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Variations in Essential Oil Biological Activities of Female Cones at Different Developmental Stages from Azorean *Cryptomeria japonica* (Thunb. ex L.f.) D. Don (Cupressaceae)

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Abstract: In the Azores Archipelago, *Cryptomeria japonica* is, currently, the most cultivated forestry tree for timber production, landscaping, and gardening, generating large amounts of foliage waste that is used for local essential oils (EOs) production. However, the existing literature on the biological potential of EOs from different *C. japonica* foliage parts, such as female cones (FC), remains limited. Thus, in the present study, EOs extracted by hydrodistillation from Azorean *C. japonica* immature and mature FC (IFC and MFC), as well as some major EO components, were screened for their: (i) antioxidant capacity, evaluated by DPPH free-radical-scavenging activity (FRSA) and β-carotene-linoleic acid bleaching activity (BCBA), (ii) antimicrobial activities, assessed by the disc diffusion method against eight bacteria and one fungus, and (iii) toxicity against *Artemia salina*. Among both FC EO samples, the IFC EO exhibited the best DPPH–FRSA, BCBA, and growth inhibitory activity against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus subtilis*, and *Penicillium italicum*, as well as a slightly increased toxic potential, due to their differential compositions, as assessed by GC–MS analysis. Thus, the FC's maturation process decreased their EOs' bioactivities. In conclusion, this finding could help in determining the optimal developmental stage for enhancing the antioxidant and antimicrobial compounds content in FC EOs. In turn, this contributes to increasing the commercial potential of *C. japonica*'s EO industry.

Keywords: Azorean *Cryptomeria japonica*; forestry waste; immature and mature female cones; essential oil; dual target agents; antioxidant properties; antibacterial activity; antifungal activity; brine shrimp lethality; sustainable circular bioeconomy

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1. Introduction

Plants, as sessile organisms, are inherently rooted in their environment, unable to flee from adverse conditions. Consequently, they have evolved intricate mechanisms to confront a myriad of environmental challenges. One remarkable adaptation is the production of an extensive range of secondary metabolites (SMs), such as terpene and terpenoid compounds, that serve as versatile tools, enabling plants to enhance their fitness through various strategies such as enticing pollinators and deterring herbivores, thereby fostering their survival and reproductive success in a co-evolving ecosystem. Therefore, due to their structural diversity, SMs from plants have a great potential to positively impact health, nutrition, and the economy on a global scale [1,2].

Terpenes (including mono-, sesqui-, and diterpene hydrocarbons) and terpenoids (oxygen-containing hydrocarbons) are the main components of essential oils (EOs), synthetized in different parts of aromatic plants. These EO components (EOCs) possess a wide range of valuable biological activities, including antimicrobial, antioxidant, anti-inflammatory, anticancer, antiallergic, immunomodulatory, antiseptic, and astringent, as well as repellent, larvicidal, and pesticidal. As a result, EOs are raw materials with potential for application in a wide variety of commercial fields (e.g., cosmetics, food, beverages, fragrances, perfumery, pharmaceutical, medical, sanitary and/or agrochemical), with manifold approaches, thus constituting a promising alternative to commercial synthetic products [3,4]. In fact, nowadays, consumers are becoming increasingly health- and safety-conscious, hence, the use of SMs in diverse commercial formulations is highly desirable, since they are, in general, less harmful to agro-ecosystems, the environment, and public health than their synthetic equivalents.

Conifers have long been recognized for their wide medicinal and/or agrochemical potential [5]. In addition, it is now well reported that conifers' EOs might be one of the promising tools for the treatment of various diseases, as well as for EO-based pesticide formulations, highlighting conifers' importance in drug development and/or in integrated pest management programs (IPMP) [5,6]. Thus, extensive research on the efficacy of EOs from conifers is of great importance to researchers who are interested in determining their health benefits, such as therapeutic agents, and/or their pest control properties. In fact, among woody plants, conifers have evolved the capacity to synthetize a complex terpenoid mixture (oleoresin), mainly accumulated in well-developed secretory resin ducts, which acts as a strong chemical defense against a wide array of biotic (e.g., pathogens and pests) and abiotic (e.g, UV radiation) stress factors, thus contributing to their evolutionary diversification and colonization success, and also has high potential economic value [7]. Conifers comprise 630 species, distributed across 8 families and 70 genera, including the *Cryptomeria* genus, comprising only 1 species, *Cryptomeria japonica* (Thunb. ex L.f.) D. Don, belonging to the Cupressaceae family and Taxodioideae subfamily [5].

