zone of 35.62± 0.44mm. The results show that all strains appear sensitive to the deferential dilutions of EO, whose diameters of growth inhibition zones range from 8.5mm to 12.33mm for *S. aureus* and reach 14.66mm for *K. pneumoniae*, while the *S. pneuminea* inhibition diameter varies between 7.63 and 11.33mm. Undiluted EO remains the most effective in inhibiting bacterial development, with areas of inhibition close to or higher than those of the antibiotic. While DMSO has no antibacterial activity, whose zones of inhibition are totally absent (Table 3).

Minimum Inhibitory and Bactericidal Concentrations

The results of the minimum inhibitory (MIC) and bactericidal (CMB) concentrations of EO and amoxicillin in relation to the strains responsible for lung infection are shown in Table 4.

In this study, we made an interesting comparison between the inhibitory activity of an antibiotic (amoxicillin) involved in the treatment of lung infections and the antibacterial effect of *R. officinalis* EO (Table 3). The results obtained showed a very remarkable efficacy since all strains showed a greater sensitivity to EO than that of ATB. This finding supports the hypothesis we have previously supported that EOs can be used as an antibacterial alternative. The MIC/CMB ratio defines the bacteriostatic or bactericidal character of an essential oil. When this ratio is less than 4, the oil is considered bactericidal. In our study, *R. officinalis* EO CMB/CMI reports were equal to 1 for all bacterial strains tested (Table 4). This essential oil, therefore, seems to exert a bactericidal action against all bacterial strains tested.

Table 3. The sensitivity of the strains*; S. pneumoniae*, *K. pneumoniae* and *S. aureus* after application of each dilution of *R. officinalis* EO, DMSO and antibiotic (ATB: Amoxicillin).

Germs	Negative control	Positive Control (DMSO)	6.25%	12.5%	25%	50%	100%	ATB
S. pneumoniae	- -	- -	-	+	+	+	+++	+++
K. pneumoniae	- -	- -	-	+	+	-	+++	+++
S. aureus	- -	- -	-	+	+	+	+++	+++

(+++): Extremely sensitive. (+): sensitive. (-): resistant. (- -): no inhibition.

Table 4. CMI and CMB of the essential oils tested expressed in mg/mL

Strains	MIC and CMB of EO *R. officinalis*		Amoxicillin 30µg	MIC/CMB of EO
	MIC	CMB	MIC	
S. pneumoniae	25.01±0.01	25.01±0.01	50.00±0.01	1
K. pneumoniae	25.02±0.02	25.02±0.02	50.0±0.01	1
S. aureus	12.50±0.02	12.50±0.01	25.00±0.01	1

Antifungal Activity

Assessment of Mycelial Growth

Faced with the problems of spoilage of vegetables by molds, much work has been done on the antifungal power of natural products extracted from plants. In this study, the antifungal activity of the EO of the plant *R. officinalis* against two fungal strains, *P. expansum* and *A. alternata* was sought in vitro. Three repeats were performed at different times. In the absence of EO, the results obtained show that the mycelial growth of *P. expansum* and *A. alternata* varies between 0 and 6.58 cm and 0 and 4.45 cm, respectively. In the presence of EO, their effect on the mycelial growth of the two strains is dose-dependent. At different concentrations of 0.01% to 1% of EO, the mycelial growth of the two strains studied was inhibited, and the maximum inhibition (100%) was obtained with the 0.5% and 1% concentrations.

Antifungal Index (AI)

The results show that the rate of mycelial growth decreases with increasing concentrations of essential oils. The antifungal index is the concentration that inhibits 50% of mycelial growth, noticing that all applied *R. officinalis* essential oil concentrations prevented, partially (0.25%, 0.05%, 0.01%) or completely (1%, 0.5%), growth of fungal strains tested (Fig. 1). The essential oil is called:

- It is very active when inhibited between 75% and 100%; the fungal strain is said to be very sensitive.
- Active when there is 50-75% inhibition; the fungal strain is called sensitive.
- Moderate activity when it has an inhibition between 25% and 50%; strains are said to be limited.
- Little or no activity when inhibited between 0% and 25%; strain is said to be insensitive or resistant.

The antifungal index is directly proportional to the increase in the concentration of EO; it is stable at both concentrations (1% and 0.5%) with a 100% inhibition percentage and between 84.41% and 70.34% for the concentration of 0.25% The decrease in AI is remarkable at 0.01 concentrations, with an inhibition percentage of 48.98% up to 5.56% after 168h. The minimum inhibitory concentration (MIC) is 0.05%, with good antifungal efficacy manifested by the essential oils of *R. officinalis.* The antifungal index of *A. alternata* at the concentration (1%) is stable at 168 hours with maximum inhibition (100%); at the concentration (0.5%), it is also stable at 120 hours, followed by a decrease up to 85.83% in the remaining hours. The antifungal index of the concentration (0.25%) is decreased faster by 90% to 24.04%. and for the last two concentrations (0.05% and 0.01%), the antifungal index is less than 50%. The minimum inhibitory concentration is of the order of some percent, with good antifungal efficacy manifested by the essential oil of *R. officinalis*. Indeed, for the latter, the MIC is 0.25%. The essential oils of rosemary exerted an important inhibitory activity vis-à-vis the two molds, *P. expansum* and *A. alternata.* The diameters, velocity, and antifungal index of mycelium growth depended on the concentration of the essential oil, with MICs of 0.05% and 0.25% for *P. expansum* and *A. alternata*, respectively.

