Abstract: The potential application of Eucalyptus globulus essential oil (EGEO) as a natural beverage preservative is described in this research. The chemical composition of EGEO was determined using gas chromatography analyses and revealed that the major constituent is 1,8-cineole (94.03% ± 0.23%). The in vitro antioxidant property of EGEO was assessed using different tests. Percentage inhibitions of EGEO were dose-dependent. In addition, EGEO had a better metal ion chelating effect with an IC50 value of 8.43 ± 0.03 mg/mL, compared to ascorbic acid (140.99 ± 3.13 mg/mL). The in vitro antimicrobial effect of EGEO was assessed against 17 food spoilage microorganisms. The diameter of the inhibitory zone (DIZ) ranged from 15 to 85 mm for Gram-positive bacteria and from 10 to 49 mm for yeast strains. Candida albicans, C. parapsilosis and Saccharomyces cerevisiae were the most sensitive fungal species to the EGEO vapor with DIZ varying from 59 to 85 mm. The anti-yeast effectiveness of EGEO alone and in association with heat processing was estimated in a real juice matrix (Orangina fruit juice) in a time-dependent manner. The combination of EGEO-heat treatment (70 °C for 2 min) at different concentrations (0.8 to 4 µL/mL) was effective at reducing S. cerevisiae growth in the fruit juice of Orangina, compared to juice preserved with synthetic preservatives. Current findings suggest EGEO as an effective and potent inhibitor of food spoilage fungi in a real Orangina juice, and might be a potential natural source of preservative for the food industry.

Keywords: natural food preservative; Eucalyptus globulus essential oil; eucalyptol; antioxidant effect; vapor phase; Orangina fruit juice

1. Introduction

Food spoilage by fungi, yeasts and bacteria is a major problem in food production, and it considerably impacts the price and availability of the food [1]. The use of synthetic and chemical antibacterial and antifungal compounds and food preservatives is considered as one of the ancient methods for reducing foodborne pathogens and contamination. The addition of synthetic antioxidant and antimicrobial food preservatives is an active way for storage to slow down food alteration and oxidation [2]. Nevertheless, due to increasing confirmation of the dangerous properties of synthetic
food additives, there is constant pressure from consumers to decrease the quantity of these chemicals in food [3] and deliver minimally processed foodstuffs without compromising food preservation, safety and quality [4].

Thus, other sources of nontoxic, bioactive and suitable natural food preservatives need to be discovered and investigated, such as plant secondary metabolites, phytochemicals and volatile oils or essential oils (EOs). A new approach to prevent and avoid the proliferation of microorganisms or protect food from oxidation is the use of EOs as antifungal, antibacterial and antioxidant preservatives. The potential application of EOs as functional components in drinks and beverages is gaining force because of growing anxiety about possibly dangerous and toxic synthetic additives [5,6]. Within the context of the extensive variety of the aforementioned foodstuffs, a collective need is the accessibility of phytochemical extracts and EOs with an agreeable flavor or scent associated with a conserving effect and that can avoid lipid alteration, oxidation and contamination by food spoilage microorganisms and pathogens [7–9].

Therefore, there has been growing attention to the discovery and investigation of safe, effective and natural antioxidant bioactive molecules because they can defend the human body from free radicals and delay the development of several chronic or acute illnesses [10]. The antimicrobial molecules found in aromatic and medicinal plants are of interest because multidrug resistant bacteria are now becoming a global community health alarm, particularly in terms of foodborne infections and nosocomial contaminations [11–13]. Several studies have reported antiseptic, anti-inflammatory, wound-healing, analgesic, antioxidant and free radical-scavenging activities [14] from aromatic and medicinal plants, herbs, spices and EOs and, in most cases, a direct food-related application has been verified [15].

The *Eucalyptus* genus is a tall shrub belonging to the family of Myrtaceae. Although some papers about *Eucalyptus globulus* essential oils (EGEOs) have been done [16–20], only a limited of them estimated *Eucalyptus* oil’s effect against pathogens and food spoilage species [7]. In spite of the well-reported in vitro antibacterial and antifungal effects, food manufacturing has used *Eucalyptus* EOs principally as flavoring agents. Consequently, the application of EOs and phytochemical extracts as natural food additives has been restricted [16].

Despite the great efficacy of EOs and their phytochemical compounds against food-related and spoilage pathogens with in vitro methods, a similar result in a food matrix is only accomplished with a greater dose of EOs [3]. This statement suggests a sensorial and organoleptic influence from changing the ordinary flavor of the food and beverages by surpassing suitable taste thresholds [7]. Therefore, to reduce the dose of EO in a real juice matrix, studies on the associated effect of EOs with traditional conservation methods such as heat processing are required. In the current research, the chemical composition of EGEO was analyzed with gas chromatography-mass spectrometry (GC-MS). Then, the in vitro antioxidant effect was carried out using DPPH radical scavenging and metal ion chelating activity. The inhibitory effects of Algerian EGE0 against several food spoilage bacterial and fungal strains were assessed in vitro (disc diffusion and disc volatilization tests) and in a real food matrix (inhibition of *Saccharomyces cerevisiae* strain for the preservation of Orangina fruit juices) and stored at laboratory temperature for 6 days. Further, for reducing the dose of EGO in the Orangina fruit juice, the associated effect of EGO with moderate heat treatment was evaluated.

2. Materials and Methods

2.1. Material

2.1.1. Distillation of *Eucalyptus globulus* Essential Oil

*Eucalyptus globulus* EO was purchased from “Ziphee-Bio” company of essential oils (Lakhdaria, Bouira, Algeria). EGEO was extracted from the aerial part with alembic steam distillation (SD). SD is a method used to obtain EOs from *Eucalyptus globulus* by passing steam generated in a pot still through the plant material. A quantity of fresh plant (leaves and small branches of the tree) was loaded
in the still and stacked in layers to allow the appropriate delivery of the steam. When the steam passed through the *Eucalyptus globulus*, tiny pockets that hold the EOs opened to release the volatile compounds. This is referred to as the distillate. The distillate will contain a mix of hydrosol (aromatic water) and EO which return to their liquid form in the condenser and are separated using a Florentine separator. EGEO was stored in air-tight sealed glass bottles at 4 °C until further use.