Cryptomeria japonica, the target species in this study, is a native species from Japan, introduced in the Azores Archipelago (an autonomous region of Portugal) in the mid-19th century, where it thrived thanks to the similar pedological and climatic conditions as those of its original country [8].

In the Azores, *C. japonica* is the most important forestry tree, not only because of its economic value, but also because its stands are a determinant element of the Azorean landscape (Figure 1A). Nowadays, about 12,500 hectares of *C. japonica* plantations are established in the Azores, representing 60% of the total wood-producing forest area [8,9], 66% of which is on São Miguel Island. This conifer species is a very large, conical, evergreen monoecious tree that can reach up to 70 m in height, with a trunk diameter of up to 4 m. Their leaves (needle-shaped), measuring 0.5–1 cm long, are densely spirally arranged, and their male cones (ovoid or ellipsoid) form axillary aggregations on branches near female cones (FC), which are globular, with a terminal distribution on down-curved branchlets with normal leaves, and often arranged in either an aggregated or solitary form (Figure 1B). Concerning FC, young or immature ones (IFC) emerge from a rosette of leaves measuring nearly 5 mm in diameter (Figure 1C). At the time of pollination, spanning from February to April, IFC possess a flat top, becoming almost globular within one month. In turn, mature FC (MFC) have a tapering apex and a 1–2 cm diameter (Figure 1C), containing 20–30 megasporophylls arranged in a spiral shape [10–12].

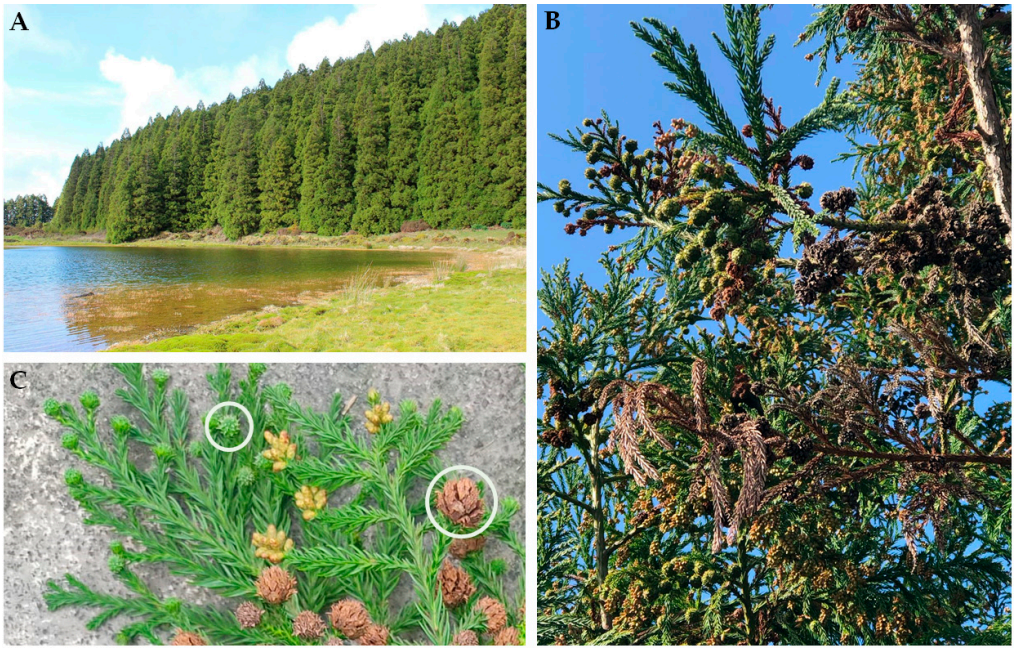

Figure 1. Azorean *Cryptomeria japonica*: (**A**) woodland; (**B**) aerial plant part; and (**C**) fresh female cones at immature and mature stages, as highlighted by a circle to the left and right sides, respectively.

