A Comparative Study between Different Tunisian Propolis Essential Oils and Their Antioxidant Activities

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ABSTRACT: *Propolis is a resinous substance collected by bees from plants with a complex and variable chemical composition. Propolis and its fractions possess multiple biological activities. This study focused on a chemical and statistical comparison between four Tunisian Propolis Essential Oils (PEO) and their antioxidant activities. Volatile oils were extracted by hydrodistillation and analyzed by GC-MS. Essential oil yield varied from 0.095% to 0.324%. A total of 59 volatile components were identified mainly dominated by sesquiterpenes and diterpenes hydrocarbons. Six major components were found in all samples collected from the four different locations α-Cedrol, Manoyl Oxide, Manool, Totarol, Tricosane, and Eicosane. The antioxidant activities of Tunisian PEO have been evaluated using two methods: β-carotene-linoleic acid bleaching and DPPH radical scavenging assays and the results were compared with those of the standard antioxidant (Triolox). PEO from Bizerte region presented a lower IC_{50} value (30.5 μg/mL) than that of the standard antioxidant Trolox (IC_{50} = 40.05 μg/mL) indicating high antioxidant capacity using DPPH assay but for the β-carotene-linoleic acid bleaching assay, PEO from Zouarine region had the lowest value of IC_{50} (26.5 μg/mL) compared to the standard (IC_{50} = 31.25 μg/mL). Our findings demonstrated that PEO possess high antioxidant activities and may be suggested as a new potential source of natural antioxidant.*

KEYWORDS: *Tunisian Propolis; Essential oil; GC/MS; Antioxidant activity; α-Cedrol.*

INTRODUCTION

Propolis is one of the hive products collected by honeybees, such as the *Apis mellifera L.* bee, from parts of plants, buds, and exudates [1]. Bees use this material to seal hive walls and its entrance, to strengthen the border of the combs, and embalm dead invaders [2]. It has been frequently used in folk medicine since around 300 BC [3].

Since propolis is gathered from various plants, its color may vary from light yellow to dark brown. It may cause staining of the comb or frame and may be found in extracted honey [1-2].

Several biological activities such as antifungal [3], antiviral [4], anti-inflammatory [5], antitumoral [6], antioxidant [7] and antibacterial [8] capacities have been reported in different constituents of the propolis making the growth of its commercial interest; it is used as a component of food additives and cosmetics [8].

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1021-9986/2020/2/217-231 15/$/6.05

Chemical studies on propolis and its essential oils concluded that its composition is complex and depends on the local plant flora and not only the region but also climatic characteristics of the area where the sample was collected [9].

Many important chemical families have been identified in propolis such as flavonoid, phenolics and aromatic compounds [4]. Propolis contains some volatile compounds which have a considerable biological effect in spite of their low proportions [3, 5, 6].

Numerous studies have demonstrated the efficiency of essential oils in low doses as biological agents [6].

The market for essential oil grows rapidly and researchers focused on the industrial development together with environmental preservation by using different techniques as HydroDistillation (HD) [12], Supercritical Fluid Extraction (SFE) [13-14], Microwave-Assisted Hydrodistillation (MAHD) [15] and Ultrasound-assisted extraction (UAE). [11-16].

Hydrodistillation is the traditional simplest, oldest and primitive process known to man for obtaining essential oils. However, this technique has been controversial for degradation of some volatile compounds due to long extraction times and thermal or hydrolytic effects [12].

As volatile compounds, various mono and sesquiterpenes are found in propolis. Other compounds were also found including alcohols, mainly aromatic alcohols, phenols, aldehydes, ketones, acids (from acetic to stearic acid), esters, many series of alkanes, alkylated benzenes and naphthalene [17, 18].

PEO have been investigated previously in European, Asian and in a few African regions since 1908 [3, 6, 9]. Concerning Tunisian propolis, to the best of our knowledge it has never been analyzed for its biological activities nor its essential oils.

For this reason the first aim of the present work is to study the chemical composition of different essential oils obtained from several Tunisian propolis localities and investigate the effect of their botanical origins and the second one is to study and compare their antioxidant activities.

Although a great deal of work has been reported on bioactivities of PEO, it is not much explored for its antioxidant capacity. Further, there is no report on PEO antioxidant activity from Tunisian origin in the literature. It's also interesting to note that there is only one recent report on the PEO antioxidant activity from China [19].

EXPERIMENTAL SECTION

Propolis samples

Tunisian propolis was collected during 2014's spring from different sites: Zouarine Kef (A_1) using a special grid, Zelligua Kef (A_2), Beni Khalled Nabeul (B) and Bizerte (C) as shown in Fig. 1, Fig. 2 and Table 1, using the scraping method and then stored at 4°C until use.

Tunisian propolis Essential oil yields

The extraction time is very important in the extraction process, which depends mainly on the vapor pressure of the aromatic compounds [50]. In order to be able to determine the optimal extraction time, follow-up kinetics of the yield by hydrodistillation extraction of PEO as a function of time. 25 g of propolis sample from Beni Khalled region, were cut into small pieces and subjected to hydrodistillation for 60, 120, 180 and 240 min in a Clevenger-type apparatus according to the standard procedure reported in the European Pharmacopoeia (1975) in order to optimize the extraction time [23].

The yield of PEO is expressed as a percentage by mass (%), it is the ratio between the mass of oil obtained and the mass of fresh material [48].

Essential oil extraction

25 g of each four propolis samples were cut into small pieces and subjected to hydrodistillation for 3h using a Clevenger-type apparatus according to the standard procedure reported in the European Pharmacopoeia [23]. Volatile compounds were separated in a separatory funnel. The obtained essential oil was so dense that we could not recover it directly and we followed the protocol quoted by *Haile et al.*, (2012) using chloroform [20]. The aqueous portion was extracted twice with Chloroform (99.96 %, analytical reagent). The organic phase obtained was dried over anhydrous sodium sulphate Na_2SO_4, filtered using Whatman filter paper No. 1, concentrated under vacuum IKA HB10 Digital, Germany), weighed and then stored at 4 °C in closed dark bottles until analysis to avoid any oxidation phenomena. Essential oils compositions were analyzed by GC-MS.

