



RESEARCH ARTICLES

Inhibitory effect of *Eucalyptus globulus* essential oil against *Botryosphaeria dothidea* and *Fomitiporia mediterranea* fungi causing wood diseases in viticulture

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Abstract

Dieback diseases are caused by *Fomitiporia* and *Botryosphaeria* two genus of fungi that colonize wood tissue in grapevine. After the prohibition of sodium arsenite in 2001, no chemical treatments are available to control these diseases. Curettage is a possible substitute solution. The results depend on many factors. The use of essential oils can be an alternative and/or complementary way. This study aims to investigate the essential oil (EO) composition of *Eucalyptus globulus* (*E. globulus*) from leaves and flowers and to evaluate its antifungal activity against *Botryosphaeria dothidea* and *Fomitiporia mediterranea* fungi. GC–MS results show that 97.79% of oil composition was identified, of which the Eucalyptol is the major compound (76.33%). Other compounds were identified such as α -Pinene (9.81%); Allo-Aromadendrene (3.07%) and Limonene (2.55%). EO had a detrimental effect *in vitro* on both developed *Botryosphaeria dothidea* and *Fomitiporia mediterranea* fungi. The effect is dose-dependent and increases with the duration of the treatment. Essential oil concentrations of 0.25% and 0.75% of *E. globulus* were efficient against *Botryosphaeria dothidea* and *Fomitiporia mediterranea*, respectively. This is the first report investigate the purpose of *E. globulus* EO to control this both phytopathogenic fungi. The results from the antifungal assays reveal that EO of *E. globulus* has an excellent inhibitory effect against both tested fungi.

Keywords *Eucalyptus* · Antifungal activity · Grapevine truck diseases · Botryosphaeriaceae · Hymenochaetaceae

Introduction

In last decade, great attention was devoted to limit pesticides in agriculture and to replace them with bio sourced products to control pests and diseases of plants. Indeed, EOs may also present antibacterial, antiviral, antifungal, insecticidal and herbicidal effects or have feeding deterrent effects against herbivores by reducing their appetite for such plants (Abbas et al. 2017; Sayed-Ahmad et al. 2017; Verdeger et al. 2020). The detection of some of these biological properties needed for the survival of plants has also been the base for searching similar properties for the combat of several microorganisms responsible for some infectious diseases in humans and animals (Abbas et al. 2017; Thiviya et al. 2022; Bendifallah and Merah 2023). Therefore, EOs may be considered as an alternative source for pest and disease control, since they constitute a rich source of bioactive compounds that are potentially suitable for use in integrated management programs (Bakkali et al. 2008; Abbas et al. 2017; Merah et al. 2020; Verdeger et al. 2020).

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Fomitiporia and *Botryosphaeria* two genus of fungi that colonize wood tissue and cause dieback. The decline of the vine was due to fatal diseases, esca and black dead arm (BDA) respectively developed in vineyards (Larignon et al. 2009; Cholet et al. 2020; Serra et al. 2021; Langa-Lomba et al. 2023). These diseases are one of the most destructive of the woody tissues of grapevine and have been found on at some hosts, causing decline and loss of their productivity (Kuntzmann et al. 2010; Úrbez-Torres 2011; Lecomte et al. 2011; Cholet et al. 2020). It should be noted that since the prohibition of sodium arsenite in 2001, no chemical control has been available against these wood diseases, prophylactic methods are mainly recommended in order to limit the development of those fungi (Ammad et al. 2018; Lecomte et al. 2021, 2022).

Eucalyptus, a native genus from Australia, belongs to Myrtaceae family and comprises about 900 species and subspecies; it is one of the world's most important and most widely planted genera. It is distributed throughout temperate, subtropical, and tropical regions. *Eucalyptus* was introduced in Algeria in 1863 (Abderahim 1983). The massive planting of these trees will be done until 1950. *E. globulus*, *E. camaldulensis*, *E. gomphocephala* are most common species in the Mediterranean region (Metro 1970). These species were used in traditionally medicine as analgesic, anti-inflammatory and antipyretic remedies for the symptoms of respiratory infections, such as cold, flu, sinus congestion for diarrhea and as an astringent in dentistry (Batish et al. 2008; Iseppi et al. 2020). More recently, *Eucalyptus* leaves extract has been found to reduce side effects of chemotherapy (Nasser et al. 2020). Moreover, EO of *Eucalyptus* species has been used in the pharmaceutical (Tsiri et al. 2003), cosmetics, food and medicinal purposes (Sefidkon et al. 2006; Iseppi et al. 2020).

