



## Chemical and Biological Investigation of leaves of *Eucalyptus Torelliana* Essential oils from Benin

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### Abstract

The essential oil obtained by hydrodistillation from leaves of *Eucalyptus torelliana* F. Muell. (Myrtaceae) growing in Benin was analyzed by GC and GC/MS. Thirty eight components, which represented 99,6 % of the total constituents of the oil were identified. The essential oil is rich in hydrocarbons monoterpenic. The major constituents found were  $\alpha$ -pinene (60.9 %),  $\beta$ -pinene (12.0 %), trans- $\beta$ -caryophyllene (9.0 %), limonene (2.9 %) and aromadendrene (2.1 %). The oil extracted reveals an average antiradical and antimicrobial activities.

**Keywords:** *Eucalyptus torelliana*, essential oils,  $\alpha$ -pinene; antiradical activity, antifungal, antimicrobial activity.

### Introduction

The genus *Eucalyptus* of the Myrtaceae family comprises more than 600 species<sup>1</sup>. Different species are used for reforestation, improvement of marshlands, and as ornamental trees and adventice in towns and on roadsides. The genus introduced in Benin, was not sufficiently studied in particular as species *E. torelliana* F. Muell. In Nigeria *E. torelliana* is used to treat gastrointestinal disorders<sup>2</sup>. In addition, a decoction of the leaves is reported to be a remedy for sore throat and other bacterial infections of the respiratory and urinary tracts<sup>3</sup>. The poultice of the leaves is applied over wounds and ulcers<sup>4</sup>. The essential oils of the leaves have been used in the treatment of lung diseases and were stated to have anti-tubercular effect<sup>5</sup>. For instance, hot water extracts of dried leaves of *E. torelliana* F. Muell are traditionally used as analgesic, anti-inflammatory and remedies cancer-related symptoms and intestinal disorders<sup>6</sup>.

*E. torelliana*, a less vigorous variety, is often associated with *E. camaldulensis*. Its hard stiff wood is used in the construction of wagons and bridge platforms. It has been little studied, the results all showing it to contain large amounts of monoterpenes together with aromadendrene or spathulenol as main sesquiterpene derivatives<sup>1,7</sup>.

*E. torelliana* is less studied. Some results available on the essential oil of *E. torelliana* revealed that it was found to be rich in hydrocarbons monoterpenic and sometimes rich in spatulenol. An oil of *E. torelliana* grown in Australia was reported to contain  $\alpha$ -pinene,  $\beta$ -pinene, ocimene and aromadendrene as its characteristic constituents<sup>1</sup>. Essential oils from the leaves of *E. torelliana* of Benin<sup>8</sup> was reported to contain  $\alpha$ -pinene (18.6%), spathulenol (16.8%), *p*-cymene (14.4%). In Mali, an oil

containing mainly  $\alpha$ -pinene (50.0-52.1%), caryophyllene oxide (7.0-7.3%) and aromadendrene (5,0-6.0%) has been observed<sup>9</sup>.

Essential oils of leaves and fruits of *E. torelliana* from Nigeria were dominated by 1,8-cineol (33.8%),  $\alpha$ -pinene (21.7%), *p*-cymene (10.7%) and  $\beta$ -pinene (10.3%) for the leaves and  $\alpha$ -pinene (55.8%), and  $\beta$ -pinene (10.8%) for the fruits<sup>6</sup>.

More recently, the essential oils of leaves of *E. torelliana* harvested in Pointe Noire (Congo-Brazzaville) allowed the identification of two chemotypes dominated by globulol and  $\alpha$ -pinene in variable relative proportions in nine samples<sup>10</sup>.

Loumouamou, *et al*, 2009 published about essential oils from leaves of *E. torelliana* pure species and *E. citriodora* x *E. torelliana* hybrids growing in Congo-Brazzaville and had identified three chemotypes dominated by globulol,  $\alpha$ -pinene and citronellyl acetate in variable relative proportions in nine samples for *E. torelliana* pure and four chemotypes dominated by  $\alpha$ -pinene, citronellal, citronellol and citronellyl acetate in variable relative proportions in six samples for *E. citriodora* x *E. torelliana* hybrids<sup>11</sup>.

The oil yield and physico-chemical characteristics of this species were determined<sup>10,11</sup>.

