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ROSEMARY ESSENTIAL OILS: EXTRACTION, GC/MS ANALYSIS, ANTIOXIDANT ACTIVITY WITH DFT AND IN SILICO INSIGHTS OF PROSPECTIVE NANO-ANTIOXIDANTS

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Docking

Abstract
The objective of this work is to identify the bioactive molecules in the essential oil of the dried leaves of Rosemary from two cities in the Eastern region of Morocco: Taourirt and Jerrada. These essential oils have already been extracted by the steam distillation method in the cooperative. the chemical composition have been determined by gas chromatography coupled with mass spectrometry (GC/MS), in which cineole 41.77%, alpha-Pinene 10.75% and Camphor 9.32% represent the majority compounds for essential oil of Rosemary from Taourirt and cineole 51.68%, alpha-Pinene 11.91 % and Camphor 10.29 % predominate in that of Jerada rosemary. Antioxidant activity in vitro was assessed in three different ways: the DPPH test, the β-carotene bleaching technique, and the reduction power measurement. The DFT analysis of the different compounds and the Docking study were also investigated in objective to study the molecular reactivity of the majority compounds, also study their affinity to various cyclodextrin as prospective encapsulation systems.

1. Introduction

Antioxidants are compounds that can delay or inhibit the oxidation of lipids or other molecules by inhibiting the initiation or the propagation of oxidizing chain reactions [1]. There are two basic categories of antioxidants, natural and synthetic. The second one has been found to cause long-term toxicological effects, including carcinogenicity [2]. Application of antioxidants is the best strategy to prevent oxidation reactions. Synthetic antioxidants such as BHT, TBHQ, and BHA have various adverse human health effects including allergy, headache, asthma, and dermatitis,

therefore, there is a general desire to replace synthetic antioxidants with natural ingredients, [3] the application of natural antioxidants (such as essential oils and plant extracts) is interesting and can be noted in an increasing number of research works [4]. A large number of medicinal plants and their purified constituents have shown beneficial therapeutic potentials. Various herbs and spices have been reported to exhibit antioxidant activity. Rosemary is a good natural source of antioxidant compounds. It is widely used in the food industry to prevent possible oxidative and microbial degradation of foods[5]. *Rosmarinus officinalis* (commonly named Rosemary) is an aromatic plant of Lamiaceae family. It is an evergreen shrub reaching a height of up to 1.5m. It grows spontaneously in many Mediterranean countries. Rosemary is used widely since antiquity in culinary, cosmetics, and medicinal products. Many studies have demonstrated that it is a very efficient plant as natural antioxidant. Rosemary is used widely since antiquity in culinary, cosmetics, and medicinal products. Many studies have demonstrated that it is a very efficient plant as natural[6]. This work was completed by a theoretically study of the major constituents of Rosemary's essential oil using density functional theory (DFT) using Gaussian 09W software [7]. The DFT studies were specifically applied to elucidate detailed understandings regarding the local/global reactivity of the studied compounds [8].

2. Experimantal details

2.1. Plant material and essential oil

The aerial parts of *Rosmarinus officinalis* were collected from region of Taourirt and Jerada in the east of Morocco in 2018 by two products Eos Cooperation.

The EO was extracted by steam distillation in two cooperations how products Eos in Jerada and Taourirt in Morocco.

2.2. Gas chromatography-Mass Spectrometry (GC-MS)

The EOs was analyzed by GC-MS in the measuring room at the Department of chemistry of the Faculty of Science in Oujda Morocco. Chromatographic analyses were performed using a GC coupled to a mass spectrometer type SHIMADZU GCMS-QP2010 series, equipped with a split/splitless injector and a column (LxDI :30m x 0.25 mm) apolar (Stationary phase :95% dimethylpolysiloxane/5% phenyl;Thickness; 0.25µm). The carrier gas used is helium.

