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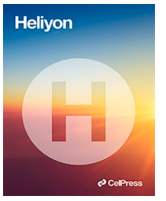

Research article

Antimicrobial, anti-enzymatic and antioxidant activities of essential oils from some Tunisian *Eucalyptus* species

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A B S T R A C T

Many plants can produce essential oils (EOs), having various biological properties. This study evaluated the antioxidant, anti-enzymatic and antimicrobial effects of the EOs derived from leaves of *Eucalyptus cladocalyx, E. angulosa, E. microcorys, E. ovata, E. diversicolor, E. saligna, E. sargentii* and *E. resinifera*. The antioxidant activity of the EOs was carried out with three different methods (ABTS, DPPH and FRAP). In addition, their anti-colinesterases, anti α-amylase and anti α-glucosidase effects were assessed by spectrophotometric assays. The antimicrobial activities were tested against six phytopathogenic bacterial strains, including two G + ve (*Bacillus mojavensis* and *Clavibacter michiganensis*) and four G-ve (*Pseudomonas fluorescence*, *P. syringae*, *Xanthomonas campestris* and *E. coli*). The current study has also investigated the inhibition of biofilm formation and the possible effect on bacterial cells biofilm metabolism of three Gram-negative (*Pseudomonas aeruginosa*, *Escherichia coli* and *Acinetobacter baumannii*) and two Gram-positive pathogenic bacteria (*Staphylococcus aureus* and *Listeria monocytogenes*). The ABTS and DPPH tests indicated that *E. diversicolor* and *E. saligna* EOs showed high antioxidant activities, whereas FRAP test suggested that *E. diversicolor* EO exhibited the better antioxidant activity. *E. resinifera* and *E. ovata* EOs were the most active against cholinesterases instead *E. ovata* and *E. sargentii* EOs were more active against enzymes involved in diabetes. Antibacterial assays revealed that *E. ovata* and *E. saligna* EOs possess significant activity closely to tetracycline. Whereas, the antifungal assay revealed that all EOs have effectively suppressed the tested fungal growth. *E. saligna* EO showed substantial efficacy inhibiting both the mature biofilm (85.40 %) and metabolic activities (89.80 %) of *L. monocytogenes*. These results demonstrate the wide range of possible uses for *Eucalyptus* EOs in both agriculture and medicine fields, suggesting potential uses as strong anti-biofilm agents and for biocontrol of phytopathogens.

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1. Introduction

Medicinal plants have always been a great resource of new compounds with important biological properties [1].

Nowadays, there is a growing interest in the use of essential oils (EOs) as a potential alternative to conventional pesticides both for the serious effects on the health and for the growing resistance to pesticides, parasites and pathogens [2]. They were also employed as a natural preservative for food because of their potency on pathogenic germs. On the other hand, a new generation of inventive and risk-free treatments for several animal, plant, and human diseases has been made possible by the application of EOs in the pharmaceutical sector [3].

Myrtaceae is among the most common commercial aromatic plant families in worldwide history and it's got excellent economic and nutritional values to treat variety of diseases [4].

It is a botanical family with commercial potential thanks to its aroma and its bioactive and antioxidant compounds [5]. The genus *Eucalyptus*, belongs to Myrtaceae and is native to Australia with over 900 species. It has been exported throughout the world, also in Tunisia [6]. *Eucalyptus* species constitute multiproduct plants par excellence. It is cultivated not just for pulp, timber, and wood, but theirs extracts present significant therapeutic and medicinal characteristics. , [7]. It has long been regarded as one of the most valuable sources of EOs. Numerous studies were carried out to establish their chemical composition. The medicinal value of *Eucalyptus* EOs is mostly determined by its content of a specific component, which is eucalyptol (1,8-cineol) [8].

The emergence of antimicrobial resistance began very soon after the introduction of antibiotics so in recent years the re-evaluation of natural substances as a source of new possible antibacterial drugs [9].

For hundreds of years, *Eucalyptus* EOs have been recognized as being antibacterial, antifungicidal, and antiseptic in nature. In fact, bibliographic research found that EOs from various *Eucalyptus* species have potential antimicrobial action against several food and plant pathogens [10,11]. They were efficient in combating both gram-positive and gram-negative bacteria [9].

Moreover, the impact on human health of synthetic antioxidants, added to food to prevent oxidative damage during production and shelf-life, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ) and propyl gallate (PG) is a topic of great controversy. Antioxidants from natural sources can be an alternative to synthetic compounds. Natural compounds are largely safer than synthetic ones and they have the advantage to display high antioxidant potential by the combination of multiple mechanisms (radical scavenge activity, metal chelation and hydrogen donation) [12].

Furthermore, it has been reported that these EOs have antioxidant and anti-diabetic properties because they scavenge reactive oxygen species (ROS) and inhibit alpha-amylase and alpha-glucosidase. Since ROS are known to exacerbate diabetes, *Eucalyptus* EOs can assist in curing it by inhibiting enzymes and scavenging ROS [13].

The aims of this study was to assess the biological properties of eight *Eucalyptus* species' EOs. Theirs antioxidant activity was studied with three different methods (DPPH, ABTS and FRAP) and their anti-enzymatic activity against cholinesterases, and against α-amylase and α-glucosidase was evaluated by spectrophotometric assays.

In addition, the antibacterial activity was evaluated against six phytopathogenic bacterial strains (*Bacillus mojavensis, Clavibacter michiganensis, Escherichia coli, Xanthomonas campestris, Pseudomonas syringae* and *Pseudomonas* fluorescens) and the antifungal activity was carried out against *Penicillium italicum, Botrytis cinerea* and *Monilinia laxa*. Finally, the biofilm inhibitory efficacy and the impact of the tested EOs on cellular metabolism in the biofilm was studied on three Gram-negative (G-ve) *Escherichia coli*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*, and two Gram-positive (G + ve) *Listeria monocytogenes* and *Staphylococcus aureus* bacterial strains.

2. Materials and methods

2.1. Essential oils

The *Eucalyptus* EOs have been obtained and characterized, as reported in Ayed et al.[14].

2.2. Antioxidant activity

2.2.1. DPPH assay

The antioxidant activity was tested using 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay test as reported by Brand-Williams et al. [15], with few modifications. Final tested concentrations of EO dissolved in methanol ranging from 5 to 20 mg/mL were obtained. . The analysis was carried out putting 25 μL of a methanol solution of the EOs in MeOH to 975 μL of a DPPH methanol solution (60 μM) prepared fresh each day and stored in the dark to have a final volume of 1 mL. Methanol was considered as a blank, and 1 mL of a solution of DPPH (60 μM) was used as a control. After 45 min, absorbance at 515 nm was read by a spectrophotometer Thermo scientific Multiskan GO (Thermo Fischer Scientific, Vantaa, Finland). The antioxidant effect of the samples was determined as the Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) equivalent's antioxidant capacity employing the calibration curve: $y = 3.7743 \times - 2.1271$, $R2 = 0.9985$. The assays were conducted in triplicate, and the results were calculated in Trolox equivalent antioxidant capacity (TEAC) with the following formula:

$$\mathrm{TEAC} = \frac{c * V * f}{m}$$

where c is the Trolox concentration (μmol/ml), V is the sample volume (ml), f is the dilution factor, and m are the grams of the sample.