Few studies have been undertaken to examine the herbicidal effects of several EO *Eucalyptus* species (Verdeger et al. 2020; Li et al. 2020; Batish et al. 2004). Among these used species, few studies have been carried out to evaluate the effects of *E. globulus* extracts as biological protective agents; it is rather the EO that has been mostly studied. Boukhatem et al. (2020) have evaluated EO of this species for its antimicrobial activities for food matrix. Several studies have highlighted an antibacterial effect of EO (Luis et al. 2016; Goldbeck et al. 2014; Boulekbache-Makhlouf et al. 2013). Two studies used *E. globulus* aerial parts for EO extraction (Ait-Ouazzou et al. 2011; Salem et al. 2018). These reports have underlined the antibacterial and antifungal potency of *E. globulus* EO (Ait Ouazzou et al. 2011; Salem et al. 2018). Nevertheless, leaves still the most used organ for EO extraction and antimicrobial studies (Shala and Gurunani 2021).

To our knowledge, there is a unique study that has studied effect of several essential oil, among them those of *E.*

globulus against four grapevine trunk diseases (Kenfaoui et al. 2023). They have reported interesting efficacy to limit the growth mycelium of several vineyard trunk diseases. No literature studies were investigating the effects of *E. globulus* EO on *Fomitiporia* and *Botryosphaeria* fungi for the control of vineyard diseases. Therefore, the aims of this study are to investigate the composition of *E. globulus* EO and evaluate their antifungal activity against *Fomitiporia* and *Botryosphaeria* fungi.

Materials and methods

Plant material

The aerial parts (leaves and flowers) of *E. globulus tree* were collected from Blida (36°29'N, 3°03'E) region in the north of Algeria country on April 2013. *E. globulus* has been authenticated and cataloged in the herbarium of the laboratory of Plant Biology, Department of Biology (University SAAD Dahleb, Blida).

Essential oil extraction

The EO was obtained by the hydro-distillation of fresh plant material (100 g of leaves and flowers in 500 ml of distilled water). The flasks containing the EO were covered with aluminum paper to protect them from light and were stored in a refrigerator at a temperature of 4 °C. The yield based on dried weight of the sample was calculated.

GC–MS analysis

GC–MS was performed using a Perkin-Elmer Clarus 600 mass spectrometer with a silica capillary column of 50 m length and 0.22 mm inner diameter with 50 µm film thickness. Chromatograms were recorded with the temperature ramp with a 4 min step at 40 °C and a further increase of the temperature up to 250 °C at a rate of 30 °C/min. Helium was used as the carrier gas at a rate of 1 ml/min. Oil sample (0.1 µl) was introduced directly into the source of the MS (mass spectrometry) via a transfer line (280 °C) with a split ratio of 1:50. EO components were identified based on their retention indices (determined with reference), calculated by using Biot's law Eq. 1 as following (Eq. 1):

$$[\alpha] = \frac{\alpha}{C \cdot l} \quad (1)$$

with $[\alpha]$ is the specific rotatory power; l is the optical pathlength of the tank; α is the optical rotation; and C the concentration of the solution in g/mL. EO components were identified based on their retention indices (determined with reference to a homologous series of normal alkanes), and by

comparison of their masses obtained by the different fragmentation patterns by the mass spectroscopic analysis with those reported in the literature (Lafferty & Stauffer 1991; Adams 2007).

Fungal material

The fungal material destined for evaluating the efficiency of treatments based on *E. globulus* EO against two fungal strains (*Fomitiporia mediterranea* and *Botryosphaeria dothidea*), were isolated from infected wood of grapevine and identified using a combination of morphological and cultural characters, and confirmed by molecular analysis (ITS and β tubuline primer) (Ammad et al. 2014). Cultures of each of the fungi were maintained on potato dextrose agar (PDA) and were stored at 4 °C.

