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Original Research Article

Climatological effects on Chemical Composition, Biological Activity of Essential oil from *Eucalyptus globulus* Leaves and Seeds

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ABSTRACT

In the last decade, there has been interest worldwide concern about the antibacterial and antioxidant properties of plants. This work aims to study the antibacterial, antioxidant activities and chemical composition of Eucalyptus globulus essential oil leaves and seeds. Essential oil was extracted from *Eucalyptus globulus* seeds and leaves by hydrodistillation giving yields of 1.8% and 0.8% respectively, Chemical composition analysis by gas chromatography coupled with mass spectrometry (GC-MS) revealed the presence of a total of 33 compounds in seeds and 28 compounds in leaves. Antibacterial activity showed that essential oils was active against all tested bacterial strains. Antioxidant propriety measured by 2,2-diphenyl-1-picrylhydrazyl (DPPH) showed that essential oils obtained from leaves have higher antioxidant activity when compared to essential oils of seeds. However, the total antioxidant capacity revealed that seeds (22,68 mg EAA/g MS) have high activity with respect to leaves (5,44 mg EAA/g MS). These results confirmed the richness of the two essential oils in phenolic compounds responsible for their biological activity. A comparative bioclimatic study was carried out in order to investigate the effect of climate on chemical composition of *Eucalyptus globulus* leaves and seeds in Morocco. Factorial component analysis between chemical composition of essential oils from different countries and climatic parameters revealed that 1.8 cineole is strongly correlated with minimal (m) and maximal temperature values (M) and Emberger quotient (Q). Whereas, essential oils extracted from seeds is not affected by these parameters.

Keywords: *Eucalyptus globulus*, essential oil, climate, antibacterial activity, antioxidant activity.

Introduction

The use of aromatic and medicinal plants in different fields (pharmaceutical, perfumery, food and cosmetics, etc.) is mainly due to their therapeutic, organoleptic and fragrant properties.[1]

Thus, these plants are rich sources of potentially bioactive compounds such as flavonoids, polyphenols, phenols, alkaloids, tannins, carotenoids and essential oils, which can be found in different parts of plants including fruits, flowers, leaves and seeds. [2]

In most plants, secondary metabolisms play a key role in biotic and abiotic interactions, including mechanisms defense against pathogens and pests.[3] Many studies focused on adding essential oils from aromatic and medicinal plants to foodstuffs to achieve an antimicrobial and antioxidant effects.[4] Indeed, essential oils have shown particular interest due to their biological and free radical scavenging properties which are associated with several pathologies such as cancer, diabetes and neurodegenerative diseases.[5]

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Another public health concern is the emergence antibiotic resistance linked to their intense and irrational use.[6] This has led to strong consumer demand for biological solutions against pathogens and has prompted researchers to look for other alternatives.[7] The antibacterial, antiviral, antifungal, antioxidant, antiparasitic and insecticidal effects of essential oils has been reported.[8] Subsequently, may be a promising solution to reduce bacterial resistance.[9]

Eucalyptus globulus is a plant of Myrtaceae family is abundant in the North-West zone of the country.[10,11] It is well known for its bioactive compounds content such phenolic compounds, flavonoids and hydrolysable tannins.[12] Due to its various biological activities, *E. globulus* essential oils are used in medicine, perfumery and pharmaceutical industry for its virtues on respiratory tract, where it facilitates bronchial mucus dissolution and elimination, anti-infective vis to bacteria and viruses.[13] In addition, an in vitro antibacterial and antioxidant effect has been reported by numerous studies.[14]

Therefore, this work targets to analyze chemical composition of essential oils *eucalyptus* extracted by hydrodistillation from seeds and leaves by using gas chromatography coupled with mass spectrometry, to investigate some biological activities, namely antioxidant activity and the antibacterial against some pathogenic bacterial strains.

Materials and Methods

Plant material

Leaves and seeds of *E. globulus L.* were harvested in the Nord-West zone (Morocco) during February 2020. The sample was identified by Dr. Chaimae Rais in The National Agency for Medicinal and Aromatic Plants, Taounate, Morocco (Document no. 059/2020).

Plant was taken to laboratory, washed with distilled water, dried in dark, and ground to obtain a fine powder with a blender (<0,1mm).[15] Obtained powders were stored at 4°C in a dry place until analysis and use.

