Yield, phytochemical characterization and anti-*Allorhizobium vitis* activity of essential oils from four Eucalyptus species growing in Morocco

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Abstract

This study aims to investigate the yield, chemical composition and inhibitory effect of four essential oils of eucalyptus collected from Morocco on the pathogenic *Allorhizobium vitis* S4 causal agent of the crown gall of grapevine. The identification of the constituents of essential oils was performed by GC-MS analysis and the antibacterial activity was evaluated using an aromatogram test. The yield of different samples of eucalyptus ranged from 2-4.5%. The results of chemical compounds analysis show that the major compounds of eucalyptus essential oils are, 1,8-Cineol (Eucalyptol) (65.55-78.11%), α-Pinene (3-15.21%), 2-β-Pinene (2.33-5.27%) and p-Cymene (0.79-11.51%). All eucalyptus essential oil exhibits an antibacterial activity *in vitro* against *Allorhizobium vitis* strain S4 with a percentage of inhibition and minimal inhibitory concentration values in the range of 13.67-20.50 % and 20-40 mg/mL, respectively.

Keywords: Eucalyptus; essential oil; chemical composition; antibacterial activity; *Allorhizobium vitis*, Morocco.

Rendement, caractérisation phytochimique et activité anti-*Allorhizobium vitis* des huiles essentielles de quatre espèces marocaines d'Eucalyptus

Résumé

Ce travail de recherche vise à étudier le rendement, la composition chimique et l'effet inhibiteur de quatre huiles essentielles d'eucalyptus collectées au Maroc sur l'agent phytopathogène *Allorhizobium vitis* la souche S4, responsable de la maladie de la galle du collet de la vigne. L'identification des constituants des huiles essentielles a été réalisée par analyse GC-MS et l'activité antibactérienne a été évaluée à l'aide d'un test de chromatogramme. Le rendement de différents échantillons d'eucalyptus variait de 2 à 4,5 %. Les résultats de l'analyse des composés chimiques montrent que les principaux composés des huiles essentielles d'eucalyptus sont le 1,8-Cinéol (Eucalyptol) (65,55-78,11%), l'α-Pinène (3-15,21%), le 2-β-Pinène (2,33-5,27 %) et le p-Cymène (0,79-1,51 %). Toutes les huiles essentielles d'eucalyptus présentent une activité antibactérienne *in vitro* contre la souche S4 d'*Allorhizobium vitis* avec un pourcentage d'inhibition et des valeurs de concentration minimales inhibitrices comprises entre 13,67-20,50 % et 20-40 mg/mL, respectivement.

Mots-clés : Eucalyptus, huile essentielle, composition chimique, activité antibactérienne, *Allorhizobium vitis*.

المحصول والتركيب الكيميائي النباتي والنشاط المضاد للبكتيريا ألورهيزوبيوم فيتيس للزيوت الأساسية لأربعة أنواع مغربية من الأوكالبتوس

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ملخص:

تهدف هذه الدراسة إلى التحقق من المحصول والتركيب الكيميائي والتأثير المثبط لأربعة زيوت أساسية من الأوكالبتوس تم جمعها من المغرب على العامل المسبب لمرض التدرن التاجي للعنب إللورهيزوبيوم فيتيس. تم تحديد مكونات الزيوت الأساسية عن طريق تحليل الكيمائي وتم تقييم النشاط المضاد للبكتيريا باستخدام اختبار كروماتوجرام. تراوحت محصول العينات بين 2 و4.5٪. وتظهر نتائج تحليل المركبات الكيميائية أن المركبات الرئيسية لزيوت الأوكالبتوس العطرية هي سينيول (اوكاليبتول) (ما بين65،55 و78،11%) أـ ـبينين (ما بين 3 و15،21%) وبيسيمين (ما بين 0،79 و1،51%). ويُظهر كل زيوت الأوكالبتوس العطرية نشاطًا مضادًا للبكتيريا في المختبر ضد سلالة إللورهيزوبيوم فيتيس ص4 مع نسبة تثبيط وأقل قيم تركيز مثبطة في نطاق 13.67و 20.50٪ و20 و40 مجم / مل، على التوالي .

