Research Article

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Chemical composition and antimicrobial activity of essential oils from *Mentha pulegium* and *Rosmarinus officinalis* against multidrug-resistant microbes and their acute toxicity study

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Abstract: This article aimed to study the antimicrobial activity, chemical composition, and acute oral toxicity of essential oils (EOs) of *Mentha pulegium* and *Rosmarinus officinalis*, two aromatic and medicinal plants widely used in the traditional Moroccan pharmacopeia. The average content of EOs was 3.2 and 2.5% for *M. pulegium* and *R. officinalis*, respectively. The chemical characterization showed a richness in some compounds identified by gas chromatography coupled with mass spectrometry (GC/MS): *R*(+)-Pulegone (45.48%), Menthone (14.2%), Piperitone (8.15%), and Isomenthone (7.18%) in *M. pulegium* and 1,8-Cineole (46.32%), Camphene (13.4%), and α-Pinene (9.52%) in *R. officinalis*. These metabolites showed a significant antimicrobial effect against the tested strains (bacteria and yeasts isolated from the hospital environment) compared to synthetic antibiotics that seem to be ineffective against resistant microorganisms. Based on lethal concentration LD_{50} >5,000 mg/kg (body weight), the oil was found to be marginally safe according to OECD guidelines and can be further explored (bio-product with low risk).

Keywords: medicinal and aromatic plants, *Mentha pulegium*, *Rosmarinus officinalis*, essential oil, chemical characterization, antimicrobial activity, acute toxicity, LD_{50}

1 Introduction

The use of medicinal and aromatic plants since ancient times in the treatment of many diseases and at the present time has increased their use in many areas, such as food preservation, cosmetics, and perfume [1–3]. It has been estimated that at least 25% of all modern medicines are derived directly or indirectly mainly from medicinal plants [4]. Indeed, in developed countries, medicinal plants have been increasingly exploited to overcome the side effects of reference drugs especially with the emergence of antibiotic resistant microorganisms and the emergence of uncommon infections that compromise the treatment of existing drugs [5]. These different difficulties have led researchers to valorize aromatic and medicinal plants (AMP) and thus find effective and accessible alternatives with fewer side effects from natural products.

Morocco is a country with a geographical position, Mediterranean climate, and geomorphological characteristics that makes it very rich in flora with an important potential in AMP, some of which being still unknown [6,7].

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The family Lamiaceae is one of the most important, about 6,900–7,200 species, belonging to 236 genera, distributed all over the world especially in the Mediterranean region and the temperate region [8]. The most important of them are *Rosmarinus officinalis* and *Mentha pulegium*.

R. officinalis grows in semi-arid and sub-humid bioclimates; it is widespread in the Eastern Rif, the Eastern Middle Atlas, the Eastern High Atlas, and the highlands of the Oriental. The dried leaves of *R. officinalis* are used as a condiment and in the composition of tea infusion as well as the creation of cosmetic products, such as shampoo, soaps, and creams [9]. *R. officinalis* essential oil (EO) is also important for its medicinal uses and its powerful antibacterial, cytotoxic, anti-inflammatory, anti-mutagenic, and chemopreventive properties [10].

M. pulegium is found everywhere in Morocco in humid places. It is an excellent digestive, as it is used effectively by the people of the sore throat, cough, bronchitis, and pulmonary infections [11–13].

The antibacterial mechanism of action by which EOs act on bacteria is still not clear. Hyldgaard et al. explained in his study that some components of EOs work to bind in the plasma membrane and thus change the permeability of the membrane [14]. Another study by Nazzaro et al. stated that some components of EOs penetrate the plasma membrane and interact with intracytoplasmic targets [15].

The present study was undertaken to determine the chemical composition and to test the antibacterial and anticandidosic activity of the EOs of *M. pulegium* and *R. officinalis* compared to the traditional antibacterial agents. Moreover, we were able to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the oils studied as well as their acute oral toxicity (LD_{50}).