2.3. Antioxidant Activities of Rosemary Essential Oil

2.3.1. DPPH radical-scavenging activity

From each extract, eight different concentrations were prepared (from 10to 70 mg / ml). 0.2 ml of each extract was mixed with 1.8 ml of DPPH (4 mg / 100 ml) methanolic solution. The mixture was then incubated for 30 min in the dark at room temperature. The absorbance was measured at 517 nm for each solution with a blank solution. Ascorbic acid was used as a standard. All measurements were performed in triplicate[9] , [10]. The percentage of the DPPH radical scavenging is calculated using the equation as given below:

$$\%Radicalscavengingactivity = \frac{(\text{Abs control} - \text{Abs Sample})}{\text{Abs control}} * 100$$

2.3.2. Reducing power determination FRAP

A volume of each extract (0.5 ml) was mixed with 1.25 ml phosphate buffer (0.2 M, pH 6.6) and 1.25 ml of potassium ferricyanide [K3Fe (CN) 6] (1% w / v). The mixture was incubated at 50 °C for 20 min. After cooling at room temperature, the reaction was stopped by adding 1.25 ml of trichloroacetic acid (10% w / v). After centrifugation of the mixture at 3000 rpm for 10 min, 1.25 ml of the supernatant was mixed with 1.25 ml of double-distilled water and 0.25 ml of ferric chloride (0.1% w / v). Absorbance was measured at 700 nm for each solution with a blank solution

containing double-distilled water. Ascorbic acid was used as a reference. All the measurements were carried out in triplicate [11], [12].

2.3.3. β-Carotene Bleaching Test

A solution of β-carotene was prepared by dissolving a quantity of 2mg in 10mL of chloroform. After that, 20mg of linoleic acid and 200mg of the emulsifier Tween-80 were mixed with the β-carotene solution. After removing the chloroform at 40°C from the final solution, 100ml of distilled water was added to the flask with vigorous stirring. 1.8 mL of this emulsion has transferred into different test tubes containing the different concentrations of essential oils dissolving in methanol (10,20,30, 40, 50, 60 and 70 mg/ml). The tubes were incubated in a water bath at 50°C for 2 hours with continuous shaking. Immediately after the addition of the emulsion, the first absorbance of samples was recorded (t_0) and then after 2 hours, both at 470 nm. BHA was utilized as a standard. All measures were performed in triplicate[13]. The inhibition of the lineolate/β-carotene radical was calculated using the following formula:

$$\textbf{Bleaching inhibition (\%)} = 100 - \left[\left(\frac{(\text{initiaial } (\beta-\text{carotene})\ (t_0) - (\beta-\text{carotene}) \text{ after 2 h})}{\text{initiaial } (\beta-\text{carotene})\ (t_0)}\right) \times 100\right]$$

2.4. DFT calculations

The electronic properties of the α-pinene, Camphor, β-pinene, Cineole and Caryophyllene compounds were investigated by the quantum chemical tools to predict the stability and the possible reactivity tendency of these compounds [7]. Full geometry optimization of the studied molecules wasper formed using DFT based on Beck's three parameter exchange functional and Lee–Yang–Parr nonlocal correlation functional (B3LYP) levels of theory using the Gaussian 09W software. The calculation was made utilizing the 6-31G (d, p) basis sets for all atoms [14],[15],[16].

2.5. Molecular docking study

The docking protocol used for this study used Cyclodextrin varieties as the target whichonly partial charge added using MMFF94 force fieldand 10/10 poses

The three-dimensional structure of cyclodextrin varieties are: α-cyclodextrin (ACD) retrieved from the structure of Suse With AlphaCyclodextrin (PDB: 4FEM[17], www.rscb.com), β-cyclodextrin (BCD) from the structure of cyclodextrin Glycosyltransferase Complexed with Its Main Product Beta-Cyclodextrin (PDB: 3CGT[18], www.rscb.com) and γ-cyclodextrin (GCD) from the structure of Michaelis complex of bacillus circulans strain 251 cyclodextrin glycosyltransferase with gamma-cyclodextrin (PDB: 1D3C[19], www.rscb.com)[15].

3. Results and discussion

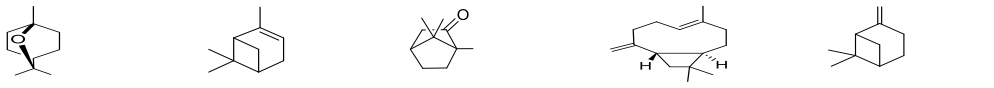
3.1. Analysis of essential oil by gas chromatography

Table 1 represent the chemical composition identified in the EO of *Rosmarinus officinalis* collected from different locations in eastern region of Morocco extracted by steam distillation in two cooperations located in Taourirt and Jerada. GC analysis of the essential oil of *Rosmarinus officinalis* from Taourirt and Jerada allowed the identification of 20 and 21 constituents respectively, of which the majority compounds in rosemary of Taourirt were1.8-cineole, followed by Alpha-Pinene, then Camphor, and caryophyllene, and Beta-Pinene which represent respectively a rate of 41.77%, 10.75%, 9.32% 6.62 % and 6.23%, and in Jerada represent as follow : 1,8-cineole(51.68%), Alpha-pinene (11.91%), Comphor (10.29%), Beta-pinene (4.98%), Caryophyllene (2.69%) as majority compounds.

