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Chemical characterization and antibacterial activity of the essential oils of *Tetraclinis articulata* (Vahl) from Morocco

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Abstract: The objective of this study was to identify new bioactive substances through the phytochemical study and evaluation of the antibacterial activity of the essential oils of *Tetraclinis articulata* (Vahl). Harvested in April 2016, in the region of Khenifra (Middle Atlas-Morocco).

Separate of hydrodistillation extracts of the three organs (branches, leaves and fruits) of *Tetraclinis articulate* were performed. The yields of essential oils are 0.92% for fruits; 0.41% for branches and 0.61% for leaves.

The GC/MS analysis allowed the identification of 33 compounds in essential oil leaves, 20 compounds in excess fruits and 58 compounds in branches. The main compounds are bornyl acetate (38.54%) and α-pinene (6.71%) for leaves. α-pinene (22.12%); 13-epi-mannol (3.58%) and retinol (3.44%) for branches. Bornyl acetate (19.96%); α-pinene (12.47%) and retinol (11.98%) for fruits.

The antibacterial properties of the essential oil of leaves of *Tetraclinis articulata* (Vahl) were tested in clinical bacterial strain. The essential oils inhibit the growth of *Escherichia coli*, *Staphylococcus aureus* and *Klebsiella pneumoniae* at 1.2 µl/ml; 2.4 µl/ml and 9.7 µl/ml respectively.

Indeed, the resistance of bacteria to antibiotics is known for its adverse effects on human health, to escape this problem, it is necessary to exploit the antibiotics from aromatic and medicinal plants.

Keywords: *Tetraclinis articulata* (Vahl), Essential oils, chemical composition, antimicrobial properties.

Introduction

Microbial infections have been successfully managed using medicinal plants. However, most drugs, such as microbially derived antibiotics, have been considered almost universal solutions to severe infections and have a low antimicrobial spectrum and side effects on human health. Their widespread and excessive use leads to resistance of microorganisms. Indeed, the resurgence of bacterial and fungal resistance is currently one of the obstacles to the successful treatment of microbial diseases by antibiotics. The side effects induced by antibiotics are of concern to users and increase the universal demand for herbal medicine, especially in developing countries.

Tetraclinis artciulata or Berber Thuya is the only species representing the genre Tertraclinis (Cupressaceae) in the northern hemisphere [1]. It is an endemic species of the southwestern Mediterranean and especially the Maghreb [2]. *T. articulata* (Vahl) plays a considerable role in the protection of the soil. Indeed, this species can grow under challenging conditions [3]. *Tetraclinis articulata* (Vahl) has been used since antiquity in traditional medicine, in the seventeenth century, it was nicknamed "tree of life" because of its medicinal properties attributed to the balsamic resin known to increase blood pressure and lower fever [4]. Indeed, many studies mention that local Moroccan populations use this plant in traditional medicine. Specifically, the population of the Khenifra region regularly uses this forest species to treat several diseases, as mentioned by Hachi [5] in their report of the floristic and ethnobotanical study of the medicinal flora used in the town of Khenifra (Morocco) that leaf decoctions of *T. articulata*(Vahl) are indicated for the treatment of gastrointestinal pain. Another ethnobotanical study on aromatic and medicinal plants (PAM) showed that this tree is

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classified as the second species for very therapeutic use [6].

Also as reported in an ethnopharmacological study conducted in Morocco that the leaves of *T. articulata* (Vahl) are used against diabetes and hypertension [7]. Different organs of the tree, especially leave and twigs, are used in the treatment of intestinal and respiratory infections [8] (Bellakhdar *et al.*, 1997).

Several studies have reported on the evaluation of the biological activities of the essential oils of *T.articulata* (Vahl). Indeed, some studies have shown that essential oils of the various organs of this tree have antimicrobial, antioxidant and anti-inflammatory properties [9].

The objective of this work is to contribute to the valorization of this species by studying the chemical composition and the antibacterial activity of the essential oils isolated from the leaves, fruits and branches of *T.articulata* (Vahl).

