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Volatile Compounds Analysis of Tunisian Propolis and Its Antifungal Activity

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Abstract

In this study, four samples of Tunisian propolis were analyzed for their volatile compounds. Static Headspace technique coupled with gas chromatography-mass spectrometry (GC/MS) was used for the volatile profile characterization. Statistical investigation of the samples was made applying hierarchical clustering method, K-Means cluster and principal component analysis. Results showed that 47, 36, 30 and 24 different compounds were identified, respectively, in the samples from Zouarine, Zelligua, Bizerte and Beni Khalled. Propolis volatiles were dominated by monoterpene hydrocarbons. *α*-pinene was the major compound representing 81.14%, 82.67% and 90.74%, respectively, of the total propolis volatiles collected from Zelligua, Beni Khalled and Zouarine and only 45.22% of the sample from Bizerte which had a very different composition. The *in vitro* antifungal activity of the volatiles from all samples against *Candida albicans* was also assayed and reported.

Keywords

Propolis, Headspace/GC-MS, *α*-Pinene, PCA, Antifungal Activity

1. Introduction

Propolis is a resinous adhesive natural substance, collected by honeybees like (*Apis mellifera* L.) from buds, leaves of trees and plants then mixed with pollen and enzymes secreted by bees [1].

Propolis is used as a purpose sealer to smooth out the hive internal walls and as a barrier against intruders [2]. It is a valuable product from ancient times to nowadays. Propolis is mostly used, in folk medicine, in pharmacy and in food

technology, as a remedy with high antioxidant, antimicrobial, antifungal, anti-inflammatory, antiviral and antitumor activities [3] [4].

Chemical composition of propolis is dependent on its botanical and geographical origin [5] [6], races of bees [7] [8] and collection season where it can be collected all year [9] [10].

Different chemical compounds including aldehydes, organic acids, esters, hydrocarbons, cyclic compounds, terpenes, flavonoid aglycones, phenolic acids and their esters, phenolic aldehydes, alcohols, ketones, sesquiterpenes, quinones, coumarins, and steroids were identified in both volatile and non-volatile propolis fractions of propolis from different botanical and geographical origin [11].

Despite the fact that volatiles are found in low contents in propolis, their pleasant aroma and significant biological activities make them very valuable for the propolis characterization [12]. Several studies showed that spread of volatile compounds identified in propolis is very wide [13] [14] [15] [16] [17]; the chemical composition depends on the specificity of the local flora at the collection region and thus on its geographic and climatic characteristics [2].

Indeed, in contrast to propolis of Europe, Algeria and Egypt, Tunisian propolis has a different botanical origin due to unique Tunisian flora which presents a generally known biodiversity with a high percentage of endemic plants [13] [18] [19] [20].

Studies regarding Tunisian propolis volatiles are very scare and limited to that of Martos *et al.* [21] investigation of flavonoids composition in Tunisian propolis and that of Kouidhi *et al.* [22] who showed that propolis ethanol extract possessed anti-cariogenic and anti-biofilm activities, and had potential protective effect against cancer cells proliferation. To the best of our knowledge, no study has been conducted concerning Tunisian propolis volatile compounds.

Candida albicans is the most well-known fungal species associated with the clinical manifestation of this pathology. It is the most prevalent and pathogenic species among all *Candida* infections which have become a serious health problem. As fungi grow increasingly resistant to the available drugs; new drugs must be researched and evaluated for their effectiveness in antifungal treatment [23] [24].

This strain has been selected for the basis of its application purpose of further formulation study.

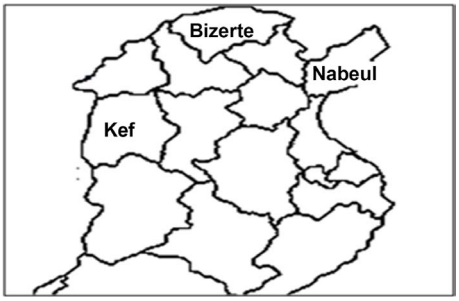
So, the present work focused on the determination of Tunisian propolis volatiles and a comparative study of the volatile composition of propolis collected from different regions was performed. Samples were analyzed using static Headspace coupled with GC/MS, applying statistical data mining techniques: principal component, hierarchical clustering and K-Means cluster analysis for classification, afterwards. The *in vitro* antifungal activity of the volatiles from all samples against human pathogen *Candida albicans* was also assayed.

2. Experimental Procedure

2.1. Propolis Samples

In this study, four samples of Tunisian propolis were collected during 2014's

spring and analyzed for their volatiles composition: two samples were collected from Kef region (200 g from Zouarine, 100 g from Zelligua), 100 g from Nabeul (Beni Khalled) and 50 g from Bizerte.



Sampling was performed based on diversity in all four regions; this difference is noticed especially with the botanical origins.

Eucalyptus, Rosemary, Thym, Citrus and Almond trees were found in both Zouarine and Zelligua sites. The Kef region is characterized by the dominance of the forests and the mountains which promoted trees diversity such as: Conifers, Birch, Ash, Yardstick, Elm, Chestnut, Poplar, Beech, Pine, Spruce, Fir, Plums and Cactus [25].

