Hydrodistillation kinetic and biological investigations of essential oils from the Tunisian *Crithmum maritimum* L.

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Abstract: This work describes the study of the chemical composition and bioactivity of the essential oils of *Crithmum maritimum* and the location of dillapiole one of their major and bioactive constituents. This compound was detected at increasing percentages in all the collected fractions during the extraction process. The highest percentage of dillapiole was obtained in the last fraction (F_9) with 100% in the roots. The essentials oils of roots and aerial parts and F_9 were assayed for their antiradical and enzymatic antioxidant properties (Catalase and Paraoxonase), the results showed that F_9 and the roots essential oil improve the activity of Catalase and Paraoxonase, respectively. The isolated oils and F_9 were tested against five bacteria and four *candida* species. The oil roots exhibited significant activity towards *Staphylococcus aureus* and *Enterococcus faecalis*. Dillapiole was found to be the strongest anticandidal. The cytotoxic activity of essential oils and F_9 was evaluated against Hela and A549 cancer cell lines. F_9 exhibited the best cytotoxic effect against Hela cell line. These results may suggest that *C. maritimum* essential oils can be used as a good source of dillapiole which could be used as a natural preservative ingredient in food and/or for pharmaceutical preparations.

Keywords: Essential oil, *Crithmum maritimum* L., Dillapiole, Antioxidant activity, Antimicrobial activity, Cytotoxic activity.

Résumé : Ce travail décrit l'étude de la composition chimique et la bioactivité des huiles essentielles de *crithmum maritimum* et la localisation du dillapiole un de leurs constituants majoritaire et bioactif. Ce composé a été détecté à des pourcentages croissants dans toutes les fractions recueillies au cours du processus d'extraction. Le pourcentage le plus élevé de dillapiole a été obtenu dans la dernière fraction (F_9) à 100% dans les racines. Les huiles essentielles des racines et des parties aériennes ainsi que F_9 ont été testées pour leurs propriétés antiradicalaires et antioxydantes enzymatiques (la catalase et la paraoxonase), les résultats ont montré que l'huile essentielle des racines et F_9 améliorent l'activité de la catalase et de la paraoxonase, respectivement. Les huiles des racines, des parties aériennes et F_9 ont été testées envers cinq bactéries et quatre espèces de Candida. Les huiles essentielles des racines présentaient une activité significative contre *Staphylococcus aureus* et *Enterococcus faecalis*. Le Dillapiole a montré la plus forte activité antifongique. L'activité cytotoxique des huiles essentielles des racines, des parties aériennes et de F_9 a été évaluée vis-à-vis des lignées cellulaires cancéreuses Hela et A549. F_9 présentait le meilleur effet cytotoxique contre la lignée cellulaire Hela. Ces résultats peuvent suggérer que les huiles essentielles de *C. maritimum* peuvent être utilisées comme une bonne source de dillapiole qui pourrait être utilisé comme un ingrédient naturel conservateur dans les aliments et / ou dans les préparations pharmaceutiques.

Mots-clés : Huile essentielle, *Crithmum maritimum* L., Dillapiole, Activité antioxydante, Activité antimicrobienne, Activité cytotoxique.

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INTRODUCTION

Crithmum maritimum L. is a halophyte belonging to the Apiaceae family and typical of coastal ecosystems. It grows wild on maritime rocks, piers, breakwaters and sandy beaches along the Mediterranean, Black Sea coasts as well as along the Pacific and Atlantic coasts under the influence of salt-rich sprays. It is rarely found on sand or gravel [1]. In temperate climates, the plant is used for ornamental decoration in rock gardens along the sea [2]. *C. maritimum* has been largely used for nutritional and medicinal purposes; it is an edible aromatic plant and has a powerful scent. It is also cultivated in many areas across Europe for several economic and industrial purposes [3,4]. In Tunisia, the leaves preserved in the vinegar, are used as condiment [5]. Its organs (roots, leaves and fruits) are rich in several bioactive substances that could be used as aromatic, medicinal, antimicrobial and insecticide [6-8]. The antioxidant activity of the crude essential oils from *C. maritimum* has been investigated using $DPPH^{\cdot}$, $ABTS^{\cdot+}$ and reducing power assays and showed a moderate activity [9-11]. It endowed with many medicinal properties, such as a tonic, diuretic, carminative and vermifuge [10]. It has been shown that the oil extracted from *C. maritimum* seeds has good nutritional quality and could be used for human consumption [12]. The essential oil is used in the formulation of cosmetics with slimming properties [13]. The demand from the medicinal and cosmetic industries for essential oils or other compounds of this halophyte species is rapidly increasing [14]. It is also known that all parts of the plant contain essential oil [15]. The main constituents are variable and depend on the region of growth [16]. The chemical composition, the antioxidant and the antibacterial activities of essential oils from *C. maritimum* have been the subject of some previous publications [1,11,12,16-18]. In some previous studies, it has been shown that the yield of the essential oil extracted from plants varies according to the duration of the extraction [19]. In the case of *Ridolfia segetum*, 78.4% of the flowers oil was recovered during the first 30 min [19], while for *Thymus vulgaris* L. 90% of the total oil was extracted in the 1h min, whereas in the case of *Lippia citriodora*, the same percentage was obtained during the first 4h 35 min, and finally, 90% of the total oil of *Artemisia campestris* L. was isolated during the first 2h [20]. Also, it has been demonstrated that the final composition of the essential oils can be influenced by the distillation time [19]. All the identified compounds in the flowers essential oil from *Ridolfia segetum* were detected in the first 10 min. Most of them were monoterpene hydrocarbons. It has been also shown that the antibacterial activity of this oil increased according to the proportion of monoterpene alcohols and phenolic derivatives contained in the tested fractions [19]. In the case of Thymus essential oil, the percentages of 1-octen-2-ol, linalool and 1,8-cineole decrease during the extraction process, whereas the proportions of γ-terpinene and carvacrol increase [20]. Recently, natural plants have received much attention as sources of biological active substances including antioxidants. To the best of our knowledge, antioxidant activities during a kinetic investigation of the essential oil of *C. maritimum* has not been previously reported, and there are no literature data about multi-method evaluations on different fractions from this species. The aim of the present study was the optimization of the yield and the chemical composition of the aerial parts and the roots of *C. maritimum* essential oils during their extraction by hydrodistillation which could lead to the isolation of pure products, allows the location of activities studied and help to understand the structure-function relationship. In the present study, the *in vitro* antioxidant capacities, using non-enzymatic $DPPH^{\cdot}$, $ABTS^{\cdot+}$ and reducing power assays and the enzymatic antioxidant property using catalase and paraoxanase of the aerial parts and the roots essential oils and of the dillapiole rich fraction (F_9) from the roots were also studied. Also the antibacterial, antifungal and cytotoxic activities against Hela and A549 cancer cell

