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Comparative Analysis of the Chemical Composition and Antimicrobial Activity of Four Moroccan North Middle Atlas Medicinal Plants' Essential Oils: *Rosmarinus officinalis* L., *Mentha pulegium* L., *Salvia officinalis* L., and *Thymus zygis* subsp. *gracilis* (Boiss.) R. Morales

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Abstract: Medicinal plants represent an inexhaustible source of traditional and effective remedies thanks to the various active ingredients they contain. Secondary metabolites present in plant extracts, such as essential oils (EO), have remarkable pharmacological properties, including antimicrobial effects. Here, the chemical composition and antimicrobial effects of four Moroccan medicinal plants, *Rosmarinus officinalis* L. (*R. officinalis*), *Mentha pulegium* L. (*M. pulegium*), *Salvia officinalis* L. (*S. officinalis*), and *Thymus zygis* subsp. *gracilis* (Boiss.) R. Morales (*T. zygis*), traditionally used in Morocco to treat microbial infections, were addressed. EO were extracted using the hydrodistillation method, and analyzed by gas chromatography coupled with mass spectrometry (GC/MS). EO yields were of 3.64 ± 0.12, 3.53 ± 0.06, 2.48 ± 0.06, and 2.34 ± 0.08%, respectively, for *M. pulegium*, *R. officinalis*, *S. officinalis*, and *T. zygis*. The main bioactive components present in these EO were piperitenone (32.9%) and pulegone (32.8%) for *M. pulegium*, 1,8-cineol (43.8%) and camphor (18.7%) for *R. officinalis*, 1,8-cineole (16.8%) and *trans*-thujone (15.9%) for *S. officinalis*, and thymol (36.4%), carvacrol (24.1%) and cymene (23.5%) for *T. zygis*. These EO showed, according to the results of their antimicrobial activities, good effectiveness against bacteria and fungi. Moreover, the *T. zygis* EO showed the most potent activity against all bacteria studied, while that of *R. officinalis*, *M. pulegium*, and *S. officinalis* showed moderate activity against the *Enterobacter cloacae* of *Streptococcus agalactiae* and *Escherichia coli*. The antifungal activity tests revealed a strong antifungal activity for the *T. zygis* EO and a moderate activity for the *S. officinalis* EO. On the other hand, the EO of *R. officinalis*, and *M. pulegium* were found to be inactive at the doses used against the selected strains. In conclusion, our results show that the medicinal plants studied contain biologically active molecules with antimicrobial effects. They can replace synthesized molecules, especially in the formulation of additives, and for therapeutic, cosmetic, and food-processing purposes.

Keywords: *Rosmarinus officinalis* L.; *Mentha Pulegium* L.; *Salvia officinalis* L.; *Thymus zygis* Subsp. *gracilis*; essential oils; GC/MS; antimicrobial activity

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1. Introduction

From the dawn of civilization, humanity has succeeded in discovering the usefulness and importance of the plants found in his environment. Medicinal plants are still considered reliable and useful sources of active ingredients known for their therapeutic properties, including antimicrobial activity [1]. At the same time, experimental science highlights the diversity of their chemical structures. Morocco has a particularly diverse climate due to its geographic location, which contributes to its wide range of ecological conditions and its rich and diverse number of plant varieties. According to the latest statistics, the vascular flora of Morocco is estimated at 4500 species grouped into 155 families and 940 genera [2]. Morocco, thus, occupies first place among the countries of the south of the Mediterranean for its wealth of endemic plants. There are nearly 800 endemic species and subspecies (19% of the total flora) because of the existence of several endemic outbreaks located at different altitudes such as Souss, Ida or Tanane, Zainane, Moulouiya, and high mountain peaks [3,4]. Of the thousands of plant species that make up the Moroccan flora, the number of medicinal plants used in traditional Moroccan herbal medicine to treat various health conditions can be estimated at about 905, belonging to 116 families and 726 genera [5]. According to this latest study, the botanical family most used in traditional Moroccan herbal medicine is the Lamiaceae family.

The Lamiaceae family is one of the largest flowering families; it includes about 250 genera and more than 7000 species. Most plants in this family are medicinal and, therefore, are an important source of essential oils (EO) [5]. These Lamiaceae species produce a wide range of aromatic compounds that have been used for a variety of medicinal purposes, including relieving stomach ailments, gas, and diarrhea [6]. Many medicinal uses are believed to be related to the terpene constituents of the EO of these plants [7]. They are also used as culinary herbs, and as ingredients in cosmetics, hygiene products, and perfumes [8]. Plants contain various important classes of phytochemicals. Generally, these compounds are classified as either primary or secondary metabolites [9]. On the one hand, these metabolites play a vital role in the defense of plants against herbivores and other interspecific defenses. On the other hand, humans also use them as medicines, flavors, pharmaceuticals, agrochemicals, perfumes, dyes, biopesticides, and food additives. Thanks to the diversity of their active compounds, these metabolites mainly include flavonoids, polyphenols, alkaloids, glycosides, terpenoids and sesquiterpenes with different antibacterial, antifungal, cytotoxic, and antioxidant activities [10].

