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Leaf essential oils chemical composition, antibacterial and antioxidant activities of *Eucalyptus camaldulensis* and *E. rudis* from korbous (Tunisia)

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Abstract
The essential oils of the two Eucalyptus species (Namely: *Eucalyptus camaldulensis* and *Eucalyptus rudis*) were obtained by hydrodistillation, and their analyses were performed by GC and GC/MS. A total of 26 different compounds were identified. Spathulenol, 1, 8-Cineole, and ρ-Cymene were the main components. The most active antibacterial essential oil is *E. camaldulensis*. The minimum bactericidal concentrations (MBC) value 0.5% (v/v) is obtained for two oils against *Staphyloccocus aureus*. The antioxidant activity is evaluated by mean of free radical scavenging assay using DPPH. IC50=342±2.5 µg/ml for *E. Camaldulensis* is obtained.

Keywords: *E. camaldulensis*; *E. rudis*; essential oils; chemical composition; antibacterial activity; antioxidant activity.

1. Introduction
Eucalyptus is one of the world's most important and most widely planted genera [1]. It belongs to the Myrtaceae family mostly found in tropical regions. Some plants of this family have medicinal value [2]. Essential oils from Eucalyptus species are also widely used in modern cosmetics, food, and pharmaceutical industries and as a fragrance additive [3]. In this regard, monoterpenoid components of the aromatic constituents of the oils are commercially available for the treatment of the common cold and other symptoms of respiratory infections [4, 5]. Many reports ascribed various biological activities to particular components of the eucalyptus essential oil and found that variations in oil composition were usually associated with substantial changes in activity, in particular the antibacterial [6-9], analgesics [10], anti-inflammatory [10], antifungal [11-13], antiviral [14], antioxidant [10] fumigant [15,16] and insecticidal effects [17]. The medicinal value of essential oil extracted from Eucalyptus is based largely in its 1, 8-cineole (eucalyptol) content. In Tunisia previous studies reported the chemical composition of essential oils of the two eucalyptus species *(Eucalyptus camaldulensis* and *Eucalyptus rudis*) according to regions and time of harvest [15, 18]. Beside, this study is designed to determine the chemical, the free-radical scavenging activity and the antimicrobial activity of two eucalyptus species.

2. Experimental
2.1. Chemicals
α- pinene, β-pinene, myrcene, α-terpinene, 1, 8-cineole, linalool, alkane standard solutions (C8-C24) were from Fluka Chemika.

2.2. Plant materials
E. camaldulensis and *E. rudis* (*Myrtaceae*) leaves were collected in March 2010 from Korbous (North East Tunisia). Taxonomic identification was performed by botanist from the institute of Research in Rural Engineering, Water and Forestry. A voucher specimen for each plant has been deposited in the herbarium of this institute.

2.3. Extraction of essential oil

The dried leaves of *E. camaldulensis* and *E. rudis* were subjected to hydrodistillation using a Dean –Stark apparatus for 3h.The yields were averaged over three experiments and calculated according to dry weight of the plant material. The essential oils were dried over anhydrous sodium sulphate until the last traces of water and then stored at 4°C [19].

2.4. Analysis of essential oil

Analyses were performed on a Hewlett-Packard gas chromatograph, Model 6890, equipped with a flame ionization detector. Analytical conditions: HP-5 MS 5% phenylmethylsiloxane capillary column (30m × 0.25mm, film thickness 0.25 µm); carrier gas, helium; flow rate, 1.3ml/min; split, 1:10; injector temperature, 250°C; detector temperature, 280°C. The oven temperature was held for 1 min at 35°C, then programmed from 35°C, to 300°C at 5°C/min. GC-MS analysis was carried out on a HP 6890 instrument coupled to a Hewlett-Packard 5973N MS computerized system, ionization voltage 70eV, electron multiplier 1670V, ion source temperature 230°C, GC conditions as above. Individual components were identified by comparison of their GC retention indices [20] and MS spectra with those reported in the literature [21] and by computer matching with the Wiley 238.L library and, whenever possible, by co-injection with authentic compounds. The percentages of the compounds were calculated from the GC peak areas, using the normalization method.