Biological evaluations were carried out by these latter had evaluated, in particular the in vitro cytotoxicity of the essential oil of leaves and fruits of *E. torelliana* and had concluded the volatile oils exhibited potent inhibitory activities against four human tumor cell lines, while showing relatively weak antimicrobial properties<sup>9</sup>. More later Adeniyi *et al*, 2009 showed that the extracts of *E. torelliana* present new therapeutic

alternative for the treatment of gastrointestinal diseases associated with *Helicobacter pylori* infections, such as gastric and duodenal ulcers<sup>12</sup>.

The aims of the present investigation were to assess the chemical composition, the antimicrobial, antiradical and acaricide activities of the essential oil of *E. torelliana* F. Muell. collected in the area of Benin.

## Material and Methods

**Plants Material:** The leaves of this plant were collected in the University Institute of Technology of Lokossa area in Benin on July 2010. The sample was authenticated by Mr. Akoègninou of the Herbarium of Abomey-Calavi, Department of Vegetal Biology where voucher specimen [AA6379/HNB] was kept for future reference.

**Isolation of Essential oil:** The essential oil was obtained by hydrodistillation of the pulverized fresh leaves (200 g) using a Clevenger-type apparatus for 2 h; after decantation the oils were dried using over anhydrous Na<sub>2</sub>SO<sub>4</sub> sodium sulphate and stored in sealed vials below 10 °C until using.

**Analysis: Gas Chromatography:** The oils were analyzed on a Varian CP-3380 GC equipped with a HP5 J and W Agilent (5 %Phenyl - methylpolysiloxane) fitted with a fused silica capillary column (30 m x 0.25 mm i.d. film thickness 0.25 µm); temperature program 50-200°C at 5°C/min, injector temperature 220°C, detector temperature 250 °C, carrier gas N<sub>2</sub> at a flow rate of 0.5 mL.min<sup>-1</sup>. Diluted samples (10/100, v/v, in methylene chloride) of 2.0 µL were injected manually in a split mode. The percentage compositions were obtained from electronic integration measurements without taking into account relative response factors. The linear retention indices of the components were determined relatively to the retention times of a series of n-alkanes (C<sub>9</sub>-C<sub>20</sub>).

**Gas Chromatography-Mass Spectrometry:** GC/MS analyses were performed using a Hewlett Packard apparatus equipped with a HP5 fused silica column (30 m x 0.25 mm; film thickness 0.25 µm) and interfaced with a quadruple detector (Model 5970). Column temperature was programmed from 70 to 200 °C at 10 °C/min; injector temperature was 220 °C. Helium was used as carrier gas at a flow rate of 0.6 mL.min<sup>-1</sup>, the mass spectrometer was operated at 70 eV. Diluted samples (10/100, v/v, in methylene chloride) of 2.0 µL were injected manually in the split mode.

The identification of individual compounds was based on the comparison of their relative retention times with those of authentic samples on the HP5 column and by matching the linear retention indices and mass spectra of peaks with those obtained from authentic samples and/on the NBS75K.L and NIST98.L libraries spectra and published data<sup>13,14</sup>.

**Antiradical activity: Free radical scavenging activity: DPPH test:** - Antiradical scavenging activity was determined using 1,1-diphenyl-2-picrylhydrazyl (DPPH) following the Mellors and Tappel method<sup>15</sup>, adapted to essential oil screening<sup>16</sup>.

1,1-diphenyl-picrylhydrazyl [1898-66-4] was purchased from Sigma-Aldrich chemistry and the solutions were prepared with analytical grade solvents purchased from standard commercial sources.

DPPH, was dissolved in ethanol to give a 100 µM solution. To 2.0 mL of the ethanolic solution of DPPH was added 100 µL of a methanolic solution of the antioxidant reference butylated hydroxytoluene (BHT) at different concentrations. The essential oils and the fractions were tested in the same method. The control, without antioxidant, is represented by the DPPH ethanolic solution containing 100 µL of methanol. The decrease in absorption was measured at 517 nm after 30 min, at 30°C.

All measurements were performed in triplicate and the concentration required for 50 % reduction (50 % scavenging concentration SC<sub>50</sub>) was determined graphically. All the spectrophotometric measures were performed with a SAFAS UV mc2 spectrophotometer, equipped with a multicells/multikinetics measure system and with a thermostated cells-case.