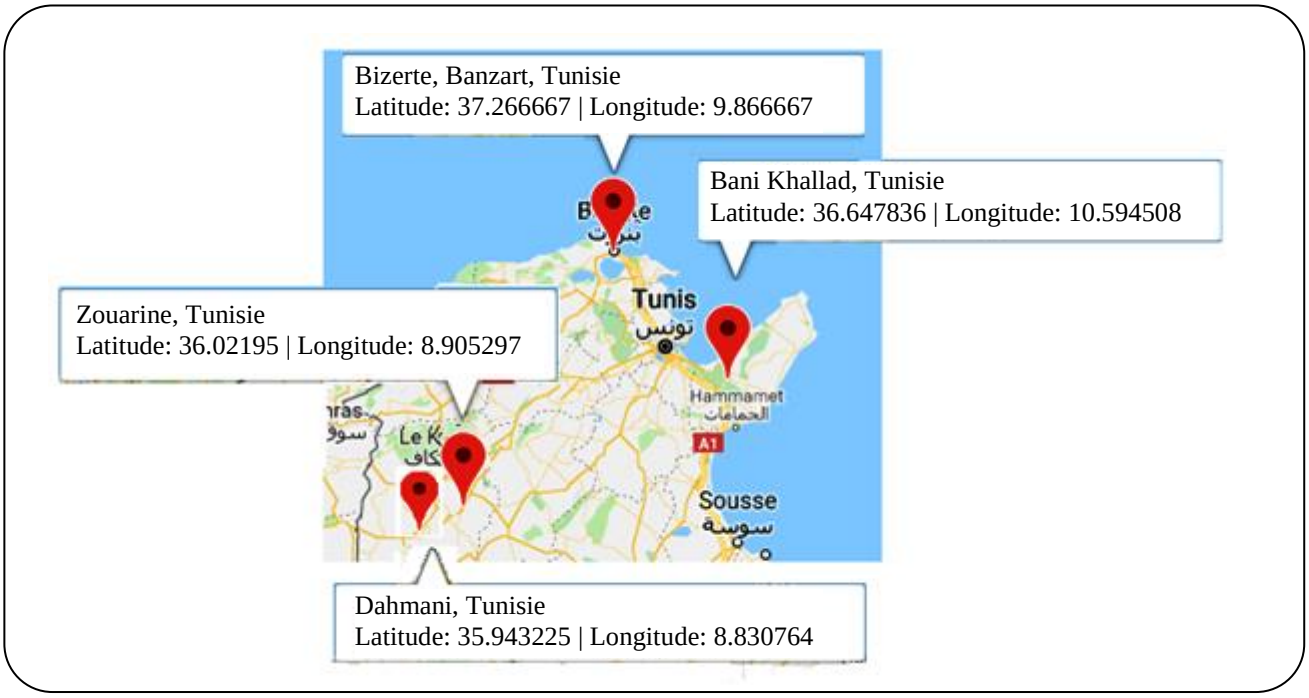

Fig. 1: Geographic location of Tunisian propolis samples.

Fig.2: Propolis samples.

Gas chromatography-mass spectrometry (GC-MS) analysis

Essential oil analysis was performed by an Agilent 7890A GC system, coupled to an Agilent 5972C mass spectroscopy detector with electron impact ionization (70eV). A HP-5 MS capillary column (30m x 0.25mm, coated with 5% phenyl methyl silicone, 95% dimethylpolysiloxane, 0.25µm film thickness; Hewlett-Packard, CA, USA) was used. The column temperature was programmed to rise from 40 to 240°C with a 5°C/min rate, the carrier gas was helium N60 with a 0.9 mL/min flow rate; split ratio was 100:1. Scan time and mass range were 1s and 50-550 m/z, respectively.

Volatile compounds identification was based on the comparison of their retention indices relative to (C_8–C_{22}) n-alkanes with those of authentic compounds available in our laboratory or those in the literature. Identification was also made by matching the compound's recorded mass spectra with those of the Wiley Registry 9th Edition/NIST 2011 edition mass spectral library.

Table 1: Characteristics of chosen regions for propolis harvest.

Region	Latitude Longitude	Climate	Popular trees
Beni Khalled	36°38'52.21"N 10°35'29.43"E	Steppe	Orange, citrus, Eucalyptus gomphocephala
Bizerte	37°16'0"N 9°52'0"E	Warm	Rosemary, Thyme, Cactus, Small plants and no eucalyptus
Zouarine	36°1'19.02"N 8°54'19.07"E	Arid	Marrubium vulgare, Amygdalus communis L, Thym, Romarins, Almonds, poplar, conifer, pines, plums and Cactus.
Zelligua	35°56'35.61"N 8°49'50.75"E	Arid	Poplar, almond, *Eucalyptus camaldulensis*, Thym, Romarins and Cactus.

The percentage determination was based on peak area normalization. All experiments were done in triplicate.

Antioxidant activity

Antioxidant activity on linoleic acid oxidation

This experiment was carried out by the slightly modified method of *Emmons et al.* (1999) [21].

β-Carotene (3 mg) was dissolved in 30 mL of chloroform and 3 mL of the solution were added to 40 mg of linoleic acid and 400 mg of Tween 40. Chloroform was removed in a Büchi Rotavapor R-200 under reduced pressure. Distilled water (100 mL) was added to the solution and mixed well. An aliquot (3 mL) of the β-Carotene/linoleic acid emulsion were mixed with 50 µL of PEO solution and incubated in a water bath at 50 °C for 1h.

Emulsion oxidation was monitored spectrometrically by measuring absorbance at 470 nm over a 60-min period. Control sample contained 50 µL of solvent in place of the extract. The antioxidant activity was expressed using the following equation:

$$AA = 100\ (DR_C - DR_S)/DR_C$$

Where AA is the antioxidant activity, DR_c is the control degradation rate (=ln (a/b)/60), DR_S is the sample degradation rate (=ln (a/b)/60), **a** is the initial absorbance at time 0, and **b** is the absorbance at 60 min. PEO samples were evaluated at the final concentration of 100 µg/mL, 50 µg/mL and 10 µg/mL. Trolox at the same concentrations was used as the reference sample. The results are expressed as IC_{50}values (µg/mL), the concentration required to cause a 50 % β-carotene bleaching inhibition. Tests were carried out in triplicate.