The remarkably increasing market value of EOs could bring about new opportunities for the sustainable management of unused forestry waste, such as that from *C. japonica*. Currently, the main source of biomass for *C. japonica* EO production in the Azores is the foliage (CJF), which constitutes the bulk of the biomass waste generated by the timber industry and forestry operations. However, it should be noted that the yield, chemical composition, and biological activities of *C. japonica* EO and, consequently, its specific commercial applications and price, can be significantly influenced by both exogenous and endogenous factors, such as species variety; the geographical region of the plant; environmental abiotic and biotic stresses; plant age; plant parts and their developmental stages; management practices (e.g., harvest period); the post-harvest processing of plant material (e.g., drying); and the extraction process (method and protocol used), among other factors [9,13–16]. It should also be highlighted that comparisons of data between different studies are very difficult when different raw materials, processing conditions, extraction protocols, analytical methods, and/or units of measurement were used, among other factors (e.g., experimental conditions of bioassays) [15].

According to the International Standard Organization on Essential Oils (ISO 9235: 2013) [17] and the European Pharmacopoeia [18], an EO is defined as the product obtained from raw vegetable material by hydrodistillation (HD), steam or dry distillation, or by a suitable mechanical process for citrus fruits. Thus, this definition excludes other aromatic products obtained by different extractive techniques, such as solvent extraction.

The chemical characterization, yield value, and bioactivity assessment of *C. japonica* biomass residues' (CJBR) EOs have focused mostly on the EO from CJF. According to our critical review [13], CJF EOs from different geographical origins are typically obtained by HD, presenting a yield range of 0.5–4.7% (*w*/*w*, dry weight basis), and are mainly constituted by a complex mixture of terpenes and terpenoids. However, the CJF EO chemotype is the α-pinene type in the Azores, while in most east Asian countries, it is mainly the *ent*-kaurene type or elemol plus *ent*-kaurene type. Nevertheless, all these CJF EO chemotypes present antimicrobial activities (which are mostly related to the individual susceptibility of the tested microorganisms) and/or other biocidal activities (including mosquito larvicidal, acaricidal, termiticidal, molluscicidal, insect repellent properties, and toxicity against brine shrimp nauplii) [13,15] with potential application in IPMP and/or human and other animals' health areas. Many other pharmacological properties of CJF EO samples from several

countries have been reported, such as: antimelanogenesis, skin whitening, antitussive, antiulcer, anxiolytic, anti-inflammatory, and antioxidant, among other important bioactivities, namely, neuropharmacological and cancer chemopreventive [9].

Currently, there is a renewed interest in the use of EOs as potential eco-friendly antimicrobial and antioxidant dual active agents for health and food preservation applications. In fact, EOs are generally accepted as natural antimicrobial and antioxidant agents, due to their generally recognized safe nature. In addition, the development of food-borne and infectious diseases represents a key issue, either due to their significantly world increased rate in recent years, with consequent social and economic problems, as well as due to the development of resistant strains to current fungicides and antibiotics. In addition, the microbial contamination of food is one of the main causes of foodstuff loss nowadays [13].

Interestingly, as a part of our continuous efforts towards the valorization of Azorean CJBR, and as incentive for the local *C. japonica*'s EO industry, we recently found that Azorean *C. japonica* FC EO is a broad-spectrum antimicrobial agent, as well as more toxic against brine shrimp nauplii, as compared to other plant parts, such as CJF EO [15]. Thus, we focus our attention on this less studied Azorean *C. japonica* plant part EO. Earlier, we demonstrated that the developmental stage of the FC collected from the same batch of Azorean *C. japonica* has a remarkable influence on the yield and chemical composition of their EOs obtained by the HD method [16].

In this context, and knowing that the bioactivities of EOs depend mainly on their chemical composition, herein we investigated the aforementioned IFC and MFC EO samples, in regard to their (i) in vitro antioxidant capacity, evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) free-radical-scavenging activity (FRSA) and β-carotene-linoleic acid bleaching activity (BCBA) assays, (ii) in vitro antimicrobial activities against eight bacteria and one fungus, and (iii) toxic effects, estimated preliminarily through a brine shrimp lethality activity (BSLA) assay. In addition, some pure EOCs (α-pinene, terpinen-4-ol, and bornyl acetate) were also screened for the aforementioned bioactivities. Overall, the results of this study will contribute to an increase in knowledge of the potentialities of *C. japonica* FC samples, which, in turn, can help *C. japonica*'s EO industry to meet different market demands compared to the typical CJF EO and, consequently, contribute to the use of Azorean forestry waste biomass in a more economic and sustainable way.