Sesquiterpenes are volatile compounds derived from three isoprene units and have the molecular formula $C_{15}H_{24}$. They constitute the second major group of active compounds in the essential oils of plants. These active compounds are in the form of hydrocarbons or oxygenated hydrocarbons such as alcohols, ketones, aldehydes, acids and lactones in the wild. They have very important properties such as antibacterial, antifungal, anti-inflammatory, antispasmodic and anti-infective [11]. Other chemical families such as monoterpenes ($C_{10}H_{16}$) are also interesting alongside sesquiterpenes. Monoterpenes represent a large group of natural organic compounds whose basic structure consists of two bound isoprene units. Monoterpenic derivatives containing hepatomes are called monoterpenoids. They play an important role in the creation of new bioactive compounds, including analgesic, anti-inflammatory, anticonvulsant, antidepressant, anti-Alzheimer, anti-Parkinson, antiviral and antibacterial compounds [12].

The region of Fez-Meknes (Morocco) has an important natural wealth likely to strengthen its economic position, thanks to its geography and the predominance of its many diverse environments: the hills of the Pre Rif (in the North), the mountains of the Middle Atlas (in the East) and Zerhoun (to the west), all of which are home to many plant species. These regions are characterized by a climate ranging from the Mediterranean to continental, cold in winter and hot in summer [13]. The northern slope of the Middle Atlas Mountains (Ifrane-Boulemane) is indeed the wettest region of Morocco after the Rif's mountain range, with a very high wealth of Lamiaceous plants, thanks to its very humid and temperate climate with an annual rainfall of 1250 mm and an average annual temperature of 13°.

The most available and widely used plants in this region are *Rosmarinus officinalis*, *Mentha pulegium*, *Salvia officinalis*, and *Thymus zygis*. They are widely used in folk medicine in many cultures. *R. officinalis* is known in Morocco under its vernacular name "*Azir*" or "*Yezri*", and is widely used in traditional Moroccan medicine for the treatment of digestive problems, kidney diseases, skin diseases, and infectious diseases [5,14]. Modern studies show the effects of rosemary on different parts of the body, such as choleretic, antifungal, antibacterial [15], antitumorigenic and antioxidant effects [16]. *M. pulegium* is called "*Fliyyû*" in Morocco, and the different parts of this plant have been traditionally used in Morocco to treat diabetes, digestive diseases, allergy, kidney problems, and diseases caused by microbes [5,17]. *S. officinalis* is popularly known in Morocco as "*Sâlmiya*". These leaves contain mainly polyphenols: flavonoids and phenolic acids responsible for its antispasmodic and choleretic actions (it augurs the secretion of bile). In addition, it is rich in EO (1 to 2.5%) with thujone (35 to 60%) and α-pinene (7 to 10%) as major compounds. These properties allow it to be particularly useful for treating digestive disorders (slow and difficult digestion, bloating, intestinal fermentation) [18]. *T. zygis* is called "*Zaitra*" or "*Azûkânnî*" in traditional Moroccan medicine, and is widely used to combat respiratory diseases and diseases caused by pathogenic germs [5]. The EO of this plant are widely used as antiseptic agents in several pharmaceutical fields and for their anti-inflammatory, anticoagulant and antioxidant properties [19]. *T. zygis* showed the improved sensory (reduced lipid oxidation) and nutritional (increased protein, fat, dry matter, and polyunsaturated fatty acid) properties of milk, as well as cheese [20].

In the context of the traditional intensive use of these species, especially for infectious diseases, and thus for their richness in bioactive compounds, this work aims to identify and compare the phytochemical composition of these four Moroccan medicinal plants and thus evaluate their antimicrobial effects.

2. Materials and Methods

2.1. Plants Material Collection and Preparation

The leaves and flowers of *R. officinalis*, *M. pulegium*, *S. officinalis*, and *T. zygis* were harvested in May 2018 for *R. officinalis*, and in June 2019 for *M. pulegium*, *S. officinalis*, and *T. zygis* (Figure 1). These plant species were collected from the Ifrane-Boulemane region of Morocco, and identified by Mohammed Fennan, a botanical expert from the Scientific Institute of the University of Mohammed V. After that, the specimens of the plants in question were prepared and deposited in the herbarium of Mohammed First University, Oujda, Morocco, under the reference numbers HUMPOM628, HUMPOM814, HUMPOM516, and HUMPOM311, respectively, for *R. officinalis*, *M. pulegium*, *S. officinalis*, and *T. zygis*. Indeed, the samples of the collected products were prepared in ventilated bags to avoid heat and the compaction of the plant material, which can lead to fermentation. The samples were quickly sent to the drying site, then to the laboratory for manual preprinting. This operation consisted of clearing the harvested plants of dirt, insects, twigs, weeds, and soil. In addition, we avoided drying in full sunlight because the direct sun causes a loss of active ingredients, the yellowing and rapid browning of plants, and a deterioration of their medical value. Drying took place in the shade, in a well-ventilated area and at a temperature of between 30 and 40 °C.