الكلمات المفتاحية: الأوكالبتوس، الزيوت الأساسية، التركيب الكيميائي، النشاط المضاد للبكتيريا، إللورهيزوبيوم فيتيس

Introduction

In recent years, many essential oils (EO) of medicinal and aromatic plants (MAP) have been investigated for their antimicrobial properties against bacterial pathogens. Among various plants, the genus Eucalyptus (Family Myrtaceae and a native of Australia) represented by over 900 species distributed throughout the world; is one of the most-extensively planted pulpwood species (Zobel, 1988). The leaf extract of eucalyptus has been reported in many studies as an important source of bioactive molecules with biological activities such as bacteriostatic and fungistatic; is also approved as food additives and used in cosmetic, medicinal, and pharmaceutical formulations (Silva et al., 2003). Eucalyptus leaves contain two important classes of secondary compounds, essential oils (Penfold and Willis, 1961), and phenolics (Hillis, 1967). Properties of compounds in both groups suggest that plants with higher concentrations will be better defended from microbial attack than those with lower concentrations. For the eucalyptus EO, the chemical compounds possess toxicity against a wide range of microbes including phytopathogens bacteria, and fungi. They have been found to reduce mycelial growth (Kissayi, 2011), inhibit spore production, the germination and bacterial growth (Oluma and Garba, 2004).

Because of its vigor, productive potential, genetic, and ecological diversity and adaptation to different climates, eucalyptus has been used all over the world in reforestations, particularly in Morocco. The first plantations were undertaken by the Division of Research and Forest Experimentation, to assess their adaptation to Moroccan climatic and soil conditions (Zrira et al., 2004). On the North of Africa, Morocco has the largest area planted with eucalyptus, and according to the high commission for water and forests and fight against desertification (HCEFLCD) he represents 40% of forestry plantation territory (0.25% of Moroccan territory) and at present about 4000 ha are being planted every year.
Many species of eucalyptus are introduced in Morocco and played an important economic role such as in the production of pulps they can also be used as wood for the mining industry, telephone posts, and charcoal. Moreover, the eucalyptus flowers are an abundant source of nectar for bees provid a considerable increase in honey production (Fiori et al., 2000). From the Moroccan collection, the most dominant species of eucalyptus are *E. camaldulensis* (48%), *E. gomphocephala* (41%), *E. sideroxylon* (4%), *E. grandis* (4%), and other eucalyptus species representing 3% (*E. tereticornis*, *E. occidentalis*, *E. globulus*, *E. robusta*, *E. brockwayi*, *E. flocktoniae*, *E. salmonophloia* and *E. salubris*) (HCEFLCD). Different chemotypes of the same species may grow in the same place and produce different oils with different activity (Kalemba and Kunick, 2003).

In this study, we investigated the chemical composition of eucalyptus leaf EO of four species using GC-MS analysis and studied their antibacterial activity against *Allorhizobium vitis*, the causal agent of grapevine crown gall. In addition, the yields of EO obtained from hydrodistilation technique were compared.

Materials and Methods

Plant material and extraction

Fresh leaves of four eucalyptus species were collected during the flowering stage from different locations in Morocco during the period between February and July 2017 (Figure 1). The essential oil extraction was done from fresh leaves (without drying) by hydrodistillation technique using a Clevenger-type apparatus. The essential oils obtained were collected by decantation and stored in a dark glass bottle at +4°C. The percentage yield of oil which was calculated as the percent of the ratio of weight of the oil to weight of the plant.

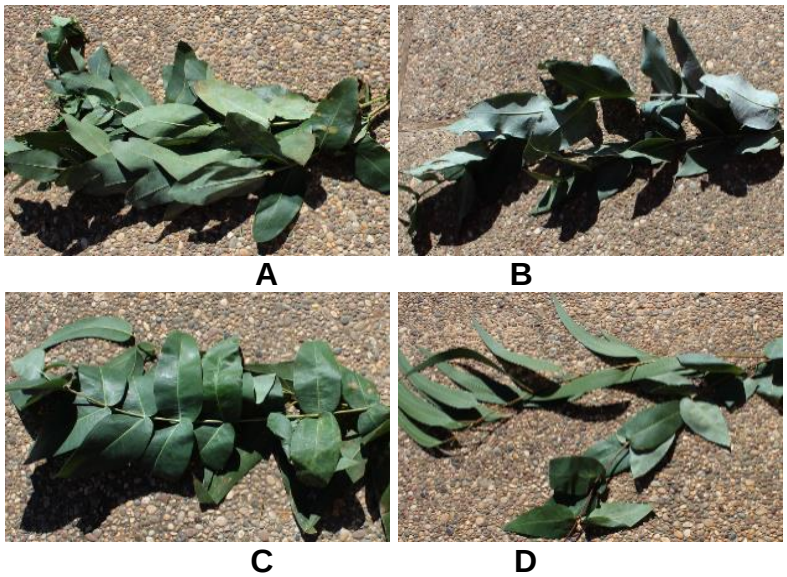

Figure 1. Samples of eucalyptus species used in this study (**A**: *E. dives*; **B**: *E. citriodora*; **C**: *E. globulus*; **D**: *E. camaldulensis*).