Essential oils extraction
Essential oils extraction from *E. globulus* seeds and leaves powders was carried out by hydro-distillation using a Clevenger-type apparatus. Powder was put in contact with distilled water, and whole was boiled at 100°C for 3h according to French standard.[16]

Chromatographic analysis
Chemical analysis of essential oils isolated from seeds and leaves was carried out by gas chromatography (Trace GC Ultra-Palarisq type) coupled to mass spectrometry (CPG/SM) at the Regional University Center of Interfaces (CURI, USMBA University) according the method of Adams *et al*.[17] Analysis was carried out after injection of 1µl of the extract into carrier gas (helium He). Programming of initial injection temperature column is 40ºC for 2 min, then rises in steps of 5 ºC/min to 280 ºC for 10 min. Mass spectra are recorded by a SCAN 50-650 type detector and a NIST type spectral library. A computer software menu controls a device. Volatile compounds were identified by their mass spectra and their relative retention index (IR) calculated from separated compounds and linear alkane's retention time.

Antibacterial activity
Antibacterial activity of essential oils extracted from *E. globulus* was tested against three bacteria isolated from altered food: Gram-positive bacteria (*Bacillus subtilis* ATTC 14579, *Staphylococcus aureus* ATTC 25923) and a gram-negative bacteria (*Escherichia coli* ATTC 25922). These microorganisms were maintained by subculturing on nutrient agar favorable to their growth. Due non-miscibility with water and therefore with culture media, they were dispersed by agar-agar (0.2%).[18] Each dilution and inoculum Quantities (10-5 CFU/ml) were added to test tubes, then incubated for 6h at 37°C and poured into petri dish containing already solidified nutrient agar and finally incubated for 24h at 37°C. Controls, consisting of liquid culture medium containing 0.2% agar plus strain tested pre-culture. Minimal inhibitory concentration was defined by observing lowest concentration of essential oil at which growth inhibition was observed.

Antioxidant activity
Samples antioxidant activity determination was tested with two methods, namely total antioxidant capacity (CAT) and DPPH antiradical capacity. CAT method was evaluated according to method reported by Nagavani *et al*,[19] using ammonium phosphomolybdate as reagent. Obtained results were expressed in equivalent mg of ascorbic acid per gram of dry matter (mg EAA/g). DPPH method was used to assess according to method described by Warsi *et al*.[20] The same procedure was repeated with ascorbic acid as positive controls. Antiradical capacity was estimated according to following equation:

$$\text{Inihibition (\%)} = \frac{Abs\ control - Abs\ sample}{Abs\ control} \times 100$$

Abs control: Absorbance (620nm) at 0 min
Abs sample: Absorbance (620nm) of sample after 6h of incubation

Climatological study
To investigate climatic effect on *E. globulus* plantations at nine sites (stations in the world), the Emberger quotient was calculated according to the following formula.[21]

$$Q = \frac{2000.P}{(M+m+546{,}4).(M-m)}$$

P: Annual precipitation in mm/m²/year
M: Maximum temperature of the hottest month in °C
m: Minimum temperature of the coldest month in °C
this comparative study was conducted to determine the correlation between climatic parameters (m, M and Q) and essential oils composition (Table 1).
To evaluate the correlations between Moroccan eucalyptus globulus leaves and seeds essential oils chemical composition compared with the eight countries (Brazil, Tunisia, India, Morocco, Algeria, Montenegro, Portugal and Tanzania), we performed a component factor analysis (FCA).

Statistical analysis
All experiments were done at least three times, and results are shown as mean ± standard deviation. Statistical significance of experimental results (significance level of $P < .05$) was calculated using ANOVA analysis. A numerical tool for performing this statistical analysis is the Excel 2013 software. Software Inc). Factor analysis (FCA) was performed using the statistical analysis software for the Past.

Table 1: Climatic parameters of nine countries studied

	Algeria	Brazil	Tunisia	Portugal	Montenegro	India	Tanzania	Morocco	Germany
m[a] (°C)	9	11,6	15	9	13	14	17	9	2
M[b] (°C)	27	24,3	32	22	31	33	22	28	20
P[c] (mm/m²)	643,5	131	309	1065	1209	720	1365	552	943

[b]M: Maximum temperature of the warmest month in °C ; [a]m: Minimum temperature of the coldest month in °C ; [c]P: Annual precipitation in mm/m²/year.