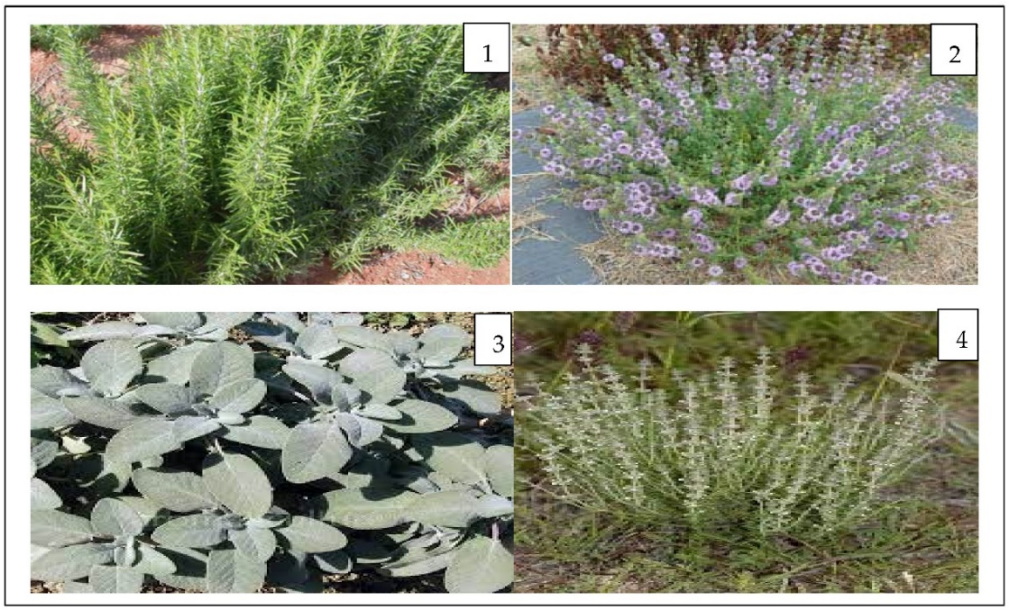

Figure 1. Plants used in our study, *R. officinalis* (**1**), *M. pulegium* (**2**), *S. officinalis* (**3**), and *T. Zygis* (**4**).

2.2. Biological Material

The determination of the antimicrobial activity of different EO was carried out on nine bacterial strains (Gram-positive Cocci and Gram-negative Bacillus) and seven fungal strains (yeasts and molds) (Table 1). These selected microorganisms are pathogenic, known for their strong resistance, and their invasive and toxic power in humans. They are frequently encountered in many infections in Morocco, which pose a clinical and therapeutic problem. These strains were isolated from a hospital environment (Provincial Hospital Mohamed V, Meknes). All strains were taken from a 20% glycerol stock at −80 °C, rejuvenated on Mueller–Hinton and Sabouraud broths, and subcultured before use.

Table 1. The test microorganisms used in this study.

	Microorganism	Reference
Fungi	*Candida albicans*	C. a
	Candida dubliniensis	C. d
	Saccharomyces cerevisiae	Sac. c
	Aspergillus niger	Asp. n
	Candida tropicalis	C. t
	Candida krusei	C. kr
	Candida parapsilosis	C. par
Bacteria	*Staphylococcus epidermidis*	5994
	Staphylococcus aureus BLACT	4IH2510
	Streptococcus agalactiae (B)	7DT1887
	Escherichia coli sauvage	3DT1938
	Escherichia coli BLSE	2DT2057
	Enterobacter cloacae	02EV317
	Klebsiella pneumoniae	3DT1823
	Proteus mirabilis	2DS5461
	Pseudomonas aeruginosa	2DT2138

2.3. Extraction and Analysis of EO Plants

2.3.1. Hydrodistillation Extraction of EO Plants

The extraction of EOs was carried out by hydrodistillation using a Clevenger-type apparatus. In a two-liter flask, 100 g of the dry matter was immersed in one liter of distilled water. This mixture was boiled for three hours, using a balloon heater to produce the water vapor which entrains the EO. The vapor produced condenses while passing through a cooler. The condensates (EO and hydrolat) were separated by decantation, and then the organic phase was dried over magnesium sulfate and stored at a temperature of 4 °C, in opaque glass bottles, hermetically sealed to protect them from air, light, and temperature variations, which are the main agents of degradation. Three replicates were performed to determine the average yield [21].

2.3.2. GC/MS Analysis of EO Plants

The analysis of the EO was carried out using the GC/MS technique and a gas chromatograph of the Thermo Electron type (Trace GC Ultra) coupled to a spectrometer system mass of the Thermo Electron Trace MS type (Thermo Electron: Trace Ultra GC, Polaris Q MS). The fragmentation was carried out by an electron impact intensity of 70 Ev. The chromatograph was equipped with a DB-5 type column (5% phenyl-methyl- siloxane) (30 m × 0.25 mm × 0.25 µm film thickness), a flame ionization detector (FID) powered by a H2/Air mixture. The column temperature was programmed at a rate of 4 °C/min from 50 to 200 °C for 5 min. The mode of injection is split (leak rate: 1/70, flow rate ml/min); the carrier gas used was nitrogen with a flow rate of 1 mL/min. The identification of the EO compounds was carried out by comparing the retention indices calculated for each of the eluted compounds (based on the retention times of a hydrocarbon standard (C7-C40) with those contained in the databases available: Adams, 2007. The mass spectra of each of the compounds were also compared with those of the databases mentioned above [22].