Much high was TEAC value more radical scavenging activity was present.

2.2.2. ABTS•+ free radical scavenging potential

The 2,2-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) test was performed following the protocol of Re et al. [16]. The ABTS and $K_2S_2O_8$ solutions with a final concentration of 7 and 2.45 mM, respectively, were mixed and stored in the dark at room conditions for 16 h to produce the radical ABTS (ABTS+). The day after, the ABTS solution was diluted with water to have an OD of 0.800, at 734 nm. In triplicate, 10 μL of the different concentrations of EOs previously dissolved in methanol (final concentrations, ranging from 5 to 50 mg/mL) and 190 μL ABTS + were put in each well for analysis. Ten microliters of sodium phosphate buffer and 190 μL of distillated water were put in three wells for the control. The results were expressed as the μM Trolox equivalent antioxidant capacity (TEAC) per gram of samples according to the formula reported by Bunea et al. [17]:

$$\text{ABTS value } (\mu\text{mol Trolox/g sample}) = \frac{c * V * f}{m}$$

where c is the Trolox concentration (μmol/ml), V is the EO volume (ml), f is the dilution factor, and m is the weight of the EO (g).

2.2.3. Ferric-reducing antioxidant power (FRAP) assay

The FRAP assay was carried out as reported by Benzie and Strain [18]. Briefly, the FRAP solution was formulated from sodium acetate buffer with a concentration of 300 mM and a pH of 3.6, a 10 mM solution of 2,3,5-triphenyltetrazolium chloride (TPTZ) in 40 mM HCl, and a 20 mM solution of ferric chloride in a volume ratio of 10:1:1, respectively. The FRAP solution was obtained fresh every day. Eight μL of the diluted sample (final concentrations ranging from 5 to 50 mg/mL), was mixed with 264 μL of the FRAP solution. The mixture was put at 37 °C for 30 min, then the absorbance was acquired at 593 nm using a Thermo Scientific Multiskan GO spectrophotometer. $FeSO_4$ solution was allowed to obtain a standard curve, and the results were calculated as μmol/L and then converted in mmol Fe (II)/g of EO.

In particular was used this formula reported by Benzie and Choi [19]:

$$FRAP\ value\ (mmol * L^{-1}) = \frac{\text{A593 nm of test sample reaction mixture}}{\text{A593 nm of } Fe^{2+} \text{ standard reaction mixture}} * Fe^{2+} standard\ concentration\ (\mu mol * L^{-1})$$

2.3. Anti-enzymatic activity

2.3.1. Cholinesterases inhibition

Cholinesterase activity was assessed by the colorimetric assay of Ellman et al. [20] with some modifications. Briefly, to 415 μL of Tris-HCl solution (0.1 M, pH 8) were added 10 μL of EO dissolved in MeOH at various concentrations (100, 10, 1 and 0.1 mg/mL) and 25 μL of AChE (or BChE) solution (0.28 U/ml) were added. The mixture was placed for 15 min at 37 °C. Then, a solution of 1.83 mM of acetyltiocoline iodide (AChI) or butyrylcholine iodide (BChI) (75 μL) and 475 μL of 5,5-dithio-bis-2-nitrobenzoic acid (DTNB) were added and the reaction was placed for 30 min at 37 °C. Absorbance were obtained at 405 nm using a spectrophotometer. Galantamine was used as a reference drug. The enzyme activity was calculated as a percentage using the below formula.

$$\% = [(A_C - A_E) / A_c] * 100$$

Where, A_C represents enzymes solution absorbance without any EOs, whereas A_E represents enzyme solution absorbance after contact with EOs. The IC_{50}, which represents the EO concentration that causes 50 % inhibition, was derived by plotting the percentage inhibition versus various samples concentrations.

2.3.2. α-Amylase inhibition assay

Amylase inhibition was evaluated following the Bernfeld methodology [21], slightly modified. One hundred microliters of EO at various concentrations (20–0.5 mg/mL) were added to 200 μL of a sodium phosphate buffer (20 mM, pH = 6.9) and 100 μL of amylase solution (10 U/mL). The reaction was placed for 10 min at 37 °C. This, was followed by the addition of 180 μL of 1 % starch water solution and incubation at 37 °C for 20 min. One hundred and 80 μL of 3,5- dinitrosalycyclic acid (DNSA) aqueous solution (96 mM) were added in the mixture and boiled 100 °C for 10 min. Finally, the mixture was cooled by adding 600 μL of distilled water and the absorbance was read at 540 nm using a UV spectrophotometer. The inhibition of the enzymatic activity of the enzymes studied was calculated as a percentage using the below formula.

$$\% = [(A_C - A_E) / A_c] * 100$$

Where, A_C represents negative control absorbance (in this mixture reaction instead of 100 μL of sample, 100 μL of buffer were added), whereas A_E represents enzyme solution absorbance after contact with EOs. The IC_{50}, which represents the EO concentration that causes 50 % inhibition, was derived by plotting the percentage inhibition vs samples concentrations.

2.3.3. α-Glucosidase inhibition assay

α-Glucosidase inhibitory activity was assessed following the method proposed by Si et al. [22] with some modifications. Briefly, the assay was performed in a 96-well plate by first adding 150 μL of 0.1 M phosphate buffer at pH 7.0 followed by the addition of 10 μL of

dissolved EO in MeOH to obtain various concentrations (20 - 5 mg/mL). Then, 15 μL of the α-glucosidase enzyme water solution (1 U/mL) were added to each well to initiate the reaction, and the plate was placed for 5 min at 37 °C; after 75 μL of the substrate 4-nitrophenyl α-D-glucopyranoside (2.0 mM) were added, then the plate was incubated for 10 min at 37 °C. The plate was read at 405 nm with a UV Spectrophotometer. Acarbose was used as a reference drug and phosphate buffer as a negative control. The results were expressed as IC_{50} values. Every assay was repeated three times.

The inhibition of the enzymatic activity of the enzymes studied was determined as a percentage using the below formula.

$$\% = \left[\frac{A_C - A_E}{A_c}\right] * 100$$

Where, A_C represents negative control absorbance (in this mixture reaction instead of 10 μL of sample. 10 μL of buffer were added), whereas A_E represents enzyme solution absorbance after addition of EOs. The IC_{50}, which represents the EO concentration that causes 50 % inhibition, was derived by plotting the percentage inhibition versus various samples concentrations.