Table 1. The main compounds of EO from *Rosmarinus officinalis*

N	Nom	Tr(min)	% TAOURIRT	% JERADA
1	Tricyclene	4.825	0.15	0.12
2	Alpha. –Thujene	4.883	0.31	0.12
3	Alpha. -Pinene	5.000	10.75	11.91
4	Camphene	5.242	4.02	3.32
5	Beta. -Pinene	5.675	6.23	4.98
6	Beta. -Myrcene	5.833	1.03	1.11
7	Alpha.-Phellandrene	6.083	-	0.24
8	(+)-4-Carene	6.275	0.65	0.61
9	m-Cymene	6.400	1.20	1.47
10	D-Limonene	6.467	4.60	4.31
11	Cineole	6.525	41.77	51.68
12	Mentha-1,4-diene	6.933	1.12	0.89
13	2-Carene	7.400	-	0.32
14	Linalool	7.533	1.19	0.90
15	Camphor	8.325	9.32	10.29
16	Borneol	8.633	4.54	2.02
17	Carvomenthenol	8.792	0.82	0.70
18	Alpha.-Terpineol	8.975	3.07	1.53
19	Bornyl acetate	10.350	1.33	-
20	Copaene	11.625	-	0.41
21	Caryophyllene	12.242	6.62	2.69
22	Alpha.-Caryophyllene	12.675	0.80	-
23	Cadina-1(10),4-diene	13.492	-	0.36
24	Caryophyllene oxide	14.308	0.49	-

These five major constituents (Figure 1) comprised more than 74.69% of the total weight of the oil in rosmary of Taourirt and 81.55% in Rosmary of Jerada, and all of the remaining individual constituents in Taourirt comprised less than 25.5% of the concentration, with a total of 100% of the constituents identified, and in Jerada the individual constituents comprised 18.43% of the concentration, with a total of 99.98% of all constituents.



1.8-Cineole Alpha - Pinene Camphor Caryophyllene Beta -Pinene

Figure 1. The main compounds of EO from Rosmarinus officinalis.

A survey of the literature reveals that our results are comparable with some research findings but different from others. M. Elyemni[20] mentioned that rosemary from the region of Fez showed that the major components were 1,8-cineole (32.18%), Alpha-pinene (15.4%), Camphor (16.20%), Beta-pinene (3.72%), Caryophyllene (0.27%).

The present study revealed that rosemary from Morocco includes 1.8-cineole and Alpha-pinene chemotypes

The main component of the Tunisian, Turkish, Moroccan and Italian oils is 1.8-cineole with usually over 40%, whereas most Spanish, French and Greek oils have 1.8-cineole, α-pinene and camphor with approximately equal ratios (20-30%) The myrcene-rich rosemary oil chemotype has been reported in South America [21].

3.2. Antioxidant Activities of Rosemary Essential Oil

In vitro antioxidant activity was assessed in three different ways: the DPPH test, the β-carotene bleaching technique, and the reduction power measurement.

3.2.1. DPPH method

This method is based on the reduction of alcoholic DPPH solution in the presence of hydrogen donating antioxidant (AH) due to the formation of non-radical form DPPH-H by the reaction DPPH + AH → DPPH- H + A. The remaining DPPH measured after a certain time, corresponds inversely to the radical scavenging activity of the antioxidant. The sensitivity of the method is determined by the strong absorption of DPPH [22].