The free radical-scavenging activity of each solution was then calculated according to the following equation<sup>17</sup>:

$$SC\% = \frac{A(blank) - A(sample)}{A(blank)} \times 100$$

Antiradical activity was expressed as SC<sub>50</sub> (mg.L<sup>-1</sup>), defined as the concentration of test material required to cause a 50 % decrease in initial DPPH absorbance.

**Antifungal activity: Preparation of the culture medium:** 11.5 g agar of yeast extract (Yeast extract AGAR) and 10 g of anhydrous glucose are mixed with 500 mL of distilled water for the preparation of culture medium. After sterilization and addition of oxytetracycline (0.1 %) 5ml, this medium was cast in limp of Petri dish 9 cm in diameter at a rate of 17 mL.

**Detection of the moulds:** A quantity of vegetable weighed from gardening culture, fresh tomato fruits and banana leaves was diluted in sterile peptone water in order to detect fungi responsible of their deterioration. 30 min after homogenizing each sample, 0.1 mL of the inocula was spread out on the sterilized mould medium (Yeast Extract Glucose Agar: YEGA) and uniformly. The present limp was incubated at 25 °C ± 1 °C five days awared from day light.

**Transplantation and mycelial growth:** The moulds detected after examination and identification then, are transplanted (subcultured) using a disc of 6 mm in diameter which carries

spores from the anamorph mould on the surface of Petri disc containing the former medium YEGA containing tested essential oils at different concentrations or no (positive control). The moulds subcultured were incubated at  $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$  the mycelial growth was appreciated every day by measuring the average of two perpendicular diameters passing by the middle of the disc, from the first day till the seventh one at least 7 days<sup>18</sup>.

The antifungal activity was evaluated by the following equation<sup>19</sup>:

$$I = \left(1 - \frac{d}{d_c}\right) \times 100$$

I : index antifungal; d : diameter of growth of Petri dish treated out of essential oil;  $d_c$ : diameter of growth of the control (witness) [Petri dish without essential oil].

**Test of determination of the fungistatic or fungicidal activity:** With the experimental concentrations where neither growth, nor germination was observed, we tested the fungistatic or fungicidal activity. This test consists in taking the mycelial disc not germinated at the end of the incubation of the Petri dish and reintroducing it in a new culture medium (former one) without natural extract. If the mycelial growth is always inhibited, the fungicidal activity of the natural extract is conformed and in the contrary case, it's spoken about fungistatic activity.

**Antimicrobial activity: Preparation of the essential oil emulsion:** 2 mL of Mueller Hinton broth added with 0.02 g/L w/v of phenol red were mixed with 40  $\mu\text{L}$  of essential oil and 2 drops of Tween 80 and has been introduced inside a hemolyse test tube. The mixture was then homogenized.

**Preparation of suspensions:** This preparation was made same manner for the three stocks of tested bacteria. A pure colony of each stock was suspended in 5 ml of Mueller Hinton broth. After incubation at  $37^{\circ}\text{C}$  during 2 hours, we obtained  $10^6$  germs/ml a microbial suspension with a turbidity equals to scale 2 of MacFarland standard.

**Determination of Minimal Inhibitory Concentration (MIC):** The method used is reported by Yehouenou *et al.*<sup>20</sup>.

100  $\mu\text{L}$  of bubble Mueller Hinton broth containing of phenol red to 0.02 g/L were distributed in all the 96 wells of microplate. We added 100  $\mu\text{L}$  extract (initial solution) to each well of the first column except that of the second line and we carried out successive dilutions of reason 2 well by well, column by column to the last well of the last column where 100  $\mu\text{L}$  were rejected. We put 100  $\mu\text{L}$  thereafter bubble Mueller Hinton not containing phenol red in the first well second-rate and carried out successive dilutions of reason 2. We sowed all the wells, except those of the first line, by introducing 100  $\mu\text{L}$  there bacterial suspension with  $10^6$  cfu. In the place of the bacterial suspension,

we put bubble Mueller Hinton broth without phenol red in the wells of the first line. The microplate one was finally covered with parafilm paper and was incubated at  $37^{\circ}\text{C}$  during approximately 18 hours.