2.4. Determination of Minimum Inhibitory Concentrations (MIC), Minimum Bactericidal Concentrations (MBC) and Minimum Fungicide Concentrations (MFC) of the EO of Plants

The determination of the minimum inhibitory concentration (MIC) was carried out according to the reference method of microdilution using 96-well microplates [23]. The MIC corresponds to the lowest concentration of the EO which produces complete inhibition of the growth of the microorganism, appreciable to the naked eye, tested after incubation. Therefore, from a stock solution of the EO prepared in DMSO (10%), a series of dilutions were carried out to obtain concentrations of 5 to 0.93×10^{-2} mg/mL of each EO. These dilutions were prepared in Mueller–Hinton broth medium for bacteria and in Sabouraud broth for fungi for a final volume of 100 µL for each concentration. Then, 100 µL of the microbial inoculum, with a final concentration of 106 or 104 CFU/mL in the case of bacteria or fungi, respectively, were added at the different concentrations of the dilution series. After incubation for 24 h at 37 °C, 10 µL of resazurin was added to each well as an indicator of bacterial growth. After a second incubation at 37 °C for two hours, microbial growth was revealed by a color change from purple to pink. The MIC value was determined to be the lowest concentration that prevents a color change of resazurin. The 11th and 12th wells were considered growth control and sterility control, respectively. The test was repeated twice for each oil. The standard antifungal studied was terbinafine (250 mg); this drug was added, after grinding, to 2 mL of DMSO (10%). To determine the minimum bactericidal concentration/minimum fungicidal concentration (MBC/MFC), 10 µL were taken from each well without visible growth and inoculated in Mueller–Hinton agar (MHA) for 24 h at 37 °C for bacteria, or in Sabouraud for fungi. MBC and MFC were defined as the lowest concentration of samples tested that produced a 99.9% reduction in CFU/mL compared to the control. In addition, the MBC/MIC or MFC/MIC ratio of each extract can be calculated to evaluate the antimicrobial power; thus, if the ratio is less than 4, the effect of the EO was bactericidal/fungicidal, and if the ratio is greater than 4, the sample has a bacteriostatic/fungistatic effect [24].

3. Results and Discussions

3.1. Yields of EOs Plants

The results relating to the yields of EO of *R. officinalis*, *M. pulegium*, *S. officinalis*, and *T. zygis* are given in Figure S1. *M. pulegium* presented the highest yield of EO, with 3.64 ± 0.12%, followed by *R. officinalis* with 3.53 ± 0.06%, *S. officinalis* with 2.48 ± 0.06%, and *T. zygis* with 2.34 ± 0.08%. Here, the EO yield obtained for *M. pulegium* was lower than that previously reported by Nadia et al. (i.e., 6.9%) [25]. The EO yield of *R. officinalis* was higher than the one reported by Makhloufi et al., for plants collected from southwestern Algeria (1.6%) [26]. The yield of EO for *S. officinalis* obtained in our study was higher than the one reported by Jack et al. (2%) [27]. Regarding *T. zygis*, a greater yield has been obtained compared to that obtained by Zekri et al. (2017) (0.3%) [28]. The difference observed in the EO yield between the literature results and our study could be explained by an adaptation of the plant to abiotic factors, such as the climate specific to the sample's origin regions, to geographical factors, such as altitude, and the nature of the soil that directly interferes with the biosynthesis towards the preferential formation of specific products [29].