Results and Discussion

Essential oils chemical composition analysis
Essential oils, obtained from *E. globulus* leaves and seeds by a hydro-distiller, were light yellow in color with a yield of 1.8% for seeds and 0.8% for leaves. Essential oils obtained from seeds is slightly higher than the value (1.57%) reported by Belkhodja *et al*.[22] For leaves, this value is lower than the value (1.2%) reported by Mohebodini *et al*.[23] Essential oils yield produced by plants, as secondary metabolites, is influenced by several biotic, abiotic and anthropogenic factors, such as temperature, maturity degree, harvesting time, extraction method, interaction with environment (climate and soil), and geographical origin.[24,25]
Chromatographic analysis essential oils of *E. globulus* leaves and seeds showed the presence of 25 compounds in leaves which represent 92.76%, and 33 compounds in seeds with 85.16% (Table 2, Figure 1 and Figure 2). The main components of essential oils isolated from *E. globulus* leaves are: 1,8 cineole (63.81%), α-Terpineol (2.73%), trans-Pinocarveol (1.85%) and Cryptone (1.55%). While essential oils from seeds were Aromadendrene (25.39%) as the main active compound, followed by 1,8-Cineole (17.13%), Globulol (17.10%), α-Gurjunene (3.15%), allo-Aromadendrene (3.04%), α-Pinene (2.62%), α-Phellandrene (2.21%) and Isovaleraldehyde (1. 15%).
These results are in agreement with previous studies of Angello *et al*.[26] who found that 1.8 cineole was the main compound of *E. globulus* leaves with a percentage 63.81%. Chemical composition *E. globulus* differs in some countries, which represents specific climatic conditions. For example, *eucalyptus globulus* leaves from India rich in limonene (10.09%), linalool (2.34%) and γ-Terpinene (2.92%).[27] *E. globulus* leaves from Algeria are rich in spathulenol (7.44%) and α-Terpineol (5.46%),[28] and from seeds are rich in 1.8 cineole (15.55%), Aromadendrene (31.17%) and Globulol (10.69%).[29] Accordingly, differences between our results could be mainly attributed to ecological conditions and plant parts used. The answer to this hypothesis will be the subject of the last part of this work.

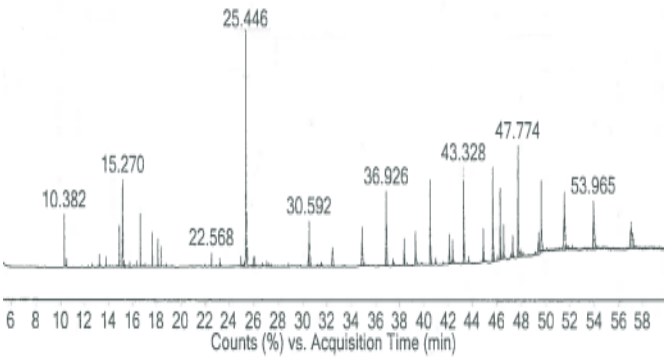

Figure 1: Chromatogram analysis (GC-MS) of the EO of *E. globulus* leaves

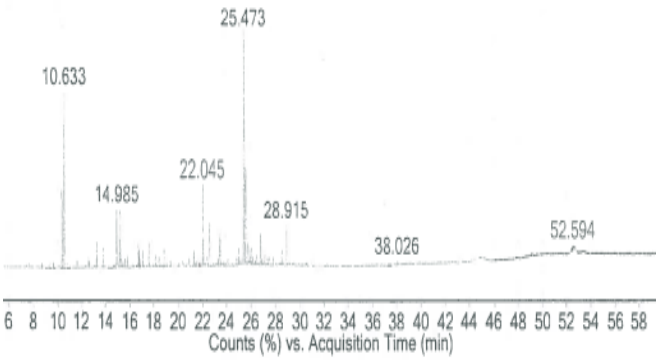

Figure 2: Chromatogram analysis (GC-MS) of the EO of *E. globulus* seeds

Antibacterial activity

Result of the antibacterial activity of essential oils extracted from *E. globulus* seeds and leaves are shown in Table 3.

According to obtained results (Table 3), *E. Globulus* seeds and leaves showed activity against strains tested. Concentrations of 8 and 10µg/mL, essential oils of seeds completely inhibited (100%) *bacillus subtilis* and *staphylococcus aureus* strains. On the other hand, essential oils extracted from leaves showed a maximum antimicrobial activity (100%) of *Bacillus subtilis* (CMI=6µg), but this inhibition is limited to 62% for *staphylococcus aureus* with a MIC greater than 10µg/ml. This finding could be justified by the difference in chemical composition.