It should be noted that the first line constitutes the negative control and the second, positive control. With the reading, obtaining a yellow color indicates a bacterial multiplication. The persistence of initial red color means the absence of growth of the microbes. The MIC is the weakest concentration for which there is no visible growth. It is thus the low concentration of the well where there is no turn with the yellow.

**Statistical analysis:** Data were subjected to analysis of variance (ANOVA). They were expressed as the mean  $\pm$  standard error of triplicate measurements; standard deviations did not exceed 5%.

## Results and Discussion

**Chemical composition:** The essential oil was obtained in 0.18 % yield from the leaves of *Eucalyptus torelliana*; this yield is similar to that already observed with the same species from the Congo-Brazzaville<sup>10,11</sup>; very low in comparison those of Benin<sup>8</sup> and Mali<sup>9</sup>. Thirty-eight compounds representing (99.6 %) of *E. torelliana* oil were identified by GC/MS (table-1). The oil was found to be rich in monoterpene hydrocarbons with a predominance of  $\alpha$  and  $\beta$ -pinene (60.9 % and 12.0 % respectively). The major compound was reported as a characteristic constituent of the leaves oil from trees growing Australia, Benin, Mali and Congo<sup>1,8-11</sup>.

**Antiradical activity:** Finally the antiradical activity of the essential oil of *E. torelliana* was evaluated by comparison with a commercial sample of butylated hydroxytoluene (BHT) which is widely used as a preservative. The following results were obtained:  $\text{SC}_{50}(\text{E. torelliana}) = (2.90 \pm 0.14) \text{ g/L}$ ;  $\text{SC}_{50}(\text{BHT}) = (7.5 \pm 0.37) \text{ mg/L}$  figures -1 and 2.

These results indicate that the oil of *E. torelliana* collected in Benin should not find any application in this field.

**Antifungal activity:** The antifungal activity of the essential oil of *E. torelliana* was evaluated. The following results were obtained (figure -3).

For 50 ml/L of essential oil concentration, we observed an abrupt increase rate reduction on the lasted 96 hours which passes from 44 to 76.85 % then progressive reduction going from 71.09 to 56.17 %; whereas for the concentrations of 25 mL/L and 20 mL/L, we noted a progressive increase this rate on the lasted 72 hours before attending its progressive reduction.

The progressive decreasing of the mycelial reduction rate observed for all concentration of *E. torelliana* essential oil

tested *Aspergillus ochraceus* may be attributed to the chemical composition of this latter which was mainly composed of 92.9% of hydrogenated terpenes, that certainly didn't possess the possibility to attach, to link to the major lipid, ergosterol in the layer of the mould membrane cell and destroy it. According to Medoff, 1988<sup>21</sup>, this combination to ergosterol localized in the layer of fungi cell membrane allow its destruction and consequently its death. The biological activity started on the first days of contact of the essential oil with the fungi cell, probably due to the presence of oxygenated sesquiterpenes and monoterpenes in low proportion respectively 5.7% and 1.0% but progressively at least within 5 to 7 days, the accumulation of hydrogenated terpenes, might provoke the decreasing of this attachment with the ergosterol and the corollary of this fact was the mycelial growth ansing.

**Antimicrobial activity:** It should be noted that the increase in ratio reduction observed on the level of (figure - 3) explains the inhibition of the mycelial growth of the essential oils during a given time whereas the intensive decreasing of the reduction ratio translates the mycelial growth. The essential oils of *Eucalyptus torrelliana* does not have a remarkable influence for the essential oil concentrations tested.

The essential oil of *Eucalyptus torrelliana* MIC raised one on *Staphylococcus aureus* ATCC25923 (8.68 ± 0.43 mg/mL) which to the double of the MIC is found equal for *Candida albicans* ATCC 14133 (4.34 ± 0.22 mg/mL) and weakest MIC was noted with regard to *Escherichia coli* (1.08 ± 0.05 mg/mL). The essential oil carried on antimicrobial average activity on *Staphylococcus aureus* ATCC 25923. It's more effective on *Escherichia coli* ATCC 25922 (MIC = 1.08 ± 0.05 mg/mL). The

result obtained on the level of antimicrobial test would be due to a synergy between the majority and minority constituents (table - 2).