In Bizerte, bees are collecting propolis only from Orange trees and the dominant climate was steppe.

In Beni Khalled region, Rosemary, Thym, Cactus and small plants are found but there was no Eucalyptus.

2.2. Volatile Compounds Analysis by Headspace Coupled with GC-MS

Volatile compounds were analyzed using a Headspace (TELEDYNE TEKEMAR HT3TM) coupled with an Agilent GC-MS system (GC with 7890A, mass detector 5975C with Triple-Axis, insert XL MSD).

1 g of propolis samples were introduced in a 30 ml headspace vials incubated for 30 min at 80˚C in headspace oven then transferred in heated line at 115˚C to avoid condensation of volatiles which were injected in the GC inlet during 1 min with a static mode. A HP-5 ms column (5% phenylmethylsiloxane) was used (30 mm × 250 μm × 0.25 μm). The carrier gas was Helium (N60 = 99.99%) with the flow rate of 1 mL/min.

Each run was performed during 24 min. The temperature was programmed at 40˚C for 1 min, raised to 100˚C with a rate of 10˚C /min then raised to 200˚C with a rate of 20˚C/min and kept constant for 5 min. It was finally raised to 300˚C with a rate of 20˚C and kept constant for 2 min. Injection was realized within 250˚C inlet with a splitless mode. The auxiliary temperature was 250˚C and the mass spectrometer was operating in EI mode (70 eV).

Quadripole and source temperature were respectively fixed at 150˚C and 250˚C with a full scan mode from 40 m/z to 550 m/z. The identification of the

propolis components was based on a long work with NIST02 mass spectra search library.

Quantification was based on the areas of total ion current (TIC) peaks. Relative area values (percentage of total volatiles) were used for characterization purposes.

2.3. Antifungal Activity: Propolis Effect on *Candida albicans* Growth

In order to study the effect of Propolis volatiles on *Candida albicans* growth, an initial suspension of the yeast was done with an optical density of 0.5 on Mac-Farland scale (corresponding to 10^8 CFU·ml^{-1}) and was decimally diluted with 0.9% (w/v) NaCl to obtain an inoculum concentration of 10^5 CFU·ml^{-1}. The antifungal assay was performed using the double-dish chamber method. 100 µl of overnight *C. albicans* culture were spread on one half of the Petri dishes containing Sabouraud dextrose agar medium. The second half containing a scale range of Propolis concentration 1 mg, 10 mg, 20 mg and 50 mg.

Plates were parafilmed and incubated for 24 h at 30˚C. The percentage of growth inhibition (GI) was calculated according to the following formula:

$$GI(\%) = \frac{N_C - N_1}{N_C} * 100$$

N_1: Number of Candida colonies/box.

N_C: Number of Candida colonies in the control box.

2.4. Statistical Data Analysis

For metrological repeatability of the measurements, each propolis sample was analyzed 3 times using Headspace coupled with GC-MS. According to GC-MS results, data matrix of [12 × 87] was formed for further analysis. Measured data were processed using ***XLSTAT*2015** software. After data preparation, the following statistical methods were applied: principal component analysis (PCA), for reduction of the space of the variables; hierarchical clustering analysis (HCA) using average linkage, in order to estimate the similarity between any pair of the clusters applying average distance between all pairs of objects in any two clusters calculation; K-Means cluster analysis (KMCA), which is an iterative technique for classification that minimizes the sum, over all clusters, of the within-cluster sums of point-to-cluster-centroid Euclidean distances [26].

3. Results and Discussion

3.1. Volatile Compounds Composition of Tunisian Propolis

Visual analysis of the propolis samples showed that their color varied from golden yellow (Zouarine), opaque green (Bizerte), glossy dark brown (Zelligua) to dark (Beni Khalled) but all samples have a strong pleasant aroma. Indeed, the propolis color and odor is dependent on its botanical source [27] [28]. Bankova *et al.* [12] concluded that the main botanical source of European, non-tropical

Asian and North American propolis is *Populus spp*.

Besides the main source, bees also use pine, linden, willow, cherry, apple and other trees [27]. In tropical regions there are no poplars; therefore bees use other plant sources for bee glue. Propolis volatiles give to the bee glue its specific pleasant aroma. It is well known that bees (*Apis mellifera*) respond to odors in several behavioral contexts [29] [30].

In Tunisia, different botanical sources can be found such as: Eucalyptus, Rosemary, Thym, Citrus and Orange trees. It is also characterized by the dominance of the forests around, which promotes diversity of trees where bees can collect propolis such as: conifers, birch, ash, yardstick, elm, chestnut, poplar, beech, apple, pine, spruce, fir, plums and cactus [20] [25]. Headspace-GC-MS analysis (**Table 1**) revealed the presence of 83 different compounds in all samples. Only 6 volatile compounds present with low proportions (0.53% - 0.082%) were not identified. Propolis sample collected from Zouarine was characterized by the highest number of compounds identified (47), followed by Zelligua (36) and Bizerte (30), while the lowest one was observed in the sample collected from Beni Khalled (24). The low quantity of volatile compounds identified in the Beni Khalled propolis could be due to the presence of waxes or may be related to flora.