2.5. Free radical-scavenging activity: DPPH assay

Antioxidant activity was determined according to [22, 23], with some modification. The hydrogen atom or electron donation ability of the oil was measured from the bleaching of purple-coloured ethanol solution of DPPH. A stock solution (10 $mg.mL^{-1}$) of the essential oil was prepared in ethanol. Dilutions are made to obtain concentrations ranging from 1 to 0.0015 $mg.mL^{-1}$, 2mL of each diluted solutions were mixed with 2 mL of freshly prepared DPPH solution in ethanol (2.10^{-4} M).The mixture was shaken vigorously and then immediately placed in a UV–Vis spectrophotometer to monitor the decrease in absorbance at 517 nm. Monitoring was continued for 30 min until the reaction reached a plateau. Butylhydroxutoluene BHT, a stable antioxidant, was used as a synthetic reference. The radical-scavenging activities of samples expressed as percentage inhibition of DPPH, were calculated according to:

$IP = [(AC (0) – AA(t))/AC(0)] \times 100$

Where AC (0) is the absorbance of the control simple (t = 0h) and AA (t) is the absorbance of the tested sample at the end of reaction t = 30 min.

2.6. Antibacterial activity

Antibacterial activity was assayed against six bacteria. Gram-positive: *Staphylococcus aureus (ATCC 25923),* and *Streptococcus A (ATCC 11700).* Gram-negative bacteria: *Escherichia coli* (obtained from stock cultures of the Faculty of sciences, Tunis), *Salmonella enteritidis (ATCC 14028)*, *Klebseilla pneumoniae (ATCC 138337)* and *Pseudomonas aeruginosa (ATCC 9027).* The effects of different concentrations of the effects of different concentrations of the oil (0, 0.05, 0.075, 0.1, 0.5%, 0.75, 1.5%, 2.5%, 3. 5 %) on the tested strains were evaluated by submerged broth culture method: *Streptococcus A* in Todd-Hewitt broth, *Staphylococcus aureus* in special staphylococcus broth. The other bacteria were tested in nutriment broth. The different solutions of oils were mixed with Tween 80 at a final concentration of 0.5% (v/v) in broth medium [23, 24]. The surviving bacteria were determined by enumeration and as colony reported as forming units per ml medium (CFU/ml) after incubation for 24h at 37°C. The colonies were developed after incubation was calculated using the following formula: percentage of inhibition growth= (I-T/I) ×100, where T is CFU/ml of test sample and I is CFU/ml of Initial cell concentration. The effects were compared with that of the standards antibiotic, Ampicillin.

The minimum inhibitory concentrations (MIC) were determined by subculture (as described above) as the lowest resulting in the maintenance of, or a reduction in, the number of organisms in the inoculums. The minimum bactericidal concentrations (MBC) were therefore defined as the lowest concentration killing ≥ 99.99% of the inoculums compared with initial viable counts. The tests were repeated at least three times and modal MIC and MBC values were selected.

2.7. Statistical analysis

The yields and the antioxidant activity of the essential oils of the two eucalyptus species were expressed as the mean ± standard error of triplicate measurements. Confidence limits were set at p<5%. Analysis of variance (ANOVA) accompanies with NEWMAN-KEULS tests were conducted to identify the significant difference between the samples (p<0.05).

3. Results and discussion

3.1. Yields and Chemical composition of the essential oils

The yields of leaf essential oils from the hydrodistillation of *E. camaldulensis* and *E. rudis* were 0.73±0.017 % and 0.73±0.03% according to their dry weight, respectively. The analysis of variance (ANOVA) indicated that the oil yield were not significantly between species (p<0.05).