α-pinene and β-pinene with respective amount of 60.9% and 12.0% and added one which is limonene 2.9% appeared to be lipophilic hydrocarbon molecules which accumulate in the lipid bilayer and distort the lipid-protein interaction, alternatively, direct interaction of the lipophilic compounds with hydrophobic parts of the protein is possible<sup>22, 23</sup>. This explains the affinity of this essential oil for the *Escherichia coli* cell membrane which allows its penetration easily inside the cytoplasm than *Staphylococcus aureus* cell membrane which don't possess the out layer membrane done by phospholipids and lipopolysaccharids absent in *S. aureus* cell membrane. This justifies the different MIC determinate on the two strains: *Escherichia coli* ATCC 25922 MIC = 1.08 ± 0.05 mgmL<sup>-1</sup> and *Staphylococcus aureus* ATCC 25923 MIC = 8.68 ± 0.43 mg/mL

### Conclusion

The essential oil extracted from the leaves of *Eucalyptus torrelliana* rich in hydrocarbons monoterpenes (77.5 %) and hydrogenated sesquiterpenes (15.4 %). The effectiveness of its reactivity of BHT with respect to the DPPH did not find any application in antiradical activity field. The antimicrobial activities of *E. torrelliana* is diversely appreciated where the MIC is high for *Candida albicans* and *Staphylococcus aureus*, it's interesting against *E. coli*. The antifungal properties show that *E. torrelliana* detains only fungistatic activity against *A. ochraceus*.