lines of the essential oils and of F_9 were evaluated and discussed.

RESULTS AND DISCUSSION

1. Influence of the hydrodistillation time on the essential oils yields

Most of the two oils were recovered during the first 135 minutes of hydrodistillation since the yields remained constant after this time, indicating that this period is sufficient to isolate practically all the oils. During the first 60 minutes, 73.7% and 70.6% of the total oils were already recovered from the aerial parts and roots, respectively. It can be concluded that the duration of the distillation influenced the yield of the oils.

2. Influence of the distillation time on oils composition

The composition of the fractions was analyzed by GC and GC-MS. The percentage composition, with Linear Retention Indices (L.R.I) calculated for each compound and the identification methods are reported in (Tables I and II). The essential oil of the aerial parts was mainly composed by phenylpropanoids, detected in all the collected fractions (68.4-99.7%). (Table I) presents their distribution and shows that all fractions were rich in these components such as dillapiole (67.7-98.4%), myristicin (0.3-0.9%) and elemicin (0.1-0.5%). Dillapiole was the major constituent of this chemical class. The percentage of phenylpropanoids increased during the extraction process. Figure 1 illustrates the distribution of this group of compounds during the extraction process. Oxygenated monoterpenes were present in all the collected fractions. Methyl carvacrol (0.1-25.9%) was found to be the major constituent of this group. These compounds were mostly detected during the first fifteen minutes (F_1), with a maximum percentage of 26.6% and then they decreased during the rest of the extraction time. Methyl carvacrol represented 25.9% of the total constituents in this fraction (F_1). Monoterpene hydrocarbons were only found in the first seven fractions (Table I). The most abundant compounds from this group were γ-terpinene (0.6-3.5%) and *p*-cymene (0.1-1.0%). A clear fluctuation of their distribution in the first seven collected fractions was observed (Table I). Similarly to the aerial parts, the essential oil from roots was also composed of phenylpropanoids, detected in all fractions (Table II). Their proportion varied from 95.0% (F_1) to 100% (F_9). This group was mainly composed by dillapiole (86.4-100%), myristicin (0.5-1.7%) and elemicin (0.1-0.4%). Dillapiole was the major constituent of these fractions and also of the whole oil. Figure 1 represents the distribution of this group of compounds during the hydrodistillation time. It shows that their amount increases during the first sixty minutes (95.0- 98.8%), decreases between 60 and 75 minutes (87.8%), then began to increase up to 100% after 120 minutes. The most abundant compounds among the phenolic derivatives were methyl carvacrol (0.2-4.4%), *p*-cymen-8-ol (0.2%) and methyl thymol (0.1%), which were distributed in the first seven fractions (Table II). Monoterpenes (γ-terpinene, terpinolene and *p*-cymen-8-ol) are not representative in this oil. They were detected only in small amounts between 15 and 60 minutes. Because the duration of the distillation process strongly influenced the composition of the resulting oil, it can be varied according to the quality of the oil required for a given use.