Identified compounds mainly belonged to the class of alcohols, aldehydes, monoterpenes and sesquiterpenes. The monoterpene hydrocarbon, *α*-pinene, was predominant in all samples. In fact, in Zouarine, it was a major constituent representing 90.74% of the total volatiles, followed by Beni Khalled (82.67%), Zelligua (81.14%) and Bizerte (45.22%). *β*-pinene was found in very low percentage of 1.75%, 1.65%, 1.18% and 1.06%, respectively, in the samples from Zelligua, Bizerte, Zouarine and BK.

The sesquiterpene, Cedrol, was only identified in samples of Bizerte amounting to 8.23% and Zelligua with 0.106%. Propolis from Bizerte contains various compounds in small amounts varied from 1.7% to 4.53%, such as 3-methyl-2-buten-1-ol, octane, tricyclene, allyl benzyl ether, 1,8-epoxy-p-menth-2-ene, *o*-cymene, *γ*-terpinene, *m* mentha-3(8),6-diene, *cis*-sabinol, 2,3-dehydro-1,8-cineole, *p*-mentha-1(7),2-dien-8-ol, 4-terpineol, *β* fenchyl alcohol, *α*-copaene, p-ethylguaiacol, *β*-copaene, junipene, *γ*-cadinene and (3e)-6-phenyl-3-hexen-2-one.

Various compounds were identified in Zouarine sample beside *α* and *β*-pinene, representing low amounts, such as: sabinene, *δ*. 3 carene and limonene. In Beni Khalled sample, we found *α*-thujene, 4-terpineol, *α*-terpinene, limonene, *γ*-terpinene, *α*-terpinolene and *trans*-verbenol. In Zelligua we found *α*-thujene, camphene, verbenene, δ. 3 carene and limonene.

It worth noting that all propolis samples collected from the four sites had 9 common compounds which were: *α*-pinene, *β*-pinene, *α*-thujene, o-cymene, *α*-phellandrene, *γ*-terpinene, *α*-copaene, tricyclene and 4-terpineol (**Table 1**) with the predominance of *α*-pinene. The two propolis samples (Zouarine and Zelligua) collected from the same region of Kef presented 6 other common

Table 1. Percentages of Tunisian propolis volatile compounds.

N°	RT	Compounds	Kovalts Index	Zouarine	Bizerte	BK	Zelligua
1	4.251	2-Buten-1-ol, 3-methyl-	789		3.248 ± 0.250[a]		
2	4.4	Propanoic acid	740				0.094 ± 0.055[a]
3	4.483	Octane	816		3.359 ± 0.046[a]	0.499 ± 0.124[b]	
4	4.972	1, 3, 3,4-Tetramethylcyclopentene	895				0.121 ± 0.039[a]
5	5.118	Ethanone, 1-(3-ethylcyclobutyl)-	937				0.388 ± 0.068[a]
6	6.23	Tricyclene	729	0.192 ± 0.019[c]	1.957 ± 0.060[a]	0.503 ± 0.124[b]	0.535 ± 0.035[b]
7	6.289	*α*-Thujene	927	0.424 ± 0.014[d]	1.752 ± 0.115[b]	2.079 ± 0.064[a]	1.137 ± 0.051[c]
8	6.427	*α*-Pinene	934	90.739 ± 1.276[a]	45.217 ± 0.007[d]	82.673 ± 2.360[b]	81.140 ± 2.310[c]
9	6.557	Dehydrosabinene	879				0.117 ± 0.054[a]
10	6.611	*α*-Fenchene	943	0.174 ± 0.019[a]			
11	6.641	Camphene	950	0.260 ± 0.019[c]		0.816 ± 0.155[b]	1.149 ± 0.067[a]
12	6.718	Allyl benzyl ether	1158		2.102 ± 0.036[a]		
13	6.72	Verbenene	1136	0.193 ± 0.019[c]		0.472 ± 0.137[b]	1.111 ± 0.046[a]
14	6.866	Benzaldehyde	982				0.360 ± 0.040[a]
15	6.97	*β*-Cymene	1024				0.426 ± 0.042[a]
16	7.005	Sabinene	973	1.805 ± 0.023[a]		0.574 ± 0.273[b]	
17	7.072	*β*-pinene	977	1.064 ± 0.018[b]	1.649 ± 0.077[b]	1.178 ± 0.194[c]	1.750 ± 0.050[a]
18	7.231	*β*-Myrcene	989	0.051 ± 0.016[b]		0.542 ± 0.168[a]	
19	7.271	2,3-Dehydro-1,8-cineole	991		2.542 ± 0.088[a]		
20	7.278	1, 3,8-p-Menthatriene	1030	0.030 ± 0.016[a]			0.443 ± 0.055[a]
21	7.354	Propylcyclohexane	979				0.313 ± 0.055[a]
22	7.464	*α*-phellandrene	1004	0.114 ± 0.019[c]	1.590 ± 0.061[a]	0.386 ± 0.119[b]	0.448 ± 0.060[b]
23	7.548	Δ-3 carene	1011	0.944 ± 0.020[b]		0.786 ± 0.167[c]	1.021 ± 0.043[a]
24	7.634	*α*-Terpinene	1017	0.103 ± 0.020[c]		0.942 ± 0.175[a]	0.374 ± 0.025[b]
25	7.739	o-Cymene	1042	0.197 ± 0.021[d]	2.056 ± 0.036[a]	0.602 ± 0.177[c]	0.752 ± 0.068[b]
26	7.801	Limonene	1029	0.543 ± 0.017[c]		1.201 ± 0.113[b]	1.416 ± 0.034[a]
27	7.867	Cis-Ocimene	1047	0.026 ± 0.012[a]			
28	8.191	*γ*-Terpinene	1059	0.151 ± 0.024[d]	4.533 ± 0.087[a]	0.993 ± 0.126[b]	0.816 ± 0.045[c]
29	8.36	Trans-Ocimene	1037	0.025 ± 0.016[a]			
30	8.563	m-Mentha-3(8) ,6-diene	1018	0.180 ± 0.005[b]	1.992 ± 0.042[a]		
31	8.567	*α*-terpinolene	1052			0.445 ± 0.109[a]	0.414 ± 0.052[a]
32	8.737	Nonanal	1081				0.285 ± 0.055[a]
33	8.999	*α*-Campholenal	1155	0.179 ± 0.020[a]			
34	9.001	3-Nonyn-1-ol	1176				0.153 ± 0.057a
35	9.123	Cis-limonene oxide	1031	0.022 ± 0.021[a]			
36	9.179	Cis-sabinol	1085		4.209 ± 0.402[a]		