2.1.2. Food Spoilage Microorganisms

Different food-spoilage bacterial strains (*Escherichia coli*, *Enterobacter sakazakii*, *Pseudomonas aeruginosa*, *Klebsiella ornithinolytica*, *Bacillus cereus* and *Staphylococcus aureus*), fungal strains (*Aspergillus niger*, *Aspergillus flavigus*, *Aspergillus fumigatus* and *Aspergillus brasiliensis*) and yeasts (*Saccharomyces cerevisiae*, *Candida parapsilosis*, *Candida albicans* and *Trichosporon sp.*) were collected and identified from different food matrices (water, milk, juices and honey) in the Laboratory of Food Microbiology (Laboratoire d’Hygiène, Blida, Algeria) and used to evaluate the microbial inhibitory effect of EGEO.

The identification of microorganisms was carried out using morphologic and biochemical characterization tests. After cell identification through Gram staining and microscopic observation was done, the traditional biochemical tests (using API 20E) were carried out to assess the tested bacteria classification following the Gram-negative bacterial identification method [21]. Different biochemical assays were used such as oxidase, TSI medium, gelatin hydrolysis, sugar assimilation, amino acid degradation, hydrogen sulfide production, citrate and Voges-Proskauer.

The fungal species were identified based on their morphological arrangements such as pigmentation, diameter of the mycelia, and microscopic determination of formation of the germ tube, spores and chlamydoconidias. Yeast strains were identified using the Auxacolor™ kit which is an identification method based on sugar digestion [22]. The growth of yeasts is assessed by the color change of a pH indicator. The Auxacolor™ system contains 16 wells in a plastic microplate. All assays with the Auxacolor™ method were carried out following the manufacturer’s guidelines. The Auxacolor™ system was stored at 4 °C and was carried to laboratory temperature before use. The fungal and bacterial species were identified with standard microbiology assays and stored in mueller-hinton agar (MHA) and sabouraud dextrose agar (SDA) for bacteria and fungi, respectively.

2.1.3. Chemicals and Reagents

Dimethyl sulfoxide (DMSO), gallic acid, butylated hydroxyanisole (BHA), L-ascorbic acid (vitamin C.), ethanol, tween 80, FerroZine™ iron reagent, 1,1-diphenyl-2-picrylhydrazyl (DPPH) and iron (II) chloride (FeCl₂) were obtained from Sigma Aldrich (St. Louis, MO, USA). Isosaline (0.9% NaCl), MHA and SDA medium were purchased from the Ideal-Labo company (Blida, Algeria). Filter paper discs (9 mm in diameter) were provided by Schleicher and Schull GmbH (Dassel, Germany). Antibiotic discs of amoxicillin-clavulanic acid (AMC, 20/10 µg), erythromycin (E, 15 µg), chloramphenicol (C, 30 µg) (Bio-Rad Laboratories, France) and antiseptic solution of Isomedine® 0.1% (Hexamidine dermal solution, Isopharma, Algiers, Algeria) were used to assess the sensitivity of isolated microorganism species.

2.2. Methods

2.2.1. Chemical Composition of *Eucalyptus globulus* Essential Oil by GC-MS Analysis

Analysis of *Eucalyptus globulus* volatile oil was done using GC-MS. Analyses were carried out on an HP 6890 gas chromatograph (Agilent Technologies, Wilmington, DE, USA) fitted with an HP-5MS fused silica column (30 m × 0.25 mm, 0.25 µm film thickness), interfaced with an HP mass selective detector 5790A (Agilent Technologies) operated by HP Enhanced ChemStation software. The oven temperature program was 45–280 °C (2 °C/min). The injector temperature was 250 °C; the carrier gas was helium, adjusted to a linear velocity of 30 cm/s; the splitting ratio was 1:20 and the detector temperature was 250 °C. Separate peaks were recognized by comparison of their Retention Index (RI) to RI of authentic models, and by comparing their mass spectra with the NIST 2007 (National Institute
of Standards and Technology, Gaithersburg, MD, USA) and Wiley 6.0 library (New York, NY, USA) mass spectral database and the literature [23].

2.2.2. In Vitro Antioxidant Activity

DPPH Radical Scavenging Assay

The radical scavenging effect was evaluated with a spectrophotometric assay based on the reduction of an ethanol solution of DPPH [24]. Briefly, a series of dilutions of E GEO was made. Then, 1.5 mL of each concentration was mixed with 1.5 mL of a 30 µg/L ethanoic DPPH solution that was incubated at laboratory temperature in a dark storeroom for 40 min. Afterward, the absorbance at 520 nm (maximum absorbance of DPPH) was noted spectrophotometrically. Separately, a negative control was made comprising all reagents except the E GEO. The free radical scavenging effect of each solution was then measured and calculated as percentage inhibition in accordance to the following equation:

\[
\% \text{ inhibition} = 100 - \left( \frac{\text{DPPH}_s}{\text{DPPH}_b} \right) \times 100
\]

where DPPHs corresponds to the absorbance of DPPH with the sample and DPPHb to the absorbance of DPPH without sample (blank).

The antioxidant ability was recorded as the IC\textsubscript{50} (medium inhibitory concentration). The IC\textsubscript{50} is defined as the quantity of antioxidant required to reduce the primary DPPH quantity by 50%. The results are expressed as the mean ± standard deviation (SD) of three tests. Ascorbic acid and BHA were used as positive standards.