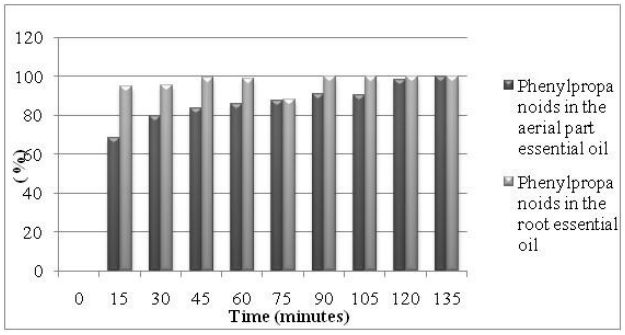

Figure 1: Evolution of phenylpropanoids yield according to the hydrodistillation time

Table I: Percentage composition of the aerial parts essential oil of *Crithmum maritimum* L. during the hydrodistillation process.

Compound	R.I[a]	R.I[b]	SM ($M^{+\cdot}$)	Fractions and duration of hydrodistillation (min)								
				F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8	F_9
p-cymene	1028	1266	134	0.5	0.5	1.0	0.7	0.9	0.1	0.4		
γ-terpinene	1063	1240	136	2.8	2.3	3.5	2.6	3.3	0.6	1.6		
4-terpineol	1178	1605	154		0.1	0.2	0.1	0.1	0.1	0.1		
methyl thymol	1236	1588	164	0.7	0.4	0.3	0.3	0.2	0.2	0.2		
methyl carvacrol	1245	1591	164	25.9	16.2	11.3	10.1	7.5	8.1	7.3	1.8	0.1
Thymol	1292	2170	150			0.1	0.1	0.1	0.1			
Carvacrol	1301	2210	150		0.1	0.1	0.1	0.1				
β-caryophyllene	1420	1603	204	0.1								
trans-α-bergamotene	1438	1575	204	0.1								
germacrene D	1482	1704	204	0.5	0.3	0.1	0.1		0.1	0.1		
α-zingiberene	1495	1717	204	0.2	0.1							
β-bisabolene	1507	1727	204	0.2	0.1							
Myristicin	1520	2261	192	0.5	0.3	0.7	0.7	0.7	0.8	0.7	0.9	0.8
β-sesquiphellandrene	1525	1771	204	0.4	0.2							
germacrene B	1558	1825	204	0.2	0.2							
Elemicin	1559	2232	208	0.2	0.1	0.2	0.3	0.3	0.3	0.3	0.4	0.5
Dillapiole	1622	2342	222	67.7	78.9	82.3	84.7	86.4	89.4	89.1	96.8	98.4
Apiole	1680	2402	222			0.1	0.1	0.1	0.1	0.1		
Total identified				100	99.8	99.9	99.9	99.7	99.9	99.9	99.9	99.8
Phenylpropanoids				68.4	79.3	83.2	85.7	87.4	90.5	90.1	98.1	99.7
Phenolic derivatives				26.6	16.8	12.0	10.4	7.8	8.5	7.6	1.8	0. 1
Yield (v/w)				0.01	0.01	0.02	0.02	0.02	0.02	0.03	0.03	0.03

[a] Order of elution and percentages of individual components are given on a HP-5 capillary column;
[b] retention indices on a HP-innowax column;
F_1: 0-15, F_2: 15-30, F_3: 30-45, F_4: 45-60, F_5: 60-75, F_6: 75-90, F_7: 90-105, F_8: 105-120, F_9: 120-135 min.

Table II: Percentage composition of the roots essential oil of *Crithmum maritimum* L. during the hydrodistillation process.

Compound	R.I[a]	R.I[b]	SM ($M^{+\cdot}$)	Fractions and duration of hydrodistillation (min)								
				F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8	F_9
γ-terpinene	1063	1238	136		0.1							
Terpinolene	1090	1282	136		0.5	0.1	0.2					
p-cymen-8-ol	1185	1847	150		0.2		0.2					
methyl thymol	1236	1585	164	0.1								
methyl carvacrol	1245	1593	164	4.4	1.6	0.3	0.6	0.3	0.2	0.2		
germacrene D	1482	1706	204	0.1								
Myristicin	1520	2260	192	0.7	1.5	1.7	1.4	1.3	1.2	1.2	0.5	
germacrene B	1558	1822	204	0.2	0.1					0.1		
Elemicin	1559	2230	208	0.4	0.1	0.1		0.1	0.1	0.1		
Dillapiole	1622	2344	222	93.9	94.7	97.6	97.4	86.4	98.3	98.3	99.3	100.0
				99.8	98.8	99.8	99.8	99.7	99.8	99.9	99.8	100.0
Phenylpropanoids				95.0	95.5	99.4	98.8	87.8	99.6	99.6	99.8	100.0
Phenolic derivatives				4.5	1.6	0.3	0.6	0.3	0.2	0.2		
Yield (mL/100g)				0.01	0.01	0.01	0.02	0.02	0.02	0.02	0.03	0.03

[a] Order of elution and percentages of individual components are given on a HP-5 capillary column;
[b] retention indices on a HP-innowax column ;
F_1: 0-15, F_2: 15-30, F_3: 30-45, F_4: 45-60, F_5: 60-75, F_6: 75-90, F_7: 90-105, F_8: 105-120, F_9: 120-135 min.