Antifungal activity assays

Antifungal activity was determined by using an in vitro volatility assay. The EO was dissolved in DMSO (dimethylsulfoxide) solution (97% and 3% respectively) and four doses of this EO were prepared (0.25, 0.50, 0.75% and 1%). Whatman paper discs of 8 mm diameter, previously sterilized by autoclaving, are first impregnated at saturation with 30 μ l of each EO solution, and then deposited on the lid of the Petri dish (Inouye et al. 2006). The control consists of discs impregnated with the same volume of DMSO. All Petri dishes were immediately closed and sealed with parafilm to prevent oil evaporation. The plates were incubated at 25 °C. Mycelial radial growth was measured from the third day of incubation (Kra et al. 2009; Soro et al. 2010). The inhibition percentage of mycelial growth was calculated as per the formula of Pandey et al. (1982).

Using the following formula:

$$PI_g = (D_t - D)/D_t \times 100$$

where PI_g is percentage of inhibition growth, D_t is mean diameter of mycelial growth in control, D is mean diameter of mycelial growth in treatment. The estimation of mycelia growth was carried for 10 days from 3 days after treatment with the EO. For a better measure of diameter of growth, all plates were taken with digital pictures and then treated with imagetool software (3.1), three (03) measures were selected for each diameter.

Minimum inhibitory concentration determination

The antifungal activity level of *Eucalyptus globulus* essential oils was determined at an initial concentration of 750 μ L/L. The minimum inhibitory concentration (MIC) was determined by starting with a stock EO concentration of 7.50 μ L.

mL^{-1} , which was sequentially diluted with dimethyl sulfoxide to give final concentrations of 3.750, 1.875, 0.938, and 0.469 $\mu\text{L} \cdot \text{mL}^{-1}$. The same methodology was applied as above but with the application of 40 μ L volumes in each of the deposits. The minimum inhibitory concentration is defined as the lowest concentration of essential oil that shows no fungal growth after two weeks of incubation (Mrvová et al. 2024).

Statistical analysis

An analysis of variance (ANOVA) was performed for antifungal potency at different doses and Duncan's test means comparison was used to determine significant differences among means of measured traits.

Results and discussion

Essential oil yield and composition

The EO extraction yield was 1.4%. This result is in analogy with literature data, which confirm that *E. globulus* EO yield varied between 0.13 and 1.87 depending on Eucalyptus species (Boukhatem et al. 2014). The maximum value of EO was obtained for the fresh leaves of *E. globulus* and *E. citriodora* (1.87 and 1.63%, respectively) while the lowest was for the fresh leaves of *E. robusta* and *E. deglupta* (0.13 and 0.15%, respectively) (Cimanga et al. 2002). Further, EO yields of different Eucalyptus from fresh plant materials of some selected Congolese trees varied significantly from one species to another. Disparity with these yields compared to those formerly reported in the literature for the Eucalyptus aromatic plants collected in other geographic areas in the world could be linked to several factors such as climate, nature of the soil, time of collection, mode of extraction and the age of the tree (Barragan et al. 2016, 2018; Sayed-Ahmed et al. 2018; Sarmoum et al. 2019; Daghbouche et al. 2020; Oulebsir et al. 2022).

According to GC–MS analysis, the major constituents of *E. globulus* EO were Eucalyptol (76.33%), α -Pinene (9.81%), Allo-aromadendrene (3.07%) and Limonene (2.55%). Oxygenated monoterpenes were the major components (80.07%) of oil followed by hydrocarbon monoterpenes (14.10%) and Hydrocarbon sesquiterpenes (3.62%). The amounts of the components from the volatile oil were determined by the peak area normalization method. The chemical constituents of *E. globulus* EO are listed in Table 1. Twenty-seven compounds were identified, corresponding to 97.79% of the total EO. The chemical composition of EO was dominated by eucalyptol compound (Table 1). Compared with previous reports, eucalyptol content is consistent with the values reported in the literature (Gilles et al. 2010).