2. Materials and Methods

2.1. Chemicals and Reagents

Kanamycin, Clotrimazole, (–)-α-pinene (≥97%), (–)-terpinen-4-ol (≥95%), (–)-bornyl acetate (≥95%), anhydrous sodium sulphate (Na_2SO_4), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, gallic acid, β-carotene, linoleic acid, Tween 20, and dimethylsulfoxide (DMSO) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Nutrient agar, Muller–Hinton Agar (MHA), and potato dextrose agar (PDA) were obtained from Merck (Darmstadt, Germany). Ethanol (96%), methanol (≥99.8%), and chloroform (≥99%) were purchased from Riedel-de Häen (Aktiengesellschaft, Seelze, Germany).

2.2. Plant Material and Collection Site

The CJF was harvested during the pollination stage (early March 2023, winter season) from healthy plants belonging to a tree population in Lomba da Maia (latitude 37°48′32.7″ N, longitude 25°20′06.5″ W, altitude 440 m), located in the northeast region of São Miguel, the largest island of the Azores, a volcanic archipelago located in the warm temperate region of the northeast Atlantic Ocean (Figure 2A−C) and distanced from the Iberian Peninsula by approximately 1500 km, calculated from Cabo da Roca (the most westerly point of the European continent). A voucher specimen (number AZB 4581) was deposited in the Herbarium AZB−Ruy Telles Palhinha of the University of the Azores. The collected plant material was then brought to a laboratory at the same university, where the FC attached to the foliage were removed, separated into IFC and MFC samples, and immediately stored at −20 °C until further use.