2.4. Antimicrobial activity

2.4.1. Tested bacteria and fungi

Six phytopathogenic bacterial strains were used, including two G + ve (*Bacillus mojavensis* and *Clavibacter michiganensis*) and four G-ve (*Pseudomonas fluorescence*, *P. syringae, Xanthomonas campestris* and *E.coli*). The above bacterial strains have been cultured on King B nutrient media (KB)for 36 h at 30 °C. In addition, five pathogenic bacterial strains were used, including three G-ve (*Acinetobacter baumannii* ATCC 19606, *Pseudomonas aeruginosa* DSM50,071 and *Escherichia coli* DSM 8579) and two G + ve (*Listeria monocytogenes* ATCC 7644 and *Staphylococcus aureus* subsp. *aureus* ATCC 25923) these strains were cultivated in Luria Broth for 18 h at 37 °C, except for *A. baumannii*, which was cultivated under these same conditions but at 35 °C. Three phytopathogenic fungal isolates were tested (*Botrytis cinerea, Penicillium italicum* and *Monilinia laxa*). All tested fungal isolates were identified by classical and molecular methods and conserved as pure cultures in the collection of the School of Agricultural, Forestry, Food and Environmental Sciences (SAFE), University of Basilicata, Potenza, Italy. They were activated on potato dextrose agar (PDA) at 24 °C ± 2°C.

2.4.2. Antibacterial assay

The antibacterial activities of the eight tested EOs were carried out following the disk diffusion method [23]. Briefly, the suspension of each bacterial strain tested was prepared at 10^6 CFU/mL (OD ≈ 0.2 nm) and adjusted by turbidimetry (Biolog, Hayward, CA, USA). Four mL of each suspension were mixed with soft agar (0.7 %) in a ratio (9:1, *v/v*) and poured into a Petri dish (Ø 90 mm). EOs were applied by soaking blank filter discs (6 mm) for 15 min in the following EOs concentrations 10000, 1000 and 100 ppm and then placed on pre-inoculated King's B plates. The negative control is a plate without EO imbibed filter discs. Antibacterial activity was evaluated by measurement of the inhibition zone diameter in mm (± SD), eventually formed around each filter disc compared to tetracycline at 1.6 mg/mL, used as a positive control.

2.4.3. Antifungal assay

The antifungal activity of the EOs was determined following the incorporation method as reported by Elshafie et al. [24]. Briefly, each studied EO was incorporated at 45 °C into Potato Dextrose Agar medium (PDA) at three concentrations, 10000, 1000 and 100 ppm. Five mm fungal-agar disc obtained from fresh fungal culture (96 h) were inoculated at the center of Petri dish (Ø 90 mm) and placed at 24 °C for 96 h. Petri dishes containing only PDA were singularly inoculated with each fungus, were considered as negative control. While, the synthetic fungicide azoxystrobin (0.8 μL/mL) was used as a positive control. The diameter fungal mycelium growth diameter was measured in mm (± SDs) and the percentage of mycelial growth inhibition (MGI %) was calculated according as reported by Petrachaianan and coworkers [25].

$$\text{MGI \%} = (\text{Gc- Gt} \ / \ \text{Gc}) \ \text{X} \ 100$$

Where, MGI is the percentage of inhibition of mycelium growth inhibition; Gc stands for the mean diameter of the fungal mycelium in the negative control PDA dish; Gt represents the fungal mycelium average diameter on treated PDA dish with EOs.

2.4.4. Biofilm and metabolic assays

2.4.4.1. Biofilm inhibitory activity. The inhibitory activity on mature biofilm was performed using Microtiter plates with 96-well flat-bottomed wells [26]. The 72-h bacterial cultures were adjusted to a 0.5 McFarland standard with fresh culture broth. Bacterial cultures were added to the wells at a rate of 10 μL per well and incubated at 37 °C (35 °C for *A. baumannii*). After 24 h, the planktonic cells were extracted and substituted with 10 or 20 μL/mL EO in each well. The final volume of each well was brought to 250 μL with various amounts of Luria-Bertani broth. Plates were closed with parafilm tape to prevent evaporation and placed at 37 °C (35 °C for *A. baumannii*) for an additional 24 h. After the removal of the planktonic cells, the sessile cells were washed two times using sterile PBS and treated with methanol (200 μL/well) for 20 min to fix the sessile cells, which were then stained with 200 μL of a violet crystal at 2 % w/v solution per well for 20 min. The staining solution was eliminated and the plates were rinsed using sterile PBS. The bound dye was released by adding 200 μL of 20 % w/v glacial acetic acid. A spectrophotometer (Cary Varian, Palo Alto, CA, USA) was used for determining absorbance at λ = 540 nm. Inhibitory activity against mature biofilm was calculated as a percentage relative to the control

(cells cultured without the samples were considered to have 0 % inhibition). Triplicate testing were conducted and average results were calculated.

2.4.4.2. EO's effect on metabolic activities in biofilm cells. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric technique was applied to assess the effect of the EOs on bacterial cells metabolism within the mature biofilm [26]. Two EOs doses (10 and 20 μL/mL) were added after 24 h of bacterial incubation, performed as above described, after eliminating the planktonic cells. The planktonic cells were taken out after a further 24 h of incubation. Subsequently, 150 μL of PBS were added, and then 30 μL of 0.3 % MTT was introduced. The microplates were then put for 2 h at 37 °C (35 °C for *A. baumannii*). The MTT solution was eliminated, followed by two gently wash steps with 200 μL of a sterile physiological solution. At the end, 200 μL of dimethyl sulfoxide (DMSO) was added to dissolved the formazan crystals, and, the plate was put at 37 °C (or 35 °C for *A. baumannii*), and then absorbance measurements were made at 570 nm (Cary Varian, Palo Alto, CA, USA).

2.5. Statistical analysis

All tests were performed in triplicate, and the results were provided as the mean ± the standard deviations (SD). Using GraphPad Prism 6.0 software, the antimicrobial activities were statistically analyzed using two-way ANOVA, followed by Dunnet's test at a confidence level of 0.05. The results of the antioxidant and anti-enzymatic analyses were statistically examined utilizing the SPSS 26 software package and one-way ANOVA. Following that, Tukey's post-hoc test was used to examine differences between means at a significance level of 0.05.

3. Results and discussion

3.1. Antioxidant activity

The antioxidant activities of the EOs were assessed using more than one test: DPPH, ABTS and FRAP to obtain a real profile of anti-radical potential. In the DPPH test, the Trolox equivalents (TE) obtained ranged from 7.8 ± 1.9 to 138.7 ± 0.8 μM/g TE. The results are in order *E. saligna* > *E. diversicolor* > *E. resinifera* > *E. cladocalyx* > *E. microcorys* > *E. ovata* > *E. angulosa* > *E. sargentii* (Table 1). In addition, all the EOs examined presented comparable activity using the ABTS test with TE values ranging between 16.1 ± 1.3 (*E. angulosa* EO) to 118.1 ± 19.7 μM/g TE (*E. diversicolor* EO). The other EOs showed an ascending TE value in this order: *E. angulosa* < *E. ovata* < *E. cladocalyx* < *E. microcorys* < *E. resinifera* < *E. saligna* < *E. diversicolor*. The EO of *E. sargentii* showed no activity. In the FRAP assay, the EO of *E. diversicolor* showed the higher FRAP values followed by *E. ovata*, *E. saligna*, *E. resinefera*, *E. cladocalys*, *E. microcorys*, *E. angulosa* and *E. sargentii*. Based on these results of the EO of *E. diversicolor* presented the highest antioxidant activities, whereas the EO of *E. sargentii* showed the lowest antioxidant activities.