The results of the chemical compositions of the essential oils were reported in Table 1. A total of 26 compounds were identified from the essential oils of *E. camaldulensis*, which represented 92.82% of the oils extracted. The major compounds detected in the oil were Spathulenol (20.2%), ρ-Cymene (14.83%), 1,8-cineole (12.16%), phellandral (6.6%), Cryptone (7.02%), Globulol (6.16%) and Terpen-4-ol (5.25%). For *E. rudis*, 25 compounds representing 90.46% of the essential oils, were identified, with Spathulenol (17.47%), ρ-Cymene (20.49%), 1,8-cineole (14.61%), Cryptone (10.38%), phellandral (4.55%), and Terpen-4-ol (5.25%) being the dominant ones. The composition analysis of the two eucalyptus leaf oils revealed that monoterpenes predominated. The seasonal variation (four seasons: May, August, November and February) in chemical composition essential oils of the leaves of the two Eucalyptus species harvest from korbous arboreta (North East Tunisia) has been previously studied [15]. Our results were in a good agreement with those of [15], who reported that Spathulenol, ρ-Cymene and 1,8- cineole were the major compounds in *E. camaldulensis* essential oil leaves collected during February period. The data analysis shows that the chemical profile of our essential oils differs from those of other origins and quantitative differences of individual compounds exist [25, 26] reported high amounts of α-phellandrene, ρ-Cymene, α-pinene, 1,8-cineole, γ-terpinene in *E.camaldulensis* oils extracted from Taiwan. It would also be noteworthy to point out that the composition of any plant essential oil studied is influenced by the presence of several factors, such as local, seasonal and experimental conditions.

3.2. Determination of the antioxidant activity

The radical-scavenging activity of the essential oils of the two Eucalyptus species is shown in table 2.

The essential oil of *E. camaldulensis* is the most potent radical scavenger with an IC50 value of 342±2.5 µg/ml. The IC50 of the standard was 9.9±0.85 µg/ml for BHT. The essential oil of *E. camaldulensis* exhibited important antioxidant activity as compared to *E. rudis* essential oil. It is very difficult to attribute the antioxidant effect of this essential oil to one or a few active principles, because an essential oil always contains a mixture of different chemical compounds. In addition to the major compounds, also minor compounds may make a significant contribution to the oils activity.

3.3. Determination of the antibacterial activity

The antibacterial activity of the essential oils was examined by submerged broth culture method against six strains bacteria selected on the basis of their relevance as food contaminants. The result, presented in table 3 and table 4, reveals that the essential oils of the two Eucalyptus species had great antibacterial activity against all six bacteria, and most activity against Gram-positive ones. This activity increased with increasing concentration of the essential oil in the wells; no activity was observed at a concentration below 0.05 % (v/v).

Overall, the essential oils exhibited a considerable antibacterial activities expressed as minimum inhibitory concentration in table 5 and table 6. The essential oil of *E. rudis* displayed remarkable antibacterial effect against all two gram-positives bacteria such as *S. aureus (ATCC 25923)*and *Streptococcus A(ATCC 11700)*, and the two gram-negatives bacteria namely *K. Pneumonia (ATCC 138337)* and *S. enteritidis (ATCC 14028)*, with MIC values of 0.1, 0.075, 0.075 and 0.5% (v/v), and the effects of the essential oil on the growth of the test bacteria demonstrated the reduced viability at MICs concentration of 99.33%; 69.69%; 63.33%; and 99.58%, respectively.

Table 1: Chemical compositions of leaf essential oils from *E. camaldulensis* and *E. rudis* collected during March period.