Metal Chelating Activity

Briefly, different concentrations of 1 mL of E GEO were dissolved in DMSO and added to a solution of 0.05 mL of 2 mM FeCl\textsubscript{2} in H\textsubscript{2}O. The reaction was started by adding 0.02 mL of 5 mM ferrozone. The mixture was vigorously shaken and incubated at room temperature for 15 min. The absorbance of the E GEO sample was then estimated and determined at 565 nm using a spectrophotometer [24]. The metal chelating activity was expressed according to the following formula:

\[
\% \text{ inhibition} = 1 - \left( \frac{\text{Abs Control} - \text{Abs Sample}}{\text{Abs Control}} \right) \times 100
\]

Gallic acid, ascorbic acid and BHA were used as positive standards. The amount of inhibition by the test samples was expressed as the percentage of concentration required to do 50% inhibition (IC\textsubscript{50}).

2.2.3. In Vitro Antimicrobial Effect of E GEO

Disc Diffusion Method

Microbial media used for culture and growth of bacterial and fungal strains were MHA and SDA, respectively. Inoculum of each bacterial and fungal species to be tested was prepared with fresh cultures by suspending the strain in isosaline (0.9% NaCl). In the first step, the antimicrobial potential of E GEO was explored with the disc diffusion assay [7]. Antimicrobial discs of amoxicillin-clavulanic acid, erythromycin, chloramphenicol and antiseptic solution of hexamidine were used to control the antibacterial of isolated micro-organisms strains according to the NCCLS guidelines [25]. Discs without E GEO were considered as a negative control. Filter paper discs (diameter 9 mm) were saturated with three different quantities (20, 40 and 60 µL per disc) of Eucalyptus EO and sited in the inoculated microbial media (MH for bacteria and SDA for fungi). After keeping at laboratory temperature for 40 min, the Petri dishes were incubated at 37 °C/24 h for bacterial strains and at 25 °C/72 h for fungal strains. The microbial inhibitory effect was calculated by measuring the diameter of the
growth-inhibition zone (DIZ) in mm (including filter diameter of 9 mm) for the tested species and compared to the antibiotic standards.

Disc Volatilization Assay

The usual assay setup as designated by Tyagi et al. [7] was followed. Briefly, a 0.1 mL portion of each suspension was spread over the surface of MHA (bacteria) or SDA (fungi) plates. A filter disc was placed on the inside external of the upper lid and 10 µL EGO was placed on each disc. The Petri dish inoculated with strains was directly overturned on top of the lid and wrapped with parafilm to avoid the escape of EGO vapor. Petri dishes were incubated at 37 °C/24 h for bacterial strains and at 25 °C/72 h for fungal strains. The microbial inhibitory effect of *Eucalyptus* volatile oil was estimated by calculating the DIZ of bacterial and fungal growth above the disc. Blank discs were used as a negative control. The quantity of EGO used was varied (20, 40 or 60 µL) by using a suitable number of sterile discs.

2.2.4. Orangina Juice Preservation by *Eucalyptus globulus* Oil and Moderate Heat Processing

Preparation of Orangina Juice Inoculated with a Yeast Strain (*Saccharomyces cerevisiae*)

Orangina fruit juices were purchased from a local company (Djaguen Company, Blida city, Algeria). The suspension of *Saccharomyces cerevisiae* was added to Orangina beverage, and the inoculated juices were moved into 250 mL sterilized glass flasks.

Orangina juice is a lightly carbonated beverage made from carbonated water, 12% citrus juice (10% from concentrated orange, 2% from an association of concentrated grapefruit, lemon, and mandarin juices), as well as 2% orange pulp. Orangina juice is sugared with high fructose corn syrup and natural orange flavors are added. Preservatives such as benzoate sodium (E211), potassium sorbate (E202) and citric acid (E330) are also added. All Orangina bottles were kept at 4 °C.

Influence of *Eucalyptus globulus* Essential Oil Alone

Tween 80 solution (0.5%) of EGO was added in the inoculated Orangina at different doses (0.8, 2 and 4 µL/mL). The Orangina sample inoculated with *Saccharomyces cerevisiae* alone was considered as a positive standard. Afterward, the flasks were kept at laboratory temperature for up to 6 days and juices were drawn on days 0, 2, 4, and 6.

The microbiological method used for yeast counts was the standard plate count (SPC) agar method. This method is used by the food industry for estimating the microbial populations in most types of juices products and samples and for determining the quality and sources of contamination at successive stages of processing. All Orangina fruit juices were successively diluted and plated on SDA medium. The petri dishes were incubated for 48 h at 25 °C and CFU counts were estimated. The effect of different doses of EGO treatment was measured in a time-dependent way by the difference in log CFU/mL of the inoculated *S. cerevisiae* [7].

Influence of *Eucalyptus globulus* Essential Oil and Moderate Heat Processing: Combined Action

Three different doses (0.8, 2 and 4 µL/mL) of EGO were added and mixed to inoculated Orangina fruit juice vials and were exposed to a medium heat treatment (70 °C) for 2 min [7]. Then, the flasks were deposited at laboratory temperature for up to 6 days and juices were drawn on day 0, 2, 4, and 6. All Orangina fruit juices were successively diluted and plated on SDA medium. The Petri dishes were incubated for 48 h at 25 °C and CFU counts were estimated. The effects of different doses of EGO in association with medium heat treatment were measured in a time-dependent way by the difference in log CFU/mL of the inoculated *S. cerevisiae*. 
2.3. Statistical Analysis

The significance of variances was analyzed using the test of one-way ANOVA followed by Tukey’s post hoc multiple comparison test. Differences with $p < 0.05$ between experimental groups were considered statistically significant. Statistical data analysis was performed using XLstat 2014 software, Addinsoft, Paris, France. For all tests, three replicates were done and the final results represent the mean of these repeats with standard deviation. The IC$_{50}$ (median inhibitory concentration) was calculated from the dose-response curve obtained by plotting percentage inhibition versus concentrations.