2 Materials and methods

2.1 Plant material

The harvest of *R. officinalis* and *M. pulegium* samples has been carried out in May 2019 in the northwestern of Morocco (Kenitra region). The plants were identified and authenticated by Professor Bari Amina, a botanist in the Department of Biology. The specimens have been deposited in the herbarium of the Laboratory of Biotechnology, Environment, Agroalimentary and Health, Faculty of Sciences, University Sidi Mohamed Ben Abdellah (USMBA) Fez, Morocco, under the reference numbers "BPRN49" for *M. pulegium* L. and "BPRN37" for *R. officinalis* L. The collected plants were washed by water, disinfected, rinsed with distilled water, and finally dried in shade and airy place for 7–10 days until the weight stabilized. The EOs have been obtained by hydrodistillation of the dried leaves of *R. officinalis* and the dried aerial parts of *M. pulegium* for 1 h 30 min at 100°C by a Clevenger apparatus. The EOs obtained were dehydrated with anhydrous sodium sulfate and stored at 4°C in dark [16,17]. The calculation of the yields was done following to the AFNOR standard as in:

$$\text{Yield EO\%} = \text{EO (g)}/\text{Dry matter (g)} \times 100.$$

2.2 GC-MS analysis of EOs

The identification and determination of the composition of the EOs was performed using GC-MS. Our samples were analyzed on a Thermo Fisher gas chromatograph coupled to the mass spectrometry system (model GC ULTRA S/N 210729). The column used for analysis was a 5% phenylmethyl silicone HP-5 capillary column (30 m × 0.25 mm × thickness 0.25 µm). The temperature was programmed from 50°C, after 5 min of initial hold, to 200°C at 4°C/min. The carrier gas was N2 (1.8 mL/min); split mode was used with a flow rate of 72.1 mL/min and a ratio of 1/50; injector and detector temperature was 250°C; and the final hold time was 48 min. Oil samples were manually injected at 1 µL diluted in hexane.

2.3 Microbiological activity

2.3.1 Microbial strains

The microbial strains tested in this study were selected based on their pathogenicity and are known to be highly antibiotic resistant. They are regularly maintained by subculturing on Mueller–Hinton (MH) agar medium for bacteria (*Staphylococcus aureus*, *Salmonella* sp., *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumonia*, and *Streptococcus* sp.) and Sabouraud medium for yeasts (*Candida albicans*, *Candida tropicalis*, and *Saccharomyces cerevisiae*). These strains were provided by the Microbiology Department of the Hassan II University Hospital of FES.

2.3.2 Antimicrobial assays

To evaluate the antimicrobial potency of our oil samples, the disk diffusion method was used [18]. Bacterial

inoculums were prepared from a 24 h broth culture in physiological saline (0.8% NaCl) to obtain an optical density ranging from 0.08 to 01 at 625 nm, equivalent to 10^6 CFU/mL. From this solution, Petri dishes containing solidified agar medium (MH agar for bacteria and Sabouraud agar for yeast) were spread, and then sterile Wattman paper disks of 6 mm diameter soaked separately with 15 µL of each EO were placed on the surface of the agar previously inoculated with the selected microorganism. For the positive control, discs of reference antibiotics Ampicillin (10 µg), Piperacillin (30 µg), Sulfamethoxazole (25 µg), Penicillin G 5units, Erythromycin (15 µg), and Streptomycin (10 µg) were applied to each Petri dish and blank discs used as negative control. The plates were stored at 4°C for 1 h. They were then incubated for culture at 37°C for 24 h for bacteria and 48 h at 30°C for yeast. The sensitivity of the strains to each product was evaluated by measuring the diameters of the growth inhibition zones in millimeters including the diameter of the 6 mm disc.

The MIC and MBC values were determined by the agar medium microdilution technique [19], cited by [20]. The EO to be tested is incorporated at varying concentrations into the agar culture medium: MH agar for bacteria and Sabouraud for yeast.