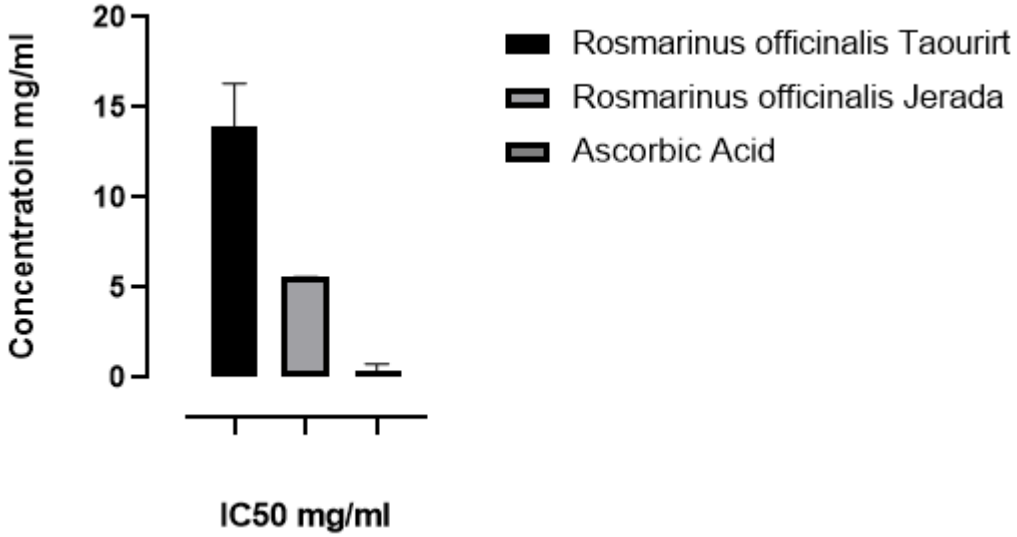


Figure 2. IC_{50} of different essential oils and ascorbic acid by DPPH

The results showed that antioxidant activity increases with increasing concentration of essential oil, and the half minimal inhibitory concentrations at 50% (IC50) of the Free radical scavenger activity of the essential oils of rosemary from Taourirt and Jerrada are respectively, IC50=13.94 ±2,37mg/ml, IC50= 5.58 ± 0.011 mg/ml, as well as that ascorbic acid is IC50=0.02165 ± 0.00074 mg/ml (Figure 2) (Note that the lower the IC50 values, the stronger the antioxidant activity).

3.2.2. FRAP

The reducing capability of the essential oil of rosemary was measured by the transformation of Fe^{3+} to Fe^{2+} in the presence of the extract. Increased absorbance of the reaction mixture indicates increased reducing power[23].

The results show that the reducing power increased with the increase of concentration of the essential oil, it is reported that the extract reaches an optical density of 0.5 at a concentration of 488.38 mg / ml for essential oil of Taourirt, and 212.75 mg/ml for essential oil of Jerada , while ascorbic acid reaches this value at a concentration of 0.06277 mg / ml (Figure 3).

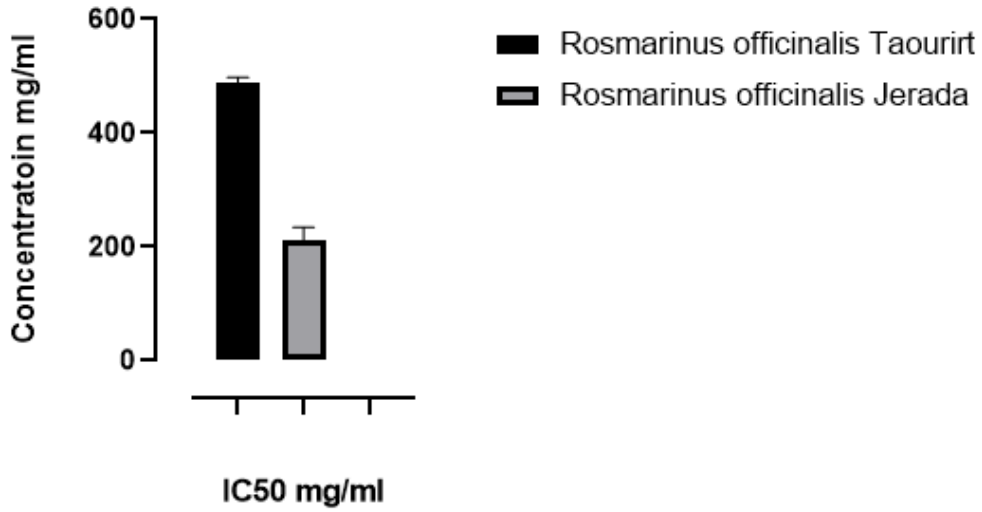


Figure 3. The IC_{50} of the different extracts for FRAP method.