Compounds	RI	Concentration (%) [a]		Identification[b]
		E. camaldulensis	*E. rudis*	
α-Thujene	928	0.3	0.59	RI,MS
α –Pinene	939	3.06	2.1	RI,MS,CO-GC
Sabinene	972	0.06	0.16	RI,MS
α-Phellandrene	1005	0.67	0.3	RI,MS
α-Terpinene	1018	0.22	0.16	RI,MS,CO-GC
ρ-Cymene	1026	14.83	20.49	RI,MS
1,8-Cineole	1033	12.16	14.61	RI,MS,CO-GC
δ-Terpinene	1063	1.36	0.81	RI,MS
Linalool	1099	0.35	0.75	RI,MS,CO-GC
α-Thujone	1114	0.31	0.26	RI,MS
ρ -Menth-2-en-1-ol	1121	1.88	1.60	RI,MS
Terpen-4-ol	1179	4.72	5.25	RI,MS
Cryptone	1186	7.02	10.38	RI,MS
Piperitone	1252	0.51	0.3	RI,MS
Phellandral	1270	6.0	4.55	RI,MS
Carvacrol	1302	1.5	1.12	RI,MS
β-Elemene	1381	0.1	0.17	RI,MS
α-Gurjunene	1409	0.35	0.02	RI,MS
(+)-Aromadedrene	1439	0.41	0.22	RI,MS
Allo-aromadendrene	1454	1.20	1.5	RI,MS
Ledene	1482	0.21	0.35	RI,MS
α-Muurolene	1492	0.03	0.4	RI,MS
γ-Cadinene	1521	0.09	0.18	RI,MS
(+)-Spathulenol	1576	20.2	17.47	RI,MS
Globulol	1583	6.16	-	RI,MS
Viridifloral	1588	0.8	0.26	RI,MS
Iso-Spathulenol	1642	1.05	0.51	RI,MS
Total (%)		92.82	90.46	
Monoterpene hydrocarbons		19.58	24.07	
Oxygenated monoterpenes		40.75	44.57	
Sesquiterpene hydrocarbons		2.26	2.43	
Oxygenated sesquiterpenes		28.70	18.14	

[a] *Percentages (mean of three analyses) obtained by FID peak area normalization, all relative response factors being taken as one.*
[b] *RI: Relative retention indices to C8-C24 n-alkanes on HP-5MS column, MS: mass spectrum, Co-GC: co-injection with authentic compounds.*

Table 2: Anti-oxidant activities on scavenging the DPPH free radical of Essential oils from *E. camaldulensis* and *E. rudis*

		IC_{50} (µg/ml)
Standards	BHT	9.9±0.85
Essential oils	*E. camaldulensis*	342±2.5
	E. rudis	>1000

Table 3: Final cell concentration of six bacteria (CFU /ml) after 24h growth in submerged culture at different concentrations of essential oil of *E. camaldulensis.*

Micro-organisms	* (10^7 CFU/ml)	Essential oil concentration%(v/v)									
		0	0,05	0,075	0,1	0,5	0,075	1	1,5	2,5	3,5
S. aureus	3.1	$5.5\ 10^{11}$	$1.8\ 10^{11}$	$3.2\ 10^{8}$	$5.1\ 10^{6}$	$2.8\ 10^{3}$	$1.2\ 10^{3}$	$3.9\ 10^{3}$	$1.0\ 10^{3}$	<1	<1
Streptococcus A	3.5	$1.8\ 10^{9}$	$1.1\ 10^{9}$	$6.7\ 10^{8}$	$1.5\ 10^{8}$	$1.1\ 10^{6}$	$1.1\ 10^{4}$	$1.9\ 10^{3}$	$3.3\ 10^{2}$	$3,3\ 10^{2}$	$3.0\ 10^{2}$
E. coli	3.6	$1.6\ 10^{9}$	$1.1\ 10^{9}$	$8.5\ 10^{8}$	$8.1\ 10^{8}$	$7.7\ 10^{8}$	$4.3\ 10^{8}$	$2.4\ 10^{8}$	$1.2\ 10^{7}$	<1	<1
S. enteritidis	3.6	$5.8\ 10^{9}$	$3.1\ 10^{8}$	$2.7\ 10^{8}$	$2.1\ 10^{8}$	$1.2\ 10^{8}$	$5.7\ 10^{7}$	$4.5\ 10^{7}$	$4.1\ 10^{7}$	$1.4\ 10^{7}$	$1.3\ 10^{7}$
K. pneumoniae	3	$3.9\ 10^{8}$	$3.3\ 10^{8}$	$2.4\ 10^{8}$	$1.1\ 10^{8}$	$8.4\ 10^{7}$	$4.8\ 10^{7}$	$2.8\ 10^{7}$	$1.4\ 10^{7}$	$1.4\ 10^{7}$	$1.2\ 10^{7}$
P. aeruginosa	4.3	$1.7\ 10^{9}$	$3.2\ 10^{8}$	$2.1\ 10^{8}$	$1.4\ 10^{8}$	$8.9\ 10^{7}$	$5.8\ 10^{7}$	$3.2\ 10^{7}$	$5.8\ 10^{7}$	$3.2\ 10^{7}$	$5.2\ 10^{5}$

** Initial cell concentration*

Table 4: Final cell concentration of six bacteria (CFU /ml) after 24h growth in submerged culture at different concentrations of essential oil of *E.rudis*.