Free radical scavenging activity on DPPH

Radical scavenging ability of PEO was measured according to the method described by *Hanato et al.*, (1988) using the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical [22]. Solutions with different extract concentrations (100µg/mL, 50µg/mL and 10µg/mL) were prepared. Trolox at the same concentrations was used as the reference sample.

2 mL of samples from different concentrations were added to 1 mL of DPPH solution (0.025 mM). After 1 h of incubation at room temperature, the absorbance was measured at 517 nm.

The antiradical activity was expressed as IC_{50} (µg/mL), the concentration required to cause a 50 % DPPH inhibition. The ability to scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = [(A_0 - A_1)/A_0]\times 100$$

Where $\mathbf{A_0}$ is the absorbance of the control reaction, and $\mathbf{A_1}$ is the absorbance in presence of the extract samples and reference. All samples were analyzed in triplicate.

Statistical Analysis

All data were reported as means ± standard deviation of three samples. Statistical analysis was performed with STATISTICA [24]. Significance of differences between samples was evaluated using the DUNCAN test and calculated by the ANOVA procedure, using a significance level of $P \leq 0.05$.

RESULTS AND DISCUSSION

Essential oils yields of the Tunisian propolis

According to the shape of the curve in Fig. 3, the operation goes through three stages:

• A first extraction step (30-75 min), corresponding to a rapid extraction step of the propolis matrix. The extraction begins when the water reaches the point of elimination after 30 minutes;

• A second extraction step, which corresponds to the depletion of the propolis matrix (75 to 120 min).

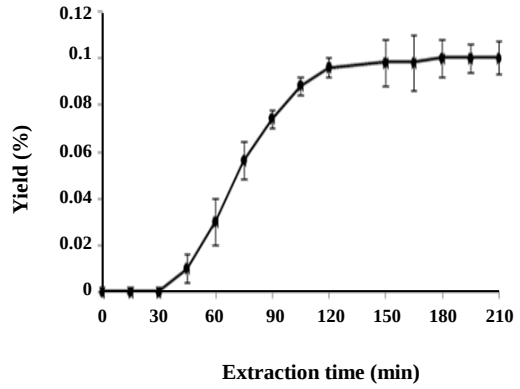

Fig. 3: Optimization of Propolis essential oil yield (%).

A third step, which results in a state of stability and which corresponds to the maximum possible yield.

Results showed that the time for obtaining an optimal PEO yield was 180 min (0.10 %). Beyond this time, a non-significant decrease ($P<0.05$) of the PEO yield was observed. However, the lower yield was obtained during the time interval from 60 to 120 min.

Propolis hydrodistillation gave a dense yellowish oil with an optimal yield of 0.324 % for the sample from Bizerte (C) followed by that from Zouarine (A_1) (0.192 %), Beni Khalled from Nabeul (B) (0.107 %) and Zelligua (A_2) (0.095 %). Respectively, 86, 70, 59 and 144 compounds were identified in the PEO.

Our results were different from those of other authors who published articles about propolis in the period 1908 -1948 [2], and reported that PEO constitute up to 10 % of the total dry weight. In addition, *Haile et al.* (2012) [20] found a higher yield of the PEO from Ethiopia (1.2- 0.92 %), whereas, lower yields were observed in the Algerian propolis (0.03-0.11 %) [9] and Greek propolis (0.03-0.10 %) [7]. These differences could be due mainly to the environmental conditions; botanic origins, kind of bee and the metabolism generating volatile compounds [25].

Chemical composition of the Tunisian propolis essential oils

Propolis essential oil composition was summarized in Table 2, where volatiles were listed in order of their retention time.

In order to recover the PEO, we used chloroform. This technique allowed us to obtain an oily extract of propolis. The chloroform used, extracted all compounds that may exist in the oil of propolis. A total of 35 compounds were identified as main constituents in all PEO samples. Only 9 major compounds were common to the four samples with different rates: α-Cedrol**,** Manoyl Oxide**,** Manool (CAS)**,** Bicyclo [5.2.0] nonane, 4-ethenyl-4, 8,8-trimethyl-2-methylene-**,** Tricosane**,** Totarol**,** Eicosane, Heneicosane and labda-8(17) ,13Z-dien-15-ol.

For example the Bicyclo [5.2.0] nonane, 4-ethenyl-4, 8, 8-trimethyl-2-methylene- represented 2.44 % of the PEO from (C) region but only traces in the sample from (A_1). Also the Manoyl Oxide constituted 11.13 % of the PEO from (C) region but only 4.46 % in the sample from (B) region.

The major constituents of the PEO from (A_1) region were Manool (CAS) representing 22.64 % of the total essential oil, followed by Totarol (14.71 %), α-Cedrol (10.06 %) and Manoyl Oxide (9.11 %). However, sample from (A_2) was mainly dominated by Dibutyl phthalate (25.72 %), α-Cedrol (20.62 %), Manool (CAS) (12.02 %), Totarol (9.88 %) and Manoyl oxide (3.93 %).

Sample from (B) region was characterized by high proportions of Manool (CAS) (28.96 %), α-Cedrol (22.10 %), Totarol (11.77 %), β. Elemene (6.20 %) and Manoyl Oxide (4.46 %). Manool (CAS) (20.88 %) was also the major compound of the sample from (C) region, followed by β-Eudesmol (12.43 %), Manoyl Oxide (11.13 %), Totarol (8.69 %) and α-Cedrol (5.73 %).

Results showed that Manool (CAS) was the dominated constituent in the samples from (A_1), (B) and (C) but Dibutyl phthalate was the main constituent of that from (A_2) region. In this study, Manool (CAS) was identified for the first time in the PEO. It was the dominant constituent in the major Tunisian PEO.

It's worth noting that the presence of Dibutyl phthalate as the major constituent of the sample from (A_2) region (25.72 %) is very interesting. Indeed, this compound was identified as a trace among the volatiles in the European (0.31 %) and Ethiopian (0.56 %) propolis [38, 39].