3.2.3. β-Carotene Bleaching Test

It consists in measuring, at 470 nm, the discoloration of β-carotene resulting from its oxidation by the decomposition products of linoleic acid. The addition of antioxidants in plant form induces a delay in the discoloration kinetics of β-carotene [24].

The result shows the evolution of antioxidant activity of essential oil of rosemary and BHA as reference according to different concentrations, and it shows that % of inhibition increased with the increase of concentration of the essential oil. The half-minimal inhibitory concentrations at 50% (IC_{50}) of the anti-discoloration of β-carotene of essential oils of Taourirt and Jerrada are respectively IC_{50}= 27.42 ±8.36 mg/ml, 12.90 ±1.27 mg/ml,and BHA is IC_{50}=0.00225 ± 0.00030mg/ml (Figure 4).

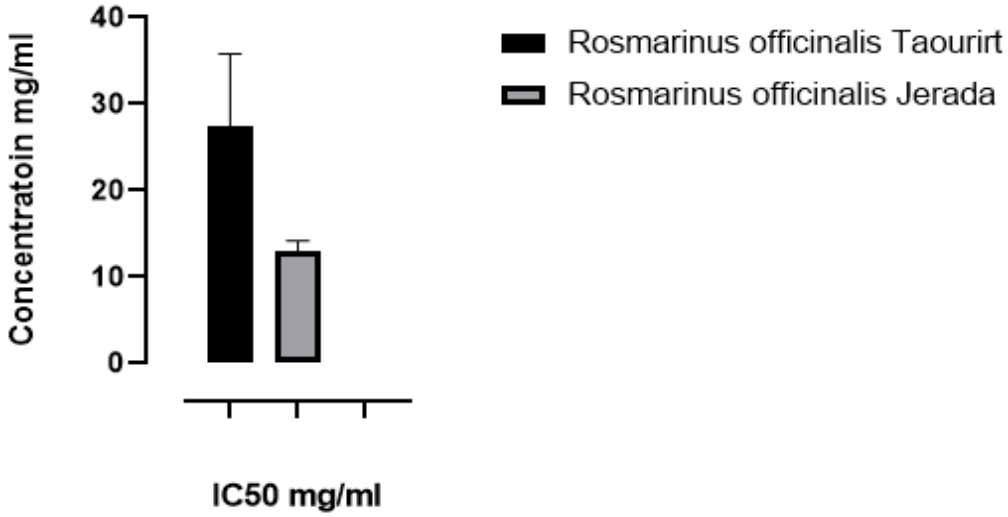


Figure 4. IC_{50} of different essential oils by β-carotene discoloration

Antioxidant activities of essential oils from aromatic plants are mainly attributed to the active compounds present in them. This can not only be due to the high percentage of main constituents, but also to the presence of other constituents in small quantities or to synergy among them [25]. IC50 values generally vary considerably among studies, which can be explained by different chemical compositions of rosemary essential oils. The chemotype of rosemary myrcene-rich essential oils has been recently shown to have the highest antioxidant activity [21].

3.3. Calculation DFT

In this study, full optimization of all geometrical variables of majority compounds in Rosemary's essential oils was described to estimate the quantum descriptors as shown in the Figure 5.