Table 1 Principal chemical composition of *Eucalyptus globulus* essential oil collected from Blida region in Algeria country

Compound	RT	Formula	(%)
Isovaleraldehyde	654	C ₅ H ₁₀ O	0.12
α-Pinene	1024	C ₁₀ H ₁₆	9.81
Camphene	1773	C ₁₀ H ₁₆	0.07
β-Pinene	1095	C ₁₀ H ₁₆	0.29
β-Mrcene	1167	C ₁₀ H ₁₆	0.23
α-Phellandrene	1160	C ₁₀ H ₁₆	0.08
α-Terpinene	1175	C ₁₀ H ₁₆	Tr
Limonene	1030	C ₁₀ H ₁₆	2.55
1,8 Cineole (Eucalyptol)	1198	C ₁₀ H ₁₈ O	76.33
Cis β-Ocimene	1250	C ₁₀ H ₁₆	Tr
γ-Terpinene	1240	C ₁₀ H ₁₆	0.06
p-Cymene	1269	C ₁₀ H ₁₄	0.96
α-Terpinolene	1276	C ₁₀ H ₁₈ O	Tr
α-diméthylstyrene	994	C ₁₀ H ₁₂	Tr
α-Gurjunene	1411	C ₁₅ H ₂₄	0.05
Linalol	1101	C ₇ H ₁₈ O ₂	Tr
Pinocarvone	1163	C ₁₀ H ₁₄ O	1.01
Terpinene-4-ol	1179	C ₁₀ H ₁₈ O	0.27
α-Terpineol	1201	C ₁₀ H ₁₈ O	0.12
Aromadendrene	1447	C ₁₅ H ₂₄	0.55
Allo-Aromadendrene	1459	C ₁₅ H ₂₄	3.07
α-Terpenyl acetate	1333	C ₁₂ H ₂₀ O ₂	0.06
Ledene	1890	C ₁₅ H ₂₄	Tr
Myrtenol	1182	C ₁₀ H ₁₆ O	0.76
Geraniol	1239	C ₁₀ H ₁₈ O	0.06
Globulol	1580	C ₁₅ H ₂₆ O	0.04
Pinocarveol	1653	C ₁₀ H ₁₆ O	1.30
Organic class			
Oxygenated monoterpenes			80.07
Hydrocarbon monoterpenes			14.10
Hydrocarbon sesquiterpenes			3.62
Total			97.79%

Kenfaoui et al. (2023) have reported the same components in different percentages. This difference can be attributed to environment parameters, genotype or age of the tree (Barragan et al. 2018; Sayed-Ahmed et al. 2018; Sarmoum et al. 2019; Daghbouche et al. 2020; Merah et al. 2020; Oulebsir et al. 2022). Moreover, Kenfaoui et al (2023) have also reported Eucalyptol, α-Pinene and Allo-aromadendrene as the most important components of *E. globulus*.