Insecticidal Activity

The essential oil of *R. officinalis* induced significant ($P<0.001$) insecticidal activity against *D. melanogaster* adults. The toxicity of the essential oil of *R. officinalis* increased significantly with concentrations. 100% adult mortality was recorded after exposure to the essential oil of *R. officinalis* with a concentration of 300 µL/Lair after 24 hours (MR%), while this concentration reaches 93.00% after 1 hour (KR%). The toxicity test showed that the essential oil of *R. officinalis* had significant adulticidal activity; the LC50 values after 1 hour of exposure are LC50 = 288.14µL/Lair and after 24 hours of exposure, LC50 = 81.32µL/Lair (Fig. 2 and Table 5).

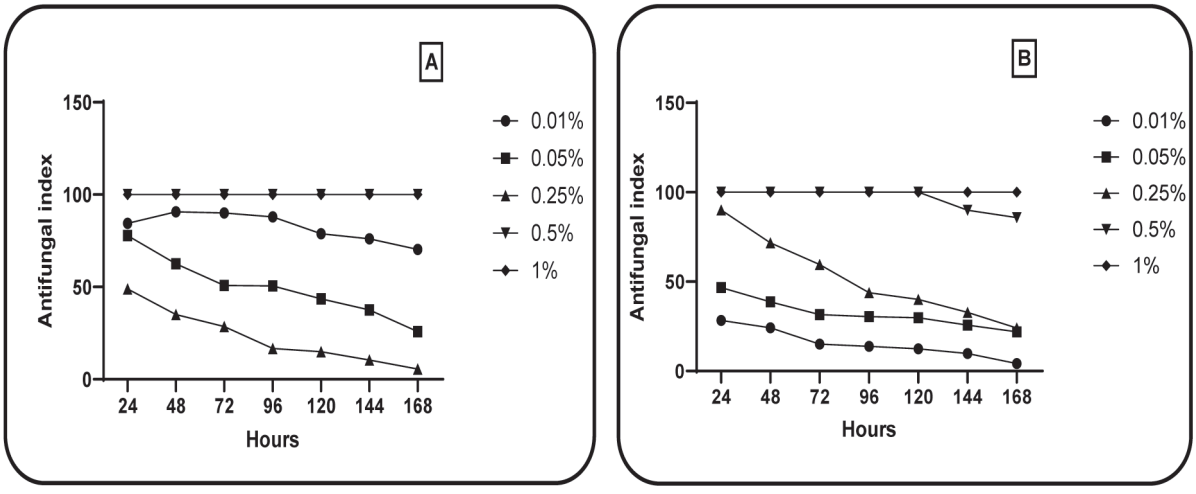

Fig. 1. Antifungal index of different EOs concentrations against *P. expunsum* (A) and *A. alternaria* (B)

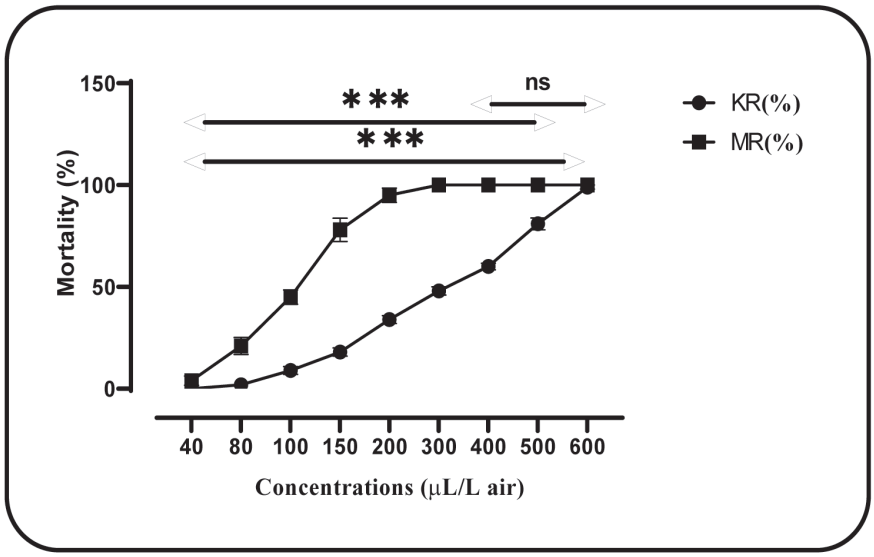

Fig. 2. Effects of R. officinalis EO applied on D. melanogaster adults: corrected mortality (%)

Table 5. Toxicity of *R. officinalis* EO applied on *D. melanogaster:* Determination of Lethal and sublethal concentrations (µL/Lair).