The results of the DPPH, ABTS, and FRAP tests underline the value of *Eucalyptus* EOs as a natural source of antioxidants. Two studies have been conducted to date to evaluate the antioxidant properties of several *Eucalyptus* EOs, including *E. oleosa* [27], *E. eugenioides*, *E. alba*, *E. stoatei*, *E. fasciculosa* and *E. robusta* [28]. However, there is a lack of available literature specifically examining the antioxidant potential of *E. angulosa*, *E. cladocalyx*, *E. diversicolor*, *E. microcorys*, *E. ovata*, *E. resinifera*, *E. saligna* and E. *sargentii* using these specific tests.

The combination of chemical constituents in the examined EOs could account for their antioxidant properties. Notably, in the EO of *E. diversicolor*, the major constituents are *p*-cymene and α-terpineol, constituting approximately 20.88 % and 17.72 %, respectively. These components likely contribute to the overall antioxidant activity of the EO. However, it is worth mentioning that previous research has indicated that *p*-cymene alone does not demonstrate substantial antioxidant activity [29]. This suggests that the higher antioxidant activity observed in *E. diversicolor* and *E. saligna* EOs may be attributed to the synergic action between these specific components and other constituents within the EO, including α-terpineol.

Table 1
Antioxidant activity of the eight EOs.

	DPPH TEAC* (μM g^{-1})	ABTS TEAC* (μM g^{-1})	FRAP (mM Fe (II)/g EO)
E. angulosa	26.8 ± 5.0^{b}	16.1 ± 1.3^{a}	12.6 ± 0.3^{a}
E. cladocalyx	82.7 ± 6.3^{d}	38.2 ± 2.7^{abc}	43.4 ± 1.1^{c}
E. diversicolor	137.5 ± 1.9^{e}	118.1 ± 19.7^{d}	471.9 ± 18.4^{f}
E. microcorys	43.6 ± 2.8^{c}	45.7 ± 3.9^{bc}	27.5 ± 0.7^{b}
E. ovata	29.4 ± 8.1^{b}	29.0 ± 2.7^{ab}	101.7 ± 3.7^{e}
E. resinifera	82.7 ± 10.2^{d}	57.2 ± 15.7^{c}	48.8 ± 1.2^{c}
E. saligna	138.7 ± 0.8^{e}	65.4 ± 5.5^{c}	65.1 ± 1.7^{d}
E. sargentii	7.8 ± 1.9^{a}	–	0.7 ± 0.0^{a}

Data are the mean ± SD determined with three experiments. Different letters indicate mean values significantly different at $p < 0.05$, according to a one-way ANOVA followed by Tukey's post-hoc test.

[a] *TEAC, Trolox equivalent antioxidant capacity, quantified as μM of Trolox equivalents per gram of EO. The conversion of absorbance values to Trolox activity per gram of samples was performed using a standard curve.

Therefore, it can be inferred that the increased presence of α-terpineol contributes to the enhanced antioxidant activity observed. Our findings can be supported by the reported potent antioxidant activity of α-terpineol, which is present in the volatile extract of the *Eucalyptus* leaves [30]. In the same research, it was observed that eucalyptol, the primary compound in *E. sargentii* and *E. angulosa*, exhibited weak antioxidant activity when evaluated using the FRAP method [30]. Furthermore, the DPPH method showed that eucalyptol had almost no free radical scavenging activity. These findings from the same study shed light on the relatively low antioxidant activity observed in these species compared to *E. diversicolor* EO.Additionally, the observed antioxidant activity in this EO may be attributed to the combined action of its components, including thymol and carvacrol, both known for their antioxidant properties [31].

3.2. Anti-enzymatic activity

Table 2 reports the anti-enzymatic activity of the eight EOs. To our knowledge this study is the first that evaluated the activities against AChE, BChE, α-amylase and α-glucosidase of these EOs.

In *E. microcorys*, *E. resinifera*, *E. sargentii* and *E. angulosa* EOs were the main active against AChE with IC_{50} values ranging from 0.3 to 0.4 mg/mL. The other EOs showed IC_{50} values following this acending order: *E. saligna* < *E. cladocalyx* < *E. diversicolor* < *E. ovata.* Instead, for that concern activity against BChE, *E. ovata* and *E. diversicolor* EOs were more active than the other EOs with IC_{50} values of 1.6 and 2.3 mg/mL, respectively, followed by *E. saligna* and *E. sargentii* EOs. The other four EOs showed no activity against BChE even if they were active against AChE.

E. ovata EO was the most active against α -amylase with an IC_{50} value of 0.2 mg/mL followed in order by *E. cladocalyx*, *E. saligna*, *E. resinifera*, *E. diversicolor*, *E. angulosa* and *E. sargentii* EOs.

Instead, *E. sargentii*, *E. microcorys*, *E. ovata*, *E. cladocalyx*, *E. angulosa* EO were the most active against α-glucosidase with IC_{50} values ranging from 7.4 to 10.5 mg/mL.

Only four *Eucalyptus* species were studied previously for their possible anti-AChE activity E. *globulus, E. camaldulensis, E. intertexta* and *E. diversifolia* [32–34]. Against BChE only the activity of *E. globulus* EO was reported [31]. The activity of the EOs against cholinesterase activities probably was due to the presence of eucalyptol that showed an IC_{50} of 0.052 mg/mL against AChE as reported by Petrachaianan and coworkers [24]. Moreover, this compound was active also against BChE with a 48.2 % inhibition at 1 mg/mL [35]. Moreover, *p*-cymene, that is present in *E. resinifera* EO as the main component and in *E. saligna*, *E. ovata* and *E. diversicolor* in lower percentages, has been shown an IC_{50} of 0.015 mg/mL against AChE and BChE [36]. Only four *Eucalyptus* EOs were analyzed for their possible inhibition on α-amylase and α-glucosidase: *E. globulus* [37,38], *E. camaldulensis* [13], *E. gunni* and *E. pulverulenta* [39].

3.3. Antibacterial activity

The data presented in Table 3 outlines the potential antibacterial activities of the EOs against the studied bacterial strains. In particular, tetracycline exhibited the most significant activity of the majority of the tested phytopathogenic bacteria. Interestingly, *E. ovata* and *E. saligna* EOs displayed notably significant antibacterial activity against *X. campestris*, with inhibition zone diameters of 36.5 and 37.5 mm, respectively, which closely resembled the effectiveness of tetracycline, having a 36.0 mm inhibition zone diameter. Moreover, *E. resenifera* and *E. microcorys* exhibited pronounced activity against *C. michiganensis*, with an inhibition zone diameter of 33.0 mm, surpassing the positive control tetracycline, which yielded a 30.5 mm inhibition zone diameter. Regarding *B. mojavensis*, it is noteworthy that *E. cladocalyx* exhibited the highest activity among the studied EOs. However, it's important to emphasize that this effectiveness, although significant, was lower compared to that of tetracycline. However, none of the studied EOs demonstrated activity against *E. coli*, *P. fluorescens* and *P. syringae*.