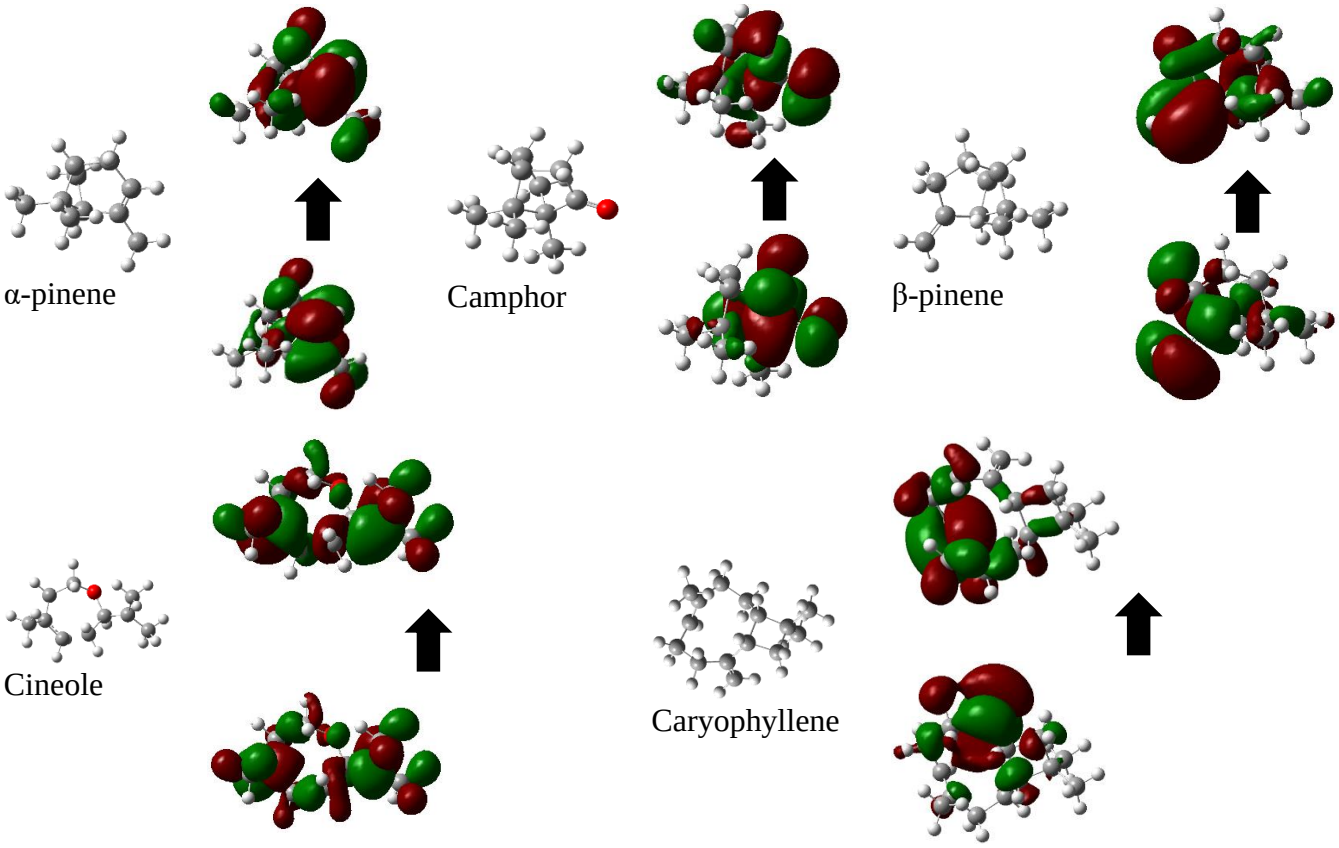


Figure 5. Optimized structure and FMO orbitals of the five compounds

Frontier molecular orbitals as HOMO (highest occupied molecular orbital) and LUMO (lowest unoccupied molecular orbital) of a molecule are very important for defining its reactivity[26][27]. The ability of the molecules to accept electrons is denoted by E_{LUMO}. The lower value of E_{LUMO}, the more probable the molecule would receive the electrons. In the same way, E_{HOMO} gives the ability to donate electrons. The higher value of E_{HOMO} would be the electron donating capacity of the molecule[15][16][27].

Table 2. HOMO, LUMO and gap energies of the main compounds of EO from *Rosmarinus officinalis*