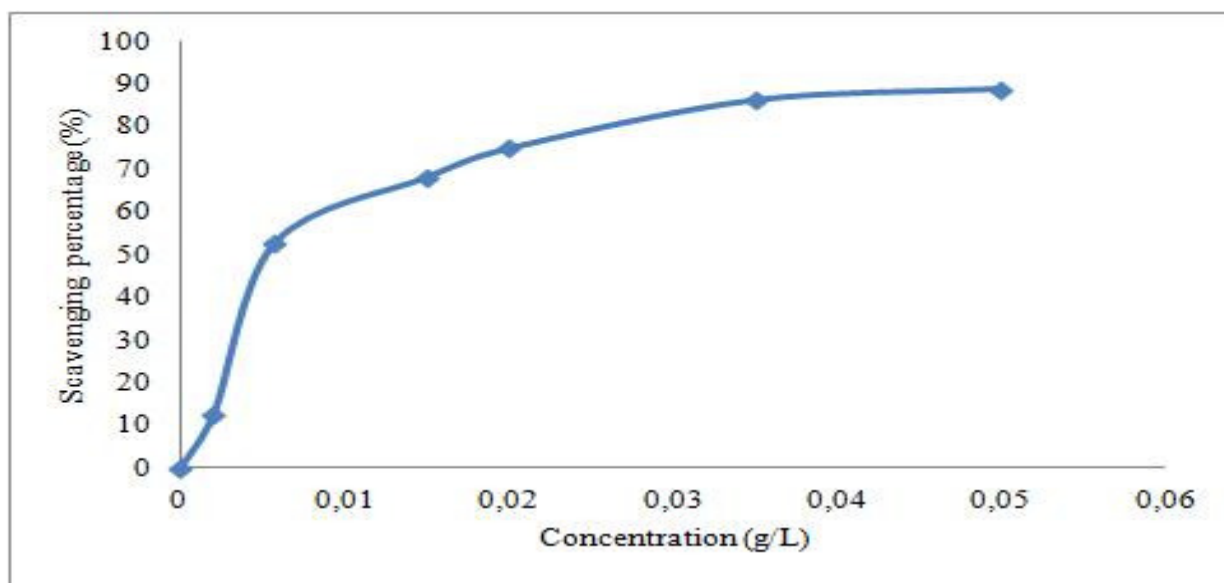


Figure- 1  
Reactivity of essential oils of BHT with respect to the DPPH

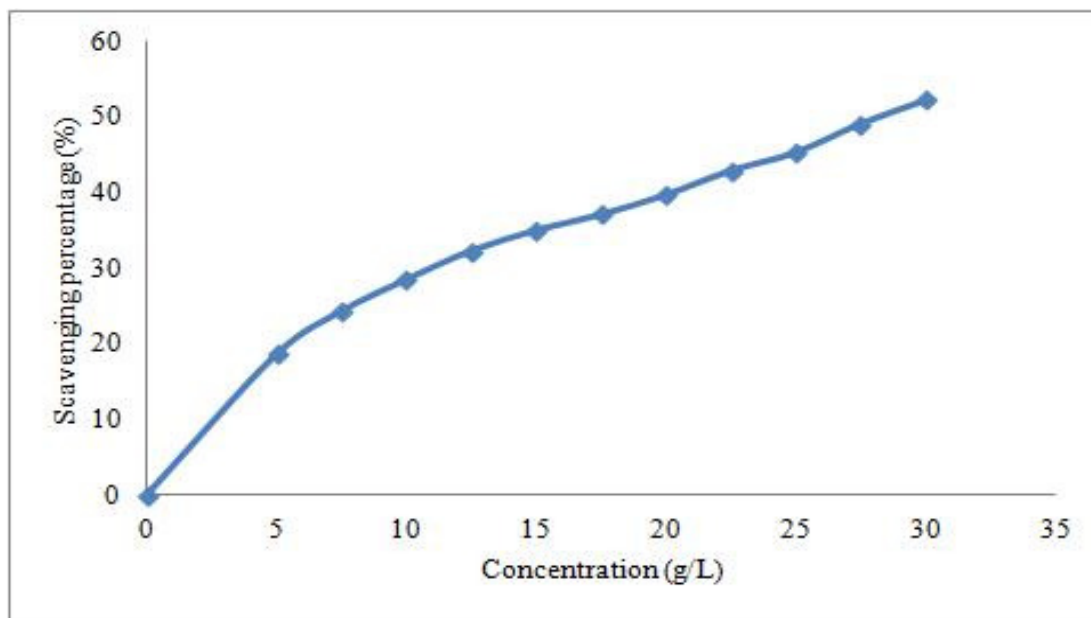
**Table-1**  
**Chemical composition of essential oils of *Eucalyptus torelliana* collected in Benin**

RI	Component	%	Mode of identification
929	$\alpha$ -Thujene	0.4	MS, RI
<b>940</b>	<b><math>\alpha</math>-Pinene</b>	<b>60.9</b>	GC, MS, RI
953	Camphene	0.1	GC, MS, RI
963	Benzaldehyde	0.1	MS, RI
<b>982</b>	<b><math>\beta</math>-Pinene</b>	<b>12.0</b>	GC, MS, RI
1008	$\alpha$ -Phellandrene	0.3	MS, RI
1020	$\alpha$ -Terpinene	0.1	MS, RI
1028	<i>p</i> -Cymene	0.1	GC, MS, RI
<b>1032</b>	<b>Limonene</b>	<b>2.9</b>	GC, MS, RI
1048	<i>trans</i> - $\beta$ -Ocimene	0.3	GC, MS, RI
1062	$\gamma$ -Terpinene	0.1	GC, MS, RI
1093	Terpinolene	0.2	MS, RI
1101	Linalool	0.2	GC, MS, RI
1174	Terpinen-4-ol	0.1	GC, MS, RI
1184	<i>p</i> -Cymen-8-ol	0.1	GC, MS, RI
1196	$\alpha$ -Terpineol	0.4	GC, MS, RI
1203	Myrtenol	0.1	MS, RI
1205	Verbenone	0.1	MS, RI
1385	Isolatedene	0.1	MS, RI
1399	$\beta$ -Elemene	0.2	GC, MS, RI
1404	$\beta$ -Longipinene	0.2	MS, RI
1407	Longifolene	0.2	MS, RI
<b>1437</b>	<b><i>trans</i>-<math>\beta</math>-Caryophyllene</b>	<b>9.0</b>	GC, MS, RI
1456	Aromadendrene	2.1	MS, RI
1470	$\alpha$ -Humulene	0.9	MS, RI
1478	$\gamma$ -Gurjunene	0.5	MS, RI
1488	Germacrene D	0.4	MS, RI
1505	$\beta$ -Selinene	1.2	MS, RI
1511	$\alpha$ -Bulnesene	0.1	MS, RI
1520	$\gamma$ -Cadinene	0.1	MS, RI
1529	$\delta$ -Cadinene	0.4	MS, RI
1597	$\beta$ -Copaen-4- $\alpha$ -ol	1.6	MS, RI
1605	Globulol	2.3	MS, RI
1614	Guaiol	0.1	MS, RI
1622	Rosifoliol	0.1	MS, RI
1650	$\alpha$ -Eudesmol	0.2	MS, RI
1674	(E)-14-hydroxy-9-epi-Caryophyllene	1.0	MS, RI
1685	Nerolidyl acetate	0.4	MS, RI
<b>Grouped components (%)</b>			
<b>Monoterpene hydrocarbons</b>			<b>77.5</b>
<b>Oxygenated monoterpenes</b>			<b>01.0</b>
<b>Sesquiterpene hydrocarbons</b>			<b>15.4</b>
<b>Oxygenated sesquiterpene</b>			<b>05.7</b>
<b>Total identified</b>			<b>99.6</b>