In summary, our study demonstrates a significant variation in the antibacterial potential of *Eucalyptus* EOs. This could be explained by the differences in sensitivity of strains within the same species; this phenomenon was previously described by Sherlock et al. [40]. Additionally, the variability of antibacterial activity can be due to the distinct compositions of each studied EO [41]. The antibacterial

Table 2
Inhibitory effects of the eight EOs on AChE, BChE, and α -amylase and α-glucosidase.

Essential oils	IC_{50} (mg/ml)			
	AChE	BChE	α-amylase	α-glucosidase
E. angulosa	0.4 ± 0.1^{a}	n.a	3.6 ± 0.5^{e}	10.5 ± 2.8^{a}
E. cladocalyx	1.3 ± 0.3^{b}	n.a	0.7 ± 0.1^{b}	7.4 ± 0.7^{a}
E. diversicolor	1.9 ± 2.4^{c}	2.3 ± 1.4^{a}	1.3 ± 0.1^{cd}	14.8 ± 2.0^{b}
E. microcorys	0.3 ± 0.1^{a}	n.a	7.1 ± 0.2^{f}	9.2 ± 2.5^{a}
E. ovata	5.4 ± 0.4^{d}	1.6 ± 0.3^{a}	0.2 ± 0.0^{a}	10.3 ± 1.2^{a}
E. resinifera	0.3 ± 0.1^{a}	n.a	1.2 ± 0.2^{cd}	14.6 ± 2.0^{b}
E. saligna	1.0 ± 0.2^{b}	5.0 ± 0.5^{b}	0.8 ± 0.0^{bc}	14.3 ± 1.1^{b}
E. sargentii	0.4 ± 0.1^{a}	13.2 ± 2.3^{c}	16.7 ± 0.0^{g}	8.0 ± 0.1^{a}
Galantamine	0.007 ± 0.002	0.05 ± 0.01	–	–
Acarbose	–	–	0.004 ± 0.001	0.8 ± 0.1

Results are reported as the mean ± SD of three experiments. Different letters indicate mean values significantly different at $p < 0.05$, according to a one-way ANOVA followed by Tukey's post-hoc test. n.a = not active.

Table 3
Bacterial Growth Inhibition (mm) of EOs against phytopathogenic strains.

Tested EOs	EO Dose [ppm]	Diameter of inhibition zone (mm)					
		Gram positive		Gram negative			
		B. mojavensis	*C. michiganensis*	*X. campestris*	*P. fluorescens*	*P. syringae*	*E. coli*
E. angulosa	10000	0.0 ± 0[d]	13.0 ± 1.2[d]	28.5 ± 1.7	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]
	1000	0.0 ± 0[d]	06.0 ± 1.2[d]	18.0 ± 3.5[c]	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]
	100	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]
E. cladocalyx	10000	16.0 ± 1.2[d]	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]
	1000	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]
	100	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]
E. diversicolor	10000	0.0 ± 0[d]	13.5 ± 1.7[d]	20.5 ± 2.9[c]	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]
	1000	0.0 ± 0[d]	04.0 ± 1.2[d]	07.0 ± 2.3[d]	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]
	100	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]
E. microcorys	10000	12.0 ± 1.2[d]	33.0 ± 2.9	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]
	1000	08.0 ± 1.2[d]	21.5 ± 1.7[b]	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]
	100	05.0 ± 0[d]	11.0 ± 2.3[d]	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]
E. ovata	10000	13.0 ± 2.3[d]	0.0 ± 0[d]	36.5 ± 1.7	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]
	1000	0.0 ± 0[d]	0.0 ± 0[d]	21.5 ± 1.7[b]	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]
	100	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]
E. resinifera	10000	11.0 ± 1.2[d]	33.0 ± 2.3	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]
	1000	0.0 ± 0[d]	27.0 ± 2.3	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]
	100	0.0 ± 0[d]	13.0 ± 2.3[d]	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]
E. saligna	10000	0.0 ± 0[d]	25.0 ± 2.3	37.5 ± 2.9	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]
	1000	0.0 ± 0[d]	13.0 ± 2.3[d]	26.0 ± 1.2[a]	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]
	100	0.0 ± 0[d]	3.5 ± 0.6[d]	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]
E. sargentii	10000	0.0 ± 0[d]	17.0 ± 2.3[d]	20.5 ± 1.7[c]	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]
	1000	0.0 ± 0[d]	08.5 ± 1.7[d]	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]
	100	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]	0.0 ± 0[d]
Tetracycline		24.5 ± 2.9	30.5 ± 1.7	36.0 ± 4.6	33.0 ± 2.3	±1.73	13.0 ± 2.3

Values are expressed as the inhibitory zone's mean diameter (mm) ± SD. In comparison to the control (tetracycline at 1.6 mg/mL), the statistical significance was determined using a two-way ANOVA complemented by Dunnet's multiple comparison test, with significance levels denoted as follows.

[a] for p 0.05.
[b] for p 0.01.
[c] for p 0.001, and,
[d] for p 0.00001, with an overall significance level set at $p < 0.05$.

activity of the studied EOs could be related to their hydrophobic properties, enabling them to cross the bacterial cell membranes which leads to the release of cellular contents [42–45]. In addition, it's worth noting that the antibacterial efficacy of *Eucalyptus* EOs can be due to their high content of 1,8-Cineole, known for its potent biological and antimicrobial activity [46]. This efficacy arises from oxidative stress and damage to bacterial cell membranes, resulting in the loss of intracellular contents [47].

Furthermore, the ability of EOs compounds synergism to improve antibacterial activity is recognized, highlighting the complex nature of EO mechanisms against microbial pathogens [48]. This hypothesis is further studied by other researchers which reported that oxygenated monoterpenes such as 1,8-Cineole and α-Terpineol are recognized for their comparatively strong antimicrobial capabilities against numerous important pathogens [49].

3.4. Antifungal activity

The obtained results of the antifungal assay were summarized in Table 4. It's evident that all studied EOs showed a significant reduction of the tested fungal growth in a dose dependent manner. In fact, all studied EOs achieved a 100 % inhibition of *B. cinerea* growth at the highest used dose (10000 ppm). However, the EOs efficacy varied according to tested species of *Eucalyptus* especially at the lower tested doses (100 and 1000 ppm). Notably, *M. laxa* was the most susceptible (100 % inhibition), particularly in the case of *E. diversicolor*, *E. saligna*, and *E. ovata* at the lowest doses. In contrast, *P. italicum* generally exhibited greater resistance, except toward *E. saligna* and *E. cladocalyx*, where these two EOs demonstrated high efficacy at the higher doses, achieving 100 % inhibition. The results of this study are in accordance with our previous research carried out by Ayed et al. , which also evaluated the antifungal properties of *Eucalyptus* EOs against *Fusarium culmorum, F. oxysporum, F. redolens* and *F. matthioli*. Particularly, it was observed an inhibition in a dose-dependent manner specially in the case of *E. cladocalyx* EO emerging as the most effective. It achieved complete suppression of mycelial growth at concentrations below 3 μL/mL [14].