Experimental

Plant material

The organs of *T.articulata* (Vahl) were harvested in the Khenifra region (Moyen Atlas-Morocco) during April (2013). The leaves, fruits and twigs harvested were dried in the shade for ten days. The taxonomic identification of *T.articulata* (Vahl) was made at the scientific institute of Mohammed University at Agdal in Rabat from the Herbarium flora of Morocco.

Hydrodistillation of essential oils

Essential oils extracted from different parts of *Tetraclinis articulata* (Vahl) was carried out by the hydrodistillation technique using a Clevenger apparatus [10]. During each test, 100 g of plant material was treated. The extraction time was three hours. Previously, the humidity content of the various samples was determined in order to express the total of essential oil (volume in ml) relative to 100 g of the dry matter. The essential oils were dried over anhydrous sodium sulphate [11], and stored at a temperature of 4 ° C in the dark until use.

Analysis and identification of the chemical composition of essential oil

The chromatographic analysis of the essential oils of *T.articulata* (Vahl) was performed using a gas chromatograph Thermo Electron type (Trace GC Ultra) coupled to a mass spectrometer type Thermo Electron Trace MS system (Thermo Electron: Trace Ultra GC, Polaris Q MS), the fragmentation is performed by electronic impact intensity of 70 eV. The chromatograph is equipped with a column of type DB-5 (5% phenyl-methyl-siloxane) (30m x 0.25mm x 0.25µm film thickness), a flame ionization detector (FID) powered by a mixture of H2 / Air gas. The temperature of the column is programmed at a rate of 4 ° C / min from 50 to 200 ° C for 5 min. The injection mode is split (leakage ratio: 1/70, ml/min flow rate); the carrier gas used is nitrogen with a flow rate of 1 ml/min.

Identification of the chemical composition of *T.articulata* (Vahl) essential oils was performed based on the comparison of their Kovats (IK) and Adams indices with those of known reference products in the literature [12,13]. It was supplemented by a comparison of indices and mass spectra with different.

The Kováts index compares the retention time of any product with that of a linear alkane of the same carbon number. They are determined by injecting a mixture of alkanes (standard C7-C40) under the same operating conditions.

Microorganisms, antibiotics and media

Commercially available antibiotics discs (Ciproflaxin 5µg). The selection of clinical microorganisms depended on their availability, thus microorganisms that have been reported to be the most frequently implicated in infectious diseases. The clinical isolates (Hospital center Mohamed V of Meknes-Morocco) were *Staphylococcus aureus* (Gram-positive), *Escherichia coli* (Gram-negative), *Klebsiella pneumoniae* (Gram-negative).

Antibacterial assays

The assay was conducted as described by Perez et al. [14]. Briefly, microorganisms from growth on nutrient agar incubated at 37 °C for 18 h were suspended in saline solution 0.9% NaCl and adjusted to a turbidity of 0.5 Mac Farland standards (10^8CFU/ml) [15]. The suspension was inoculated onto 90 mm diameter Petri plates with a sterile, nontoxic cotton swab on a wooden applicator. Wells with a diameter of five millimetres were punched in the agar and filled with 5µl of essential oil. The dissolution of the extract was added in 0.5% (v/v) DMSO, which did not affect microorganism growth, according to our control experiments. Commercial antibiotics were used as positive reference standard to determine the sensitivity of the strains. Discs were directly placed onto the bacterial culture. Plates were incubated aerobically at 30 or 37°C for 24 h. Antimicrobial activities were evaluated by measuring inhibition zone diameters (IZD).

Determination of minimum inhibitory MIC concentration and minimum bactericidal concentration (MBC)

The broth dilution method was used to determine minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) of the essential oils against the test microorganisms as recommended by the National Committee for Clinical Laboratory Standards. The tests were performed in test tubes. Essential oils were dissolved in 0.5% DMSO and transferred in tubes to obtain a two-fold serial dilutions ranging from 32, 8; 16; 9,7, 4,86; 2,4 and 1,2 µl/ml. The tubes were inoculated with microbial suspensions diluted from the same 0.5 Mac Farland standards to have 10^8 CFU/mL in each tube [15]. After 24 h incubation in the air at 37°C, MIC was recorded as the lowest extract concentration demonstrating no visible growth in the broth. MBC was recorded as the lowest extract concentration that kills 99.9% of bacterial inocula. MBC values were determined by removing 100 µL of bacterial suspension for subculture demonstrating no visible growth and by inoculating nutrient agar plates. Plates were incubated aerobically at 37 °C for a total period of 48 h.