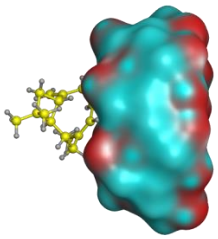
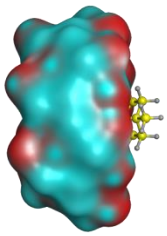
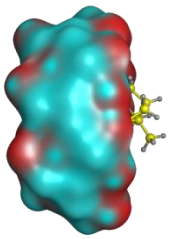
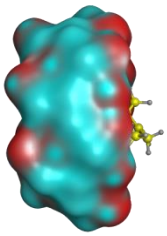
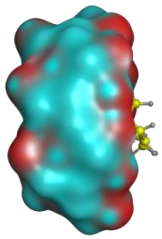
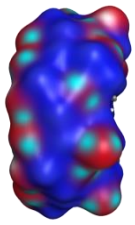
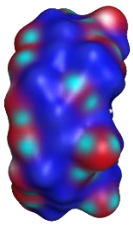
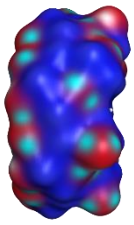
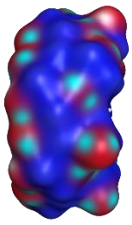
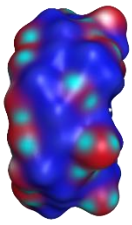
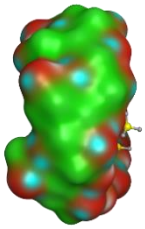
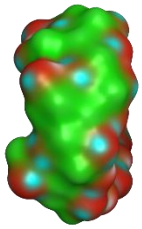
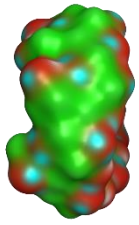
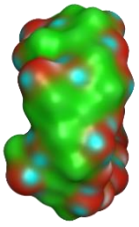
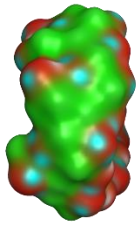
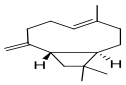
Molécule	HOMO (eV)	LUMO (eV)	ΔE_{gap} (eV) $\Delta E_{gap} = E_{LUMO} - E_{HOMO}$
α-pinene	-5.938848	0.803488	6.742336
Camphor	-6.232064	-0.227392	6.004672
β-pinene	-6.23288	0.777648	7.010528
Cineole	-3.367088	-1.9362864	1.4308016
Caryophellene	-5.924432	0.417248	6.34168

These two quantum descriptors give indications about the reactivity of our compounds. With the highest value for Cineole: E_{HOMO}= -3.367088 eV and the lowest value of E_{LUMO}= -1.9362864 eV, it is in the same time donor and acceptor of electrons.
The gap of energy is an important parameter that gives indication about the reactivity and the stability of the studied molecules. It was calculated using the equation:
$\Delta E_{gap} = E_{LUMO} - E_{HOMO}$
From the results represented in the table 2, Cineole has the lowest value of gap energy Egap= 1.4308016 eV, so it is the most reactive and the lowest stable compound compared to other molecules.
The three quantum descriptors (E_{HOMO}, E_{LUMO}, Egap) can gives also information about the biological activity of molecules[7], [28], [29], [30],[31].

3.4. *Molecular docking study*

Cyclodextrins (CDs) are natural compounds produced from starch by enzymatic degradation with three native varieties ACD,BCD and GCD, molecular docking was done for the five majority compounds with those three varieties as shown in Table

Table 3. 3D binding modes and binding affinities ofthe main compounds in Rosmarinus EO

ACD($\Delta G_{binding}$ in Kcal/mol)	 -3.2176	 -4.8068	 -4.3658	 -4.5549	 -4.5507
BCD($\Delta G_{binding}$ in Kcal/mol)	 -3.4777	 -4.9423	 -4.6891	 -4.5128	 -4.5670
GCD($\Delta G_{binding}$ in Kcal/mol)	 -5.0443	 -4.4218	 -4.4211	 -4.2686	 -4.2920
Compound	 Caryophyllene	 1.8-Cineole	 Camphor	 Beta -Pinene	 Alpha -Pinene

From all the compounds, only ACD doesn't cover Caryophyllene, others bind with cyclodextrin and totally cover the molecule in it molecular surface which makes the complex ligand/CD theoretically stable in neutral environment (pH=7), but in term of binding affinities only BCD have higher values with the majority of the compounds due its cavity size of 11.01 A° which

makes the complex compound/BCD prospective nano-antioxidants. In summary Rosmary is healthy plant and it has lot of nutritionals benefits. This study is a part of many others works on the valorizationof natural product since 2006 [32–58]. The Chemical composition of Romary oil depends on the climate as well as the water stress. Chetouani et al. showed recently that a decrease in the essential oil yield of Rosmarinus officinalis as a function of the increase in the intensity of water stress at the juvenile and adult stages, with significant increases in camphor and 1,8-cineole [59].

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Conclusion

The extraction of essential oils from rosemary carried out by steam. GPC/MS analysis showed these EOs is predominantly composed of Cineole alpha-Pinene and camphor We compare the three methods we see that the DPPH method is the most efficient because it has the small value of the IC_{50} for tow EOs (Taourirt and Jerada) and rosemary of Jerada more antioxidant then of Taourirt.

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