3. Results

3.1. Chemical Composition of Eucalyptus globulus Essential Oil

Six compounds were identified with GC-MS (Table 1). The major constituent of the EGO was 1,8-cineole or eucalyptol (94.03% ± 0.23%), followed by $\alpha$-pinene (94.03% ± 0.23%) and $\gamma$-terpinene (94.03% ± 0.23%). The concentration of other compounds in the EGO was less than 1%.

| Retention Time (RT, min) | RI § | Name # | Concentration (%) |
|--------------------------|------|--------|-------------------|
| 8.777                    | 925  | $\alpha$-Pinene | 2.93 ± 0.1 |
| 9.685                    | 969  | $\beta$-Pinene | 0.20 ± 0.02 |
| 10.110                   | 983  | Myrcene | 0.19 ± 0.07 |
| 10.282                   | 1000 | $\alpha$-Phellandrene | 0.59 ± 0.02 |
| 10.886                   | 1033 | Eucalyptol (1,8-Cineole) | 94.03 ± 0.23 |
| 11.282                   | 1053 | $\gamma$-Terpinene | 1.93 ± 0.17 |

Oxygenated monoterpenes 94.03

Monoterpane hydrocarbons 05.86

* § Compounds listed in order of elution from a non-polar column. § RI (retention index) calculated relative to C6–C17 n-alkanes.

3.2. Antioxidant Effect of the EGO

The antioxidant effect was determined by DPPH radical scavenging and metal ion chelating activity (Table 2). The capacity of the EGO to scavenge DPPH radical and its reducing ability was assessed on the basis of its dose giving 50% inhibition (IC$_{50}$). In addition, its ability to chelate Fe$^{2+}$ metal ions was evaluated and calculated using different doses. The radical scavenging activities of positive controls (BHA and ascorbic acid) were more active than those obtained from EGO. However, EGO had a superior metal ion chelating activity with an IC$_{50}$ value of 8.43 ± 0.03 mg/mL, followed by BHA (104.73 ± 7.30 mg/mL) and gallic acid (136.97 ± 9.09 mg/mL). Current results show that EGO has the best chelating activity.

Table 2. Antioxidant property of Eucalyptus globulus volatile oil in vitro.

| Sample                                      | DPPH Radical Scavenging IC$_{50}$ (mg/mL) | Chelation IC$_{50}$ (mg/mL) |
|---------------------------------------------|-------------------------------------------|----------------------------|
| Eucalyptus globulus Essential Oil           | 2.48 ± 2.24 $^C$                          | 8.43 ± 0.03 $^A$            |
| Positive Control (BHA)                      | 0.13 ± 0.05 $^B$                          | 104.73 ± 7.30 $^B$          |
| Positive Control (Ascorbic acid)            | 0.018 ± 0.004 $^A$                        | 140.99 ± 3.13 $^C$          |
| Positive Control (Gallic Acid)              | —                                         | 136.97 ± 9.09 $^C$          |

IC$_{50}$ = medium inhibitory concentration; values are given as mean ± SD (n = 3). Means within the same column followed by the same capital letters are significantly not different ($p > 0.05$) from one another according to the ANOVA test followed by Tukey’s comparison tests.
3.3. In vitro Antibacterial and Antifungal Effects

3.3.1. Disc Diffusion Assay

The microbial inhibitory effect of EGOE was assessed against different food spoilage microorganisms: six yeast, six bacteria and five fungal strains. The results of the antibacterial tests of EGOE are presented in Table 3. The EGOE displayed a dose-dependent inhibition effect. EGOE was found to be active against all the Gram-positive bacteria species. The EGOE inhibited the growth of *Staphylococcus aureus*, *Bacillus cereus* and *Escherichia coli* with DIZ ranging from 11 to 18 mm at the lower volume of EGOE (20 µL/mL) and from 19 to 85 mm at the higher amount (60 µL/mL). The DIZ increased with the increasing quantity (20, 40 and 60 µL) of EGOE in each paper disc. However, no inhibitory action was found in the case of *Pseudomonas aeruginosa*. For the fungal strains, the DIZ varied from 10 to 49 mm (Table 4). The highest DIZs were shown by *Candida albicans* ATCC (40 mm) and *Saccharomyces cerevisiae* (49 mm) at a greater volume of EGOE (60 µL). The sensitivity of these bacteria to chloramphenicol and EGOE can be explicated by the fact that they have a similar mechanism of action on Gram-negative strains.

The highest susceptible yeast was *Saccharomyces cerevisiae* (49 mm), followed by *Candida albicans* ATCC (40 mm), *Trichosporon* sp. (39 mm) and *Candida parapsilosis* (20 mm) (Table 4). Further, the DIZ due to the *Eucalyptus* volatile oil was bigger for yeast and Gram-positive strains than for mycelial species and Gram-negative bacteria.