Concentrations µL/Lair		LC_{10} (LCL-UCL)	LC_{25} (LCL-UCL)	LC_{50} (LCL–UCL)	LC_{90} (LCL-UCL)	Hill slope	R^2
R. officinalis	Shock	118.81 83.36 to 161.52	185.00 148.00 to 226.81	288.14 249.55 to 330.71	698.72 543.92 to 968.37	2.48	0.97
	Mortality	31.88 20.35 to 46.87	50.91 37.87 to 66.11	81.32 67.11 to 97.22	207.42 153.25 to 294.61	2.35	0.97

Discussion

Our yield was consistent with that obtained from *R. officinalis* EO [26]. Our result is high compared to the EO yield of *R. officinalis* grown in Morocco (0.54%) [27] and lower than those of [22], which are of the order of 0.6% and 0.82%, respectively. Indeed, the variation in essential oil yield depends in particular on the geographical origin [28], the phenological stage, and environmental factors. The stage of plant development [29] and the EO extraction method [30]. The analysis of essential oils of rosemary from the Boulhaf AL-Dair region is very interesting, it allowed us to note that the number of components obtained by chromatographic analysis (CPG/ SM) is important, either 20 chemical components, However, in their study on the analysis of rosemary essential oils from geographically different origins in Algeria (Algiers, Bibans, Djelfa, and Laghouat), Boutekedjiret and his collaborators [31] listed between 28 and 61 chemical components. The bacterial strains studied (*K. pneumoniae*, *S. pneumoniae*, and *S. aureus*) appear sensitive to different dilutions of *R. officinalis* essential oil. While undiluted, EO shows optimal efficacy in inhibiting bacterial development with inhibition zones close to or greater than those of the antibiotic. The antibacterial activity of rosemary EO is higher than that obtained by Lograda and his collaborators [32]. In this study, the authors tested rosemary oil from several regions of eastern Algeria (Kherrata (Bedjaia), Boutaleb (Sétif), Bibans (Bourdj Bou-Arriridj), Agmeroual and N'gaous (Batna), and Boussaâda (M'sila). The diameters of the inhibition zones obtained did not exceed 20 mm. The results obtained from this study show that antibacterial activity is mainly due to the presence of antibacterial molecules. In fact, EO is rich in monoterpene hydrocarbons, and terpinenes, which are endowed with great antibacterial activity against the bacteria Gram- and Gram + [33]. These chemical components exert their antimicrobial activity on microorganisms by disrupting membrane integrity and according to Wang and colleagues [34], the presence of borneol, l-Verbenone, β-linalol, camphor, and other phenolic compounds in rosemary essential oil is responsible for this activity. The antifungal activity of the EO of the plant *R. officinalis* vis-à-vis two fungal strains, *P. expansum and A. alternata*, by the direct contact method, was sought in vitro. In the absence of EO, the results obtained show that the mycelial growth of *P. expansum* and *A. alternata* varies between 0 and 6.58 cm and 0 and 4.45 cm, respectively. In the presence of EO, their effect on the mycelial growth of the two strains is dose-dependent. At different concentrations of 0.01% to

1% of EO, the mycelial growth of the two strains studied was inhibited, and the maximum inhibition (100%) was obtained with the 0.5% and 1% concentrations. Antifungal activity is probably due to the type and molecular structure of the active components present in EO, such as terpenes that affect not only permeability but also other functions in cell membranes [35, 36]. These results are comparable to those of Campo et al. (2000) [37], who announced that low essential oil concentrations of some citrus have a partial inhibitory effect due to inhibition of respiration and altered cell permeability. The antifungal power of rosemary essential oils could be attributed to the presence of antifungal components classified in the list of constituents with antifungal activity of Mehmet and Chalchat, 2008 [38], such as myristicin, curcumin, caryophyllene, elemicin, pinene, terpinene, and terpinolene in different proportions. The toxicological test carried out using the fumigation method revealed an effective insecticidal activity of *R. officinalis* EO against the adults of *D. melanogaster*. Bouabida and Dris, 2022a, [39] reported that plant extracts have a biocidal effect on insects. In the same sense, Bouabida and Dris, 2022b [40] have proven that the essential oil of *Ruta graveolens* cultivated in the region of Tebessa (Eastern Algeria) has pesticidal activity against the larvae of *D. melanogaster*. Among the plant extracts, we distinguished essential oils. The work of Sharma et al. 2023 [41] shows that citral EO had an insecticidal effect and was followed by an increase in development time in larvae, pupae, and adults of *D. melanogaster*. *Cymbopogon winterianus* essential oil may have larvicidal activity in a Diptera species (*D. melanogaster*) and can be developed into an herbal insecticide [42].

Conclusions

This scientific research shows that *Rosmarinus officinalis* essential oil has antibacterial activity against three bacterial strains: *Staphylococcus aureus, Klebsiella pneumoniae,* and *Streptococcus pneumoniae*, and antifungal activity against two strains, *Penicillium expansum and Alternaria alternate*, and exhibits insecticidal activity against adult *Drosophila melanogaster*. Therefore, *R. officinalis* essential oil, based on these results, we can suggest the use of *R. officinalis* essential oil as a natural alternative against lung bacteria, fungi, and insects.

Declaration of Competing Interest

None.

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