Furthermore, *Escherichia coli* strains is shown to be resistant. Monteiro *et al*.[30] confirmed a high gram-negative bacteria resistat when compared to gram-positive bacteria. Gram-positive bacteria tend to be more sensitive to antibacterial agents due to their simple cell wall structure than Gram-negative bacteria.[31] Studies carried out on mechanism action revealed that essential oils increase bacterial membrane permeability, facilitating their entry inside bacteria, reducing their activity, and leading to cell death. Essential oil effectiveness on Gram positive bacteria was slightly higher, indicating that they are more sensitive to bioactives antibacterial effect.

Gram negative bacteria lower susceptibility may be due to presence of an outer membrane limiting the entry of hydrophobic compounds into lipopolysaccharide layer.[32] Essential oils MIC against *Staphylococcus aureus* and *bacillus subtilis* are much higher than that reported by Bey-Ould *et al*.[33] Unlike that of leaves, we obtained lower MICs equal to 9.35mg/mL against *bacillus subtilis* and *staphylococcus aureus*.

Observed antimicrobial activity may be due to active compounds constituting such as terpene phenols (1,8 cineole, linalool, borneol, etc.) which can cause rupture of plasma membrane or alter mitochondria structure.[34] Thus, it has been suggested that hydrophobic nature and chemical structure or their majority compounds may have a very important role in antimicrobial activity, they allow them to distribute among lipids of bacterial or fungal cell membrane and mitochondria, this disrupts cell structures and makes them more permeable, leading cell bacterial to death.[35]

Table 2: Composition of essential oils extracted from *E. globulus* leaves and seeds

Compounds	Leaves	Seeds
Isovaleraldehyde	5,02	1,15
2-pentanone-4-hydroxy-4- methyl	0,845	0,46
α –Pinene	8,305	2,62
β -Pinene	0,035	0,00
β-myrcène	0	0,05
α –Phellandrene	0	2,21
α –Terpinene	0	0,05
p –Cymene	0,5	0,2
o-Cymene	0	0,2
Limonene	1,15	0,10
1,8-Cineole	54,495	17,13
β –Ocimene	0,915	0
γ-Terpinene	0	0,14
Linalool	0,05	0
Trans Pinocarveol	1,85	0
cis-Verbenol	0,35	0
trans-dihydro-α-Terpineol	1,65	0
Pinocarvone	0,9	0
δ-Terpineol	0,35	0
Borneol	0	0,16

Terpinen-4-ol	0	0,89
p-Cymen-8-ol	0,225	0
Cryptone	1,55	0
α-Terpineol	2,73	0,2
Neral	0,315	0
Piperitone	0,125	0
Carvenone	0	0,1
p-menth-1-en-7-al	0,05	0
α-Terpinyl acetate	0	0,59
Isoledene	0	0,36
α-Gurjunene	0	3,15
β-Gurjunene	0	0,15
Aromadendrene	1,86	25,39
Allo-Aromadendrene	0,02	3,04
γ-Gurjunene	0	0,55
β –Selinene	0	0,05
α-Selinene	0	0,37
δ-Cadinene	0	0,62
Spathulenol	3,72	0
Caryophyllene oxide	0,83	0
Globulol	3,83	17,10
Viridiflorol	0,5	0,07
Guaiol	0	0,35
Ledol	0,1	5,74
γ-Eudesmol	0	0,57
β-Eudesmol	0	0,11
α-Eudesmol	0,49	1,09
α-Cadinol	0	0,2

Antioxidant activity

Anti-radical capacity

Results of seeds and leaves essential oils antioxidant activity evaluated by DPPH method are shown in figure 3. Inhibition percentages are revealed by an appearance of yellow color.[36]

At first glance, it can be seen that ascorbic acid presented maximum antioxidant activity at low doses (0 to 0.5 mg/ml) compared to that of two essential oils studied. Beyond concentration of 0.5mg/ml, we find that seed essential oil antioxidant activity remains stable but a gradual increase in essential oil antioxidant activity extracted from leaves. It is deduced that leaves essential oil antioxidant activity has a higher activity than those of seeds at high concentration (Figure 3). Antioxidant activity stabilization of extracted from seeds from 0.5 to 2 mg/ml may be due to existence of pro-oxidants in these extracts (seeds). According to Saleh *et al*,[37] low inhibition rate of DPPH radical could be attributed to monocarbon compounds inefficiency. Following monoterpenes: α pinene, β pinene, limonene, β myrcene, sabinene and terpinolene have antioxidant properties. A strong antioxidant activity of essential oils is attributed to their phenolic groups such as thymol, carvacrol and probably to 1,8 cineole.[38]

Total antioxidant capacity (CAT)

To verify the above-mentioned observations, we use another technique for evaluating antioxidant activity. This is total antioxidant activity (CAT) that affects polar and non-polar molecules (Figure 4).