3.3.2. Disc Volatilization Method

The in vitro antifungal and antibacterial effects of EGOE were assessed according to the absence or the presence of inhibition zones. The DIZ resulting from the exposure to EGOE vapors is shown in Tables 3 and 4. The DIZ due to the same quantity of EGOE was larger for yeast than for mycelial species (Figure 1A,B).

| Table 3. Susceptibility of bacterial strains to *Eucalyptus globulus* essential oil and to classic antibiotics (positive controls). |
|---------------------------------------------------------------|
| **Bacterial Strains** | **Disc Diffusion Technique** | **Disc Vapor Technique** | **Positive Control**<sup>b</sup> |
| | **Volume of EGOE (µL) per Disc** | **Gram-negative bacteria** | **Volume of EGOE (µL) per Disc** | **Gram-negative bacteria** |
| | | 20 | 40 | 60 | 20 | 40 | 60 | AMC | E | C |
| *Pseudomonas aeruginosa* | - | - | - | - | - | - | - | 16 | 14 |
| *Enterobacter sakazakii* | 12 | 15 | 25 | - | 85 | 85 | - | 19 | 27 |
| *Klebsiella ornithinolytica* | 11 | 19 | 34 | - | - | - | - | 22 | 12 |
| *Escherichia coli* | 11 | 19 | 34 | - | 36 | 36 | 12 | 19 | 7 |
| **Gram-positive bacteria** | | | | | | | | | |
| *Bacillus cereus* | 15 | 35 | 50 | 24 | 40 | 59 | 25 | 35 | 24 |
| *Staphylococcus aureus* | 18 | 48 | 85 | 41 | 85 | 85 | 26 | R | 16 |

<sup>a</sup> Diameter of inhibition zone (mm) comprising a disc diameter of 9 mm; <sup>b</sup> Amoxicillin-clavulanic acid (AMC, 20/10 µg), erythromycin (E, 15 µg) and chloramphenicol (C, 30 µg) were tested as a positive control for microbial species. EGOE: *Eucalyptus globulus* essential oil. (-) No activity.
Table 4. Susceptibility of fungal strains to *Eucalyptus globulus* essential oil and antiseptic solution.

| Fungal Strains                  | Disc Diffusion Assay Quantity of EGO (µL) per Disc | Disc Volatilization Assay | Hex b |
|---------------------------------|---------------------------------------------------|---------------------------|-------|
|                                 | 20 | 40 | 60 | 20 | 40 | 60 | 60 | 40 |
| Yeasts                          |    |    |    |    |    |    |    |    |
| Candida albicans ATCC           | -  | 11 | 40 | -  | -  | -  | -  | 30 |
| Candida albicans (Ca1)          | 10 | 25 | 27 | -  | -  | -  | 53 | 85 |
| Candida albicans (Ca2)          | 14 | 19 | 24 | 85 | 85 | 85 | 13 |    |
| Candida parapsilosis            | 12 | 17 | 20 | 85 | 85 | 85 | 30 |    |
| Saccharomyces cerevisiae        | 11 | 37 | 49 | -  | -  | -  | 59 | -  |
| Trichosporon sp.                | 16 | 34 | 39 | 21 | 85 | 85 | 13 |    |
| Molds                           |    |    |    |    |    |    |    |    |
| Aspergillus niger (An1)         | 12 | 21 | 29 | -  | -  | -  | -  | -  |
| Aspergillus niger (An2)         | -  | 10 | 15 | -  | -  | -  | -  | -  |
| Aspergillus fumigatus           | -  | -  | -  | -  | -  | -  | -  | -  |
| Aspergillus flavus              | -  | -  | -  | -  | -  | -  | -  | -  |
| Aspergillus brasiliensis ATCC   | -  | -  | 14 | -  | 43 | 85 | 14 |    |

a Diameter of inhibition zone (mm) including a disc diameter of 9 mm; b Antiseptic solution (Hoxamidine 0.1%) used as a positive control for fungal strains. EGO: *Eucalyptus globulus* essential oil; (-) No inhibitory effect; ATCC: American Type Culture Collection.

Figure 1. *In vitro* inhibitory activity of *Eucalyptus globulus* essential oil against bacterial and fungal species: Disc diffusion (A) versus vapor diffusion (B) methods using three different quantities of EO (20, 40 and 60 µL/disc). Red arrows are the diameter of the inhibitory zone (DIZ).
As detected in the previous analyses using EGO in the liquid phase, the DIZ due to EGO vapors augmented with an increasing amount of the EGO and followed a similar tendency with respect to the different microbial species. Nevertheless, in comparison with the liquid phase, the EGO vapors resulted in considerably superior DIZ in all the yeast strains tested, except Candida albicans ATCC. In addition, a total inhibitory effect (85 mm) of EGO vapors was found in the case of Staphylococcus aureus and Enterobacter sakazakii bacterial strains. However, no inhibitory action was found for Aspergillus species, Pseudomonas aeruginosa and Klebsiella ornithinolytica. Candida albicans (Ca2) and Candida parapsilosis were the most sensitive yeasts to EGO vapors because total inhibition zones were generated using 20, 40 and 60 µL of EO per disc.

3.4. Orangina Fruit Juice Preservation

3.4.1. Effect of Varying Dose of EGO

As EGO was active to inhibit different food-borne spoilage bacteria and fungi in in vitro methods, its effect in a fruit juice matrix (Orangina juice) was also studied (Figure 2). The reduction in viability of Saccharomyces cerevisiae due to EGO use in a concentration-dependent way (0.8, 2 and 4 µL/mL) and a time-dependent manner (i.e., 0, 1, 2, and 6 days) was evaluated. Complete growth inhibition was observed in Orangina fruit juice only when a high concentration of EGO (4 µL/mL) was used. However, the doses of 2 and 4 µL/mL did not show an important decrease in the final amount of yeasts (2.8 log CFU/mL and 2 log CFU/mL, respectively) in comparison to untreated Orangina juice (2.3 log CFU/mL).

![Figure 2](image)

Figure 2. Effect of different doses of EGO (0.8, 2 and 4 µL/mL) on the viability of Saccharomyces cerevisiae cells in Orangina beverage during storage. The fungal growth was followed up to 6 days after the application. HT: Heat treatment at 70 °C for 2 min; PG: Positive control or juice with synthetic antimicrobial additives (sodium benzoate and potassium sorbate); EGO: Eucalyptus globulus essential oil; CFU: Colony-forming unit. * significant difference (p < 0.05) according to ANOVA one-way analysis followed by Tukey’s post hoc multiple comparison tests.