Moreover, Cholestan-3-ol, 2-methylene- and 3-Oxatricyclo [3.2.1.0 (2, 4)] octane, (1α, 2β, 4β, and 5α) have never been previously reported as propolis constituents.

In this study, it was identified only in the sample from (C) region.

Thunbergol existed also only in the sample from (C) region but it was detected before for the first time in the Turkish propolis from Kazan [46]. There are some constituents who figure only in one place and not in the others, like Oleic acid in (A_1), β-Eudesmol in (C), Isobutyl phthalate in (A_2) and (-)-Neoclovene-(I), dihydro-Dodec-2-en-10-ynedial in (B). This difference could be related to the environmental conditions, botanic origins, kind of bee and the metabolism generating volatile compounds [25].

Results in Table 2 showed that the main volatile compounds are common to all four samples. The amounts of four compounds are similar between all samples: α-Cedrol, Manoyl Oxide, Manool (CAS) and Totarol but there is a gap concerning Dibutyl phthalate.

α-Cedrol was found in PEO from (A_2) and (B) with almost same amounts (20.62 % and 22.10 %; respectively) followed by the PEO from (A_1) (10.06 %) and finally the lower amount was found in PEO from (C) (5.73 %).

Manoyl Oxide was found in PEO from (A_1) and (C) with almost same amounts (9.11 % and 11.13 %; respectively) followed by lower amount in the PEO from (A_2) and (B) (3.94 % and 4.46 %, respectively).

Manool (CAS) was found in PEO from (A_1), (B) and (C) with almost same amounts (22.64 %, 28.96 % and 20.88 %; respectively) followed by lower amount in PEO from (A_2) (12.02 %).

Totarol was found in PEO from (A_2), (A_1), (B) and (C) with almost same amounts (9.88 %, 14.71 %, 11.77 % and 8.69 %; respectively).

Dibutyl phthalate was only found in PEO from (A_2) with high amount (25.72 %) followed by the PEO from (A_1) (1.74 %) and finally as a trace in PEO from (B) (0.19 %) Table 2 showed that there was a relative similarity among the four samples from various regions of Tunisia. In fact, four main compounds are always present in high amount in all regions. However, the number of compounds identified in all samples was different. Sample from (A_2) region was characterized by the highest number of compounds identified (144 compounds) while only 59 volatile components were detected in the sample from (B) region.

In addition, samples from two sites (A_1) (A_2) collected from the same region (A), presented different essential oil composition. This was mainly due to the collect method; in fact, propolis from (A_1) was collected by a grid while that from (A_2) was hand collected by scraping the hive. Consequently, differences are related to the propolis purity itself.

Botanical origins of Propolis

In the current study, propolis samples have different plant origin due to different geographical positions. In such cases, bees cut fragments of vegetative tissues to release the substances used in propolis production. The specificity of the flora determines the chemical composition of propolis, including volatile compounds [25].

In fact, Eucalyptus, Rosemary, Thym, Citrus and Almond trees were found in both A_1 and A_2 sites. A region was 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 [26].

In B region, bees are collecting propolis only from Orange trees and the dominant climate was steppe. In C region, Rosemary, Thym, Cactus and small plants are found but no Eucalyptus; this region was characterized by warm and temperate climate due to its location near the sea.

Cedrol is a sesquiterpene alcohol found generally in cedar wood and other conifer essential oils. Being identified as a major volatile constituent in all propolis samples proved that pine coniferous tree such as spruce and yellow pine was the main plant collected by bees for propolis elaboration. Cedrol was also reported as a major component (4.3 to 6.3 %) of Greek propolis, Algerian one (2.57 %) and Turkey one (7.0 -15.6 %) [7, 9, 27].

Loizzo et al. (2013) [28] reported that most abundant diterpene in endemic plants in Tunisia was Manoyl oxide which is in accordance with our result since this compound was identified in all regions. Moreover, *Hanana et al.* (2014) [30] proved that some volatile components are specific to pin species, such as Manoyl oxide for *Pin Caribaea* and Calamanene, α-Phellandrene and α-pinene for *Pin Coulteri* [30, 29, 21]. This may give us an idea of the existence of pine trees in all regions where those constituents were mentioned.

Totarol was present in high amount in all propolis samples. This compound was first isolated in

Table 2: Chemical composition of Tunisian propolis essential oils analyzed by GC-MS.