In addition, the obtained results are also in agree with some previous studies that have emphasized the antifungal potential of *Eucalyptus* EOs against various pathogenic fungi. The EOs from the leaves of Tunisian *Eucalyptus* species, including *E. polyanthemos*, *E. pimpiniana* and *E. oleosa*, exhibited a remarkable antifungal activity against five different *Fusarium* species [50]. In particular, *E. oleosa* EO achieved a complete inhibition of mycelium growth at 6 μL/mL for all tested *Fusarium* species. Similarly, a study conducted by Gakuubi et al. [10] reported the fungicidal properties of *E. camaldulensis* EOs even at low doses equal to 7–8 μL/mL. As

Table 4
Effects of the eight EOs on the growth of three phytopathogenic fungi.

	Dose [ppm]	Mycelium Growth Inhibition (MGI %)		
		B. cinerea	P. italicum	M. laxa
E.angulosa	10000	100 ± 0.0[d]	64.44 ± 3.14	100 ± 0.0[d]
	1000	35.55 ± 0.0	40.00 ± 6.29[b]	100 ± 0.0[d]
	100	17.78 ± 6.29[d]	10.00 ± 4.72[d]	28.89 ± 6.29
E. cladocalyx	10000	100 ± 0.0[d]	100 ± 0.0[d]	100 ± 0.0[d]
	1000	94.44 ± 7.86[d]	3.34 ± 4.72[d]	100 ± 0.0[d]
	100	25.55 ± 11.0[c]	0.0 ± 0.0[d]	31.11 ± 18.86
E. diversicolor	10000	100 ± 0.0[d]	43.33 ± 1.57[a]	100 ± 0.0[d]
	1000	36.67 ± 4.72	10.0 ± 4.72[d]	100 ± 0.0[d]
	100	13.33 ± 3.14[d]	0.0 ± 0.0[d]	100 ± 0.0[d]
E- microcorys	10000	100 ± 0.0[d]	28.89 ± 9.43[d]	100 ± 0.0[d]
	1000	34.44 ± 1.57	11.11 ± 0.0[d]	100 ± 0.0[d]
	100	8.89 ± 3.15[d]	0.0 ± 0.0[d]	24.44 ± 12.57
E- resinifera	10000	100 ± 0.0[d]	23.33 ± 1.57[d]	100 ± 0.0[d]
	1000	40.0 ± 6.29	8.89 ± 3.15[d]	61.11 ± 7.86
	100	25.55 ± 11.0[c]	0.0 ± 0.0[d]	30.0 ± 26.71
E. ovata	10000	100 ± 0.0[d]	40.0 ± 6.29[b]	100 ± 0.0[d]
	1000	82.22 ± 3.14[d]	20.0 ± 3.15[d]	100 ± 0.0[d]
	100	68.89 ± 3.15[b]	0.0 ± 0.0[d]	100 ± 0.0[d]
E. saligna	10000	100 ± 0.0[d]	100 ± 0.0[d]	100 ± 0.0[d]
	1000	100 ± 0.0[d]	42.22 ± 3.14[b]	100 ± 0.0[d]
	100	67.78 ± 7.86[a]	8.89 ± 6.29[d]	100 ± 0.0[d]
E. sargentii	10000	100 ± 0.0[d]	72.22 ± 7.86	100 ± 0.0[d]
	1000	32.22 ± 1.57[a]	23.33 ± 4.7[d]	100 ± 0.0[d]
	100	17.78 ± 6.29[d]	0.0 ± 0.0[d]	0.0 ± 0.0
Azoxystrobine 0.8 µL/mL		49.52 ± 5.41	59.23 ± 4.74	42.84 ± 2.64

Values are means ± SD of three esxperiments. In comparison to the control (Azoxystrobine), the statistical significance was determined using a two-way ANOVA complemented by Dunnet's multiple comparison test, with significance levels denoted as follows.
[a] for p 0.05.
[b] for p 0.01.
[c] for p 0.001, and,
[d] for p 0.00001, at a significance level of $p < 0.05$.

suggested by Camele et al. [51], the efficacy of EOs against phytopathogenic fungi may depend on the specific biological properties of their primary active constituents and the potential synergistic effects resulting from their combined action. In summary, these findings provide substantial evidence supporting the potential use of *Eucalyptus* EOs in natural fungicidal applications.

3.5. *Eucalyptus EOs' biofilm and its metabolism inhibition efficacy*

The results summarized in Table 5 show that the antimicrobial effects of EOs showed significant variations between eucalyptus species and bacterial strains ($p < 0.05$). As shown in Table 5, the MIC values ranging from 30 to 45 µL/mL. Since bacteria form biofilms to resist commonly used antibiotics, we used a microtiter biofilm plate assay to evaluate the biofilm inhibitory activities of the EOs

Table 5
Minimum inhibitory concentration (MIC) of *Eucalyptus* EOs against the tested bacterial strains.

	Gram negative			Gram positive	
	A. baumannii	*E. coli*	*P. aeruginosa*	L. monocytogenes	*S. aureus*
E. angulosa	36 ± 2[d]	32 ± 1[c]	32 ± 2[b]	32 ± 2[c]	30 ± 1[a]
E. cladocalyx	36 ± 2[d]	36 ± 2[d]	>45[d]	30 ± 2[a]	30 ± 2[a]
E. diversicolor	38 ± 2[d]	30 ± 2	32 ± 1[b]	30 ± 1[a]	36 ± 2[d]
E. microcorys	30 ± 1[b]	34 ± 2[c]	34 ± 1[c]	32 ± 1[c]	30 ± 1[a]
E. ovata	34 ± 1[d]	36 ± 2[d]	>45[d]	30 ± 1[a]	30 ± 1[a]
E. resinifera	34 ± 2[d]	34 ± 2[c]	>45[d]	30 ± 1[a]	30 ± 1[a]
E. saligna	34 ± 2[d]	30 ± 1	32 ± 1[b]	30 ± 1[a]	30 ± 1[a]
E. sargentii	>45[d]	32 ± 2[b]	32 ± 2[b]	30 ± 2[a]	30 ± 1[a]
Tetracycline	24 ± 2	26 ± 2	28 ± 2	26 ± 2	26 ± 2

The results are the mean ± SD of three experiments. In comparison to the control (tetracycline 8 µg/mL), the statistical significance was determined using a two-way ANOVA complemented by Dunnet's multiple comparison test, with significance levels denoted as follows.
[a] for p 0.05.
[b] for p 0.01.
[c] for p 0.001, and.
[d] for p 0.00001, at the significance level of $p < 0.05$.

against five biofilm-forming clinical pathogenic bacterial strains.

The comprehensive outcomes of these experiments can be found in Tables 6 and 7. The assays were carried out using both crystal violet and MTT tests, utilizing two different concentrations: 10 μL/mL and 20 μL/mL, lower than the minimum inhibitory concentration (MIC) determined through the resazurin test.

The investigation unveiled varying levels of inhibitory effects of the EOs on mature biofilm, with variations noted among different bacterial strains. Notably, *L. monocytogenes* displayed a heightened sensitivity to the EOs, consistently exhibiting substantial levels of inhibition in response to several EOs. At the higher concentration, the EOs consistently maintained their inhibitory effectiveness, with *E. angulosa* achieving no less than 60.30 ± 4.45 % inhibition, and *E. saligna* impressively reaching 85.40 ± 2.05 % inhibition.