3.4.2. Combined Effect of EGO and Moderate Heat Processing

The log decrease in CFU count of the Saccharomyces cerevisiae due to the associated action of EGO at different doses along with medium heat treatment at 70 °C for 2 min of Orangina juices was calculated for a specific time interval (0, 1, 2 and 6 days). In juices treated using an association of medium heat processing and all EGO doses, total fungal inhibition of Saccharomyces cerevisiae was recorded on first sampling after 2 days (Figure 3). Therefore, the association of medium heat processing
with EGO reduced the EGO concentration requirement considerably. Even in the Orangina juices treated with a lower quantity of EGO, the association of medium heat processing at 70 °C for 2 min improved the log reduction by 3.5 log CFU/mL in comparison to EGO-treated juices. Therefore, the application of moderate heat processing with EGO can offer improved Orangina juice preservative.

![Figure 3. Effect of EGO at different doses (0.08, 0.2 and 0.4 µL/mL) in association with moderate heat processing (70 °C for 2 min) on the viability of Saccharomyces cerevisiae cells in Orangina beverage during storage. The fungal growth was followed up to 6 days after the heat processing. HT: Heat treatment at 70 °C for 2 min; PG: Positive control or juice with synthetic antimicrobial additives (sodium benzoate and potassium sorbate); EGO: Eucalyptus globulus essential oil; CFU: Colony-forming unit. * significant difference (p < 0.05) according to ANOVA one-way analysis followed by Tukey’s post hoc multiple comparison tests.](image)

4. Discussion

As published in previous reports, the EO of the Eucalyptus genus was described by a great quantity of 1,8-cineole (eucalyptol). Current data are in accordance with those published by Elaissi et al. [18] and Goldbeck et al. [26], who found that the principal chemical element of EGO was eucalyptol. The chemical composition of a diversity of other Eucalyptus species have been reported [7,19,27] and are also in accordance with our data.

Different percentages of eucalyptol in Eucalyptus globulus leaf EO have been shown: 53.7% in Tunisia, 14.5% in Germany, 33.6% to 66.7% in India and 71% in Brazil. The majority of EO extracted from Eucalyptus trees contains at least 10% to as high as 97% eucalyptol (1,8-cineole). Ecological position (Table 5), climatic conditions and extraction methods have been invoked as reasons for the chemical composition disparities and variations [18,20].
A few investigations have been completed to study the antioxidant activity of different *Eucalyptus* EO such as *E. globulus* [28,30,34–37]. Our IC$_{50}$ values are not in agreement with those published for the DPPH radical scavenging method [34]. The scavenging activity on the DPPH radical reported as IC$_{50}$ value was 57 µg/mL for Tunisian EGEO, but this result is inferior as compared to the BHT value obtained with the same test (11.5 µg/mL) [34]. *E. tereticornis* EO presented powerful DPPH, OH and O$_2^-$ radical scavenging activity [35]. In opposition, *E. camaldulensis* and *E. radiata* EOs have been reported to display a medium DPPH scavenging effect [38]. This variance in antioxidant power may be related to the variation in chemical composition, distillation methods and environmental aspects, age of the trees, storage conditions and geo-climatic situations [12].

In addition, Singh et al. [35] tested the *Eucalyptus* EO and its three main oxygenated terpenes (isopulegol, β-citronellol and citronellal) for antioxidant and scavenging effect. The authors reported that the EO extracted from *Eucalyptus* displayed medium to powerful antioxidant effect in terms of metal chelating (877.3% ± 9.27% inhibition), DPPH radical (IC$_{50}$ = 425.4 ± 6.79 mg/mL) and lipid peroxidation inhibition. This research revealed that *Eucalyptus* EO leaves contain oxygenated monoterpenes rich EO presenting antioxidant action.

The chelating property on metal ions is one of the important mechanisms of antioxidant activity. *Eucalyptus* EO showed a better chelating effect (8.43 ± 0.03 mg/mL) on ferrous ions in comparison with standards (BHA = 104.73 ± 7.30 mg/mL and ascorbic acid = 140.99 ± 3.13 mg/mL). Analysis of metal ion chelating activities revealed that all EOs extracted from *Eucalyptus* species were capable of