Gas chromatography analysis (GC-MS)

The chemical composition of the EOs was analyzed using a Hewlett Packard model HP6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a DB-5MS capillary column (30 m × 0.25 mm i.d., film thickness 0.25 µm; Agilent Technologies, USA) and coupled to an HP model 5973 mass selective detector. The oven temperature was initially maintained at 50°C and then increased, by 7°C/min, to 300°C. The injector temperature was 290°C. Purified helium was used as the carrier gas, with a flow rate of 1 mL/min, and the split ratio was 60:1. Mass spectra were obtained, in EI mode, at 70eV ionization energy and the mass range was from m/z 35 to 400. For each EO, a sample of 10 µL was diluted in 990 µL of pure hexane, and 1 µL was injected for the analysis. The device was managed by a computer system type "HP ChemStation Software" G1701BA, version B.01.00, and the data reworks were carried out with the same software. The identification of each compound was based on the comparison of its retention index (RI) (calculated using n-alkanes series between C9 and C31) and its mass spectra (MS) spectra with those described in the literature, and by computer matching with standard reference databases (NIST98, Wiley275 and CNRS libraries).

Antibacterial activity

Strain and culture conditions

The bacterial strain used in this study is *Allorhizobium vitis* strain S4 (sequenced strain) isolated from black raspberry in Hungary (Popoff et al., 1984). *A. vitis* S4 was cultivated on MG medium (Moore et al., 2001) (D-mannitol, 5 g/L; L-glutamic acid, 2 g/L; KH_2PO_4, 0.5 g/L; NaCl, 0.2 g/L; $MgSO_4(7H_2O)$, 0.2 g/L; Yeast extract, 0.5 g/L; Agar, 15 g/L; pH=7) and incubated, for 24 hours, at 28°C.

Disc diffusion method

A 100 µL sample of the *A. vitis* strain S4 suspension (10^7CFU/mL) was inoculated, using the flooding method, on YPGA medium. Sterile filter paper discs (5 mm diameter) were soaked with either 2 µL of pure EO or 2 µL of sterile distilled water (negative control). The filters were placed in Petri dishes, either directly onto the center of the culture medium, to evaluate the EO antibacterial activity (aromatogram test). After incubation at 28°C, for 24 hours, the inhibition zone around each disc was measured and the percentage inhibition was calculated using the following formula (Kalemba and Kunick, 2003):

$$\textbf{Percent of inhibition \% = [(T1-T2)/T1] × 100}$$

where: T1: diameter of the bacterial load with treatment by sterile distilled water (SDW); T2: diameter of the bacterial load with treatment by essential oil.

Minimum inhibitory concentration (MIC)

Bacterial growth was analyzed using a Microbiology Bioscreen C Reader (Labsystems®, Helsinki, Finland) according to the manufacturer's instructions. The essential oils of eucalyptus were diluted in YPG (Yeast extract, 5 g/L; Peptone, 5 g/L; Glucose, 10 g/L; pH=7), supplemented with 0.01% dimethylsulfoxide (DMSO), to obtain EO concentrations ranging from 40 mg/mL to 0.075 mg/mL (YPG+EO). Bacterial suspensions, from exponential cultures, were inoculated at OD_{600nm}= 0.01 in 200 µL YPG-EO in Bioscreen honeycomb 100-well sterile plates. Cultures were incubated for 3 days, at 28°C, with shaking at medium amplitude. Growth measurements (OD_{600nm}) were taken at 20 min intervals. The MIC corresponds to the lowest concentration of EO which resulted in 100% growth inhibition.

Statistical analysis

The significant effect of essential oils of eucalyptus on growth inhibition of *A. vitis* S4 was evaluated by analysis of variance (ANOVA1) (factor: treatment), performed with the SPSS 20 statistical software (IBM Corporation, Somers, NY, USA). The arcsin of the inhibition percentage was used for statistical analysis and was calculated using the formula:

$$Arcsin=\sqrt{(\%I/100)}$$

Where: %I is the rate of bacterial growth inhibition.