The EOs are emulsified in a 0.2% agar solution to disperse the compounds and improve their contact with the germs being tested. Dilutions are then prepared: 100, 40, 20, 10, 5, 3, and 2 µL/mL in this agar solution.

Then, 1.5 mL of each dilution was aseptically added into test tubes containing 13.5 mL of culture medium to obtain final concentrations of the EO of 10, 4, 2, 1, 0.5, 0.33, and 0.2 µL/mL. The tubes were shaken before pouring into the petri dishes. Control dishes, containing the culture medium plus the 0.2% agar solution alone, were also prepared.

After solidification, the dishes were inoculated with the inoculum. Inoculation is done by striations using a calibrated platinum loop to collect the same volume of inoculums.

The incubation was set at 37°C for 24 h for bacteria and 30°C for 48 h for yeast. Each test was repeated three times under the same experimental conditions [21,22]. The results give the MIC as the lowest concentration of EO for which we do not observe growth with the naked eye.

To determine the MBC, from the plates that did not show microbial growth, we re-inoculate on MH agar for bacteria and Sabouraud agar for yeast, reseal, and incubate the plates under the same conditions as before. The oil is said to be bacteriostatic when the strains grow and bactericidal when they did not.

2.4 Acute toxicity study

To evaluate the LD_{50} of *R. officinalis* and *M. pulegium* EOs, adult female rats [23] were used according to the guidelines of the Organization for Economic Cooperation and Development 423 [2,3]. Before starting the experiment, the rats were deprived of food for 4 h, and then, they were divided into groups [24,25]. For each EO, the division was done by randomly putting six rats per group. Three groups received orally 300, 1,000, and 2,000 mg/kg dose of EOs, and the control group received corn oil. After the administration of EOs, mortality and toxicity symptoms, change in body weight, food intake, respiration, and convulsions were evaluated during the first hours after gavage and then daily for 14 days.

2.5 Statistical analysis

The comparison of the averages is done by the SPSS software.

3 Results and discussion

3.1 Extraction and analysis of the chemical composition of volatile extracts

The average yields of EOs by hydrodistillation were expressed in milliliter per 100 g of dry plant matter. These rates were 3.2% for the *M. pulegium* and 2.5% for the *R. officinalis*. In Sardinia, Italy, work has shown that the yield of Rosemary varies between 0.2 and 1.3% [9], which is lower than what was found in our study. Other researchers have stated that the EO content of the aerial parts of wild Rosemary in Tunisia was varied between 1.17 and 2.7% [26]. Concerning *M. pulegium*, the yield of the dried aerial parts was 3.30% in the North of Morocco [21]. These results were confirmed in our study and were in contrast with those done in the region of Khenifra (6.2%), Azrou (5.9%), and M'rirt (5.29%) [22].

Chromatographic analyses of EOs have identified 29 compounds, which presented approximately 99.13% for *M. pulegium* against 16 compounds (93.52%) for *R. officinalis* (Table 1). The major compounds of the *R. officinalis* EO were 1,8-Cineole (46.32%) followed by Camphene (13.4%) and α-Pinene (9.52%). This composition is relatively comparable to that found by other researchers in Morocco on samples of *R. officinalis* from different regions