Name	Rt	Kef				Beni Khalled		Bizerte	
		Zouarine		Zelligua					
		%	µg/mL	%	µg/mL	%	µg/mL	%	µg/mL
Monoterpenes hydrocarbons									
α-pinene	6.927	-	-	0.44±0.03[a]	0.09±0.02	-	-	-	-
l-Phellandrene	24.612	-	-	-	-	-	-	1.09±0.06[a]	0.11±0.01
Camphene	18.098	-	-	2.74±0.10[a]	0.57±0.001	0.74±0.12[c]	0.16±0.01	1.38±0 .14[b]	0.15±0.10
Sesquiterpene hydrocarbons									
1s. cis-Calamenene	21.096	0.64±0.01[b]	0.01±0.03	-	-	-	-	1.03±0.02[a]	0.13±0.003
β. Elemene	31.018	6.41±0.59[a]	0.17±0.02	2.93±0.03[b]	0.61±0.01	6.20±0.15[a]	1.53±0.005	-	-
γ-eudesmol	22.584	0.74±0.06[c]	0.02±0.005	-	-	0.92±0.05[b]	0.20±0.01	2.55±0.12[a]	0.84±0.01
β-Eudesmol	22.854			-	-	-	-	12.43±1.08[a]	2.45±0.22
α-Eudesmol	22.867	7.69±0.94[a]	0.20±0.02	-	-	1.55±0.18[b]	0.34±0.02	-	-
α-Cedrol	22.248	10.06±2.15[b]	0.27±0.05	20.62±0.13[a]	4.69±0.06	22.10±0.15[a]	4.71±0.15	5.73±0.52[c]	0.60±0.43
β-Bourbonene	28.105	-	-	2.39±0.03[a]	0.50±0.01	2.07±0.26[b]	0.63±0.03	2.09±0.01[b]	0.41±0.06
Manoyl Oxide	27.851	9.11±1.01[b]	0.24±0.03	3.94±0.43[c]	1.15±0.01	4.46±0.16[c]	0.98±0.04	11.13±0.14[a]	2.22±0.36
Manool (CAS)	28.676	22.64±0.71[b]	0.61±0.06	12.02±1.29[d]	15.25±0.36	28.96±0.48[a]	6.01±0.21	20.88±0.52[c]	4.17±0.64
Totarol	31.411	14.71±0.58[a]	0.39±0.05	9.88±0.32[c]	2.09±0.07	11.77±1.78[b]	2.81±0.20	8.69±0.16[c]	1.19±0.36
Others									
Cholestan-3-ol. 2-methylene-	27.188	-	-	-	-	-	-	1.28±0.06[a]	0.22±0.01
1. 3.5-Trihydroxy-9(10H)-acridinone	27.585	-	-	1.72±0.02[b]	0.89±0.05	-	-	3.67±0.07[a]	1.34±0.01
2-Aminochrysene	27.586	-	-	3.02±0.01[b]	1.09±0.01	3.59±0.04[a]	0.96±0.01	-	-
Pentadeca-2.13-diyne	27.928	-	-	-	-	1.92±0.07[a]	0.93±0.01	1.67±0.04[b]	0.33±0.05
Heneicosane	29.012	0.45±0.12[d]	0.01±0.01	1.17±0.01[a]	0.24±0.01	0.77±0.01[b]	0.15±0.01	0.71±0.01[c]	0.07±0.01
Oleic acid	29.826	1.35±0.06[a]	0.03±0.01	-	-	-	-	-	-
Ethyl Oleate	29.830	-	-	-	-	-	-	1.29±0.02[a]	0.45±0.01
(-)-Neoclovene-(I). dihydro-Dodec-2-en-10-ynedial	29.899	-	-	-	-	1.14±0.01[a]	0.25±0.01	-	-
3-Oxatricyclo[3.2.1.0(2.4)]octane.(1α.2β.4β.5α)	30.525	-	-	-	-	-	-	1.44±0.01[a]	0.48±0.05
7-[(1E)-prop-1-enyl] cycloocta-1. 4-diene	30.537	2.81±0.10a	0.07±0.02	1.16±0.01c	0.77±0.02	2.54±0.09b	0.72±0.04	-	-
labda-8(17) .13Z-dien-15-ol	30.703	2.47 ±0.45[b]	0.06±0.01	2.50±0.01[b]	0.64±0.05	3.03±0.10[a]	0.83±0.22	1.34±0.03[c]	0.27±0.04
Nonadecane	30.714	2.21±0.27[a]	0.06±0.01	-	-	0.20±0.01[b]	0.04±0.01	-	-
Bicyclo[5.2.0]nonane. 4-ethenyl-4.8.8-trimethyl-2-methylene-2-vinyl-	31.01	-	-	0.16±0.05[c]	0.62±0.03	1.28±0.01[b]	0.26±0.01	2.44±0.01[a]	0.48±0.01

Table 2: Chemical composition of Tunisian propolis essential oils analyzed by GC-MS. (Contimued)

Thunbergol	31.024	-	-	-	-	-	-	5.90±0.04^{a}	1.33±0.25
Isobutyl phthalate	26.347	-	-	3.88±1.76^{a}	1.03±0.18	-	-	-	-
Dibutyl phthalate	27.139	1.74±0.47^{b}	0.05±0.01	25.72±0.02^{a}	5.77±0.04	0.19±0.006^{c}	1.72±0.01	-	-
Tricosane	31.135	5.18±0.35^{a}	0.14±0.01	2.31 ±0.06^{c}	0.59±0.03	2.08±0.02^{c}	0.63±0.05	3.33±0.94	0.87±0.15
Eicosane	32.805	3.08±0.54^{a}	0.08±0.01	0.18±0.006^{c}	0.04±0.01	1.66±0.03 ^{b}	0.34±0.02	0.58±0.01^{c}	0.14±0.01
Pentacosane	32.815	-	-	1.63 ±0.02^{b}	0.34 ±0.01	-	-	3.01±0.97^{a}	1.75±0.83
Heptacosane	34.216	-	-	0.99±0.01^{c}	0.20±0.01	1.2±0.07^{b}	0.24±0.01	3.96±0.14^{a}	0.80±0.15
Tetracosane	34.219	-	-	-	-	0.32±0.01^{b}	0.07±0.01	2.96±0.94^{a}	0.80±1.20
Total (Main constituents)		93.32±4.95^{a}	2.48±0.12	98.95±2.40^{a}	37.65±0.24	98.71±2.02^{a}	24.54±0.33	97.17±0.40^{a}	16.71±1.44
Others		6.675^{a}	2668.65	1.05^{c}	23766.24	1.29^{c}	20751.5	2.83^{b}	23783
Groupe (%)									
Monoterpene hydrocarbons		-		3.18^{b}		0.74^{c}		2.47^{a}	
Sesquiterpene hydrocarbons		17.12^{c}		25.94^{b}		30.376^{a}		8.85^{d}	
Oxygenated Sesquiterpenes		8.432^{b}		0.16^{d}		3.75^{c}		17.43^{a}	
Diterpenes hydrocarbons		37.35^{b}		24.64^{d}		41.47^{a}		30.95^{c}	
Oxygenated Diterpenes		-		-		-		5.90^{a}	
Aliphatic hydrocarbons		7.84^{b}		6.11^{c}		4.57^{d}		14.05^{a}	
Diterpene labdane		11.58^{a}		6.43^{b}		7.49^{b}		12.47^{a}	
Fatty acids		3.67^{a}		-		-		1.56^{b}	
Oxygenated aromatic		1.74^{c}		32.66^{a}		3.78^{b}		-	
Others		2.81^{c}		2.88^{c}		5.60^{b}		8.07^{a}	

RT: Retention Time; Zouarine Kef (A_1). Zelligua Kef (A_2). Beni Khalled Nabeul (B). Bizerte (C).

A) 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).

- : not detected.