Results

Yield and chemical composition

Essential oils obtained by hydrodistillation of the samples of eucalyptus are light yellow color with a characteristic odor. Their oil yields of eucalyptus species obtained from dry leaves is shown in the Figure 2. According to the results, the yield of Eucalyptus essential oils varies among the species, with a range of 2 to 4.5%. The *E. dives* has recorded the high yield with 4.5% followed by the *E. citriodora* with 4.3%, and the *E. dives* showed the lowest oil yields with 2%.

The main components of the different essential oil of eucalyptus tested in this study are shown in the Table 1 and illustrated in Figure 3. According to gas chromatography (GC-MS) analysis, the studied essential oil presents a quite similar complex chemical composition. In total, 23 compounds were identified belonging majorly to monoterpenes and sesquiterpenes hydrocarbons; representing between 86.55 and 98.49% of the total oil of studied eucalyptus oils.

For all the samples, their essential oils contained 1,8- Cineol (Eucalyptol), an oxygenated monoterpene, as the main constituent ranged from 65.55%-78.11%. The α-Pinene, a non-oxygenated monoterpene was the second most abundant constituent with concentration ranged from 3 to 15.21% followed by 2-β-Pinene (2.33-5.27%) and p-cymene (0.79-11.51%).

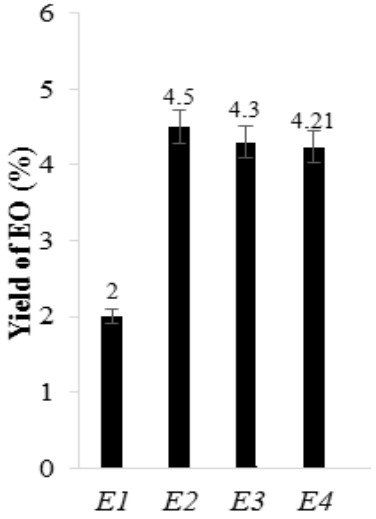

Figure 2. Yield of Essential oil extraction by hydrodistillation (calculated as the percent of the ratio of weight of the oil to weight of eucalyptus leaves).

Table 1. Chemical composition of essential oil of eucalyptus tested in this study (identification by **RI**: identification by Kovats indices. Retention index relative to C9–C31 on DB-5; **MS**: capillary column).

Compounds	**RI***	***E. dives***	***E. globulus***	***E. citriodora***	***E. camaldulensis***
α-Pinene	930	**3**	**15.21**	**12.21**	**10.24**
Isoamylisovalerate	1100	0.47	-	-	-
β-Pinene	973	0.24	0.54	0.58	0.44
Myrcene	988	-	0.32	0.4	-
α-Phellandrene	1004	-	0.33	-	-
1-8Cineole (Eucalyptol)	1029	**67.08**	**71.69**	**78.11**	**65.55**
γ-Terpinene	1077	0.94	-	-	0.26
Pulegone	1000	1.62	-	-	-
ρ-Cymene	1025	**11.51**	**3.39**	**0.79**	**3**
β-Fenchyl alcohol	1158	**3.12**	-	-	-
Camphor	1145	0.34	-	-	-
Borneol	1168	0.51	-	-	0.72
Terpinen-4-ol	1181	1.01	0.7	-	0.98
β-Pinene-2	1200	-	**3.73**	**5.27**	**2.33**
Thymol	1288	0.53	-	-	-
Carvacrol	1301	0.76	-	-	-
Trans-Caryophyllene	1500	0.26	-	-	-
Isoaromadendrene	1501	0.27	-	0.33	0.33
Aromadendrene	1654	0.95	0.65	1	0.73
Viridiflorol	1609	0.43	-	-	-
γ-Gurjunene	1700	2.28	0.93	-	1.97
δ- Gurjunene	1700	0.35	-	-	-
Total		**95.67**	**97.49**	**98.69**	**86.55**