Results and Discussion

Yields of essential oils of *Tetraclinis articulata* (Vahl)

The yields of essential oils from the leaves, branch and fruits of *T. articulata (*Vahl) are respectively 0.61% 0.41% and 0.92%. The essential oil content obtained from the leaves is high compared to that of the same organ of *T.articulata*(Vahl) from Khemisset-Morocco [9] and Wilaya of Tiaret (0.35%) (Algeria) [16]. However, the yield of essential oil isolated from the branches of *T.articulata* (Vahl) of the Khemisset region (Morocco) [17] is 0.41%, similar to that obtained during this study.

The difference in yield can be attributed to several factors including which organ was used, the origin of the species, the period of harvest and the duration of drying.

The essential oils of leaves, branch and fruits of *T.articulata* (Vahl) were analyzed by gas chromatography coupled with mass spectrometry. The chromatograms for the three essential oils of the species are presented in Figures 1, 2 and 3.

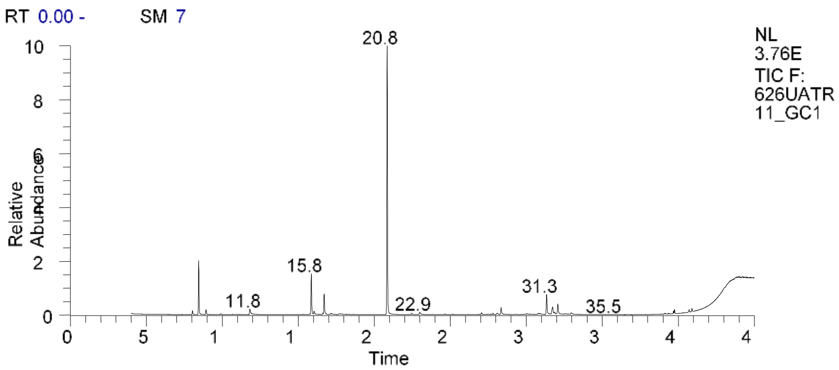

Figure 1. Chromatogram of the essential oils of the leaves of *T.articulata* (Vahl)

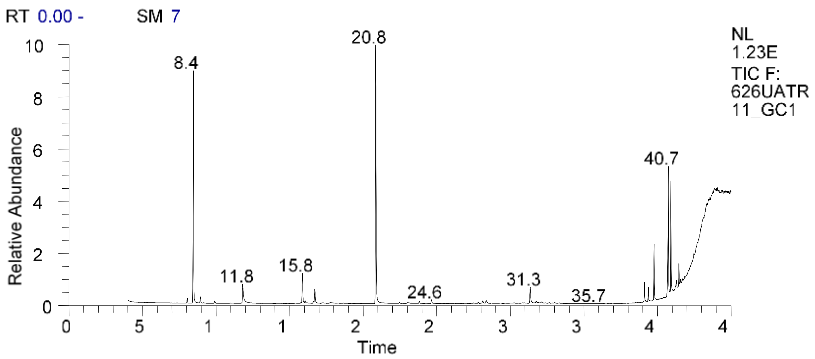

Figure 2. Chromatogram of the essential oils of the fruits of *T. articulata* (Vahl)