Table 1: Chemical composition of EOs of *R. officinalis* and *M. pulegium*

IK	Compounds	*M. pulegium* (%)	*R. officinalis* (%)
924	α-Thujene	—	1.6
936	α-Pinene	0.49	9.52
940	Camphene	—	13.4
967	Sabinene	—	3.18
993	Myrcene	0.17	—
998	Octanol-3	1.85	—
1,013	*p*-Cym	—	1.87
1,021	1,8-Cineole	—	46.32
1,031	Limonene	2.6	—
1,072	*p*-Mentha-3,8-diene	1.5	—
1,088	α-Terpinolene	—	0.8
1,144	Camphor	—	4.76
1,154	Menthone	14.2	—
1,167	Pinocarvone	1.35	—
1,169	Borneol	—	6.48
1,170	Isomenthone	7.18	—
1,173	Menthol	0.92	—
1,176	Terpinene-4-ol	—	3.56
1,188	1-α-Terpineol	—	0.66
1,194	Dihydrocarvone	5.16	—
1,238	*R*(+)-Pulegone	45.48	—
1,242	Carvone	5.2	—
1,245	Eucarvone	4.02	—
1,253	Piperitone	8.15	—
1,287	Bornyl acetate	—	0.27
1,398	Caryophyllene	—	0.69
1,420	β-Caryophyllene	0.86	—
1,437	Humulene	—	0.17
1,465	γ-Murolene	—	0.15
1,514	β-Himachalene	—	0.09
		99.13	93.52

IK: Kováts indices; –: absence.

of the North East (Aknoul) and the East (Rchida and Berkine) [23]. In Tunisia, studies have confirmed the same results [27].

In Algeria, studies conducted on three samples collected in three different stations showed that 1,8-Cineole is the most abundant compound, with 72.91% sample of Youkous, 32.59% sample of Draa Hammam, and 32.76% sample of Ammacha [28]. In northeastern Spain, the chemical composition showed some differences from our work. In this case, the most abundant compounds were Camphor and α-Pinene [29]. Regarding the EO of *M. pulegium*, the plant is characterized by the presence of *R* (+)-Pulegone (45.48%), Menthone (14.2%), Piperitone (8.15%), and Isomenthone (7.18%) as the main constituents. These results are similar to the majority of the other studies done in Morocco [21,30,31]. Similarly, in the North-East of Algeria, studies have shown that Pulegone (38.815%) presents the highest percentage in comparison to other components, such as Menthone (19.240%), Piperitone (16.528%), Piperitone (6.348%), Isomenthone (6.096%), Limonene (4.293%), and Octaan-3-ol (1.854%) [32]. In Italy, phytochemical analyses showed that oxygenated monoterpenes were the most abundant class in all the EOs investigated (92.2–97.7%), with two different chemotypes, Pulegone/Isomenthone and Piperitone/Isomenthone [33]. This variability in the chemical composition results of the *R. officinalis* and *M. pulegium* EOs qualitatively and quantitatively may be due to the EO method of extraction, extrinsic or intrinsic factors, origin, species, the duration of drying, the part of the plant used, age of the plant and the period of the vegetative cycle, or genetic factors [34–37].

3.2 Bioassays

The results of the antimicrobial activity of the EOs of *M. Pulegium* and *R. Officinalis* are summarized in Table 2. The comparison of the averages of the zones of inhibition shows that there is variability between the classes; the EOs and the antibiotics did not react in the same way toward the tested microorganisms.

M. pulegium EO exerted a moderate antimicrobial effect against the bacteria tested, except *Salmonella* sp., which appeared resistant; the most important zone of inhibition is that of *S. aureus* with value is 17.33 ± 2.52 mm. In the case of yeasts, *C. tropicalis* showed no sensitivity to the effect of oil, but the average inhibition zones for the other two strains reached values of 16.33 ± 5.13 mm for *C. albicans* and 15 ± 4.36 mm for S. *cerevisiae*.

Based on previous research, yeasts are more resistant than pathogenic bacteria to mint oil [38]. On the other hand, screening of *M. pulegium* EOs for their antagonistic activity against pathogenic bacteria reveals that they do not show appreciable activity except for that observed against *Streptococcus pyogenes* (16 mm) [32]. In the present work, it was found that *M. pulegium* EO has an effect on yeast (*C. albicans* and *S. cerevisiae*) and also has an effect on all bacteria were studied with the exception of *Salmonella* sp.