3.2. The Chemical Analysis of the Plant EO

The chemical composition of the EO extracted from the studied species, *R. officinalis*, *M. pulegium*, *S. officinalis*, and *T. zygis*, was analyzed using GC/MS, whose percentages are, respectively, in the order of 99.2%, 98.2%, 98.1%, and 99.8%. Table 2 shows all the chemical composition results for each species. According to our results, the analysis of the chemical composition of the EO of *R. officinalis* (Figure S2A) made it possible to identify 29 compounds, whose content was greater than or equal to 0.01%. These 29 compounds represent 99.16% of all the constituents of the EO. The latter was characterized by the majority presence of 1, 8-cineol (43.8%), camphor (18.7%), α-pinene (9.5%), borneol (5.9%), and β-pinene (5.18%) (Figure S4). Finally, it should be noted that the chemical compounds of this species were strongly dominated by oxygenated monoterpenes (75.3%), hydrocarbon monoterpenes (22.4%), and sesquiterpene compounds (0.8%). These results were consistent with those reported by Ben Abada et al., (2020) whose main constituents were 1,8-cineole (44.8%), followed by camphor (9.2%), α-pinene (9.2%), borneol (5,2%), camphene (3.2%) and β-pinene (3.3%) [30]. The analysis of the chemical composition of the *M. pulegium* EO (Figure S2B) made it possible to identify 30 compounds whose content is greater than or equal to 0.01%. These 30 compounds represent 98.2% of all the chemical components of the EO. This oil was characterized by the majority presence of piperitenone (32.9%), pulegone (32.84%), menthone (10.9%), piperitone (7.9%), and limonene (2.0%) (Figure S4). The chemical compounds of *M. pulegium* were strongly dominated by oxygenated monoterpenes (94.8%), hydrocarbon monoterpenes (2.49%), and sesquiterpene compounds (0.1%). The results are consistent with those reported by Ahmed et al. (2018). They demonstrated the presence of the predominant components with different percentages: pulegone (57.8–62.8%), menthone (9.5–15.0%), limonene (4.9–6.9%), δ-germacrene (3.1–3.3%), and *p*-menthane-3-ol (2.3–3.6%) [31]. Regarding the chemical composition of the EO of *S. officinalis*, we have been able to identify 31 compounds in total, representing 98.08% of the chemical components of the EO, with 1, 8-cineole (16.8%), *trans*-thujone (15.9%), camphor (11.7%), *trans*-caryophyllene (12.56%), α-humelene (8.2%), and viridiflorol (7.4%) being major compounds (Figure S4). These components have been grouped into four classes: monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, and oxygenated sesquiterpene. The results showed that oxygenated monoterpenes (50.5%) constitute the main class of compounds, followed by sesquiterpene hydrocarbons (21.8%), then monoterpene hydrocarbons (16.0%), and finally oxygenated sesquiterpene (9.7%). The data from the analysis of *S. officinalis* (Figure S2C) was consistent with those previously reported by Hassan et al., (2018). However, the results of the chemical compositions of nonacosan (3.1%) and pentacosan (2.4%) are absent in our samples [32]. Finally, the chemical composition of the EO of *T. zygis* made it possible to identify 34 compounds

whose content was greater than or equal to 0.01% (Figure S2D). These 34 compounds represent 99.8% of the components of the EO. This oil was characterized by the majority presence of thymol (36.4%), carvacrol (24.1%), cymene (23.5%), and terpenen-4-ol (2.5%) (Figure S4). The chemical compounds of *T. zygis* were strongly dominated by oxygenated monoterpenes (67.5%), hydrocarbon monoterpenes (29.9%), sesquiterpene (1.5%), and oxygenated sesquiterpene compounds (0.8%). However, *T. zygis* from Spain found the same major compound (thymol), with a content of (68.1%) [33]. The results showed that the chemical profile of our EO varies from those of other origins, by the existence of qualitative and quantitative differences in the individual components. Our EO have various biological effects on body cells, acting, for example, as infectious agents. The effects and targets are multiple, because of each chemical component and their multiplicity. These biological effects can be attributed to the most abundant compounds, including piperitenone (active principle of *M. pulegium*) [34], and 1,8-cineole (the active ingredient of *S. officinalis* and *R. officinalis*), being particularly useful for treating digestive disorders [18], as an antimycotic [15], and an antibacterial [16]. It is noted that these active ingredients belong to the carbonyl group, comprising a double bond between a carbon atom and an oxygen atom, with the carbon atom being commonly bonded to the hydrogen atoms or the carbon. Indeed, the whole class of polyphenols is mainly used in phytomedicines for the treatment of different diseases.

Table 2. Chemical composition of *R. officinalis*, *M. pulegium*, *S. officinalis*, and *T. zygis* EO.

	IK	Chemical Compositions	*R. officinalis*	*M. pulegium*	*S. officinalis*	*T. zygis*
1	926	Tricyclene	0.2	—	—	—
2	930	α-Thujene	0.3	—	—	0.8
3	939	α-Pinene	9.5	0.2	8.1	—
4	952	3-Methyl cyclohexanone	—	0.1	—	—
5	954	Camphene	4.5	0.3	3.2	0.7
6	960	Thuja-2,4-diene	—	—-	—-	—
7	967	Verbenene		—	—	—
8	979	ß-Pinene	5.2	—	1.1	0.1
9	983	Octanone		0.1	—	0.4
10	990	Myrcene	0.5	—	1.0	0.6
11	991	Octanol		0.3	—	—
12	1002	α-Phellandrene	0.1	—	—	0.1
13	1011	δ-3-Carene	–	—	—	—
14	1017	α-Terpene	0.5	—	0.3	—
15	1024	*p*-Cymène	1.2	—	—	—
16	1026	Cymene		—	0.5	23.5
17	1029	Limonene	—	2.0	1.3	0.4
18	1031	1,8-Cineole	43.8	0.1	16.8	0.5
19	1152	Menthone	—	4.3	—	—
20	1155	Isopulegol		0.3	—	—
21	1059	γ-Terpinene	0.7	—	0.4	2.9
22	1162	Menthone		10.9	—	—
23	1070	2-Methyl-5-propan-2-ylbicyclohexan-2-ol	0.2	—	—	0.1
24	1072	Linalool oxide-cis				0.1
25	1086	Fenchone	—	—	—	0.1
26	1088	Terpinolene	0.2	—	0.1	0.3
27	1096	Linalool	0.6	—	—	—
28	1102	Thujone-cis	—	—	3.1	—
29	1114	Thujone-trans	—	—	15.9	—
30	1022	Dehydrosabinaketone	—	—	—	—
31	1133	2-(4-Méthyl-2,4-cyclohexadiényl)-2 propanol	—	—	—-	—
32	1138	Iso-3-Thujanol	—	—	—	—
33	1146	Camphor	18.7	—-	11.7	2.2
34	1153	Neo-3-Tujanol	—	—-	0.1	—-