Micro-organisms	*(10^7 CFU/ml)	Essential oil concentration%(v/v)									
		0	0,05	0.075	0,1	0,5	0,75	1	1,5	2,5	3,5
S. aureus	3	$5.5\ 10^{11}$	$1.3\ 10^{9}$	$7.0\ 10^{8}$	$2.0\ 10^{5}$	$5.5\ 10^{2}$	$3.3\ 10^{2}$	<1	<1	<1	<1
Streptococcus A	3.3	$7.8\ 10^{9}$	$3.4\ 10^{7}$	$1.0\ 10^{7}$	$1.7\ 10^{6}$	$1.7\ 10^{5}$	$1.8\ 10^{4}$	$1.4\ 10^{4}$	$1.4\ 10^{4}$	$1.7\ 10^{3}$	$5.2\ 10^{2}$
E .coli	3	$1.6\ 10^{9}$	$8.2\ 10^{8}$	$2.9\ 10^{8}$	$1.9\ 10^{8}$	$1.6\ 10^{8}$	$1.6\ 10^{8}$	$1.6\ 10^{7}$	$4.2\ 10^{7}$	$2.9\ 10^{7}$	$2.5\ 10^{7}$
S. enteritidis	5.3	$1.9\ 10^{12}$	$1.3\ 10^{12}$	$1.4\ 10^{11}$	$1.1\ 10^{10}$	$2.2\ 10^{5}$	$1.5\ 10^{5}$	$1.2\ 10^{5}$	$6.4\ 10^{4}$	$4.6\ 10^{4}$	$9.4\ 10^{3}$
K. pneumoniae	3	$2.6\ 10^{8}$	$5.4\ 10^{7}$	$1.1\ 10^{7}$	$1.2\ 10^{7}$	$1.1\ 10^{7}$	$1\ 10^{7}$	$1\ 10^{7}$	$2.0\ 10^{6}$	$1.8\ 10^{6}$	$4.1\ 10^{5}$
P. aeruginosa	4.4	$1.1\ 10^{10}$	$2.2\ 10^{9}$	$1.1\ 10^{9}$	$7.7\ 10^{8}$	$4\ 10^{7}$	$1.9\ 10^{6}$	$7.1\ 10^{5}$	$3.7\ 10^{5}$	$2.0\ 10^{5}$	$5.8\ 10^{4}$

**Initial cell concentration*

Table 5: Minimum inhibitory concentration (MIC) values % (v/v), growth inhibition (%), and minimum bactericidal concentration (MBC) % (v/v) of the essential oil of *E. camaldulensis* against spoilage bacteria.

	CMI (%)		Growth inhibition (%)		CMB (%)	
Micro-organisms	Essential oil	Ampicillin	Essential oil	Ampicillin	Essential oil	Ampicillin
S.aureus	0.1	0.05	83.81	85.62	0.5	0.075
Streptococcus A	0.5	0.075	97.14	62.42	1	0.1
E.coli	1.5	0.5	80	92.36	2.5	1
S.enteritidis	1	0.5	6.25	56.82	_	1.5
K.pneumonia	1	0.5	6.66	62.46	_	1.5
P.aeruginosa	1	0.5	25.58	41.84	_	1.5

Table 6: Minimum inhibitory concentration (MIC) values % (v/v), growth inhibition (%), and minimum bactericidal concentration (MBC) % (v/v) of the essential oil of *E. rudis* against spoilage bacteria.