Podocarpustotara in New Zealand but later it has also been identified in *Podocarpaceae* and *Cupressaceae* family. Recently it has been isolated in Rosemary. This result confirmed the existence of rosemary in all the studied regions. Similar results were found by *Zammit et al.* (2013) [31] who reported that the diterpenoide Totarol was the predominant constituent in all samples of Maltese propolis. These authors proved that propolis cytotoxicity variation against an ovarian cancer cell line and a human breast tumor cell line correlated with Totarol content [32].

The major volatile terpenes identified from all samples, were also recognized for their medicinal properties. Indeed, the antimicrobial activity of propolis is accounted to the presence of compounds such as heptacosane, heneicosane, labda-8, 13Z-dien-15-ol, nonadecane, β-bourbonene, Manoyl oxide, isobutyl phthalate, Totarol, Eicosane and dibutyl phthalate [10]. Biological activities of identified constituents are summarized in the Table 3.

The diterpenes (mainly acids of labdane type) were the major constituents of Tunisian propolis essential oils. These compounds were also characteristic of the Mediterranean propolis from Sicily, Greece, Crete and Malta. As reported by *Melliou et al.* (2007) [7], the plant sources of this kind of propolis are *Cupressaceate Ferula* and *Pinaceae* and the bee specie is *Apis mellifera*.

Chemical classes of volatiles characterizing Tunisian Propolis

In all studied essential oils, several volatiles groups existed mainly: Monoterpene hydrocarbons, sesquiterpene

Table 3: Propolis volatiles from different geographic origin.

Geographic origin	Main constituents	Reference
Hungary	β-eudesmol, benzyl benzoate	(19)
Algeria	2-hexenal (8%), myristic acid (3.11%), linoleic acid (3.06%), alpha-Cedrol (2.57%)	(6)
Croatia	Limonene (6.4 – 10,5%), benzyl alcohol (3,1 – 18,2%), benzyl benzoate (3,6 – 4,4%)	(3)
Canary islands	nerolidol (3.2 – 11.0%), spatulenol (3.2 – 8.4%), ledol (1.6 – 3.8%)	(14)
Greece	α –pinene (7.9 – 45.8%), trans-β-terpineol (2.2 – 6.6%), Junipene (1.5 – 11.7%), δ-cadinene (0.3 – 8.4)	(4)
India (Maharashtra state)	Tricosane (13.6%), hexacosane (11.5%), palmitic acid (8.5%), linalool (6.7%), methyleugenol (6.0%)	(1)
Italy (Northern)	Benzoic acid (3.1 – 30.1%), benzyl benzoate (0.2 – 13.1%), β-eudesmol (2.9 – 12.9%), δ-cadinene (1.3 – 13.3%), γ-cadinene (1.4 – 8.9%), T-cadinol (2.7 – 10.0%), α-cadinol (4.8 – 9.7%)	(20)
Portugal	Viridiflorol (9.0 – 39.0%), n-tricosane (5.3%), n-Nonadécane (4.0 – 18.0%)	(21)
China (Inner Mongolia)	3-methyl-2-buten-1-ol (26.8%), phenylethyl alcohol (17.1%), 2-methoxy-4-vinylphenol (9.5%)	(22)
Brazil	Spatulenol (3.0 – 13.9%), (2Z, 6E)-farnesol (1.6 – 14.9%), prenyl-acetophenone (0.2 – 8.7%), benzyl benzoate (0.3 – 18.3%)	(19)
Brazil	β-caryophyllene (12.7%), acetophenone (12.3%)	(12)
Brazil	Nerolidol (6.6%), trans-caryophyllene (4.1), spatulenol (3.6%)	(23)
Brazil (Minas Gerais State)	(E)-nerolidol (17.1%), β-caryophyllene (13.4%), selina-3, 7(11) diene (10.4%)	(24)
Brazil (Teresina, Piaui State)	1, 8 – cineole (24.0%), exo-fenchol (11.3%), terpinen-4-ol (7 .7%)	(25)
Brazil (Piaui State)	α-pinene (0.3 – 34.4%), E-caryophyllene(2.6 – 17.4%), α-copaene (3.6 – 7.5%)	(26)
Brazil (Rio de Janeiro State)	α-pinene (18.3%), β-pinene (6.5%), δ-cadinene (7.0%)	(27)
Brazil (Rio Grande do Sul State)	α-pinene (57.0 – 63.0%), β-pinene (12.5 – 30.8%), limonene (1.5 – 11.2%)	(7)
Ethiopia (Assela)	5,6,7,8-tetramethylbicyclo [4,1,0] hept-4-en-3-one (15.0%), acoradiene (13.8%), epicedrol (6.8%)	(28)
Ethiopia (Haramaya)	Calamenene (13.8%), 4-terpineol (8.6%), epi- bicyclosesquiphellandrene (8.4%)	(13)
Turkey	Cedrol (7.0– 15.6%), α-bisabolol (14.3%), δ-cadinene (2.7 – 5.6%)	(29)
China (Inner Mongolia)	Heptadecane (7.0%), phenantrene (4.0%)	(22)
Estonia	Eucalyptol (25.9%), α-pinene (20.6%), benzaldehyde (10.8%), β-pinene (8.9%)	(29)
France	β-eudesmol (30.0%), guaiol (10.0%), benzylbenzoate (8.0%)	(30)

hydrocarbons, oxygenated sesquiterpenes, diterpenes hydrocarbons, oxygenated diterpenes, aliphatic hydrocarbons, diterpenes labdane and oxygenated aromatic compounds. Diterpenes hydrocarbons such as: Manool (CAS), Totarol, camphene and sesquiterpene hydrocarbons like α-Cedrol and β-Elemene were the predominant groups in all essential oils samples.

Tunisian propolis VS other propolis

Our results were compared with those of others and the differences were illustrated in the Table 3. The Iranian PEO was found to be rich in α-pinene (43.9 %), 1.8-cineole (11.1 %), camphene (8.6 %), broneol (3.4 %), camphor (2.4 %) and verbenol (2.3 %). The PEO from Kerman contained a similar composition revealing high amounts of α-pinene (46.1 %), 1.8-cineole (11.1 %),

camphene (9.6 %), camphor (5.3 %) but also other important compounds such as: sabinene (4.6 %), broneol (3.4 %), bornyl acetates (2.8 %), verbenone (2.3 %) and linalool (2.1 %) [29].