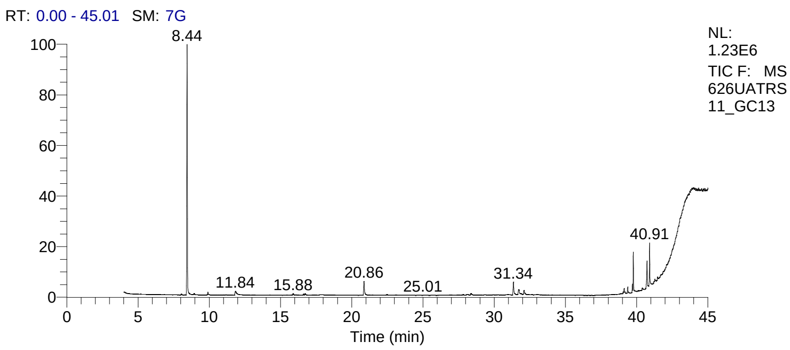

Figure 3. Chromatogram of the essential oils of the branches of *T.articulata* (Vahl)

Table 1. Chemical composition of leaves, branches and fruits of *T. articulata* (Vahl).

N	Constituents	IR (Calculate)	IR (Adams)	Percentage %		
				leaves	*branches*	*Fruits*
1	Tricyclene	924	926	0.48	0.28	0.11
2	α-Pinene	933	939	6.71	12.47	22.12
3	Camphene	948.92	954	0.70	0.40	0.27
4	Sabinene	973.62	975	0.16	0.22	0.43
5	Limonene	1028.33	1029	1.61	2.32	1.23
6	α-Campholenal	1122.33	1126	5.93	2.10	0.51
7	Borneol	1165.25	1169	3.52	1.42	0.25
8	Terpinen-4-ol	1177.06	1177	0.42	--	-
9	α-Terpineol	1187.92	1188	0.52	-	-
10	γ-Terpineol	1198.85	1199	-	-	0.45
11	Dihydrocarveol	1194	1193	-	0.39-	
12	Neo-dihydrocarveol	1195	1194	-		0.16
13	Bornyl acetate	1285.25	1285	38.54	16.96	2.10
14	Neoiso- dihydrocarveol acetate	1352.32	1359	-	0.25	-
15	α-Terpinyl acetate	1334.98	1349	0.78	-	-
16	α -Copaene	1375.85	1376	0.07	0.22	-
17	Sibirene	1401	1400	0.15	0.44	-
18	(E)-Caryophyllene	1419.34	1419	0.11	-	-
19	Germacrene D	1481.31	1481	0 .43	-	-
20	Trans-Muurola-4(14),5-diene	1492.45	1493	0.06	-	-
21	γ- Amorphene	1495.23	1495	0.13	-	-
22	α- Muurolene	1501	1500	0.11	-	-
23	Cubebol	1516.83	1515	0.39	0.28	0.25
24	δ-Cadinene	1525.08	1523	1.35	0.36	0.49
25	γ -Cuprenene	1533.08	1533	0.21	-	-
26	β-Vetivenen	1556.07	1555	0.09	-	-
27	Caryophyllene oxide	1583.16	1583	0.57	0.88	-
28	β-Oplopenone	1609	1607	0.24	1.06	-
29	β-Atlantol	1614	1608	0.30	0.11	0.27
30	1-Epi-Cubenol	1629	1628	3.49	1.41	1.83
31	Torreyol	1642.90	1646	2.38	0.38	1.36
32	Valerianol	1656.36	1658	2.14	0.24	0.95
33	Eudesma-4(15),7-dien-1β-ol	1689.09	1688	0.10	0.29	0.33
34	(3Z)-Cembrene A	1994	1966	0.19	1.00	0.81
35	β -epi-Dolabradiene	2021.68	2000	0.11	0.71	0.59
36	13-epi-Mannol	2068.67	2060	-	2.62	3.58
37	Retinol	2240,35		0.36	11.98 98	3.44
38	Trans-Ferrugunol	2340	2332	-	1.07	-
	Monoterpenes en %			9.66	15.69	24.16
	Sesquiterpenes en %			2.71	1.02	0.49
	Diterpenes en%			0.3	1.71	0.59
	Oxygenated monoterpenes en %			11.18	4.26	1.46
	Oxygenated sesquiterpenes en %			9.61	4.65	4.99
	Oxygenated diterpenes en %			0.36	15.67	6.98
	Esters en %			39.32	17.21	2.10
	Total			73.14	60.16	40.77

The results obtained made it possible to identify 33 constituents representing approximately 73.14% of the chemical composition of the essential oil from the leaves of *T.articulata* (Vahl) (Figure 1) with the main compounds being: Bornyl acetate (38.54 %); α-pinene (6.71%); α-campholenal (5.93%), borneol (3.52). 28 compounds representing a total of 60.16% of the essential oil isolated from the branches of *T.articulata* (Vahl) (Figure 2) with the main compounds being: Bornyl acetate (16.96%); α-pinene (12.47%); retinol (11.98%).