Figure 4 illustrates total antioxidant capacity (CAT) essential oils of *E. globulus* plant extracted from leaves and seeds. Seeds Antioxidant power is 22.68 mg EAA/g DM which is a much higher value than that obtained from essential oils leaves (5.44 mg EAA/g DM). This can be explained by difference in composition between two essential oils because seeds are rich in sesquiterpene which is (34.27%) and (1.88%) for leaves. Same as the presence of hydrophobic molecules in essential oils from seeds. These results are in agreement with Bey-Ould *et al*.[33] According to Jayaprakash *et al*.[39] ammonium phosphomolybdate reducing power depends on phenolic compounds content, position as well as number of hydroxyl groups. Multiple studies have shown that antioxidant activity of an essential oil is influenced by many parameters, such as growth and development phase of plant, harvest season and environment, as well as major compounds and structures phenolics presence.[40]

Climatological analysis of essential oils variability

Table 4 illustrate climatic parameters of various studied countries. Maximum temperatures (M in °C), minimum temperatures (m in °C) and precipitation (mm) are measured to calculate Emberger quotient (Q). The latter makes it possible to determine bioclimatic stage where vegetation is grown.

Climatic parameters analysis (Table 4) shows that different studied sites are characterized by various thermal and water profiles. India locations, Tunisia and Montenegro have highest minimum and maximum temperatures (33, 32 and 31°C, respectively). In precipitation terms, Tanzania, Montenegro and Portugal are highest. These parameters reveal a difference between studied sites. Emberger quotient expresses relationship between amount of precipitation and temperature (warmest

and weakest temperature month). Our results show that Moroccan eucalyptus belongs to sub-humid bioclimatic stage. Climatic analysis carried out makes it possible to characterize plant studied and to identify it according to phenolic compounds and their biological properties. It can be deduced that *E. globulus* is largely characterized by a secondary metabolism associated with climatic properties. Figure 5 illustrates studied sites (country) location in Emberger diagram according to formula developed by Mokhtari *et al*.[21]

According to Figure 5, climatic parameters could show that Brazil locations, Tunisia, India, Morocco, Algeria, Montenegro, Portugal and Tanzania in Emberger diagram. Germany, which had lowest minimum temperature (2°C), is in humid stage. Thus, Portugal, Tanzania and Montenegro are benchmarks in same Stage with higher minimum temperatures. Brazil and Tunisia are in arid stage where temperature of coldest month sets them apart. For Morocco and Algeria, we notice that minimum temperature is comparable and located in Sub-Humid stage. India is characterized by a hot climate and is located in Sub-humid stage. Bioclimatic indices have a strong influence on essential oils chemical composition from provenances studied. This could mean that essential oil from *E. globulus* leaves depends on climatic conditions. On other hand, seeds essential oil is not influenced by these conditions. Altitudinal gradient is associated with variation of an environmental factors set, such as air temperature, precipitation, sunlight intensity, UV radiation and air humidity. Climatic conditions at high altitude, mainly low average temperatures and high light intensity, make it possible to modify morphology, physiology and productivity of plants in order to protect them and adapt them to stress conditions.[41] In our study, a significant difference was observed in yield of essential oils and in chemical composition of plants.[42]

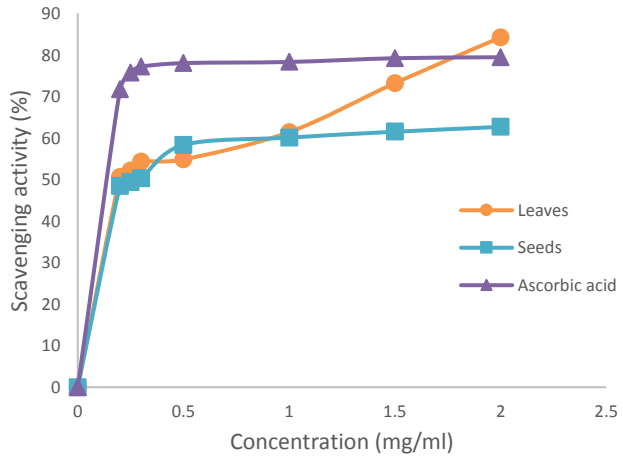

Figure 3: Scavenging activity of essential oils extracted from *eucalyptus globulus* leaves and seeds and ascorbic acid used as control