Table 2. *Cont.*

	IK	Chemical Compositions	*R. officinalis*	*M. pulegium*	*S. officinalis*	*T. zygis*
35	1164	1,3,4-Trimethyl-3-cyclohexene-1-carboxaldehyde	—	0.4	—-	—-
36	1169	Borneol	5.9	—-	0.9	0.1
37	1171	Menthol	—	0.3	——	——
38	1177	Terpenen-4-ol	1.0	0.3	0.2	2.5
39	1188	α-Terpineol	3.7	2.7	—	0.6
40	1243	Carvone	—	0.50	—	—-
41	1254	Piperitone epoxy	—	0.10	—-	—-
42	1229	Pulegol-cis	—	1.0	—-	—-
43	1237	Pulegone	—	32.8	—-	0.1
44	1239	Isobornylformate	—	—	—	—
45	1252	Piperitone	—	7.9	—-	—-
46	1285	Bornylacetate	1.6	—	0.5	—-
47	1290	Thymol				36.4
48	1299	Carvacrol	—	—	0.5	24.1
49	1304	Methyl acetate		0.03	—	—-
50	1309	4-Vinylgaïacol		0.04	—	—-
51	1343	Piperitenone		32.93	—	—
52	1388	ß-Bourbonene		0.02	—	—
53	1408	Caryophyllene-cis		0.15	—	—
54	1419	Caryophyllene-trans	0.5	—	12.6	1.4
55	1441	Aromandendrene	—	—	0.6	—
56	1454	α-Humelene		0.3	8.2	—
57	1481	Delta-Germacrene	—	0.1	—	—
58	1496	Viridiflorene	—	—	0.3	—
59	1513	Cadinene	—	—	—-	0.1
60	1515	Cubebol	—	—	—	
61	1523	ß-Cadinene	—	—	0.2	0.1
62	1578	Spathulenol	—	—	0.2	—
63	1583	Caryophyllene oxide	0.2	—	0.5	0.5
64	1592	Viridiflorol	—	—	7.4	—
66	1608	Humulene epoxide II		—	0.6	—
67	1641	Aromandendrene epoxy	—	—	0.2	—-
68	1644	Selina-3,11-dien-6-α ol	—	—	0.2	—
69	1686	Germacra4(15),5,10(14)-trien-1-α-ol				0.1
70	1729	Longifolol				0.1
71	2060	13-Epimanool	—	—	1.3	—
72	2100	Heneicosan		0.2	—	—
73	2200	Docosene		0.2	—	—
Percentage of monoterpene hydrocarbons %			22.38	2.49	16.006	29.94
Percent Sesquiterpene Hydrocarbons %			0.24	0.54	21.82	1.54
Percentage of oxygenated monoterpenes %			75.3	94.83	50.54	67.51
Percentage of oxygenated sesquiterpene %			0.84	0.14	9.71	0.83
Total %			99.16	98.19	98.076	99.82

As shown in Figure S3, the chemical compounds isolated from these EOs are classified into four types (monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons and oxygenated sesquiterpenes). Indeed, the results show that in the case of *T. zygis* monoterpene hydrocarbons exhibited the highest percentage (29.9%), followed by *R. officinalis* (22.4%), *S. officinalis* (16.0%), and *M. pulegium* (2.5%). In the case of oxygenated monoterpenes, *M. pulegium* presented the highest percentage (94.8%), followed by *R. officinalis* (75.3%), *S. officinalis* (50.5%), and *T. zygis* (67.51%). In the case of sesquiterpene hydrocarbons, *S. officinalis* presented the highest percentage (21.8%), followed by *T. zygis* (1.5%), *M. pulegium* (0.5%), and *R. officinalis* (0.2%). In the case of oxygenated sesquiterpene, *S. officinalis* presented the highest percentage (9.7%), followed by *R. officinalis* (0.8%) and *M. pulegium* (0.1%). These results indicate that these four EOs are of great value in different

fields. These plants belonging to the Lamiaceae family have antioxidant, antimicrobial and anti-inflammatory properties. Their biological activities are determined by their active components. Then, they can be considered very important natural resources.