| Plants          | Country            | Periode          | Method                                      | Composition (%) | Authors                     |
|-----------------|--------------------|------------------|---------------------------------------------|-----------------|-----------------------------|
| *E. globulus*   | Spain              |                  | Hydrodistillation of leaves and small branches of the tree. Certified as biological products to be used in humans. | Eucalyptol = 63.81 α-Pinene = 16.06 Aromadendrene = 3.68 α-Cymene = 2.35 | Luis et al. [28] |
|                 |                    |                  |                                             | Limonene = 68.51 α-Terpinol = 8.60 α-Terpinyl acetate = 6.07 α-Pinene = 3.01 |                |
| *E. radiata*    | Australia          |                  |                                             | Eucalyptol = 4.6% Metileugenol = 3.5 α-Pinene = 2.9 Globulol = 3.2 Terpine-4-ol = 2 | Vieira et al. [29] |
| *E. globulus*   | Alentejo (Portugal)| Spring 2014      | Hydrodistillation in a modified Clevenger-type apparatus | Eucalyptol = 23.6 Eucalyptol = 19.8 α-Pinene = 3.8 iso-Valeradehyde = 2.4 α-Phellandrene = 1.9 | Si Said et al. [30] |
| leaves          |                    |                  |                                             | Globulol = 26.5 Eucalyptol = 19.8 α-Pinene = 3.8 iso-Valeradehyde = 2.4 α-Phellandrene = 1.9 |                |
| Fruits          | Bejaia (Algeria)   | 2013             | Hydrodistillation using a Clevenger type apparatus | Eucalyptol = 13.2 p-Cymene = 12.5 α-Pinene = 12.1 β-Pinene = 10.6 | Salem et al. [31] |
| Aerial parts    | Takelsa (Tunisia)  |                   |                                             | Eucalyptol = 32.1 p-Cymene = 10.4 Eucalyptol = 7.7 β-Pinene = 7.4 |                |
|                 |                    | Full flowering    | Hydrodistillation by Clevenger apparatus     | p-Cymene = 37.8 α-Pinene = 13.3 Eucalyptol = 10.3 L-Phellandrene = 8.2 |                |
|                 |                    | Stages (Feb. 2017)|                                             |                 |                |
|                 |                    | Fructification    |                                             |                 |                |
|                 |                    | stages (May 2017) |                                             |                 |                |
| Fresh leaves    | Ankober Ethiopia   |                  | Hydrodistilled in a Clevenger-type apparatus | Eucalyptol = 63.001 α-Pinene 16.101 Camphor 3.422 | Mekonnen et al. [32] |
| Fresh stems     | Nakhon Nayok,      | Rainy Season     | Hydrodistilled                              | Eucalyptol = 44.54 α-terpinene 19.83 α-Pinene 4.95 Terpine-4-ol = 3.49 | Soonwera and Sittichok [33] |
| and leaves      | (Thailand)         | June-Aug. 2018   |                                             |                 |                |
chelating iron (II) in a dose-dependent way [9]. Another study reported that the antioxidant action of phytochemicals in lemon eucalyptus oil may be due to their redox activities of the phenolic compounds and oxygenated terpenes and that make them good reducing, scavenging and chelating EO [8]. One of the probable modes of action of the antioxidative effect is the chelation of transition metals.

There are very limited published studies about the antioxidant property of eucalyptol as the principal chemical compound of the E GEO [36]. Consequently, antioxidant property detected for the volatile oil could be linked to the remaining compounds [35]. El-Ghorab et al. [37] revealed a medium antioxidant property by preventing the oxidative alteration of linoleic acid (20%) for 12 days for the EO of Eucalyptus camaldulensis. Instead, EOs extracted from aerial parts of Eucalyptus camaldulensis, growing wild in different regions of Sardinia Island (Italy), have exhibited an in vitro antioxidant potential that varied between 0.5 and 5.8 mM [38]. The mode of action involved in the lipid peroxidation and inhibition of DPPH assay is not analogous, thus, the results of the current experiment are not similar to those formerly published by El-Ghorab et al. [37] and Barra et al. [38] because the positive controls and units used in both assays for the determination of the IC50 values are not identical.

Numerous published reports have assessed and confirmed the in vitro antibacterial, anti-yeast and antifungal potential of E GEO against a varied collection of pathogens [39,40]. For example, Eucalyptus citriodora EO has been demonstrated to have a wide range of anti-yeast action. Moreover, Eucalyptus urophylla and Eucalyptus camaldulensis EOs are also recognized for their microbial inhibitory effect [38]. Though much research has been dedicated to the antifungal and anti-yeast effects of E GEO [40], only a limited number of studies have estimated their bio-activity against foodborne bacteria and fungi [7]. Damjanović-Vratnica et al. [41] reported 85.8% of eucalyptol in E GEO from Montenegro and demonstrated its important and significant effect in the inhibition of different yeast and bacteria growth. Furthermore, another study [18] revealed powerful antiviral, antiseptic and antimicrobial activities of eight EGEOS from Tunisia.

The microbial inhibitory action of E GEO has been shown to diverge considerably within microorganisms and species. The higher antiseptic potential could be directly linked to their main chemical constituents detected in the E GEO (such as eucalyptol and α-pinene) or with the interaction among the minor and major components [18]. Previously published studies revealed that Gram-positive strains are more vulnerable than Gram-negative bacteria; the inhibitory effect against yeasts (C. albicans and S. cerevisiae) and fungi (Aspergillus, Mucor, and Penicillium species) has also been investigated [7]. According to one of the reports, Eucalyptus odorata volatile oil presented the strongest in vitro inhibitory effect against micro-organisms and Eucalyptus bicostata EOs possess the greatest antiviral action [18].

The E GEO vapor could be extremely active against food spoilage microorganisms in minor doses in comparison with the liquid diffusion, thus producing less influence on the organoleptic and sensorial food properties [42]. Hence, estimating the in vitro antifungal and antibacterial actions of E GEO vapor might open up a promising dimension with many possible uses, especially in the food industry.

Current data obtained by both vapor diffusion and agar disc diffusion tests were not similar. The efficiency of the E GEO had previously been described but their use in the vapors phase requires an advanced approach [20]. This could be related to the variance in the chemical composition profile of the vapors and liquid oil because the oil must be enriched in terms of its volatile compounds [7]. A supplementary clarification that has been proposed for the vapor phase being more active is that the hydrophobic compounds in the aqueous phase associate to create micelles and thus restrain that connection of the EO to the microorganism, while the vapor phase permits free attachment [43]. Despite published papers on the in vitro antibacterial and antifungal properties of E GEO, few published articles exist on the bioactivity of E GEO vapors. The research done by Goni et al. [44] showed that the in vitro antibacterial activity of a combination of cinnamon and clove volatile oils presented a greater inhibitory action with less active doses in the vapor phase in comparison to the liquid phase.