Antifungal activity of *E. globulus* EO

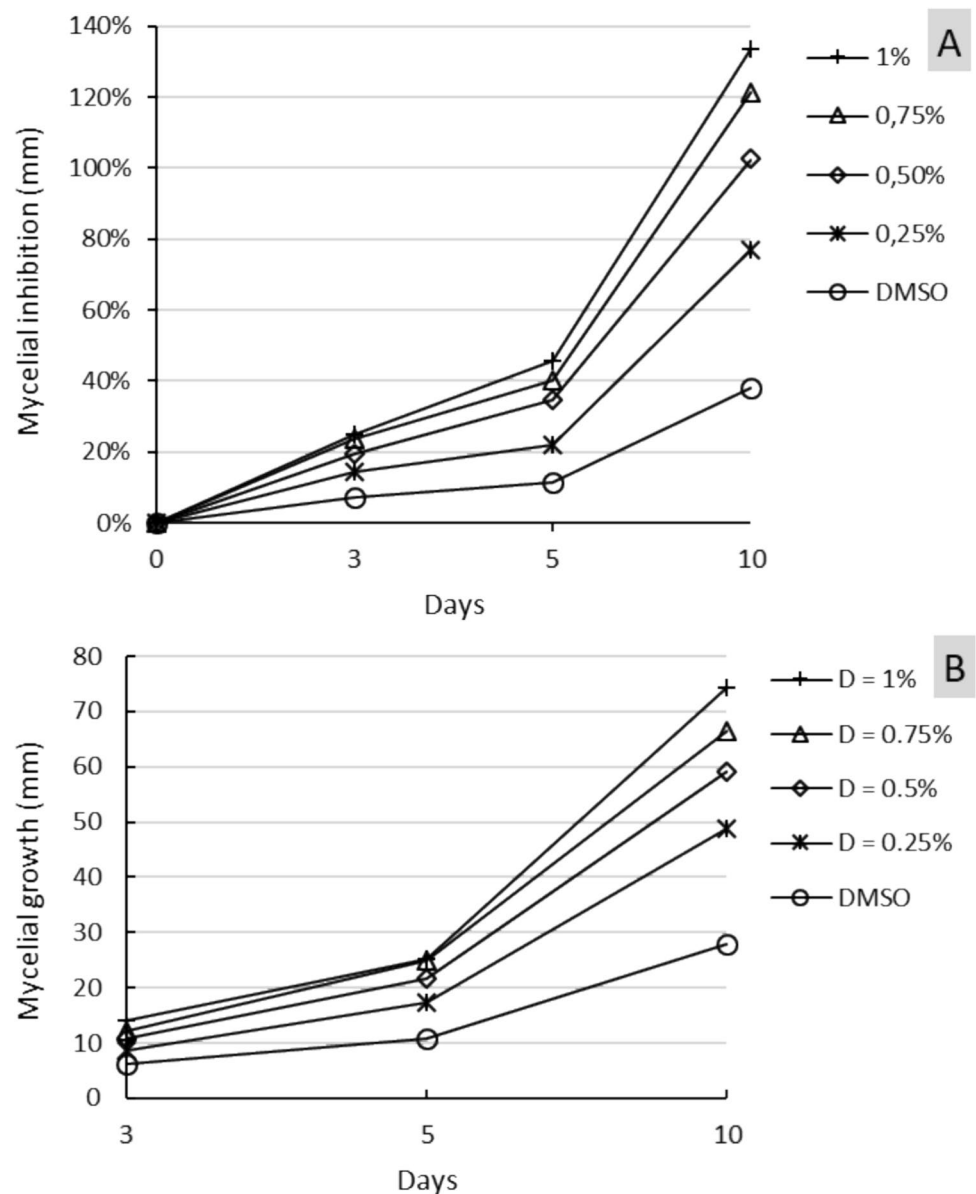
The *E. globulus* EO was tested for its antifungal activity against two important fungal species causing esca and BDA (black dead arm) wood diseases in viticulture. The results

showed that the EO reduces significantly the growth of the fungal species (Fig. 1A and B).

Mycelial inhibition was more marked at 10 days after application whatever the fungus (Fig. 2A,B). The doses of EO, D4 (1%), D3 (0.75%) and D2 (0.50%) exhibited the highest inhibitory effect compared to the lowest dose D1 (0.25%) (Fig. 2C,D). Whereas no inhibition was registered, even after 15 days with the control test. D4, D3 and D2 are evolving for the average toxicity at the beginning of the application of treatments to a high toxicity up to (05 days) (Fig. 2B). The dose D1 (0.25%) showed low toxicity at the beginning of its application to an average toxicity at the end of treatment on both strains: *Botryosphaeria* and *Fomitiporia* exhibited a net efficiency (Fig. 2C,D). This tendency is verified by testing the variance or difference was highly significant (Fig. 2C,D). The dose of treatment and its duration indicated the existence of a highly significant difference in inhibition of mycelial growth rate for (*Botryosphaeria dothidea* and *Fomitiporia mediterranea*) with the respective.