S. aureus exhibited significant sensitivity to the inhibitory actions of the EOs, particularly at higher concentrations. The EOs consistently demonstrated robust inhibitory effects against *S. aureus*, with inhibition rates consistently exceeding 39.98 ± 2.32 % (*E. diversicolor* EO) and reaching as high as 78.24 ± 3.13 % (*E. saligna* EO). Similarly, *E. coli* showed sensitivity to the EOs, especially at elevated concentrations. The EOs effectively curtailed *E. coli* biofilm formation, with inhibition rates consistently surpassing 23.95 ± 2.04 % for *E. ovata* EO and reaching a maximum of 76.49 ± 2.21 % for *E. saligna* EO.

A. baumannii also appeared to be sensitive to the inhibitory effects of most of the EOs, except for *E. sargentii*. At the higher concentration, the EOs consistently maintained a strong inhibitory capacity against this strain, with the inhibition never dropping below 28.48 ± 2.21 % (*E. diversicolor* EO) and reaching as high as 80.04 ± 2.16 % for *E. microcorys* EO.

P. aeruginosa, on the other hand, exhibited varying degrees of sensitivity to the different EOs. At the higher concentration, certain EOs, such as *E. diversicolor* and *E. sargentii*, demonstrated significant inhibitory effects, with inhibition rates of 72.96 ± 3.41 % and 66.54 ± 4.42 %, respectively. However, *E. cladocalyx*, *E. resinifera*, and *E. ovata* EOs, were less effective and did not display any activity against *P. aeruginosa*.

An examination of the metabolic impact on bacterial cells within mature biofilms was explored, with a specific emphasis on utilizing the MTT test. The outcomes, as illustrated in Table 7, unveiled that the EOs demonstrated notable inhibitory effects on the metabolic processes of microbial cells within biofilms. In these tests, inhibition rates reached an impressive value of 95.86 % (*E. resinifera* EO *vs A. baumannii*). What's particularly noteworthy is that in all instances where EOs had a discernible effect, the inhibition rate consistently remained above 34.07 % (*E. diversicolor* EO *vs P. aeruginosa*), These findings underscore the consistent and

Table 6
Percent inhibition of two doses of *Eucalyptus* EOs on the mature biofilm.

	A. baumannii	*E. coli*	L. monocytogenes	*P. aeruginosa*	*S. aureus*
E. angulosa	0.00	31.95	54.77	58.56	60.19
10 μL/mL	±0.00	±2.97****	±4.31****	±4.23****	±3.67****
E. angulosa	32.92	54.79	60.30	62.92	74.13
20 μL/mL	±2.37****	±4.01****	±4.45****	±3.87****	±2.57****
E. cladocalyx	25.30	24.59	58.89	0.00	64.77
10 μL/mL	±1.78****	±2.22****	±4.13****	±0.00	±3.23****
E. cladocalyx	48.59	42.67	73.55	0.00	71.78
20 μL/mL	±2.09****	±5.04****	±2.43****	±0.00	±2.09****
E. diversicolor	25.85	67.92	76.11	0.00	37.27
10 μL/mL	±2.05****	±2.12****	±3.97****	±0.00	±2.71****
E. diversicolor	28.48	74.14	81.86	72.96	39.98
20 μL/mL	±2.21****	±3.67****	±2.02****	±3.41****	±2.32****
E. microcorys	77.85	57.50	74.46	32.24	64.07
10 μL/mL	±3.25****	±3.92****	±3.41****	±2.42****	±4.12****
E. microcorys	80.04	61.20	84.56	61.98	74.17
20 μL/mL	±2.16****	±3.42****	±2.77****	±2.91****	±3.14****
E. ovata	16.22	12.37	72.89	0.00	24.62
10 μL/mL	±1.04****	±1.17***	±2.67****	±0.00	±2.21****
E. ovata	52.06	23.95	73.27	0.00	52.78
20 μL/mL	±4.43****	±2.04****	±1.13****	±0.00	±3.98****
E. resinifera	24.05	47.29	74.84	0.00	61.86
10 μL/mL	±2.55****	±4.41****	±2.55****	±0.00	±3.99****
E. resinifera	36.77	49.01	77.45	0.00	67.94
20 μL/mL	±2.87****	±4.02****	±3.04****	±0.00	±4.04****
E. saligna	20.37	75.40	78.05	52.67	60.19
10 μL/mL	±1.51****	±2.36****	±2.44****	±2.32****	±4.07****
E. saligna	47.68	76.49	85.40	65.41	78.24
20 μL/mL	±3.31****	±2.21****	±2.05****	±2.87****	±3.13****
E. sargentii	0.00	19.32	75.38	34.94	34.75
10 μL/mL	±0.00	±1.67****	±5.01****	±3.01****	±2.04****
E. sargentii	0.98	52.22	84.78	66.54	71.28
20 μL/mL	±0.12	±3.78****	±2.21****	±4.42****	±3.89****

Results are the mean ± SD of three experiments. The percentage of inhibition was determined using the formula: (ODC - ODS)/ODC * 100, where ODC corresponds to the optical density of untreated bacteria and ODS denotes the optical density of bacteria treated with samples. At a significance level of $p < 0.05$, * denotes $p < 0.05$, ** signifies $p < 0.01$, *** represents $p < 0.001$, and **** indicates $p < 0.00001$ compared to the control (inhibition = 0). These comparisons were made using a two-way ANOVA followed by Dunnet's multiple comparison test.

Table 7
Assessment of *Eucalyptus* Species Essential Oils' Inhibitory Effects on Metabolism within mature Biofilm: Results from MTT Testing.