3. Antioxidant activity

The antioxidant activity of the different fraction collected during the hydrodistillation process of the aerial parts and roots of *C. maritimum* has been determined by complementary three complementary different tests, namely the DPPH$^{\cdot}$, ABTS$^{\cdot+}$ and reducing power assays. As a reference antioxidant, BHT (butylated hydroxytoluene) was used. The results are resumed in (Tables III-V).

3.1. DPPH Radical scavenging assay

An interesting antioxidant activity of the various fractions and whole essential oils was proved by the DPPH$^{\cdot}$ Assay (Table III). This activity increases with the increase of the proportion of dillapiole. The last fraction (F_9) from both aerial parts (IC_{50} = 0.042 ± 0.001 mg/mL) and essential oils roots (IC_{50}= 0.038 ± 0.001 mg/mL) was the most active one. The activity was certainly due to the high amount of dillapiole, which practically represented the unique component of this fraction and by its specific hydrogen and/or electron donating power. Table III also shows that fraction F_9 in both cases (aerial parts and roots) was clearly more active than the corresponding essential oils (IC_{50} = 0.91 ± 0.1 mg/mL, aerial parts; IC_{50} = 0.048 ± 0.002 mg/mL, roots). The abrupt increase in the antioxidant activity starting from the fraction F_8 can be explained by an antagonism phenomenon that occurs in the preceding fractions. In particular, the possible formed allyl radical from dillapiole could be neutralized by the presence of other chemicals, which can play the role of a donor of electron or an hydrogen radical.

3.2. ABTS Radical scavenging assay

In the ABTS assay (Table IV), the same phenomenon was observed in both cases (IC_{50} = 0.022 ± 0.004 mg/mL, aerial parts; IC_{50} = 0.331 ± 0.04 mg/mL, roots) for the dillapiole-rich fraction F_9. Again, the antioxidant activity using the ABTS^{+} radical cation increased with the proportion of dillapiole. Table IV also proved that dillapiole, which exclusively formed fraction F_9, in both cases (aerial parts and roots) was almost more active than the corresponding essential oil (IC_{50} = 0.0514 ± 0.002 mg/mL, aerial parts; IC_{50}= 0.643 ± 0.034 mg/mL, roots). The high antioxidant activity of F_8 and F_9 can be explained by the donation of a H$^{\cdot}$ by the methylene of the allyl moiety in dillapiole, leading to the formation of a conjugated stable tertiary radical.

Table III: Antioxidant activity of different fractions of aerial parts of *Crithmum maritimum* mesuared in DPPH assay (IC_{50} in mg/mL)[a,b]

Sample	Aerial parts	Roots
EO[c]	0.910 ± 0.100	0.048 ± 0.002
F_1	0.786 ± 0.001	0.046 ± 0.001
F_2	0.713 ± 0.002	0.044 ± 0.001
F_3	0.689 ± 0.002	0.044 ± 0.002
F_4	0.680 ± 0.004	0.045 ± 0.001
F_5	0.596 ± 0.001	0.044 ± 0.020
F_6	0.508 ± 0.001	0.043 ± 0.014
F_7	0.478 ± 0.001	0.044 ± 0.001
F_8	0.044 ± 0.002	0.038 ± 0.002
F_9	0.042 ± 0.001	0.038 ± 0.001
BHT	0.018 ± 0.01	

[a] Values were expressed as mean ± SE;
[b] IC_{50} (mg/mL): the concentration at which 50% is inhibited;
[c] Essential oils tested in previous work[11]; BHT: butylated hydroxytoluene.

Table IV: Antioxidant activity of different fractions of aerial parts of *Crithmum maritimum* mesuared in ABTS assay (IC_{50} in mg/mL) [a,b]

Sample	Aerial parts	Roots
EO[c]	0.051 ± 0.002	0.643 ± 0.034
F_1	0.048 ± 0.001	0.573 ± 0.010
F_2	0.045 ± 0.002	0. 610 ± 0.060
F_3	0.038 ± 0.003	0.589 ± 0.020
F_4	0.030 ± 0.004	0.555 ± 0.010
F_5	0.030 ± 0.008	0.513 ± 0.020
F_6	0.024 ± 0.003	0.428 ± 0.030
F_7	0.024 ± 0.006	0.379 ± 0.030
F_8	0.022 ± 0.007	0.339 ± 0.020
F_9	0.022 ± 0.004	0.331 ± 0.040
BHT	0.050 ± 0.010	

[a] Values were expressed as mean ± SE;
[b] IC_{50} (mg/mL): the concentration at which 50% is inhibited;
[c] Essential oils tested in previous work[11]; BHT: butylated hydroxytoluene.

3.3. Reducing power assay

Reducing power assay is often used to evaluate the ability of natural antioxidant to donate electrons [21,22]. Many reports have revealed that there is a direct correlation between the antioxidant activity and the reducing power of certain plant extracts [23,25]. Table V shows the reducing power (as indicated by the absorbance at 700 nm) of different fractions of the essential oils from the aerial parts and roots of *C. maritimum* compared with butylated hydroxytoluene (BHT). The two last fractions F_8 and F_9 from the essential oil of the aerial parts showed the highest activity. Again, this could be explained by the ability of dillapiole, which practically was the unique constituent of these fractions, to convert the ferric ion Fe^{3+} to the ferrous ion Fe^{2+} by donating an electron [26]. Increasing the absorbance at 700 nm indicates an increase in the reductive ability. Table V shows the dose-response value for the reducing powers of the tested fractions. With respect to the reference standard BHT (0.02 mg/mL), the IC_{50} values of the *C. maritimum* essential oils fractions showed moderate values.