The results mirrored that inhibition is dose dependent manner, that the highest concentration inhibits more efficiently the fungi. According to the statistical analysis, the *E. globulus* EO exhibited different degrees of inhibition on the growth of the tested fungi. The MIC were 7.5, 1,875 μL.mL⁻¹ against *Fomitiporia mediterranea* and *Botryosphaeria dothidea*, respectively. This result confirms those of Kenfaoui et al (2023) who reported the efficacy of the EO of *E. globulus* against grapevine trunk diseases. The same results showed that EO prevented the growth of the tested microorganisms with an inhibition zone medium diameter increasing proportionally with the applied concentration. According to Jacobson (1989), the most promising plants belong to the Meliaceae, Rutaceae, Asteraceae, Myrtaceae, Abiateae, and Canellaceae families. A wide variety of EOs are known to possess antimicrobial properties and in many cases this activity is due to the presence of active constituents, mainly attributed to isoprenes such as monoterpenes, sesquiterpenes and related alcohols, other hydrocarbons and phenol. This study shows that *E. globulus* EO were characterized by the high proportion of oxygenated monoterpenes, monoterpenes and oxygenated sesquiterpenes were mainly represented by 1,8-Eucalyptol and α-Pinene, 1, 8-Eucalyptol (C₁₀H₁₈O) was the major metabolite present in the EO extract from leaves. In addition, study showed a significant concentration (76.33%) compared to the other previously identified compounds (Rosato et al. 2007; Micucci et al. 2020; El Khetabi et al. 2021). These reports are further supported by our finding about 1,8-Cineole which showed high inhibitory activities against *C. albicans* and *P. vulgaris* with MIC values of 31.3 and 62.5 μg/mL respectively. However, it has been shown that the oils have greater potential for antimicrobial activities than those of 1,8-Cineole as their main

Fig. 1 Inhibitory effect of *E. globulus* EO against *B. dothidae* **A** and *F. mediterranea* **B** fungi, showing the mycelial growth rate versus time and applied dose



component. Otherwise, other compounds such as Limonene, α -Pinene, *P*-Cymene, and Terpineol-4-ol, which have relatively strong antimicrobial activities may be responsible for this activity. Therefore, the synergistic effects of these active chemicals with other constituents of the EOs should be taken into consideration for the antimicrobial activity.

In this work, we studied the EO obtained from adult leaves collected in 2013 at spring period.). Several components detected in the Algerian *E. globulus* EO have been reported as efficient antibacterial or antifungal agents, such as 1,8-cineole (Cimanga et al. 2002), α -Terpineol, Terpinen-4-ol, α -Phellandrene (Dorman & Deans 2000). It has been demonstrated in the literature that the inhibitory activity of an EO results from a complex interaction between its different constituents, which may produce additive, synergistic or

antagonistic effects, even for those present at low concentrations (Xianfei et al. 2007), 1,8-Cineole in combination with camphor has shown higher antimicrobial effects (Viljoen et al. 2003; Kenfaoui et al. 2023). On the other hand, the compounds present in the greatest proportions are not necessarily responsible for the greatest share of the total activity, and then, the activity could be attributed to the presence of minor components such as Camphene, Paracymene, β -Pinene and γ -Terpinene known already to exhibit antibacterial activity (Viljoen et al. 2003). Results obtained from the antifungal tests, using the EO from *E. globulus* had excellent inhibitory effects against both *B. dothidae* and *F. mediterranea*. The type of action of antimicrobial agents depends on the kind of microorganism and evidence designated that in the case of EOs, it is largely associated with cell membrane damage.