Continued

37	9.18	Trans-Pinocarveol	1131	0.164 ± 0.022[b]			0.430 ± 0.058[a]
38	9.185	Trans-Verbenol	1118			0.534 ± 0.095[a]	
39	9.238	Camphor	1121	0.030 ± 0.013[a]			
40	9.269	Cis Verbenol	1119				0.350 ± 0.024[a]
41	9.279	*α*-Terpineol	1143		2.188 ± 0.053[a]		
42	9.286	p-Mentha-1,5-dien-8-ol	1125	0.056 ± 0.017[a]			
43	9.397	Pinocarvone	1114	0.035 ± 0.017[a]			
44	9.402	6-Nonynoic acid, methyl ester	1200				0.229 ± 0.042[a]
45	9.471	Borneol	1166				0.518 ± 0.042[a]
46	9.479	p-Mentha-1(7) ,2-dien-8-ol	1120		3.005 ± 0.145[a]		
47	9.563	4-Terpineol	1137	0.477 ± 0.017[d]	2.366 ± 0.571[a]	1.591 ± 0.133[b]	0.922 ± 0.040[c]
48	9.732	*β*-Fenchyl alcohol	1115		3.567 ± 0.035[a]		
49	9.746	Myrtenol	1191	0.024 ± 0.014[a]			
50	9.755	NI 1	0		2.975 ± 1.191[a]		0.613 ± 0.028[b]
51	10.122	Carvacrol Methyl Ether	1245			0.370 ± 0.144[a]	
52	10.234	NI 2	0		1.443 ± 0.023[a]		0.119 ± 0.042[b]
53	10.285	p-Ethylguaiacol	1303		2.258 ± 0.068[a]		
54	10.519	Bornyl acetate	1277	0.184 ± 0.018[a]			
55	10.52	*α*-Fenchyl acetate	1278			0.235 ± 0.102[a]	0.139 ± 0.039[b]
56	10.619	NI 3	0	0.032 ± 0.017[a]			
57	10.696	Isopulegol	1196	0.052 ± 0.041[a]			
58	10.727	NI 4	0			0.083 ± 0.144[a]	
59	10.887	Isocaryophyllene	1494		1.760 ± 0.019[a]		
60	10.928	Methyl m-tolyl carbinol	1169	0.033 ± 0.045[a]			
61	11.035	*α*-Copaene	1376	0.336 ± 0.038[b]	2.335 ± 0.290[a]	0.231 ± 0.113[b]	0.285 ± 0.054[b]
62	11.263	*β*-Copaene	1433	0.044 ± 0.021[b]	3.203 ± 0.030[a]		
63	11.541	Longifolene	1398	0.158 ± 0.021[b]	1.781 ± 0.016[a]		
64	11.573	*γ*-curcumene	1524			0.752 ± 0.103[a]	0.478 ± 0.053[b]
65	11.574	*α*-Ylangene	1369	0.069 ± 0.015[a]			
66	11.64	Germacrene-d	1515	0.024 ± 0.026[a]			
67	11.642	Valencene	1474			0.191 ± 0.100[a]	
68	11.943	Junipene	1401		2.806 ± 0.027[a]		
69	11.945	NI 5	0	0.055 ± 0.024[a]			
70	11.951	NI 6	0				0.076 ± 0.036[a]
71	11.976	NI 7	0	0.017 ± 0.022[a]			
72	12.113	*γ*-Cadinene	1435	0.023 ± 0.037[b]	2.085 ± 0.016[a]		
73	12.264	Δ-Cadinene	1469				0.052 ± 0.037[a]