	CMI (%)		Growth inhibition (%)		CMB (%)	
Micro-organismes	Essential oil	Ampicillin	Essential oil	Ampicillin	Essential oil	Ampicillin
S.aureus	0.1	0.05	99.33	85.62	0.5	0.075
Streptococcus A	0.075	0.075	69.69	62.42	2.5	0.1
E.coli	2.5	0.5	3.33	92.36	_	1
S.enteritidis	0.5	0.5	99.58	56.82	3.5	1.5
K.pneumonia	0.075	0.5	63.33	62.46	_	1.5
P.aeruginosa	0.5	0.5	9.09	41.84	_	1.5

On the other hand the minimum inhibitory of the oil of *E. camaldulensis* ranged from 0.1 to 1.5% (v/v) against all the test organisms. The most susceptible organism was *S. aureus (ATCC 25923)*, with CMI of 0.1% (v/v) and the oil exerted it the significant reduction in microbial counts of 83. 76%, flowed by *Streptococcus A* with MIC of 0.5% (v/v) and the essential oil MIC revealed potential effect of antibacterial activity as remarkable decrease in CFU numbers of 97.14%. However, moderate antibacterial activity of the essential oil tested was noted against *K. Pneumonia (ATCC 138337), S. enteritidis (ATCC 14028)* and *P. aeruginosa (ATCC 9027).*
The results from viable count essay revealed that the MBC concentration from the leaf essential oils of the two Eucalyptus species had a severe effect on the cell viability of the tested bacteria (Table V-VI). All the strains of the Gram-positive bacteria, *S. aureus* and *Streptococcus A* were found to be the most sensitive strains tested to the oils of the two Eucalyptus species. The essential oils exerted a similar bactericidal activity against S. *aureus (ATCC 25923),* with CBM values of 0.5% (v/v) and maximum bactericidal proprieties against *Streptococcus A (ATCC 11700),* with MBC values of 1.0 and 2.5% (v/v) by *E. camaldulensis* and *E. rudis*, respectively. For the Gram-negative bacteria, *E. coli* appears to be more sensitive to the essential oil of *E. camaldulensis*, with MBC value of 2.5% (v/v). Also, higher concentration of the oils was needed for the bactericidal action against *P. aeruginosa (ATCC 9027)* and *K. Pneumoniae (ATCC 138337).*
Compared to the activities obtained by the tested antibiotic (Ampicillin), the most active antibacterial essential oil is *E. camaldulensis*. The Gram positive were more sensitive than the Gram negative. This higher resistance among Gram-negative bacteria could be ascribing to the presence of their outer phospolipidic membrane, almost impermeable to lipophilic compounds [27]. The essential oils of the two Eucalyptus species harvest from Korbous arboreta (North East Tunisia) have been previously studied for their antibacterial activities using the agar disc diffusion method [9].Our results confirmed the observations of Elaissi et al.[8], demonstrated that *S.aureus* was found the most sensitive bacteria strain. Also, we have been reported that *E.camaldulensis* exhibited a moderate inhibition against *S. aureus* (11.7±0.6mm, zdi) comparate to the same antibiotics standards (24.5±7.5-34.3±11.5, zdi), while the lowest activity was mostly evident with essential oils of *E.rudis.*
According to [28], the antibacterial activity of the essential oils of the two eucalyptus species against studies bacteria can be attributed to the presence of (+) - Spathulenol present in the greatest proportion and ρ -Cymene, which represented 14.83-.49% of the essential oils tested, the biological precursor of carvacrol is hydrophobic and causes welling of the cytoplasmic membrane [29].

Conclusion

The monoterpenes, confer the chemical profile of analyzed essential oils eucalyptus samples. The both essentials oils were characterized by Spathulenol, ρ-Cymene and, 1,8-cineole as a major compounds. The bioassay comfirm that gram positive bacteria are more sensitive compared to gram negative ones, *S.aureus* being in general the more sensitive strain (MBC= 0.5% (v/v). *E. camaldulensis* disclosed substantial bioactivity (IC_{50}= 342±2.5 µg/ml). This plant many be suggested as a new potential source of natural antioxidant and antibacterial agents.

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