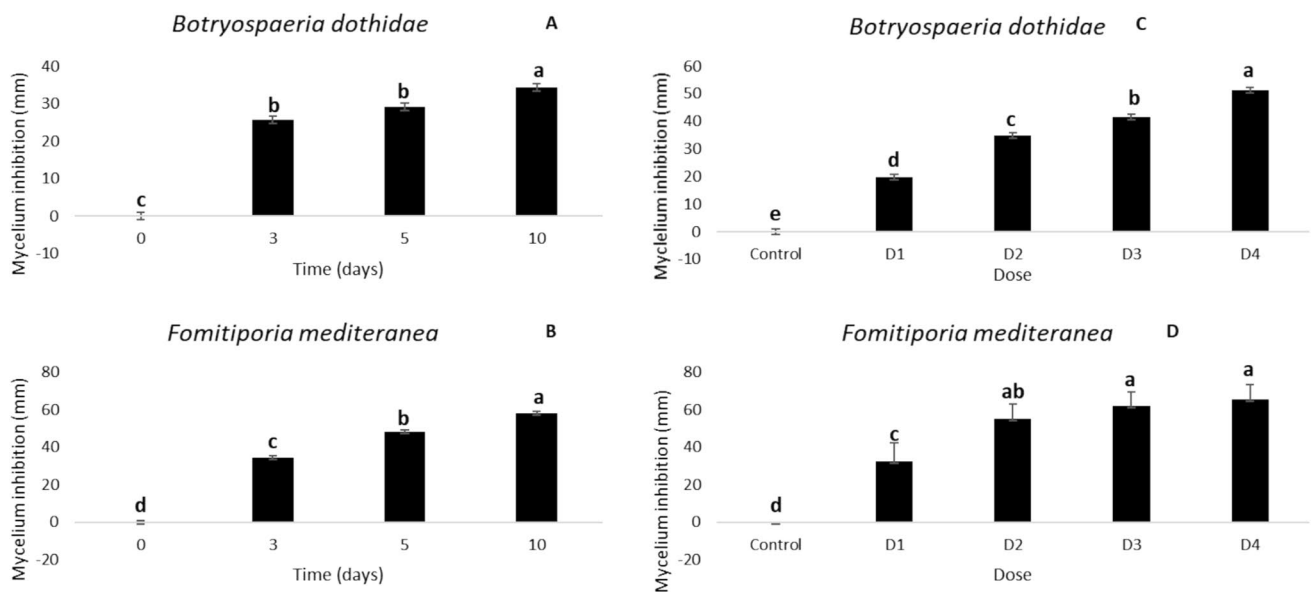


Fig. 2 The EO effect of *E. globulus* during the duration of treatment **A** against the *Botryosphaeria dothidae* **B** *Fomitiporia mediterranea* and versus applied dose **C** and time **D**. D1: 0.25%, D2: 0.50%, D3: 0.75% and D4: 1%, control: T1: 03 days, T2: 05 days and T3: 10 days. For each histogram, columns with the different letters are significantly different according to Duncan means comparison test at the probability level of $P < 0.05$

The nature of their chemical components facilitates their accumulation in lipid-rich structures and causes structural and functional damage to the membranes.

The essential oil of *E. globulus* presented an interesting fungal inhibitory activity, due to the composition of these EOs in active compounds. Several studies have already reported efficient activities of essential oils on both fungi and bacteria (Hur et al. 2000; El Khetabi et al. 2021; Bendifallah and Merah 2023; Ammad et al. 2018).

Conclusion

The results in this study revealed the presence of 27 constituents in the EO composition of *E. globulus* growing at Blida region in North Algeria. The comparison with literature data, showed that the major constituents of studied EO were similar to those reported elsewhere, although there were differences in the percentage composition. The antifungal activity of EO from leaves and flower parts of *E. globulus* may be directly associated with their major constituents or the presence of synergy between the major and minor constituents within the oil. As a result, the antifungal activity was probably associated with the high percentage of oxygenated monoterpene (80.07%), which exhibited also moderate to strong activities against the assayed microorganisms. To the best of our knowledge, this study reports for the first time the antifungal activity of *E. globulus* EO against wood fungi in viticulture field. Our finding suggested that

Eucalyptus oil can be regarded as a new original vine biopesticide that is highly efficient, broad spectrum and safe. The practical application of Eucalyptus oil as a biological pesticide should involve consideration of the application modes and time according to the different fungistatic rates (i.e., weather, temperature, and other properties). Besides, some additives (adjuvants) to the Eucalyptus oil also should be developed to reduce its volatilization. Further investigations for the mode of the constituents' actions, on alteration of mycelium and field evaluation are necessary. These results are useful in selective, biodegradable and naturally produced antifungal compounds.

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Data availability Data will be available upon request.

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