Finally, 20 chemical compounds representing 40.77% of the essential oil from the fruit of *T.articulata* (Vahl) (Figure 3) with main compounds being: α-pinene (22.12%); 13-*epi*-mannol (3.58%); retinol (3.44%).

The essential oils of leaves and fruits have the same major compound (bornyl acetate) in different concentrations. Some of the compounds such as *neoiso*-dihydrocarveol acetate, dihydrocarveol, *trans*-ferruginol are absent in the essential oils of the leaves and fruits. By against that of the twigs, contains the percentages of 0.25%, 0.39% and 1.07% respectively. Also, we notice the absence of 13-*epi*-mannol in the essential oil of the leaves.

The absence of other compounds was found for the essential oils of fruits and branch such as β-vetivenen, γ -cuprenene, α-muurolene, γ-amorphene, muurola-4 (14),5-diene, germacrene D, *(E)*-caryophyllene, α-terpinyl acetate, α-terpineol and terpinen-4-ol compared to the leaves. It should be noted that the fraction of esters is the most abundant group of all the compounds identified for the leaves and twigs (39.32%, 17.21% respectively) whereas the most abundant group for fruits is monoterpene with a percentage of 24.16%.

Several studies show that there is a difference in plant chemical composition by region. The leaves of *T.articulata* (Vahl) from the Khemisset region show a high concentration of α-pinene and camphor (23.54% and 17.27 respectively) [9]. The chemical composition of essential oils of *T.articulata* (Vahl) leaves of Tetouan origin (Morocco) identified by Barrero et al. in 2005 is characterized by the dominance of camphor (19.10%), bornyl acetate (16.50%) and bornéol (9.60%) [18], different from the results of this study. Also, essential oils isolated from leaves of *T.articulata* (Vahl) from El Haçaiba region (Algeria) identified by Toumi et al. in 2010 contains camphor (26.67%), bornyl acetate (25.79%). %), borneol (12.96%), and α-pinene (3.65%) [18]. In Algeria, in the region of Ouled Mimoun the essential oils contained camphor (31.60%), bornyl acetate (17.12%), borneol (14.27%), Limonene (3.0%) and α-pinene (6.49%) [16]. The results of another study also show that the essential oil isolated from the branches of *T.articluata* (Vahl), harvested in the region of Khemisset (Morocco) is characterized by the dominance of α-pinene (30.22%), Limonene (22.29%), widdrol (5.41%), bornyl acetate (4.76%) and humulene (3.49), [17], which is different from the results of this study. All the results obtained have shown that the chemical profile of the essential oil of the leaves, branch and fruits of *T.articulata* (Vahl) varies qualitatively and quantitatively according to the organ. Those who demonstrate the importance of the plant material used to extract the essential oil. The difference observed between the chemical composition of the essential oils of *T.articulata* (Vahl) of the Khénifra region with other regions of Morocco and those of Algeria, could be explained by the adaptation of *T.articulata* (Vahl) to several factors such as altitude, climate and soil type, all of these factors can direct the biosynthesis to the formation of specific products.

Antibacterial activity of the essential oil

The results of the susceptibility tests are shown in Table 2. The antibacterial activity of essential oil from the leaves of *T.articulata* (Vahl) against the bacteria used was qualitatively and quantitatively evaluated by the presence or absence of zones inhibition. It was found from the analysis of the results obtained (Table 2) that all three microorganisms studied are sensitive to the essential oil of the leaves of *T.articulata* (Vahl). The inhibition diameters are for *S. aureus* (14. 23± 0,22 mm) *E. coli* (11,91± 0,11 mm) and *K. pneumoniae* (10.41±0.27 mm).