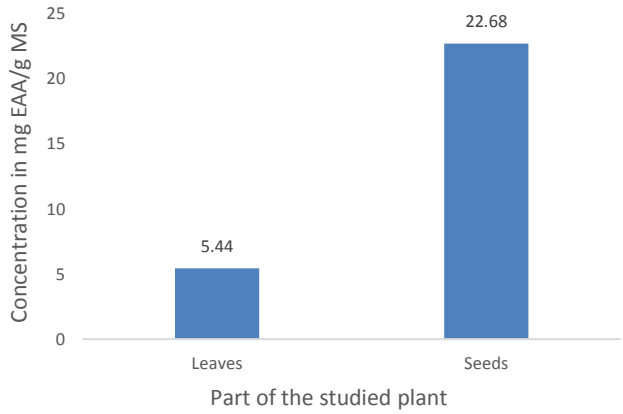

Figure 4: Total antioxidant capacity of essential oils from *eucalyptus globulus* leaves and seeds

Factorial Correspondence Analysis (FCA)

Figure 6 illustrates components factor analysis (FCA) of the essential oils chemical composition of *Eucalyptus globulus* leaves and seeds from nine countries and climate parameters.

This analysis shows that the first axis summarizes climatic parameters and chemical compounds (component 1) while the second axis summarizes essential oils origin (component 2). Figure 6 shows that chemical compound of essential oil extracted from seeds contributes to positive part of component 1 and the negative of component 2. Essential oil of leaves from Algeria, Brazil, Tunisia, Morocco, and Montenegro contributes positively in component 1 except India, which contributes negatively. while Tanzania contributes to positive part of Component 2 and the negative of Component 1. Montenegro and Portugal were positively correlated with Terpinyl acetate <α-> (Ter yl acet <α->). while Algeria, Brazil, Tunisia and Morocco were correlated with 1.8 cineole, Globulol, Limonene, and Pinene <α->. Tanzania is correlated with Cineole <1,4-> and Pinene <β->.

Chemical composition of leaves from different countries is strongly correlated with minimum temperature, maximum temperature and Emberger quotient. On other hand, seeds essential oil is not affected by these parameters. In addition, we observe a significant correlation of 1.8 cineole to minimum (m) and maximum (M) temperature. On other hand, we find that minimum and maximum temperatures are strongly correlated in Brazil, Montenegro and Portugal. Where in addition, essential oils of the latter are more correlated with 1,8 cineole synthesis which varies according to climate. According to Li *et al*,[43] 1,8 cineole synthesis increases with increasing temperature between 20 and 46°C. According to Sonigra *et al*,[44] species found in warm climates such as tropical and Mediterranean countries possess essential oils. Morshedloo *et al*.[45] demonstrated that several factors affect plant secondary metabolites such as environmental conditions, harvest season and/or plant age. Secondary metabolites biosynthesis is also correlated with elevated temperature in plants.[43] However, *E. globulus* secondary metabolism was found to be more sensitive to climatic variability. Indeed, a hot and sunny dry climate favors essential oil formation.[46] Results obtained from our study show that *eucalyptus globulus* essential oils chemical compositions are strongly influenced by climatic factors. The oils were obtained from in countries with different climates, each of which has its own rainfall and edaphic characteristics.[46]