Table V: Iron (III) to iron (II)-reducing activity of different fractions of aerial parts of *Crithmum maritimum* (IC_{50} in mg/mL)[a,b]

Sample	Aerial parts	Roots
EO[c]	-	-
F_1	1.87 ± 0.01	1.52 ± 0.01
F_2	1.55 ± 0.03	1.12 ± 0.06
F_3	1.57 ± 0.02	1.10 ± 0.02
F_4	1.28 ± 0.02	1.12 ± 0.01
F_5	1.28 ± 0.01	1.06 ± 0.0 2
F_6	1.38 ± 0.01	1.02 ± 0.03
F_7	1.28 ± 0.004	1.00 ± 0.03
F_8	0.69 ± 0.01	0.78 ± 0.02
F_9	0.53 ± 0.007	0.62 ± 0.04
BHT	0.02 ± 0.01	

[a] Values were expressed as mean ± SE;
[b] IC_{50} (mg/mL): the concentration at which 50% is inhibited;
[c] Essential oils tested in previous work[11]; -: not tested, BHT: butylated hydroxytoluene.

3.4. Catalase activity

In the present study we demonstrated a significant activity of the enzyme catalase in the presence of the fraction F_9 (dillapiole) (597.907 ± 0.124 µmol of H_2O_2 destroyed/ min/mg protein) compared with vitamin C (747.384 ± 0.016), followed by roots and aerial parts essential oils which showed a moderate effect (149.476 ± 0.068 and 119.581 ± 0.062 µmol of H_2O_2 destroyed/min/mg protein, respectively) (Table VI). The decrease of catalase activity may cause the

Table VI: The enzymatic antioxidant (Catalase and paraoxonase) and cytotoxic (Hela and A549 cells lines) activities of aerial parts, roots and F_9 (dillapiole) of *Crithmum maritimum*

Sample	Catalase (Units/mg protein)[a]	Paraoxonase (PON 1) (IU/L)[b]	Cytotoxic activity[c]	
			Hela	A549
Aerial parts	149.476 ± 0.068	8 ± 0.2	185.21 ± 1.35	578.68 ± 8.42
Roots	119.581 ± 0.062	17 ± 0.5	1928.45 ± 6.44	620.59 ± 5.69
Dillapiole	597.9072 ± 0.124	6 ± 0.3	12.02 ± 0.13	
ascorbic acid (Vitamin C)	747.384 ± 0.016	55 ± 0.2		

a: Units/mg protein: µmol of H_2O_2 destroyed/min/mg protein;
b: IU : µM/min
c: IC_{50} (µg/mL)

accumulation of the $O_2^{\cdot-}$, H_2O_2 or accumulation of hydrogen peroxide correlates with cancer metastasis [27]. Catalase plays a protective role against cardiovascular diseases.

3.5. Paraoxonase activity (PON1)

PON1 significantly decreases lipid peroxidase generation during LDL oxidation in the presence of HDL modification by lipid peroxidase. Increased PON1 activity is associated with decreased risk of cardiovascular disease; so that promoting PON1 activity has become an important goal in the search for drug treatments to reduce cardiovascular risk. In the present study we demonstrated a significant activity of the enzyme paraoxonase associated with HDL in the presence of the roots essential oil (17 ± 0.5 µM/min/L) compared with the vitamin C (55 ± 0.2 µM/min/L) (Table VI). The aerial parts essential oil and dillapiole fraction (F_9) showed a moderate effect on the activity of this enzyme (8 ± 0.2 and 6 ± 0.3 µM/min/L, respectively) (Table VI). These findings confirm that the roots essential oils of *C. maritimum* was useful for assaying PON1 activity and play a role in the higher risk for several diseases, especially as PON1 has been implicated in many important afflictions affecting health, including organophosphorus sensitivity, and in the etiology of various disorders such as diabetes, atherosclerosis, Parkinson's, and Alzheimer diseases.

4. Antibacterial activity

Because of the small volume of the other fractions, essential oils of the aerial parts and roots, fraction F_9 (dillapiole) from roots of *C. maritimum* were assayed *in vitro* against *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27950), *Enterococcus faecalis* (ATCC 29212), *Staphylococcus aureus* (ATCC 25923) and clinical strain: *Acinetobacter sp.* for determination of their antibacterial activity. From the MIC values (Table VII) it can be noted that fraction F_9 (roots) was less active against *Escherichia coli* (MIC = 5.0 mg/mL), *Staphylococcus aureus* (MIC = 2.5 mg/mL), *Enterococcus faecalis* (MIC = 2.5 mg/mL) and *Acinetobacter* (MIC = 5.0 mg/mL) than the whole essential oil roots. The activity of the latter could be explained by a synergistic association of some of the constituents. Fraction F_9 did not show any activity against *Pseudomonas aeruginosa*. On the other hand, essential oil of the roots showed a moderate antibacterial activity against *S. aureus* (MIC = 0.31mg/mL) and *E. faecalis* (MIC = 0.62 mg/