Current results revealed that Algerian E GEO was effective against all yeast and Gram-positive bacteria strains. Previous data already showed that E GEO has in vitro inhibitory activity against several microorganisms [7,18]. For example, E GEO displayed powerful action (with 14.3 to 18.2 mm
DIZ) against different bacterial species (\textit{S. aureus}, \textit{E. coli}, and \textit{A. faecalis}) and no inhibitory effect was reported against yeast strain (\textit{C. albicans}) [45]. Vilela et al. [42] tested the \textit{in vitro} antifungal property of both the EGO and its main compound (eucalyptol) against two \textit{Aspergillus} strains. They obtained complete inhibition when using the EGO, while less antifungal activity was obtained when testing eucalyptol alone. This proves that the potential synergistic influence of minor and main chemical compounds defines the final \textit{in vitro} inhibitory potential of the volatile oils [3]. Based on the chemical profile of EGO, it can be suggested that the \textit{in vitro} antiseptic potential is seemingly due to its great concentration of oxygenated monoterpenes (94.09%).

Tserennadmid et al. [46] assessed the antifungal activity of different volatile EOs such as lemon, clary sage, marjoram and juniper in fruit juices. The minimum inhibitory concentration (MIC) of these EOs in fruit juices was considerably greater than \textit{in vitro} MIC values. As great doses of several EOs are necessary to accomplish a suitable fungal inhibitory activity, undesirable levels of unsuitable tastes and smells may exist [47]. To more decrease the necessary EGO dose for monitoring the \textit{S. cerevisiae} load in Orangina juices, the interaction between EO and heat processing was evaluated.

To avoid the development of spoilage bacteria and yeasts in foods, numerous protection methods, such as thermal processing, the additions of acids or salts, and drying have been investigated in food production [48–52]. In this background, several volatile molecules and EOs containing oxygenated monoterpenes (linalool, geraniol, citronellol, citral, limonene and pinene), combined with moderate thermal processing, were tested to decrease the growth of \textit{S. cerevisiae} in beverages [48].

In the last decade, numerous reports have revealed the incidence of additional activities when associating EOs with moderate heat and high hydrostatic pressure actions [49,50,53,54]. Synergistic effects between EOs with high hydrostatic pressure or moderate heat have been linked to the existence of lethal damages in the external membrane of surviving microorganisms, which may help the entrance of oil’s compounds into the cells.

The research of Cherrat et al. [50] revealed the superior effect of EOs extracted from \textit{Myrtus communis} and \textit{Laurus nobilis} in association with medium thermal processing and high hydrostatic pressure to accomplish a greater level of bacterial inhibition of food spoilage microorganisms, and as result, decrease undesirable effects on sensorial food properties. Belletti et al. [48] have proved that the addition of citral and \textit{Citrus} EO to soft juices in association with moderate heat processing can avoid yeast spoilage in beverages. In addition, the bacterial growth of \textit{Listeria innocua} in orange juice was inactivated by the addition of vanillin in relation to the entity of heat processing [51].

As the required EO concentration against foodborne and spoilage bacteria and yeast is affected by the interactions of the EO chemical constituents with the food medium and ingredients, higher quantities are necessary to achieve appropriate food preservative action. This undesirably influences the organoleptic and sensorial properties of the foodstuff [7,52]. To resolve this problem, an interesting alternative is the application of an association of moderate heat treatment with EOs, which improves the antibacterial and antifungal effects of the EO influencing the vapor pressure of the compounds [55–57]. Consequently, the association of EOs with medium heat processing can be investigated for emerging food conservation technologies. Few studies have focused on the association of EOs (\textit{Eucalyptus} and \textit{Mentha}) with thermal processing at 55 °C [7,52]. This approach considerably decreases the EOs concentration necessity, offers a very valuable interaction, as the rise of heating increases the quantity of EO in the vapor phase, thus it improves its anti-yeast effect.

Belletti et al. [58] detected that neither the presence of the EO at their greater doses alone nor the heat treatment alone, was able to maintain or decrease the yeast and fungal stability of the drinks against \textit{Saccharomyces cerevisiae}. In opposition, when applied in association, moderate heat processing (55 °C, 15 min) improved the activity of EO compounds (pinene, linalool and citral) and made an increase in their vapor pressure, which in turn amplified their chance to solubilize in the fungal cell membrane. The current research also revealed the improvement in the antifungal effect of EGO on association with moderate heat action. The association of medium heat treatment with EGO application has not been formerly published for avoiding Orangina juice contamination. Current
findings demonstrated that EGEO can be used with moderate heat action for the food protections of Orangina juices.

5. Conclusions

Current findings revealed that EGEO could be used as a possible antifungal and antibacterial agent against foodborne and food spoilage microorganisms. The chemical composition of the different compounds characterizing the EGEO showed the predominance of oxygenated terpenes responsible for the microbial inhibitory effect against pathogens. The use of the EGEO in association with moderate heat processing effectively reduced the growth of spoilage yeast strain in Orangina fruit juices. Current data offer an outstanding record of EGEO as an antifungal agent and propose its possible use for beverage preservation. Supplementary research should be done to determine the effectiveness of EGEO in order to use it as a natural additive in different food matrices and/or improvement of food shelf life.

Author Contributions: Conceptualization, M.N.B.; methodology, M.N.B., A.B., H.G.N.; GC-MS analysis, M.N.B., M.R.; formal analysis, M.N.B., A.B., H.G.N.; resources, S.A.M.; writing—original draft preparation, M.N.B., S.A.M.; writing—review and editing, M.N.B., S.A.M.; All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Acknowledgments: The authors would like to thank Kelly Keating (The Pharmaceutical Research Institute (PRI), Albany College of Pharmacy and Health Sciences, Rensselaer, NY, USA) for proofreading, constructive criticism and English editing of the manuscript. Also, we would like to thank the “Laboratoire d’Hygiène de Blida (Blida, Algeria)”, especially Djamel Teffahi and Abdenacer Hmida for their help and making the facilities available for carrying out this research. M.N.B. sincerely thanks Noureldien Darwish and Thangirala Sudha from the PRI for their continuous support, advice and scientific interactions.

Conflicts of Interest: The authors declare no conflict of interest.

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