Continued

74	12.938	Cedrol	1543		8.232 ± 0.105[a]		0.106 ± 0.026[a]
75	13.14	NI 8	0	0.015 ± 0.029[b]	1.407 ± 0.028[a]		
76	13.238	1,3-Diphenylpropane	1665	0.030 ± 0.027[a]			
77	13.423	Guaiazulene	1472	0.013 ± 0.021[a]			
78	13.626	NI 9	0		1.696 ± 0.132[a]		
79	13.711	2-Undecene	1123	0.019 ± 0.004[a]			
80	18.902	NI 10	0	0.027 ± 0.008[a]			
81	19.548	NI 11	0	0.040 ± 0.032[a]			
82	21.428	2-Pentene, 5-phenyl-	1199	0.077 ± 0.027[a]			
83	21.431	(3E)-6-Phenyl-3-hexen-2-one	1435	0.012 ± 0.002[b]	2.373 ± 0.020[a]		
Total number of compound				47	30	24	36
Total %				99.461%	100%	98.677%	99.078%

Values are given as mean ± SD (n = 3). Values followed by the same letter did not share significant differences at $p < 0.05$ (Duncan's test). NI: non identified compound, RT: Retention time.

compounds which were: camphene, verbenene, *α*-terpinene, Δ.3 carene, *trans*-pinocarveol and limonene. These similarities may be due to the close floral origin of both sites especially the distance between them that not exceed 20 km.

Compounds such as *β*-pinene, limonene, *α*-pinene, *γ*-terpinene, Cedrol, *β*-myrcene, *β*-ocimene are known to possess antimicrobial, antioxidant and antifungal activity [14] [31]. Melliou *et al.* [14] analyzed the antibacterial activity of Greek propolis and determined that higher antibacterial activity was observed in samples with high content of *α*-pinene (45.8%). Based on these results and since our study revealed a high content of *α*-pinene, varying from 45.22% to 90.74%; Tunisian propolis could possess an important antibacterial activity.

Our results on the propolis from Bizerte are in accordance with those mentioned by Melliou *et al.* [14] who used hydrodistillation and GC-MS for volatile extraction and analysis of Greek propolis; they showed that *α*-pinene was the major constituent (45.8%). These results were lower than our findings for the three other samples. In addition, our findings are totally different from those found on tropical propolis in which *α*-pinene was present in low proportion up to 1.6% [32] [33]. However, Nunes and Guerreiro [34] performed Headspace analysis and attributed higher amounts of *α*-pinene, *β*-pinene, sulcatone, carene, limonene, eucalyptol, *α*-ocimene, *β*-ocimene, acetophenone and nonanal to the green Brazilian propolis. Moreover, *α*-pinene composed more than a half of all volatiles in Brazilian and Uruguayan propolis and over 40% in Iranian, Kerman and Greek ones which is lower than our results in Tunisian propolis. In the other hand, *β*-pinene was found in Uruguayan propolis with a higher amount (27.44%) than *α*-pinene (22.96%), in Brazilian propolis (20.85%), in Estonian propolis (8.86%) and even in Greek propolis (2.2%); these results were higher than ours [35].

Our results showed that Cedrol was identified in the Bizerte sample with an amount of 8.23%. This was in accordance with Greek propolis [14], higher than Ethiopian propolis (2.48%) and much lower than Brazilian one (33%). 4-Terpineol was found in all Tunisian regions in low amounts which is different from Ethiopian propolis where it composed one of the predominant compounds (8.57%) [15].

In Tunisian propolis, dl-limonene was found in small amounts of 0.54%, 1.42% and 1.20%, respectively, in the samples of Zouarine, Zelligua and Beni Khalled. These results were different comparing to Croatian [13] and Brazilian propolis from Jaguari region [36], where high percentages of dl-limonene were detected, using hydrodistillation method. This compound is known with its anti-anxious and antidepressant benefits [35] [36] [37]. β-methyl crotonaldehyde was identified as an important compound in Brazilian (10.1%) and also in Chinese propolis [28], using Headspace analysis, but was not found in our samples.

Moreover, Eucalyptol presented a high amount of 25.95% in Estonian propolis but wasn't found in our samples. Benzaldehyde was only identified in the Tunisian propolis from Zelligua with a low amount (0.360%). Greek propolis presented a close amount of this compound (0.1% - 0.3%), whereas Estonian sample contained high proportion about 10.85%.

Chinese propolis was totally different than samples from Europe and South America but characterized by high amounts of 3-methyl-3-buten-1-ol and 3-methyl-2-buten-1-ol, (40.33% and 11.57%, respectively), 4-penten-1-yl acetate (9.04%) and α-longipinene (9.41%) [28]. That last compound was found only in Chinese and Greek propolis and was also reported in Brazilian green propolis in high amount (24.89%) [38]. All these compounds were not identified in our study and this might be due to the botanical, geographical and floral differences. Comparing to different European samples, a lot of similarities were found with the volatile composition of propolis from Brazil, Uruguayan, China and Greece but the nearest one to that of Tunisian propolis is the volatile composition of the Greek propolis [5] [37]. In fact, from 83 compounds, 37 are similar.

3.2. Chemometric Analysis

For the statistical analysis standardized, data matrix [12 × 87] was prepared as described above.