Table VII: MIC of aerial parts and roots essential oils and F_9 (from roots) of *Crithmum maritimum*

Bacteria	MIC[a] (mg/mL)			
	Aerial parts	**Roots**	**F_9 (roots)**	**Imip.**
Escherichia coli ATCC 25922	5.0 ± 0.1	1.25 ± 0.02	5.0 ± 0.2	0.25
Staphylococcus aureus ATCC 25923	1.25 ± 0.02	0.31 ± 0.01	2.50 ± 0.04	0.0156
Enterococcus faecalis ATCC 29212	10.0 ± 0.2	0.62 ± 0.01	2.50 ± 0.05	0.5
Pseudomonas aeruginosa ATCC 27950	10.0 ± 0.1	>10	>10	-
Acintobacter	1.25 ± 0.01	0.31 ± 0.01	5.0 ± 0.1	-

[a] MIC: Minimum Inhibitory Concentration (mg/mL);
[b] Data represent mean±standard deviation of three replications;
values with different letters differ significantly at $p < 0.05$;
-: not tested, ATCC: American Type Culture Collection; Imip: Imipenem as antibiotic.

Table VIII: Antifungal activity of aerial parts and roots essential oils and F_9 (from roots) of *Crithmum maritimum*

Candida	MIC[a] (mg/mL)			
	Aerial parts	**Roots**	**F_9 (roots)**	**Fluconazole**
***Candida glabrata* ATCC 90030**	0.25 ± 0.01	0.125 ± 0.01	0.062 ± 0.002	0.156± 0.01
***Candida parapsilosis* ATCC 22019**	0.125 ± 0.01	0.25 ± 0.01	0.12 ± 0.02	0.156± 0.01
***Candida krusei* ATCC 6258**	0.25 ± 0.001	0.25 ± 0.01	0.12 ± 0.01	0.169± 0.01
***Candida albicans* ATCC 90028**	0.25 ± 0.03	0.25 ± 0.01	0.12 ± 0.001	0.172± 0.01

[a] MIC: Minimum Inhibitory Concentration (mg/mL);
[b] Data represent mean±standard deviation of three replications;
values with different letters differ significantly at $p < 0.05$;
ATCC: American Type Culture Collection

mL). However, the essential oil of the aerial parts showed a weak antibacterial activity against all used bacteria.
This result may due to the relatively complex chemical composition of this essential oil with respect to the one extracted from the roots.

5. Antifungal activity

The results of the *in vitro* antifungal activity of essential oil from the aerial parts, roots of *C. maritimum* and F_9 (dillapiole) against the employed pathogenic yeasts are presented in table VIII.
These results showed that the two essentialoils exhibited the same antifungal activity against all the Candida tested (MIC = 0.25 mg/mL). On the other hand, the dillapiole rich fraction F_9 displays the same activity against *Candida parapsilosis*, C. *krusei* and *C. albicans* (MIC = 0.12mg/mL) whereas *C. glabrata* was found to be more sensitive towards the same fraction (MIC = 0.062 ± 0.002 mg/mL).

6. Cytotoxic activity

Table VI shows the IC_{50} values of cells treated with aerial parts, roots and the fraction F_9 (dillapiole) essential oils of *C. maritimum*. The results of our studies suggest that F_9 (dillapiole) of *C. maritimum* possess the strongest cytotoxic effects against Hela cell. The lower IC_{50} of the essential oil is the more effective it is for cancer. The American National Cancer Institute declared that there is a significant cytotoxic effect of anticancer product when its value IC_{50} value < 30 µg/ml [28].

The results showed that the fraction F_9 (dillapiole) presented the best cytotoxic effect against the Hela cell lines with IC_{50} value of 12.02 ± 0.13 µg/mL. The same fraction (F_9) showed a moderate activity against the A549 with IC_{50} value of 228.44 ± 3.22 µg/mL, this result can be explained by the selectivity phenomenon of dillapiole against the cancer cells lines. On the other hand, the essential oils of aerial parts of *C. maritimum* exhibited a moderate activity against Hela and A549 cells with IC_{50} values of 185.21 ± 1.35 and 578.68 ± 8.42 µg/mL, respectively. Whereas, The essential oil of roots of *C. maritimum* found to be less active against the Hela and A549 cells lines with IC_{50} values of 1928.45 ± 6.44 and 620.59 ± 5.69, respectively.

EXPERIMENTAL

1. Plant material

Crithmum maritimum L. was collected in October 2010 in Monastir (Tunisia).

Identification was performed at the Laboratory of Genetic, Biodiversity and Valorisation of Bioresources, Higher Institute of Biotechnology of Monastir, University of Monastir, Tunisia. A voucher specimen (C.M-10) has been deposited in our laboratory.