Table 2. Average of diameters of the zones of inhibition of the essential oils relative to the clinical bacterial strains:

Strains	**Inhibition zone diameters (mm)**	
	T. articulata (Vahl) (5µl)	Ciprofloxacin (5µl)
Staphylococcus aureus	14.23 ± 0.22	18 ± 0
Escherichia coli	11.91 ± 0.11	17.00 ± 0
Klebsiella pneumoniae	10.41 ± 0.27	15.00 ± 0

Table 3. Minimal inhibitory and bactericidal concentrations (MIC and MBC) for essential oil leaves

Concentrations (µl/ml)		**32.8**	**16**	**9.7**	**4.86**	**2.4**	**1.2**	**control**
S. aureus	**MIC**	-	-	-	-	-	+	+
	MBC	-	-	-	-	+	+	+
E. coli	**MIC**	-	-	-	+	+	+	+
	MBC	-	-	+	+	+	+	+
K. pneumniae	**MIC**	-	-	-	+	+	+	+
	MBC	-	-	+	+	+	+	+

During our investigations, the determination of minimal inhibitory concentration was evaluated by observing the inhibitory power of our *T. articulata* (Vahl) essential oil sample at different concentrations to the bacteria tested (Table 3).

Indeed, the essential oil of leaves of *T. articulata* (Vahl) has shown an important inhibitory effect against the microorganisms studied. All microbial strains were inhibited at the concentration of 9.7 µl/ml and were bactericidal at the concentration of 16 µl/ml. The most sensitive microorganism to leave essential oil was *Staphylococcus aureus* mestizo whose growth was inhibited at the low concentration of 2.4 µl/ml for the MIC and 4.86 µl/ml for the MBC. Following these results, the essential oil of *Tetraclinis articulata* (Vahl) from the Khenifra (Morocco) region exhibited very interesting antibacterial characteristics on the microorganisms tested. This is consistent with investigations by Bourkhiset al., who have shown that the species *Tetraclinis articulata* (Vahl) inhibits the mycelial development of *Staphylococcus aureus* and has significant antimicrobial activity [9] (Bourkhiss M *and al*., 2007). The antimicrobial properties of the essential oils of several aromatic and medicinal plants have been attributed to their chemical profile and especially to terpene alcohols [19-21].

The antibacterial activity of the essential oil *T. articulata* (Vahl) of the Khenifra-(Morocco) region is due to its chemical profile. It should be noted that the essential oils are characterized by the dominance of esters with a percentage of 39.32% more precisely related to the presence of bornyl acetate with a percentage of 38.34%, the latter is known for its antibacterial power. Indeed, a study showed that the essential oils of *Thymus longicaulis*, having two chemotypes rich in geranyl acetate for the first and a-terphenyl acetate for the second, possess a high antimicrobial activity [20]. The essential oil of *T.articulata* (Vahl) from the region of Essaouira (Morocco), which presents as chemotypes bornyl acetate and α -pinene, has marked a significant inhibitory activity and Bourkhis and al., have reported that α-pinene has no inhibitory power, this confirms that bornyl acetate is the active ingredient, responsible for the antimicrobial activity observed by Bourkhis M et al. [9].

However, the synergistic effect between all these chemical constituents should also be taken into account for the antimicrobial activity [21].

According to our results, the MBC / MIC ratios are less than 3 for all the strains, so the essential oil of the leaves of *T. articulata* (Vahl) has a bactericidal capacity towards the strains tested.

Conclusion

The detailed analysis of the essential oils of the leaves, twigs and fruits of *T. articulata* (Vahl) from Morocco led to the identification of 33 compounds for the leaves, 28 for the branches and 20 for the fruits.

The results obtained show differentiation in yield and composition of the essential oil of *T.articlata* Vahl) from one part to another. These results are different from those reported in the literature which shows the influence of biotic and abiotic factors on *T.articulata* (Vahl), The antibacterial activity of the essential oil of the leaves of *T.articulata* (Vahl) shows activity via the strains tested.

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