3.3. Antimicrobial Activity

3.3.1. Antibacterial Activity

The antibacterial activity of the EO of the four Moroccan medicinal plants was evaluated by the agar dilution method against nine bacterial strains (Gram-positive bacteria: *S. epidermidis*, *S. aureus*, *S. agalactiae*; and Gram-negative bacteria: wild-type *E. coli*, *E. coli* BLSE, *E. cloacae*, *K. pneumoniae*, *P. mirabilis*, and *P. aeruginosa*). The results were expressed in MIC and MBC (Table 3). Previously published data indicate that a plant extract with MIC values below 600 µg/mL is considered adequate and has excellent antibacterial activity [35]. Based on this criterion, we can say that the EO tested have significant antibacterial activity. They inhibit the growth of bacterial strains tested to varying degrees. The EO isolated from *T. zygis* has very strong activity against *S. agalactiae*, *E. coli*, and *E. cloacae* with MICs ranging from 40 to 300 µg/mL, and has moderate activity against *S. epidermidis*, *S. aureus*, *K. pneumoniae*, *P. mirabilis*, and *P. aeruginosa* with MIC values of 1200, 1200, 600, 2500, and 2500 µg/mL, respectively. The presence of high levels of thymol (36.4%), carvacrol (24.1%), cymene (23.5%), and terpenen-4-ol (2.5%) as major components in the *T. zygis* essential oil, and the potential synergistic phenomenon between them, could explain the strong antibacterial activity of this oil. It was reported in a previous study that an essential oil rich in thymol (23.49%) and carvacrol (21.31%), has a very high antibacterial activity [36,37].

Table 3. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the studied species' EOs (µg/mL).

	Microrganisms	Reference	*R. officinalis*		*M. puleguim*		*S. officinalis*		*T. zygis*	
			MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Gram-positive bacteria	*S. epidermidis*	5994	5000	5000	>5000	>5000	5000	>5000	1200	2500
	S. aureus BLACT	4IH2510	1200	2500	2500	5000	5000	5000	1200	2500
	S. agalactiae (B)	7DT1887	>5000	>5000	>5000	>5000	600	1200	300	600
Gram negative bacteria	Wild-type *E. coli*	3DT1938	>5000	>5000	1200	2500	>5000	>5000	300	600
	E. coli BLSE	2DT2057	5000	5000	>5000	>5000	2500	5000	5000	5000
	E. cloacae	02EV317	600	1200	300	600	150	300	40	70
	K. pneumoniae	3DT1823	>5000	>5000	>5000	>5000	>5000	>5000	600	1200
	P. mirabilis	2DS5461	>5000	>5000	2500	5000	>5000	>5000	2500	2500
	P. aeruginosa	2DT2138	>5000	>5000	1200	2500	2500	5000	2500	5000

Staphylococcus epidermidis: S. epidermidis, Staphylococcus aureus: S. aureus, Streptococcus agalactiae: S. agalactiae, Escherichia coli: E. coli, Enterobacter cloacae: E. cloacae, *Klebsiella pneumoniae: K. pneumoniae, Proteus mirabilis: P. mirabilis, Pseudomonas aeruginosa: P. aeruginosa.*

Carvacrol and thymol act by disrupting the bacterial wall and plasma membrane, through interaction with membrane proteins. Specifically, thymol appears to disrupt the production of ATP by the cell, inhibiting the Krebs cycle, preventing it from recovering its normal functioning after exposure to thymol [38]. For its part, carvacrol acts essentially on the plasma membrane, where it modifies the membrane fluidity, and disturbs the ionic balance of the bacteria [38]. In addition, the isolated EOs of R. officinalis, *M. puleguim*, and *S. officinalis* have very high activity with MIC values of 600, 300, and 150 µg/mL, respectively, against *E. cloacae*. Generally, Gram-positive bacteria are more sensitive to the impact of the examined EOs than Gram-negative bacteria, according to our results. The cell wall structure of Gram-positive bacteria is known to make them susceptible to EO activity. Furthermore, the greater resistance of Gram-negative bacteria can be explained by reason

of the complexity of their cell wall, containing a double membrane as opposed to the single glycoprotein/teichoic acid membrane of Gram-positive bacteria [39]. In fact, this can be attributed to the fact that Gram-negative bacteria have a rigid outer membrane, rich in lipopolysaccharide (LPS) and more complex, thus limiting the diffusion of hydrophobic compounds through it, while this extra-complex membrane is absented in Gram-positive ones that are rather surrounded by a thick peptidoglycan wall not dense enough to resist small antimicrobial molecules, facilitating access to the cell membrane [40,41]. In addition, Gram-positive bacteria can facilitate the infiltration of the hydrophobic compounds of EO due to the lipophilic ends of lipoicheoic acid present in the cell membrane [42]. Efflux systems such as the RND (Resistance-Nodulation cell division) family represent the main cause of resistance and multi-resistance by efflux in Gram-negative bacteria.

The bioactivity of the chemical compounds in each oil, the functional groups of the majority substance (alcohols, phenols, aldehydes), and synergistic effects between components might explain the differential in antibacterial activity between the EOs in question. Phenols, alcohols, aldehydes, and ketones are well known as the most efficient chemical substances with a broad spectrum of antibacterial activity [43,44]. Phenols are the most active chemicals in bacteria owing to the acid nature of their hydroxyl substituent [39]. Because alcohols are soluble in aqueous media and inflict severe damage to microorganisms' cell walls, they are particularly effective against bacterial strains [45]. Rather than being bacteriostatic, alcohols are bactericidal [46]. The phytochemical analysis of the examined EO revealed the presence of these components in differing quantities amongst the EO in our study. This might explain where the antibacterial action comes from, as well as the differences in activity amongst the oils analyzed.