The essence of *R. officinalis* exerted a significant inhibitory effect compared to antibiotics; it inhibited the growth of all bacteria except *P. aeruginosa*. It inhibited even *E. coli* and *Salmonella* sp. resistant to all antibiotics tested. Yeasts showed equally high inhibition zones; between 14.73 ± 1.73 mm for *C. albicans* and *S. cerevisiae* and 11.67 ± 2.08 mm for *C. tropicalis*. Similar work conducted in Tunisire on *R. officinalis* samples of different

Table 2: Effect comparison of substances (essential oil and antibiotics), all species (bacterial and yeast), on inhibition area diameter

Strains	Inhibition area diameter (mm)							
	Eos		Antibiotics (µg/disc)					
	RO	MP	PRL30	AMP10	RL25	E15	P5	S10
Salmonella sp.	10.33 ± 1.15^{a}	0	0	0	0	0	0	0
S. aureus	10 ± 1^{c}	17.33 ± 2.52^{b}	0	13.33 ± 1.53^{c}	0	19.67 ± 0.58^{ab}	22.67 ± 0.58^{a}	12.33 ± 0.58^{c}
E. coli	8.67 ± 0.58^{a}	7 ± 0.0^{a}	0	0	0	0	0	0
K. pneumonia	9.33 ± 0.58^{b}	7.33 ± 0.58^{c}	0	0	0	8 ± 0.0^{bc}	0	12.33 ± 1.15^{a}
P. aeruginosa	0	7 ± 0.0	0	0	0	0	0	9 ± 0.0
Strypto sp.	11.33 ± 0.58^{c}	9.67 ± 0.58^{c}	0	0	20 ± 0.0^{b}	21.67 ± 0.58^{ab}	22.67 ± 0.58^{a}	21.66 ± 1.53^{ab}
C. tropicalis	11.67 ± 2.08^{a}	0	*	*	*	*	*	*
C. albicans	14.73 ± 1.73^{a}	16.33 ± 5.13^{a}	*	*	*	*	*	*
S. cerevisiae	14.73 ± 1.73^{a}	15 ± 4.36^{a}	*	*	*	*	*	*

Data are presented through the means (±SE). RO: *R. officinalis;* MP: *M. pulegium*; AMP10: Ampicillin (10 µg); PRL30: Piperacillin (30 µg); RL25: Sulphamethoxazole; P5: Penicillin G 5units, Erythromycin 15 µg (E15), and Streptomycin 10 µg (S10); *: not tested.

origins also proved that *P. aeruginosa* bacteria was the most resistant to all oils compared to other strains [26,39].

The quantitative evaluation of the *in vitro* antimicrobial activity of the EOs of *R. officinalis* and *M. pulegium* against the tested microorganisms was determined through their MIC and MBC (Table 3).

The presence of *R. officinalis* oil in the culture medium caused antimicrobial effect on (*Salmonella* sp., *S. aureus*, *E. coli*, *K. pneumonia*, *C. tropicalis*, and *C. albicans*) and a bacteriostatic/fungistatic effect on (*Strypto* sp. and *S. cerevisiae*). *P. aeruginosa* was resistant to even the highest 1% concentration. Other works have proven the same thing; *P. aeruginosa* is the most resistant strain [40].

The EO of *M. Pulegium* showed a bactericidal effect on *S. aureus* since 1% of the oil killed the strain (MIC = MBC = 1%). In the case of yeasts, the oil exerted a fungistatic effect on *C. albicans* and *S. cerevisiae*. The antimicrobial effect of *M. Pulegium* has been demonstrated in other studies [29], including on *C. albicans* strains, as well as on *Staphylococcus epidermis* and *Acinetobacter baumannii* strains [41].