First, the PCA was applied to reduce variables in space and therefore the correlation between them. The number of principal components that will represent the data, by explaining the corresponding part of the total variance of the initial variables and will be used in further statistical analysis was chosen according to Kaiser's eigenvalue criterion [29]. In order to cluster the samples, two techniques were used: Hierarchical clustering Analysis (HCA) and K-Means clustering. The calculated distances and the results of the HCA are presented as a dendrogram in **Figure 1** and **Figure 2**.

In fact, **Figure 1** shows hierarchical cluster analysis HCA of all propolis volatile compositions. Dendograms revealed that propolis samples from Beni

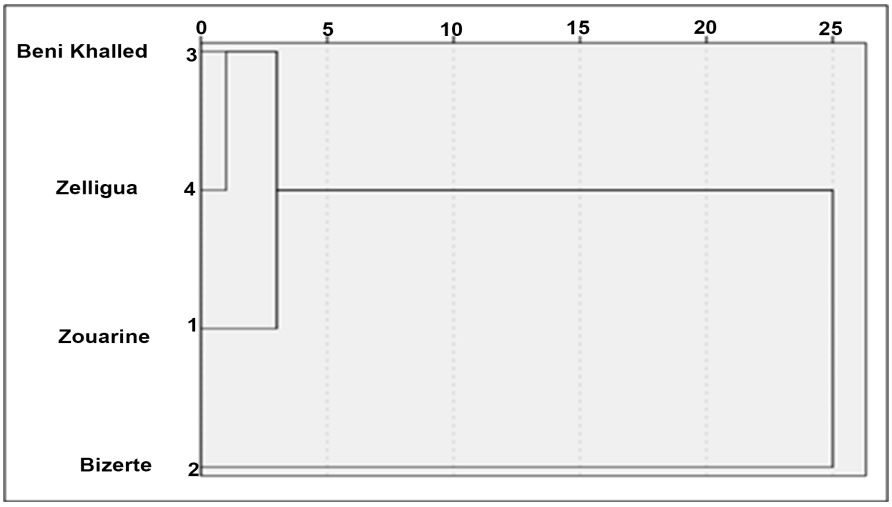


Figure 1. Dendograms of hierarchical cluster analysis HCA.

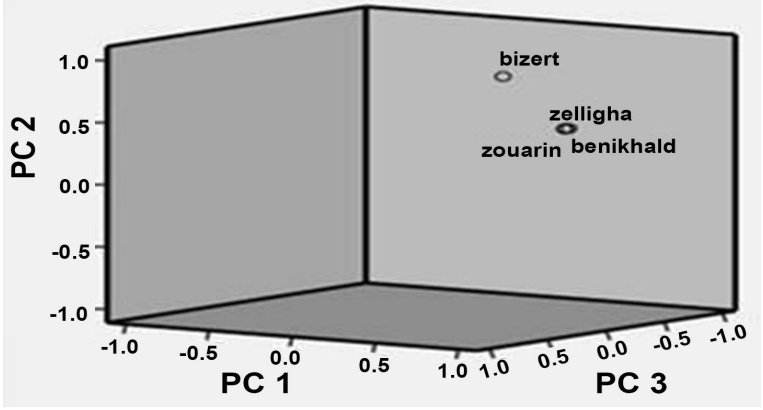


Figure 2. Plots of K-Means cluster analysis.

Khalled and Zelligua have close similarities in their composition. Propolis sample from Zouarine is different but still have some similarities with both previous samples. Finally, this analysis proved that propolis sample from Bizerte form a unique cluster, far from the others which mean, that its volatile composition is so different.

Before classification using KMCA, it is required to specify the clusters number and the iterations number to the algorithm of this analysis. The first parameter was calculated using a simple rule of thumb

$$k = \lceil \sqrt{(n/2)} \rceil \quad (1)$$

where operator ⌈ ⌉ means rounding the result to the nearest larger integer number and n is the number of the initial variables, n = 87. The iterations number was not constant as it depends on more critical parameter (minimum Euclidean distances sum of particular classification result).

In order to find the global minimum, KMCA was programmed to run 500 times. The minimum value of the calculated minimums of the Euclidean distances sum of each run was chosen as the best classification result. The results of

KMCA, when k = 7, are presented as data scatter plots in three-dimensional space of first three PCA components in Figure 3.

Dendrogram in Figure 2, scatter plot in Figure 3 and diagram in Figure 4 presented clustering results. It is clearly seen that Bizerte, Zouarine, Zelligua and Beni Khalled propolis samples fall into 3 separate clusters, meaning that their volatile composition was very different. Beni Khalled and Zelligua samples formed one cluster since their volatiles composition was very similar. The sample from Zouarine formed the second cluster which is not very far from this group.

Bizerte sample had a very different composition from all other samples and constitute the third cluster. This classification is confirmed by looking to data scatter plots of K-Means cluster analysis Figure 3 which showed that Zelligua, Beni Khalled and Zouarine samples were near to each other and only the sample of Bizerte was removed from the group. For better certitude, the 9 common compounds cited above were selected for the statistical analysis and another data matrix [12 × 9] was made. PCA for common compounds was applied and results are presented in Figure 5.