2. Extraction of the essential oils

Fresh aerial parts and roots were separately cut in small pieces, weighed before extraction and subjected to hydrodistillation using a Clevenger-type apparatus. The essential oils obtained during 135 min of the isolation process were collected at intervals of 15 min and dried over sodium sulfate, weighed and stored in sealed glass vials in a refrigerator at 4-5°C until future use.

3. Analytical Gas Chromatography

Gas chromatograph: HP 5890-series II equipped with flame ionization detector (FID), HP-5 (30m × 0.25 mm ID, 0.25 µm film thickness) and a HP-Innowax column (polyethylene glycol column, 0.25mm internal diameter, 30m length and 0.25 µm film in thickness) fused silica capillary column, carrier gas nitrogen (1.2 mL/min). The oven temperature was programmed from 50°C (1 min) to 280°C at 5°C/min. The injector and detector temperatures 250°C and 280°C, respectively. Volume injected: 0.1µL of 1% hexane solution. The identification of the components was performed by comparison of their retention times with those of pure authentic samples and by mean of their Retention Indices (R.I.) relative to the series of *n*-hydrocarbons.

4. Analytical Gas Chromatography-Mass Spectrometry

GC/EIMS analyses were performed with a Varian CP-3800 gas-chromatograph equipped with a HP-5 capillary column (30 m × 0.25 mm; coating thickness 0.25 µm) and a Varian Saturn 2000 ions trap mass detector. Analytical conditions: injector and transfer line temperatures 220 and 240°C respectively; oven's temperature programmed from 60°C to 240°C at 3°C/min; carrier gas helium at 1 mL/min; injection of 0.2 µL (10% hexane solution); split ratio 1:30. The identification of the constituents was based on comparison of the retention times with those of authentic samples, comparing their linear retention indices relative to the series of *n*-hydrocarbons, on computer matching against commercial (NIST 98 and ADAMS) and home -made library mass spectra built up from pure substances, components of known oils and MS literature data [29-34]. Moreover, the molecular weights of all the identified substances were confirmed by GC/CIMS, using MeOH as CI ionizing.

5. Antioxidant activity

5.1. DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay

The DPPH radical scavenging capacity was measured from the bleaching of purple colored ethanol solution of $DPPH^{\cdot}$. Method described by Hanato et al. [35] has been used. Each sample concentration (0.5 mL) was mixed with the same volume of $DPPH^{\cdot}$ ethanol solution. After 30 min incubation in the darkness at 25°C, the absorbance of the sample at 520 nm was read. A mixture of 0.5 mL of DPPH. solution and 0.5 mL of ethanol was used as a blank. The decrease in absorption induced by the samples was compared to that of the positive control, BHT (butylated hydroxytoluene). The calculated IC_{50} values denoted the concentration required to scavenge 50% of DPPH radicals. The results were expressed in inhibition percentage versus samples concentrations ($mg.mL^{-1}$) at 30 min. All the measurements were performed in triplicate.

5.2. ABTS (2,2'-azinobis-3-ethylbenzothiazoline- 6-sulfonic acid)radical -scavenging assay

The radical scavenging capacity of antioxidant for the ABTS radical was determined as described by Re et al. [36] $ABTS^{\cdot+}$ was generated by mixing 7 mM of ABTS at pH 7.4 (5 mM NaH_2PO_4, 5 mM Na_2HPO_4 and 154 mM NaCl) with 2.5 mM

potassium persulfate (final concentration) followed by storage in the dark at room temperature for 16 h before use. The mixture was diluted with ethanol to give an absorbance of 0.70 ± 0.02 units at 734 nm using a spectrophotometer (Helios, Unicam, Cambridge UK). For each sample, the diluted methanol solution of essential oil (100 µL) was allowed to react with fresh $ABTS^{\cdot+}$ solution (900 µL), and then the absorbance was measured 6 min after initial mixing. BHT (butylated hydroxytoluene) was used as a positive standard. The capacity of free radical scavenging was expressed as IC_{50} (mg/mL) value, which represents the concentration required to scavenge 50% of ABTS radicals. The capacity of free radical scavenging IC_{50} was determined using the same equation previously used for the DPPH method. All measurements were performed in triplicate.

5.3. Reducing power assay

The reducing power was determined according to the method of Oyaizu [37]. Various concentrations of different phases of the aerial parts and roots (0.03, 0.06, 0.125, 0.25, 0.5 and 1 mg/mL) were mixed with 1 mL of a 200 mmol/L sodium phosphate buffer (pH 6.6) and 1 mL of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min. Then 1 mL of 10% trichloroacetic acid (w/v) was added. The upper layer solution (2.5 mL) was mixed with 2.5 mL of deionised water and 0.5 mL of freshly-prepared ferric chloride solution (0.1%). The absorbance was measured at 700 nm: a high absorbance indicates a high reducing power.

5.4. Catalase activity

Catalase activity was measured according to Aebi's method [38]. Hydrogen peroxide (H_2O_2) disappearance was monitored kinetically at 240 nm for 1min at 25°C. The enzyme activity was calculated using an extinction coefficient of 0.043 $mM^{-1}cm^{-1}$. One unit of activity is equal to one µmol of H_2O_2 destroyed/min/mg protein.