	A. baumannii	*E. coli*	L. monocytogenes	*P. aeruginosa*	*S. aureus*
E. angulosa 10 μL/mL	94.11 (±2.12)****	64.64 (±4.91)****	74.57 (±3.75)****	75.50 (±2.15)****	86.74 (±1.17)****
E. angulosa 20 μL/mL	95.62 (±2.01)****	84.28 (±3.17)****	86.07 (±2.09)****	82.02 (±2.01)****	89.25 (±1.05)****
E. cladocalyx 10 μL/mL	93.70 ±1.01****	76.69 ±2.52****	82.36 ±2.96****	69.84 ±3.04****	78.09 ±2.44****
E. cladocalyx 20 μL/mL	94.48 (±2.22)****	79.65 (±2.15)****	85.85 (±1.75)****	76.07 (±1.24)****	79.43 (±2.45)****
E. diversicolor 10 μL/mL	0.00 (±0.00)	42.94 (±3.97)****	19.02 (±1.12)****	30.88 (±2.57)****	0.00 (±0.00)
E. diversicolor 20 μL/mL	31.77 (±2.98)****	47.41 (±4.15)****	34.45 (±3.17)****	34.07 (±3.12)****	0.00 (±0.00)
E. microcorys 10 μL/mL	79.91 (±2.43)****	72.80 (±3.25)****	49.11 (±3.03)****	81.98 (±1.76)****	86.12 (±2.34)****
E. microcorys 20 μL/mL	85.54 (±2.31)****	82.41 (±2.43)****	60.51 (±4.44)****	85.86 (±2.12)****	90.48 (±2.04)****
E. ovata 10 μL/mL	86.63 (±2.04)****	59.85 (±3.12)****	47.51 (±3.23)****	2.63 (±0.05)	60.48 (±3.34)****
E. ovata 20 μL/mL	94.65 (±1.64)****	70.14 (±3.03)****	58.90 (±4.06)****	34.72 (±3.24)****	89.56 (±1.42)****
E. saligna 10 μL/mL	86.99 (±2.08)****	87.34 (±1.45)****	89.80 (±2.11)****	85.35 (±2.32)****	86.58 (±1.15)****
E. saligna 20 μL/mL	89.94 (±1.33)****	90.26 (±3.13)****	90.46 (±2.07)****	87.30 (±1.05)****	89.85 (±2.01)****
E. sargentii 10 μL/mL	81.15 (±1.99)****	51.34 (±4.27)****	37.70 (±3.22)****	76.12 (±2.04)****	88.35 (±2.12)****
E .sargentii 20 μL/mL	85.52 (±2.08)****	79.47 (±2.38)****	77.22 (±3.13)****	79.38 (±1.03)****	90.58 (±2.09)****
E. resinifera 10 μL/mL	76.13 (±3.87)****	60.86 (±3.07)****	80.22 (±2.92)****	76.51 (±3.65)****	79.97 (±4.82)****
E. resinifera 20 μL/mL	95.86 (±1.14)****	67.44 (±3.92)****	82.89 (±3.06)****	78.97 (±1.67)****	82.44 (±2.13)****

The percentage of inhibition was determined using the formula: (ODC - ODS)/ODC * 100, where ODC corresponds to the optical density of untreated bacteria and ODS denotes the optical density of bacteria treated with samples. At a significance level of $p < 0.05$, * denotes $p < 0.05$, ** signifies $p < 0.01$, *** represents $p < 0.001$, and **** indicates $p < 0.00001$ compared to the control (inhibition = 0). These comparisons were made using a two-way ANOVA followed by Dunnet's multiple comparison test.

substantial efficacy of these *Eucalyptus* EOs in affecting the metabolic processes of sessile cells within mature biofilms. For example, when scrutinizing *A. baumannii*, the inhibition rates consistently showed remarkable figures. E. sargentii achieved inhibition rates exceeding 85.52 %, while others, including *E. cladocalyx*, *E. resinifera*, *E. ovata*, and *E. angulosa*, attained inhibition rates surpassing 94 %. Concerning *E. coli*, the application of higher EO concentrations resulted in significant inhibitory effects, with *E. saligna* EO yielding an impressive 90.26 % inhibition, and *E. diversicolor* EO never falling below 47.41 %.

The primary reason behind the inhibitory effects of *Eucalyptus* EOs can be attributed to their influence on the metabolic processes of bacterial cells. This was particularly evident in the case of *E. saligna* EO, which not only inhibited the adhesion process by 78.05 % but also significantly influenced the metabolism of sessile cells by 89.80 %. However, when examining *E. diversicolor* EO's impact on *S. aureus*, no metabolic influence was detected.

Our data agree with previous research [11,50], and supports the effectiveness of *Eucalyptus* EOs in inhibiting biofilms across various pathogens.

Moreover, this study highlighted the increased susceptibility of the Gram-positive *L. monocytogenes* to the samples used. EOs tend to impact Gram-positive bacteria due to differences in their cell membrane compositions, a factor supported by our findings [52].

Emphasizing the significance of *L. monocytogenes* in the food industry is crucial, given its association with severe foodborne illness, particularly listeriosis. This pathogen's ability to form biofilms provides protection against typical environmental elimination methods [53].

The effectiveness of the tested EOs against biofilms seems associated with their composition, notably the presence of eucalyptol, a significant component in the EOs, as established in our earlier research [14]. Eucalyptol, known for its antibacterial properties, impacts bacterial cells by altering their shape and size. This substance induces programmed cell death in *S. aureus* and cell death due to injury in *E. coli*. Its efficacy against *E. coli* is due to its ability to disrupt the cell wall and membrane, a vital finding in combating Gram-negative bacteria where standard antibiotics often encounter impediments [54].

Moreover, the variations in the antibacterial impacts of the EOs might result from the collective effects of the complex compounds within *Eucalyptus* EOs, rather than being solely attributed to a single major component [55].

From a metabolic perspective, it appears that EOs primarily targeted the metabolism of sessile cells. However, the inability of *E. diversicolor* EOs to affect *S. aureus* metabolism suggests a different mode of action, as highlighted in previous studies on *Eucalyptus*

EOs add the reference. This indicates that the EO might have a certain impact on mature biofilm through diverse mechanisms, potentially involving interactions with DNA, bacterial cell surfaces, or other structural components [56].

Unveiling the specific alternative mechanisms behind this inhibitory effect requires further investigation. Analyzing genes associated with bacterial adhesion at a molecular level is necessary to understand the transition from the initial to the mature biofilm stage.

4. Conclusion

This work greatly improves our knowledge about the properties of several Tunisian *Eucalyptus* EOs, with particular emphasis on their antioxidant, enzymatic and antibacterial activities against bacteria affecting humans and plants. The antioxidant capacities of EOs, measured by different techniques, demonstrate notable capacities, implying a promising use as natural antioxidants in various fields. Additionally, the anti-cholinesterase activity identified in *E. ovata* and *E. resinifera* suggests a possible impact on neurological function, potentially influencing diseases such as Alzheimer's disease. The effects on α-amylase and α-glucosidase open new ways of using *Eucalyptus* EOs in medical contexts, particularly in the control of diabetes. The EOs tested have also been shown to have significant antifungal characteristics. The EOs of *E. ovata* and *E. saligna* showed robust antibacterial activity against plant pathogenic bacteria comparable to tetracycline, highlighting their potential applications in agriculture for disease management. The EO of *E. saligna* inhibited mature biofilm and metabolic activities with exceptional efficiency, indicating its importance as a potent antibiofilm agent. Further research is needed to reveal the full applications of *Eucalyptus* EOs, providing a better understanding of their benefits and expanding their potential uses to other sectors.

CRediT authorship contribution statement

Amira Ayed: Writing – original draft, Methodology, Investigation, Formal analysis. **Lucia Caputo:** Writing – original draft, Methodology, Investigation. **Vincenzo De Feo:** Writing – review & editing, Validation, Supervision, Conceptualization. **Hazem S. Elshafie:** Writing – original draft, Methodology, Investigation. **Florinda Fratianni:** Methodology, Data curation. **Filomena Nazzaro:** Investigation, Formal analysis, Data curation. **Lamia Hamrouni:** Methodology, Investigation, Formal analysis. **Ismail Amri:** Supervision, Data curation, Conceptualization. **Yassine Mabrouk:** Validation, Methodology, Formal analysis. **Ippolito Camele:** Writing – review & editing, Formal analysis, Data curation. **Flavio Polito:** Writing – original draft, Methodology, Investigation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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