In this case, all non-common compounds with small contributions take values close to zero, and the highest common compounds having the biggest impact on the classification results are analyzed. The samples of Bizerte, Zouarine, Zelligua and Beni Khalled propolis again fall into three separate clusters. Zelligua and Beni Khalled samples are close to each other despite the difference of geographical positions of the collection sites. However, Zelligua and Zouarine samples regardless of their belonging to the same region, presented some differences in volatiles composition.

3.3. Antifungal Activity: Propolis Effect on *Candida albicans* Growth

The volatiles of all samples were also studied for their antifungal activity against a human pathogen fungus (*Candida albicans*).

Results in Table 2 and Figure 5 and Figure 6 showed that only volatiles from Beni Khalled and Zelligua were active against *C. albicans*. The strongest fungicidal activity was exhibited by propolis from Beni Khalled in which 10 mg inhibited 65% ± 1.96% of the growth of *C. albicans* while only 20 mg of Zelligua's propolis inhibited 50% ± 4.08% of *C. albicans*'s growth; those results were compared to a control. *Candida albicans* growth was completely inhibited with propolis volatiles from both Beni Khalled and Zelligua at a concentration about 50 mg, respectively: 100% ± 3.20% and 100% ± 1.60%.

The chemometric analysis revealed that volatile composition of propolis from Beni Khalled and Zelligua were similar and have 18 common compounds, as proved in Figure 4 from the PCA analysis, this similarity may explain the antifungal activity of both propolis samples compared to the propolis samples from other regions in which no activity were observed even in higher concentrations.

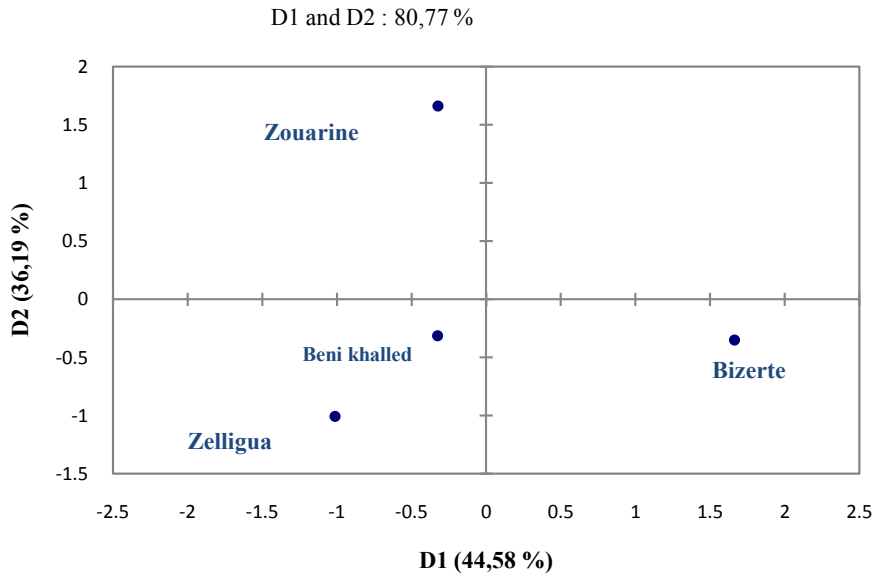


Figure 3. PCA presentation of compounds in propolis.

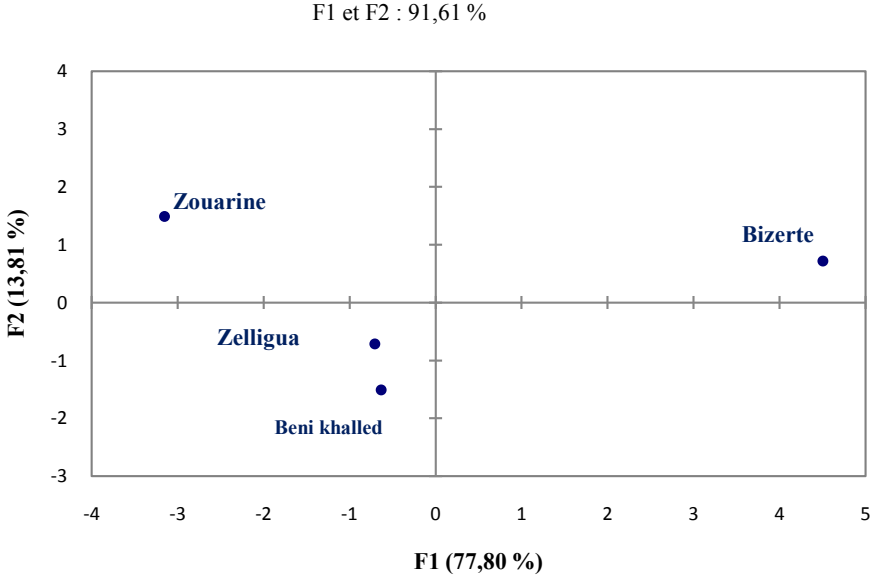


Figure 4. PCA for common compounds in all propolis samples.