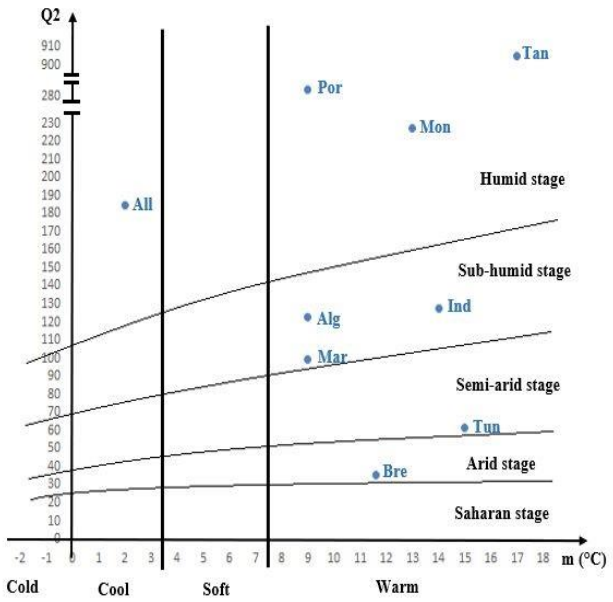

Figure 5: Emberger diagram of bioclimatic stages of nine countries

Alg: Algeria ; Bre: Brazil ; Tun: Tunisia ; Por: Portugal ; Mon: Montenegro ; Ind: India ; Tan: Tanzania ; Mar: Morocco ; All: Germany

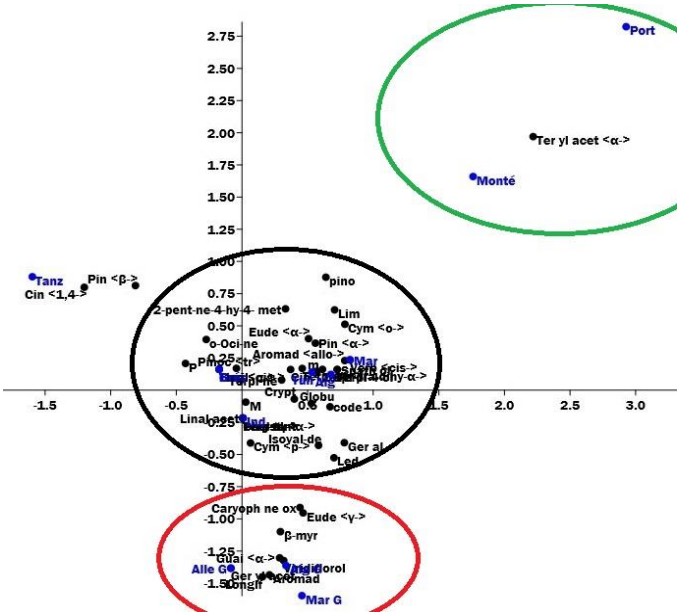

Figure 6: Factorial component analysis (AFC) of *Eucalyptus globulus* essential oil composition associated with climatic parameters

Conclusion

This study permitted us on the first hand to identify chemical compounds in E. globulus leaves and seeds of and on the other hand to illustrate the antibacterial potential of these essential oils since gram-positive bacteria showed a remarkable activity while gram-negative bacteria were resistant. Moreover, we have shown that E. globulus EO has a very important antioxidant effect and can be applied against some diseases that generate free radicals. These activities may be due to their major contents, namely α- pinene, 1,8- cineole, Aromadendrene and Globulol.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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Table 3: Inhibition percentage of bacteria caused by essential oils extracted from *eucalyptus globulus* leaves and seeds

	Seeds (%I)			Leaves (% I)		
C (µg/mL)	***E. coli***	***B. subtilus***	***S. aureus***	***E. coli***	***B. subtilus***	***S. aureus***
0	0	0 ± 0,00	0 ± 0,00	0	0 ± 0,00	0 ± 0,00
2	0	0 ± 0,00	92 ± 1	0	0 ± 0,00	0 ± 0,00
4	0	8 ± 1	98 ± 1	0	97 ± 1	0 ± 0,00
6	0	36 ± 1	99 ±	0	100 ± 0,00	34 ± 1,73
8	0	60 ± 2	99,66 ± 0,57	0	100 ± 0,00	45 ± 2
10	0	100 ± 0,00	100 ± 0,00	0	100 ± 0,00	62 ± 1
12	0	100 ± 0,00	100 ± 0,00	0	100 ± 0,00	78 ± 1
14	0	100 ± 0,00	100 ± 0,00	0	100 ± 0,00	100 ± 0,00

[a]C : Concentration ; %I : Inhibition percentage
Mean ± SD in the same column with different superscript letters differs significantly (P < .05).

Table 4: Emberger quotient of different countries studied

	Algeria	**Brazil**	**Tunisia**	**Portugal**	**Montenegro**	**India**	**Tanzania**	**Morocco**	**Germany**
Q[a]	122,77	35,43	61,26	283,77	227,53	127,72	904,87	99,60	184,33

[a]Q: Emberger quotient

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