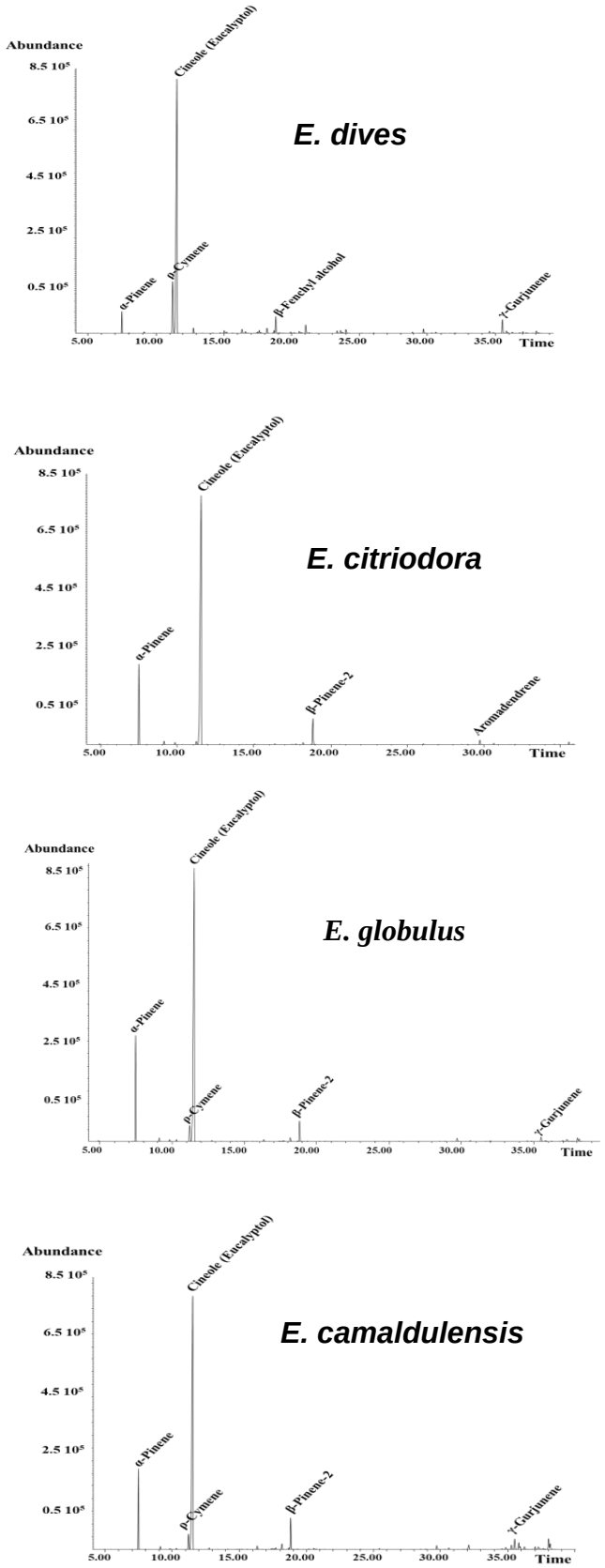

Figure 3. GC-MS chromatograms of the different EOs analyzed in this study (the name of the peaks correspond to the major compounds cited in Table 1).

Antibacterial activity

The antibacterial activity of essential oils extracted from 4 samples of eucalyptus was evaluated against the phytopathogene strain *A. vitis* S4. According to results of aromatogram tests (Figure 4), all the tested essential oil inhibits the growth of *A. vitis* S4 *in vitro* with differences in the level of activity. The percentage of inhibition of tested essential oils is ranged from 13.67% and 20.50% (Figure 5). The essential oil of *E. dives* exhibits the highest antibacterial activity with a percentage of inhibition equal to 20.50 % followed by the *E. globulus* with 14.25%, the *E. camaldulensis* with 14.01% and the *E. citriodora* with 13.67%.

Concerning of the minimal inhibitory concentration, the essential oils of *E. dives* present a MIC equal to 20 mg/mL while the other eucalyptus oils (*E. globulus*, *E. citriodora*, and *E. camaldulensis*) present the same MIC equal to 40 mg/mL

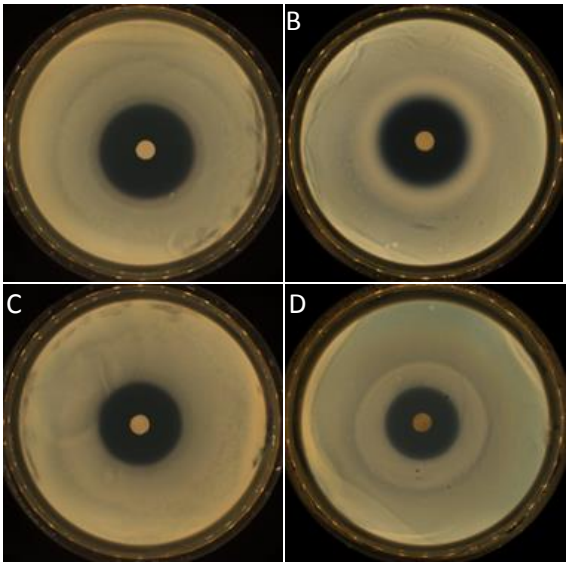

Figure 4. Aromatogram test of essential oils of eucalyptus against *A. vitis* S4. **A**: *E. dives*; **B**: *E. citriodora*; **C**: *E. globulus*; **D**: *E. camaldulensis.*