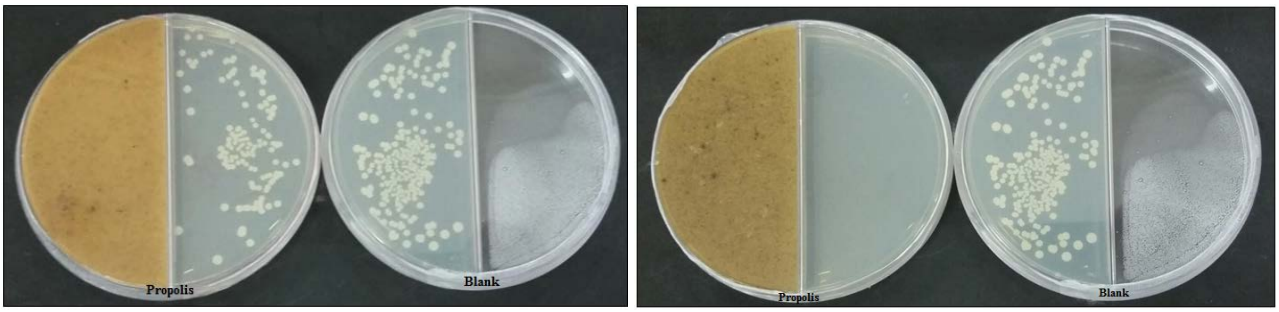
The observed antifungal activity is probably due to the high amounts of *α*-pinene in propolis from Zelligua and Beni Khalled, respectively (81.140% - 82.673%) which is well known to possess similar antifungal and antimicrobial activities [39]. This hypothesis cannot be confirmed since volatiles in propolis from the region of Zouarine contained higher amount of *α*-pinene reaching 90.739% and although it was not active against *Candida*. Those conclusions may give us the confirmation that only a synergy between compounds found in propolis from both regions can lead to the antifungal activity observed.

Comparing all compounds between active and inactive propolis samples, we found that three compounds were found in common in both Beni Khalled and

Table 2. Growth inhibition of *C. albicans* by propolis VOCs.

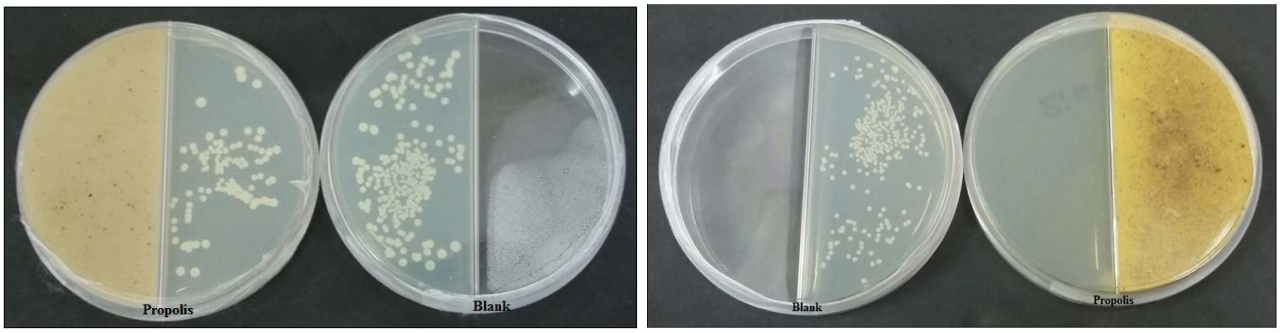
Propolis from Beni Khalled		Propolis from Zelligua	
Concentration (mg)	**Growth inhibition (%)**	**Concentration (mg)**	**Growth inhibition (%)**
10	65 ± 1.96	20	50 ± 4.08
50	100 ± 3.20	50	100 ± 1.60

Values are the means of inhibitory rate SD, means based on 3 replicates.



(a) (b)

Figure 5. Effect of VOCs of Beni Khalled propolis against *C. albicans*. (a) Effect of 10 mg of propolis against *C. albicans* growth; (b) Effect of 50 mg of propolis against *C. albicans* growth.



(a) (b)

Figure 6. Effect of VOCs of Zelligua propolis against *C. albicans*. (a) Effect of 20 mg of propolis against *C. albicans* growth; (b) Effect of 50 mg of propolis against *C. albicans* growth.

Zelligua samples which are α-terpinolene, γ-curcumene and α-Fenchyl acetate (0.445%, 0.414%; 0.752%, 0.478% and 0.235%, 0.139%, respectively). Those compounds are known for their strong antifungal activities as shown in some previous studies [37] [40].

Further tests on the anti candida albicans activity of common compounds in active propolis samples may prove to us weather this antifungal activity is due only to one of those compounds, a synergy of all three or a synergy between all volatile compounds in propolis.

4. Conclusions

The present work provides first data about the Tunisian propolis volatiles and reveals its interesting character.

Headspace GC-MS analysis showed the presence of different compounds in all propolis samples with the predominance of *α*-pinene which is known for its several biological activities. The statistical analysis applied for the propolis volatiles composition showed a notable variation between all samples. This difference is mainly related to the local flora, geographic and climatic characteristics of the site. As far as it concerns the antifungal activity, it should be noted that the active samples showed minor differences in their activities independently from their geographic origin or chemical consistency. Results showed that due to the important number and the diversity of its volatile compounds, Tunisian propolis could be used as a potential source of natural volatiles which play important role by contributing to the pleasant aroma and biological activity of propolis.

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Competing Interest

The authors declare that they have no competing interests.

Funding Statement

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