5.5. Paraoxonase activity (PON1)

Paraoxonase 1 activity was determined using paraoxon (1.2 mmom/L) as substrate in 0.1 M tris-HCl buffer at pH 8.0 containing 2 mM $CaCl_2$. The sample to be tested was added (5µL) to start the reaction, and the increase in absorbance at 405nm was recorded [39]. PON1 activity was measured by a simple and rapid automated method adapted on Konelab 30™ (Thermo Electron Corporation, Ruukintie, Finland). Paraoxonase 1 activity is defined as 1 µmol p-nitrophenol formed per minute per L (µM/min/L).

6. Antibacterial activity

6.1. Microrganisms

The antibacterial activity was tested against five microorganisms, including reference strains consisting of Gram-negative rods: *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853) and Gram-positive cocci: *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212), and clinical strains: *Acinetobacter* sp. The bacterial strains were cultured over night at 37°C in Muller Hinton agar.

6.2. Micro-well dilution assay

The MIC was defined as the lowest concentration able to inhibit any visible bacterial growth. MIC values were determined by a microtitre plate dilution method [40] dissolving the sample in 10% DMSO solution. Sterile 10% DMSO solution (100 µL) was pipetted into all wells of the micro-titre plate before transferring 100 µL of stock solution to the microplate. Serial dilutions were made to obtain a concentration ranging from 10 to 0.0775 mg/mL. Finally, 50 µL of 10^6 colony forming units (cfu/mL) (according to Mc-Farland turbidity standards) of standards microorganism suspensions were inoculated on to microplates and incubated at 37°C for 24h. At the end of incubation period, the plates were evaluated for the presence or absence of growth. MIC values were determined as the lowest concentration of the sample at which the absence of growth was recorded. All the samples were screened three times against each microorganism. Imipenem was employed as a positive control against Gram-positive and Gram-negative bacteria. The final

concentration of DMSO in the well had no effect on bacterial growth.

7. Antifungal activity

Antifungal activity was Analyzed by the micro-dilution method Against human pathogenic Candida strains oven were tested *Candida albicans* American Type Culture Collection ATCC 90028, *Candida glabrata* ATCC 90028, *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019. The Minimal inhibitory concentration (MIC) Was Measured in 96-well microtiter plates After 48 h of incubation samples (at 37 °C) of fungi suspensions adjusted to 0.5 McFarland turbidity standards with a Densimat (bioMérieux). Was defined as MIC the lowest extract concentration inhibiting visible growth of a microorganism Each. For studies antifungal powers the fungal inoculum was prepared in sterile saline (NaCl 9%) from colonies of fungal culture after 24 hours of incubation, and then adjusted to a value of 0, 5 MacFarland using a densitometer (Bio-Mérieux). The ethanol was used for the dilution cascade extracts and as a negative control, fluconazole as positive control [41].

8. Cytotoxic activity

8.1. Cell cultures

The human A549 lung epithelial carcinoma and HeLa cervix cells lines were obtained from the American Type Culture Collection (ATCC, Rockville,MD, USA) and cultured in DMEM medium (Dulbecco's Modified Eagle, Gibco) containing 10% (v/v) fetal calf serum, 2 mM glutamine and antibiotics (200 U/L of penicillin and 50 mg.L^{-1} of streptomycin). Cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere.

8.2. Cell viability assay

The cytotoxicity assay was performed according (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (MTT) method with slight modifications [42]. Briefly, the Cells were seeded at $5x10^3$ cells/well in 100 µL of growth medium and incubated at 37 °C for 24 h to adhere. The cells were treated by essential oils and incubated for 48 h; then 10 µL of MTT (5 mg/mL) was added to each well and the incubation lasted 2 h. After this, 100 µL of DMSO were added to each well. The absorbance (A) was measured at 540 nm by a Multiskan Ascent (Ascent Software version 2.6) microplate reader. This assay was conducted in triplicate and the percentages of cell growth were calculated as follow:

Cell growth (%) = [A (sample)/ A (control)] x 100.

CONCLUSION

From the present work, we can conclude that the chemical composition of the different fractions of the aerial parts and roots essential oils of *Crithmum maritimum* varied during the distillation process. Dillapiole was the major constituent of these fractions and also of the whole oil. The antibacterial and antifungal activities of the aerial parts, roots and the last fraction (F_9) which contains only dillapiole were tested in vitro. All fractions were tested for their antioxidative abilities using non-enzymatic (DPPH, ABTS and reducing power assays) methods. It has been found that the antioxidant activity of the collected fractions increases with the increase of the concentration of dillapiole. It has been also found that the fraction F_9 rich in dillapiole displayed an interesting antioxidant enzymatic (catalase) property. The same fraction also showed a cytotoxic against the Hela cell line. On the other hand, the roots essential oil has proved capable of improving the activity of Paraoxonase (PON1).

These results may suggest that *C. maritimum* essential oils can be used as a good source of dillapiole, a compound endowed with good antioxidant and cytotoxic properties, which could also be used as a natural preservative ingredient in food and/or for pharmaceutical preparations.

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