Table 3: MIC and MBC of *R. officinalis* and *M. pulegium* oils

Bacterial strains	*R. officinalis*		*M. pulegium*	
	MIC	MBC	MIC	MBC
	µL/mL		µL/mL	
Salmonella sp.	4	4	Nd	Nd
S. aureus	2	2	10	10
E. coli	4	4	Nd	Nd
K. pneumonia	4	4	Nd	Nd
P. aeruginosa	Nd	Nd	Nd	Nd
Strypto sp.	2	4	Nd	Nd
Yeast				
C. tropicalis	1	1	Nd	Nd
C. albicans	2	2	10	Nd
S. cerevisiae	1	2	0.5	Nd

Nd: not determined; it means that the concentrations that were used are ineffective.

According to our results, the sensitivity of microorganisms can vary depending on the germ tested and the oil used. The inhibitory power of our EOs is attributed to the most abundant compounds (pulegone 45.48%, 1,8-Cineole 46.32%). Researchers have linked the antibacterial activity of rosemary oil to its high 1,8-Cineole content [42,43].

Duru et al. demonstrated the strong antimicrobial activity of pulegone against a range of bacterial strains [44]. This compound causes damage when it passes through the bacterial membrane and disrupts the structure of their different layers of fatty acids, polysaccharides, and phospholipids [12,45]. According to the literature, the oils of some Lamiaceae possess strong antibacterial activity compared to standard drugs [46,47].

Cimanga et al. stated that the antimicrobial activity of EOs is not only related to the compounds present in greater quantities but also less-abundant constituents [48]. In addition, synergistic and antagonistic effects can occur between the components of the oils depending on the microorganism tested [49].

3.3 Study of the acute toxicity

Behavioral changes depend on the oil and the dose administered. In our work (for both EOs), no signs of toxicity,

Table 4: Study of the acute toxicity of EO of *R. officinalis* and *M. pulegium* oil administered by gavage to rats

EOs	Number of rats by batch	Dose (mg/kg)	Mortality	Signs of toxicity	LD_{50}	Category GHS (mg/kg)
R. officinalis	6	300	0	—	>5,000	5
	6	1,000	0	—		
	6	2,000	0	—		
M. pulegium	6	300	0	—	>5,000	5
	6	1,000	0	—		
	6	2,000	0	—		
Control	6	—	0	—	—	—
	6	—	0	—	—	—
	6	—	0	—	—	—