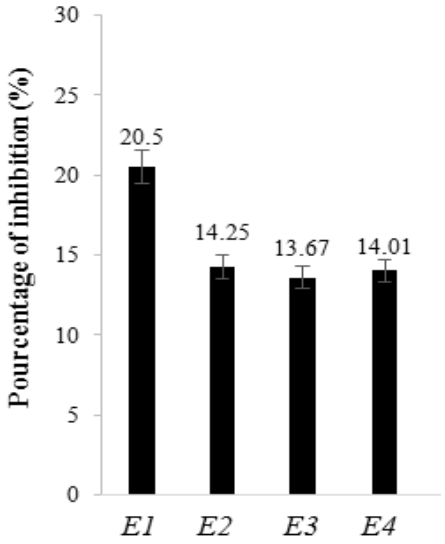

Figure 5. Antibacterial activity of different essential oils of eucalyptus against *A. vitis* S4 using the aromatogram test.

Discussion

Medicinal plants generate a tremendous amount of chemicals required for their efficiency and enhancement. Medicinal plants and secondary metabolites are increasingly used as complementary medications. Several researchers evaluated the application of essential oils as new alternatives and ecofriendly management of phytopathogenic bacteria attacks. However, the oil yield, and the difference chemical composition from a qualitative and quantitative point of view depend on many factors such as the species used, the part of plant tested, the stage of plant development, the harvesting area, the climate and the technique of extraction used for isolation of essential oil have all been found to significantly affect the two essential oil quality and quantity (Zrira et al., 2004; Asili et al., 2007; Babu and Kaul, 2007; Okoh et al., 2008).

According to our results, the yield of eucalyptus essential oils varies among the species, with a range of 2 to 4.5%. These findings were in agreement with those reported by Karamian et al. (2015), they found that the isolation yield of eucalyptus varies with the species from 1.12% to 5.38%. The variation in essential oil yields are as a result of several biotic and abiotic factors involved in determining the amount and constituents of essential oil (Kissayi, 2011).

Concerning the gas chromatography (GC-MS) analysis, the studied essential oil presents a quite similar complex chemical composition. Their essential oils contained 1,8-Cineol (Eucalyptol), an oxygenated monoterpene, as the main constituent and the α-pinene, 2-β-Pinene and p-Cymene a non-oxygenated. The obtained results of this study were also founded in numerous research work of chemical composition of many species of eucalyptus (Kalemba and Kunick, 2003; Fiori et al., 2008; Subramanian et al., 2012; Farah et al., 2013; Karamian et al., 2015). They indicate that the 1,8-Cineole (Eucalyptol) is the major compounds of eucalyptus which he α-Pinene was

characterized as the second major compounds of eucalyptus oils according to others observations (Hasegawa et al., 2008; Zewdie et al., 2009).

Considering the antibacterial activity of essential oils, our results showed an interesting anti anti-*Allorhizobium vitis* effect. The activity of eucalyptus essential oils was shown against many pathogens, such as *Pseudomonas* spp., *Acinetobacter* spp., *Enterobacter* spp., *Candida albicans*, *Escherichia coli* and others (Hillis, 1967; Cimanga et al., 2002; Rahon and Benali, 2012; Traoré et al., 2014). The antibacterial activity of essential oil has already been explained that depend to the chemical composition. In our results, the variation of the antibacterial activity of eucalyptus essential oils is probably due to the difference in the percentage of major compounds in the oils. On the other hand, and according to the literature, the antimicrobial activities of eucalyptus essential oil is not due to the major compound, 1,8-Cineole, since this compound cannot exhibit an antimicrobial activity against pathogens even with a highest concentration; and the antibacterial activity depends on the synergistic combination of the minor and major compounds (Oluma and Garba, 2004; Warot, 2006).

Conclusion

In conclusion, the results obtained in the present work revealed that the essential oils of eucalyptus exhibit an antibacterial effect against *A. vitis* S4. This remarkable activity can be due to several major and minor compounds particularly 1,8- cineole, α-pinene, 2-β-pinene and p-cymene. Therefore, the eucalyptus can be a reliable source of bioactive organic substances that can be used as a less expensive alternative to control pathogens and the same time more respectful of human health and, environment.

Acknowledgements

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Conflict of interest

There is no actual or potential conflict of interest in relation to this article.

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