3.3.2. Antifungal Activity

The antifungal activity of the EO in question were evaluated against seven fungal strains, namely *C. albicans*, *C. dubliniensis*, *C. tropicalis*, *C. krusei*, *C. parapsilosis*, *S. cerevisiae*, and *A. niger*. Results were expressed in MIC and MFC (Table 4). The EOs of *T. zygis* and *S. officinalis* showed a very good antifungal power, referring to the reading established by [47]. Indeed, the results of this activity revealed a strong activity in the EO of *T. zygis* and moderate activity in the EO of *S. officinalis*. On the other hand, the EO of *R. officinalis* and *M. puleguim* were inactive concerning the strains studied and the concentrations used. However, the most sensitive strains are *C. parapsilosis* and *A. niger*. Both strains were inhibited by the *T. zygis* EO at a concentration of 300 µg/mL. These results are consistent with those of Rota et al., (2008), which showed greater sensitivity of *S. aureus* compared to *E. coli* to the EO extracted from *T. zygis* [33]. Previous studies on the *R. officinalis* EO reveal antimicrobial activity and indicate a similarity with our results obtained specifically in the *S. aureus* and *E. coli* strains in this work [48–50]. The presence of major phenolic chemicals such as carvacrol and cymene, which are renowned for their antibacterial characteristics, might explain the high antifungal activity of *T. zygis* [51]. These compounds have been shown to enter the microorganism and interact with active enzymatic sites and/or cellular emitted, while others involve cell membrane disruption and pro-oxidant effects [52]. Because of the variability in the amounts and profiles of essential oil components, it is likely that their antimicrobial activity is not due to a single mechanism, but to several sites of action at the cellular level. The mode of action of essential oils depends primarily on the type and characteristics of the active components, in particular their hydrophobic properties, which allow them to penetrate the double phospholipid layer of the microbial cell membrane. This can induce a change in membrane conformation, a chemo-osmotic disturbance and an ion leak (K^+) [53]. This mechanism was observed with tea tree oil on yeast (*Candida albicans*) in vitro. Some phenolic compounds of essential oils interfere with the membrane proteins of microorganisms such as ATPase, either by direct action on the hydrophobic part of the protein, or by interfering with the translocation of protons in the membrane preventing phosphorylation of ADP [54]. Essential oils can also inhibit the synthesis of DNA, RNA, proteins, and polysaccharides [55]. In addition, it has been shown that the antifungal

activity of the essential oil rich in thymol and carvacrol against *Candida* isolates is due to inhibition of the biosynthesis of ergosterol and the disruption of membrane integrity [56,57]. Additionally, it has been reported that the fungicidal effect of coriander essential oil is the result of damage in the cytoplasm membrane and the subsequent leakage of intracellular components, such as DNA [58]. Similarly, the disruption of the endomembrane system of fungal cells, including the plasma membrane and mitochondria, that is, the inhibition of the synthesis of ergosterol, dehydrogenase malate, Mitochondrial ATPase, and succinate dehydrogenase activities, was related to the antifungal activity of the natural essential oil derived from turmeric against *Aspergillus flavus* [59]. Based on these results, it can be said that the antifungal activity of the studied essential oils may be due, with a strong possibility, to the chemical compounds that constitute them, especially thymol and carvacrol.

Table 4. Minimum Inhibitory Concentration (MIC) and Minimum Fungicide Concentration (MFC) of EOs of Studied Species (µg/mL).

Micro-Organisme	*R. officinalis*		*M. puleguim*		*S. officinalis*		*T. zygis*	
	MIC	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**
C. albicans	>5000	>5000	>5000	>5000	>5000	>5000	1200	1200
C. dubliniensis	>5000	>5000	>5000	>5000	>5000	>5000	1200	1200
C. tropicalis	>5000	>5000	>5000	>5000	>5000	>5000	1200	1200
C. krusei	>5000	>5000	>5000	>5000	>5000	>5000	2500	2500
C. parapsilosis	>5000	>5000	>5000	>5000	2500	5000	300	600
S. cerevisiae	>5000	>5000	>5000	>5000	>5000	>5000	1200	1200
A. niger	>5000	>5000	>5000	>5000	1200	2500	300	600

4. Conclusions

This work on these plants, *R. officinalis*, *M. pulegium*, *S. officinalis* and *T. zygis*, was based on their traditional uses by the Moroccan population. *M. pulegium* has the highest EO yield followed by *R. officinalis*, then *S. officinalis,* and finally *T. zygis*. It has been proven that these essential oils contain very important components that are different from each other, with different effects. They also contain the same components, such as α-pinene, camphene, and limonene. These EO have varying degrees of antibacterial and antifungal action, with the essential oils of *T. zygis* being particularly potent in this regard. Therefore, these results support the possibility of using the *T. zygis* essential oil as a natural agent in several fields such as the food and pharmaceutical industries to fight against antibiotic resistance.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/chemistry4040115/s1. Figure S1: Yields of essential oils plants, Figure S2: GC-MS chromatogram of the EO of *R. officinals* (A), *M. pulegium* (B), *S. officinals* (C), and *T. zygis* (D), Figure S3: Chemical families of the four plants' EO, Figure S4: Major chemical compounds isolated from essential oils of *R. officinalis*, *M. pulegium*, *S. officinalis*, and *T. zygis*.

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