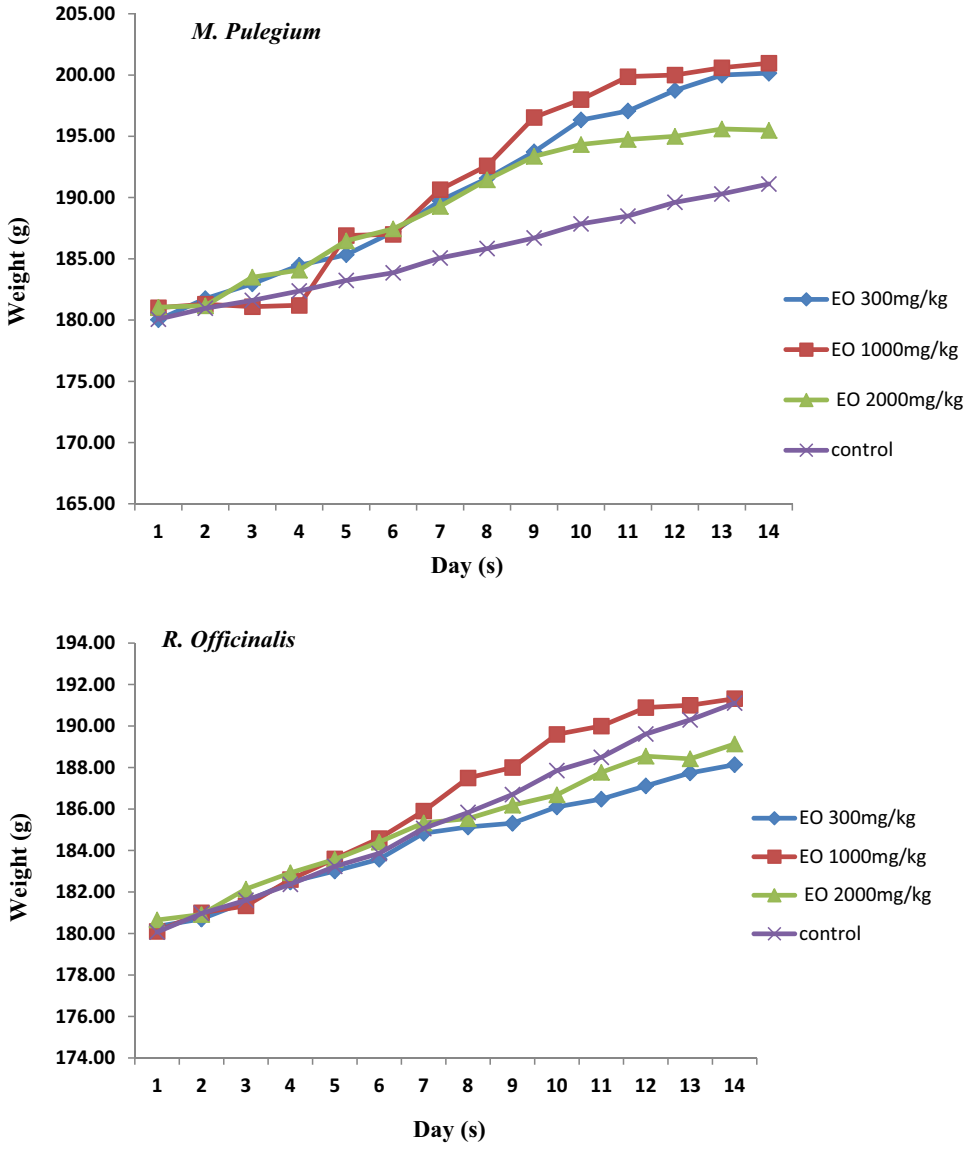

Figure 1: Influence of *R. officinalis* and *M. pulegium* EOs on the weight change of rats during 14 days.

behavioral alterations, death, or even loss of body weight were recorded in the rat groups during 14 days (Table 4 and Figure 1). According to the OECD, the LD_{50} of both oils is higher than 5,000 mg/kg; hence, the oils are classified into category 5 of the Harmonized System of Classification (GHS) as nontoxic byoral route [24].

Farias et al. found the LD_{50} of *R. officinalis* was greater than 2.0 g/kg, and none of the treated animals died or showed symptoms associated with toxicity [50]. *R. officinalis* was marginally safe according to OECD guidelines with LD_{50} >5,000 mg/kg [51].

Previous research on another mint species confirmed that the LD_{50} is still above 5,000 mg/kg [52]. Indeed, 2,000 mg/kg of *Mentha mozaffarianii* EO had no negative effect on the behavioral responses of test animals up to 14 days of observation. Physical observations also revealed no signs of change in skin, coat, eyes, mucous membranes, behavior, tremors, salivation, and diarrhea. Similarly, no mortality was observed at the dose tested [52].

4 Conclusions

Due to the specified therapeutic virtues, their antimicrobial activity, and diversity of composition of their EOs, *M. pulegium* and *R. officinalis* could be considered a source of bioactive molecules in the pharmaceutical industry for the production of new synthetic agents in the treatment of infectious diseases caused by the studied strains. Their use as broad-spectrum antibiotics can also be encouraged. However, further research through *in vivo* tests is needed to better understand the basic biotechnological values of applied phytotherapy and to develop a natural biological means of control. It would also be desirable to complete this study with other ones on the chronic oral toxicity of these EOs and their pharmacodynamic characteristics.

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Conflict of interest: Authors state no conflict of interest.

Ethical approval: The ethical institutional committee, Faculty of Sciences; Ibn Tofail University, Kenitra, Morocco, approved the protocol. All the experimental proceedings achieved in laboratory animals followed the internationally accepted standard guidelines for animal care.

Data availability statement: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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