Moreover, the most abundant components in Brazilian PEO were β-caryophyllene (12.7 %), acetophenone (12.3 %), farnesene (9.2 %) and linalool (6.47 %) [26].

β-Eudesmol was found to be the major constituent of PEO from France, Hungary, Bulgaria and Northern Italy [36, 37]. These results were different from ours since this compound was identified as trace in the Tunisian PEO.

However, similar results were found in previous works on Greek propolis in which the terpenoids was detected as major volatile compound and on Brazilian green propolis which was characterized by the predominance of Sesquiterpenes [7- 38].

Naik et al. (2013) [8] have reported essential oil composition of Indian propolis and showed that long-chain alkanes (Tricosane, hexacosane, heptacosane) and terpenoids were the main groups of volatile compounds, while oxygenated monoterpenes, sesquiterpenes and oxygenated aliphatic hydrocarbons were the most abundant constituents of the Ethiopian propolis [27-39].

Propolis volatiles from Canary Island belong mainly to sesquiterpenes (nerolidol, spatulenol, ledol) and long chain hydrocarbons [40]. However, Algerian PEO was characterized by the dominance of 2-hexenal (8 %), myristic acid (3.11 %), linoleic acid (3.06 %) and α-Cedrol (2.57 %) [8,9].

In the most of European propolis volatiles, sesquiterpenes were predominated. In the other hand, essential oils of Argentinean propolis [46], contained high percentage of monoterpenoids and major constituents were o-cymene and limonene.

It important to note that chemical composition of propolis essential oils was characterized by its diversity, resulting from the specificity of the local biodiversity. In fact, the chemical consistency of propolis is highly dependent on the flora of the region from which it is collected.

All samples from Tunisian propolis were free of Thymol. This result proved that hives chosen in this study were never been exposed to a chemical treatment and they are totally natural. In fact, hives must be treated against problems like Varroa mites, with volatile compounds, mainly Thymol. In such case, the profile of propolis essential oils is completely unnatural and is dominated up to 70 – 80 % by Thymol, which is only a micro component in untreated hives from the same region [47].

α-pinene has been identified as a trace (0,45 %) in the Tunisian PEO from A_2 region but was totally absent in A_1, B, and C regions. This is in accordance with what was reported in some European and tropical propolis samples (6-4-40) but different from other results on Greek (7.9-45.8 %), Mexican (Yucatan) (11.9 %) and Brazilian propolis (Rio Grande do Sul State) (57.0-63.0 %) in which α-pinene was a major compound.

The diterpene Manool present with high proportion in the Tunisian PEO, was isolated before from Greek propolis and was evaluated as the most active compound against the proliferation of HT-29 human colon adenocarcinoma cells, with the advantage of not affecting the normal human cells [33-35]. Simple eye inspection of the distribution of hydrocarbons, diterpenes and sesquiterpenes of PEO from those four different regions of Tunisia enabled detection of consistent differences between samples. The comparison of complex data sets and their statistical treatment requires computerized means of analysis.

Chemometrics, a multivariate method of statistical analysis, has proved to be an effective way to deal with complex chemical data.

In this study, and in order to treat chemical data of PEO and to detect chemical differences and similarities between samples, Hierarchical Cluster Analysis (HCA) was used.

HCA provided a dendrogram of chemical affinities among the PEO samples (Fig. 7), permitting to conclude that samples from A_1 and C regions were characterized by a close similar composition which was different from those obtained from A_2 and B regions. Also, as it can be shown, sample from B region was completely different from all other samples.

These differences and similarities could be explained by the differences in the botanic origins, climate and geographic positions.

Antioxidant activity

Antioxidant activity on linoleic acid oxidation

The antioxidant assay, using β-carotene discoloration is widely used, because this one is extremely susceptible to free radical-mediated oxidation. It decolorized easily

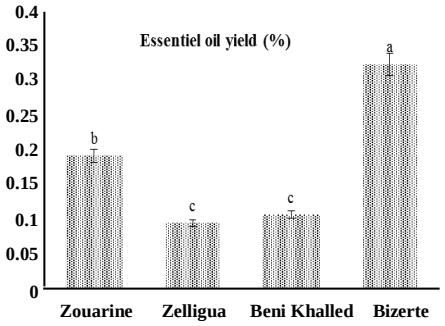

Fig. 4: Oil yield of Tunisian propolis (%). Values are given as means ± SD (n = 3). Means followed by the same small letter did not have significant differences at p<0.05 (Duncan test).

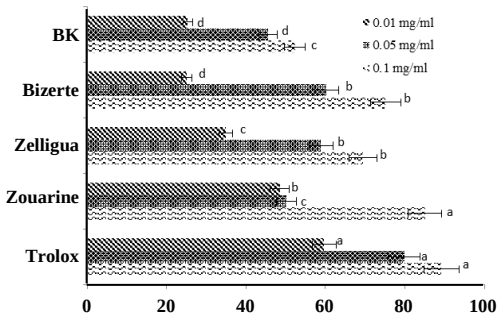

Fig. 5: Antioxidant activity on linoleic acid oxidation (%). Values are given as means ± SD (n = 3). Means followed by the same small letter did not have significant differences at p<0.05 (Duncan test).

antioxidants, due to its double bonds being sensitive to oxidation [41]. This test measures the sample's potential for inhibiting conjugated dienehydroperoxides formation from linoleic acid oxidation [42].

Fig. 5 presented the antioxidant activity of Tunisian propolis essential oils, determined by the β-carotene – linoleic acid system. For a concentration of 0.1 mg/mL, PEO from A_1 region had the strongest antioxidant activity of 85 %, which is almost near that of the positive control at the same concentration. However, sample from B region presented the lowest activity of 52 %. For a concentration of 0.05 mg/mL, PEO from C region had the highest antioxidant activity, over 60 %, while PEO from B region had the weakest one.

Finally, for a concentration of 0.01 mg/mL, the PEO from A_1 region showed an activity similar to that of the positive control. However, the antioxidant activity of PEO from C region was very low.