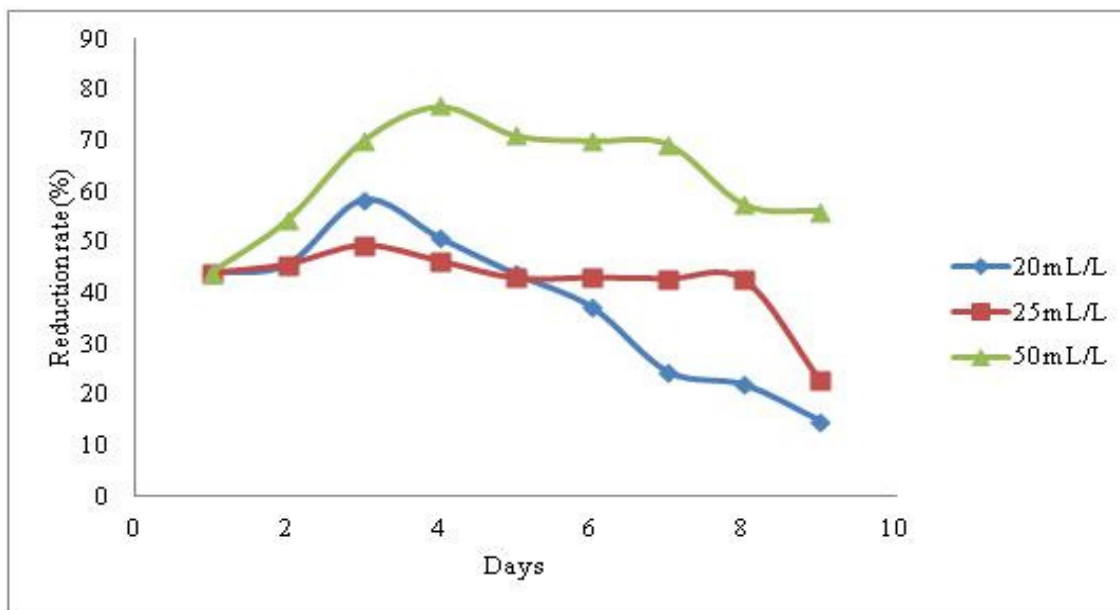
RI : Retention index relative to n-alkanes (C<sub>9</sub>-C<sub>20</sub>) on a column HP5; GC, identification was based on retention times of authentic compounds on a Varian CP-3380GC with a fused silica capillary column; MS, identification was based on comparison of retention index of the computer matching of the spectra of peaks with ESSENCES, NBS75K.L and NIST98.L libraries and published data<sup>13,14</sup>; RI, tentatively identified based on comparison of retention index of the compounds compared with published data<sup>13,14</sup>.

**Table- 2**  
**Antimicrobial activity (Minimal Inhibitory Concentration: MIC value, mg/mL) of**  
**Essential oil of leaves of *Eucalyptus torelliana***

Microbial stock	Minimum Inhibitory Concentration (MIC) (mg/mL)
<i>Escherichia coli</i> ATCC 25922	1.08 ± 0.05
<i>Staphylococcus aureus</i> ATCC 25923	8.68 ± 0.43
<i>Candida albicans</i> ATCC 14133	4.34 ± 0.22



**Figure – 2**  
**Reactivity of essential oils of *E. torelliana* with respect to the DPPH**



**Figure- 3**  
**Action of the oil essential of *Eucalyptus torelliana* with various concentrations on the mycelial**  
**Growth of *Aspergillus ochraceus***

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