Results led us to conclude that PEO form A_1 region had the strongest antioxidant activity at 0.1 mg/mL and 0.01 mg/mL concentrations while those from B and C were characterized by the lowest one. In fact, basing on IC_{50} values, (Table 4), A_1 had the lowest value of (IC_{50}=26.5µg/mL) compared to those of all regions and especially to that of Trolox (IC_{50}=31.25 µg/mL), while, the highest value of IC_{50} (83µg/mL) was observed in the PEO from B region.

Naik et al. (2013) has reported higher value of IC_{50} (0.32 mg/mL). This difference may be due to the differences in the PEO's composition [8].

Effect of various propolis samples on DPPH free radical

The model system of scavenging DPPH free radical is a simple method for evaluating the antioxidant activity of compounds due to their hydrogen-donating ability [32]. The antiradical activity of various PEO samples is presented in Fig. 6. For a concentration of 0.1 mg/mL, 0.05 mg/ml and 0.01 mg/mL, PEO from C region had the strongest antioxidant activities of 84 %, 68 % and 34 %, respectively. This sample presented a lower IC_{50} value (30.5 µg/mL) than that of the standard antioxidant Trolox (IC_{50} = 40.05 µg/mL) indicating high antioxidant capacity. However, PEO from A_1, A_2 and B regions showed higher IC_{50} values (60.6 µg/mL, 86.9 µg/mL and 56 µg/mL, respectively) than that of the standard antioxidant Trolox (IC_{50} = 40.05 µg/mL) indicating a low antioxidant capacity.

To the best of our knowledge this is the only comparative study between PEO antiradical activities. However, *Naik et al.* (2013) [8] confirmed that the PEO possess antiradical activity. According to the results in Figs. 5-6, the essential oils presented antioxidant activity and it is evident that antioxidant activity is dose-dependent; in fact, higher antioxidant activity was observed when essential oil concentration was the highest. This antiradical activity may be related to the presence of bioactive compounds in PEO such as phenolic and volatile compounds. In fact, polyphenols are thought to promote optimum health partly via their antioxidant and free radical scavenging

Table 4: Antioxidant activity of Propolis.

Region	IC_{50} (µg/mL) β-Carotene	IC_{50} (µg/mL) DPPH
Zouarine	26.5	60.6
Zelligua	41	86.9
Beni Khalled	83	56
Bizerte	46.9	30.5
Trolox	31.25	40.05

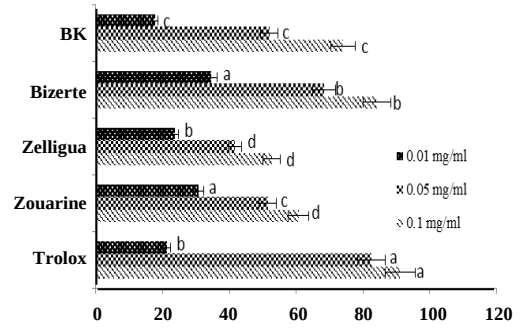

Fig. 6: DPPH free radical scavenging activity (%). Values are given as means ± SD (n = 3). Means followed by the same small letter did not have significant differences at p<0.05 (Duncan test).

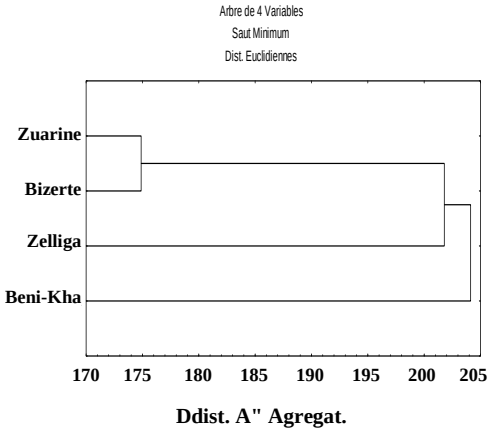

Fig. 7: Dendrogram of HCA cluster analysis.

effects thereby protecting cellular components against free radical induced damage. But due to their diverse chemical structures, they are likely to possess different antioxidant capacities (44). In addition, according to *Tepe et al.* (2004) [43], monoterpenes act as radical scavenging agents.

The antioxidant activity of individual compounds identified as constituents of PEO has been previously reported as shown in Table 3. Sesquiterpene alcohols like Bicyclo[5.2.0]nonane, 4-ethenyl-4,8,8 trimethyl-2-methylene-, α and γ-eudesmol, had low antioxidant activity. Moreover, it was well known that compounds such as: tridecane, pentadecane, nonadecane, heneicosane, Tricosane, pentacosane, heptacosane, and palmitic acid, present in the PEO, do not possess antioxidant properties. Contrarily, α-Cedrol and β-Eudesmol had a notable antioxidant activity. In addition, it was proved that Totarol is a nature super antioxidant, three times stronger than Vitamin E, and has potent force to neutralize free radicals linked to skin ageing [45]. Highly purified, Totarol can be used in cosmetic and health care preparations for its antibacterial, antioxidant and anti-inflammatory effects [49].

CONCLUSIONS

This study revealed the presence of 9 major volatile compounds in all propolis samples which could be used as chemical markers permitting to classify and identify their botanical origins. It is worth noting that the knowledge of Tunisian PEO is far from being exhaustive especially that its composition and biological proprieties have never been studied.

The difference in percentage and composition of essential oils of Tunisian propolis from collection sites could be due to the botanical origin, physical and chemical characteristics of soil, plant age and parts of plant that was collected by bees to make propolis.

This finding also illustrated that PEO possess antioxidant activities. On the basis of these

results, PEO could be used as an easily accessible source of natural antioxidant, food supplement or in the pharmaceutical industry.

Acknowledgments

The authors are grateful to Mr. Farid Limam chief head of the biological department of Borj Cedria Tunisia. We also thank Mr. Bilel Garbouj from the Bee House Beni Khalled, Mr. Anis Mejri and Mr. Khaled Zaghmouri for providing the Propolis samples.

Received : Oct. 12, 2018 ; Accepted : Feb. 5, 2019

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