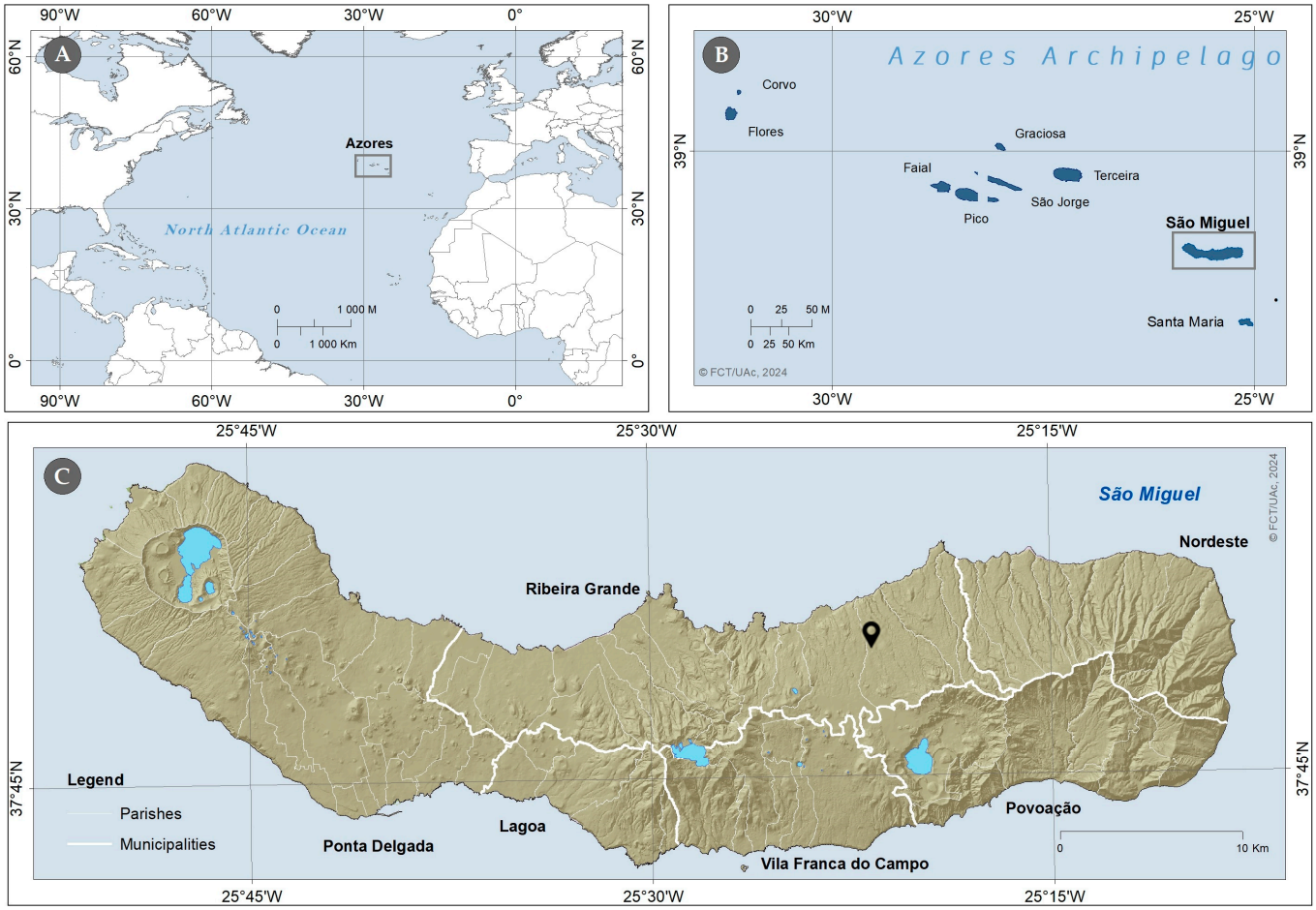

Figure 2. Geographical location of the collection area of *Cryptomeria japonica* female cones: (**A**) the Azores Archipelago in the northern Mid-Atlantic Ridge highlighted by a square; (**B**) São Miguel Island in the Azores Archipelago highlighted by a square; and (**C**) the collection site marked with a black pin (maps from Faculty of Science and Technology, University of the Azores, 2024).

2.3. Essential Oil Extraction by Hydrodistillation Method

The EOs from the Azorean *C. japonica* IFC and MFC samples were obtained by HD through a Clevenger-type extractor, according to the European Pharmacopoeia [18], as detailed in Janeiro et al. [16]. Briefly, the ratio of the plant material sample to water was 1.5:10 g/mL, and the time of distillation was approximately 3 h, starting from the first distillate drop. The isolated EOs were dehydrated with anhydrous Na_2SO_4 and stored in sealed amber vials at 4 °C until further analysis. Each HD process was performed in triplicate.

2.4. Essential Oil Composition Analysis

Gas chromatography–mass spectroscopy (GC–MS) analyses were carried out with a GCMS–QP2010 Ultra gas chromatograph–mass spectrometer (Shimadzu, Tokyo, Japan), using a ZB–5MSPlus capillary column (5% phenyl, 95% methyl siloxane) with a dimension of 60 m length × 0.25 mm i.d. and a film thickness of 0.25 µm (Phenomenex Inc., Torrance, CA, USA). An aliquot of 0.1 µL of an EO sample diluted in methylene chloride (0.1 g/mL) was injected by split mode (24.4:1), and helium was used as the carrier gas, with a flow rate maintained at 36.3 cm/s. The temperature of the oven was programmed from 50 °C to 260 °C at 2 °C/min ramp rate, where it was then maintained at 260 °C for 5 min. The injector and MS detector temperatures were kept at 260 °C. The transfer line and ion source temperatures were 300 °C and 260 °C, respectively. The MS was obtained at 70 eV, where the mass scan range (m/z) was 40–400 amu with a scan time of 0.3 s. The

identification of the EOCs was performed by comparing their retention indices (RI), relative to *n*-alkane standard indices, and their GC/MS spectra with two MS databases: (i) a lab-made library with commercially available standards and components of reference EOs, and (ii) other libraries (FFNSC4.0, NIST11, and Wiley10). For quantification, the EOCs' raw percentages were calculated by integrating total ion current (TIC) chromatogram peaks without correction factors as mean values of three injections from each sample [16].

2.5. In Vitro Antioxidant Activity Evaluation

2.5.1. DPPH Free-Radical-Scavenging Activity (FRSA) Assay

The FRSA of the EOs or EOCs at different concentrations (0.15–150 mg/mL) and ascorbic acid (positive control) samples was determined by measuring their ability to quench the DPPH stable free radical, according to Chen et al.'s [19] procedure, with some modifications. Briefly, 0.1 mL of each sample was mixed with a 0.1 mL DPPH solution (0.08 mg/mL in methanol) in the well of a 96-well plate. The mixture was shaken vigorously and kept in the dark at room temperature for 30 min. The absorbance (Abs) was then measured at 520 nm against methanol as the blank in a Multiskan FC microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). All assays were performed in triplicate. The FRSA was calculated as a percentage of the inhibition of DPPH discoloration using the following Equation (1):

$$\text{FRSA } (\%) = 1 - \left(\frac{\text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \right) \times 100 \quad (1)$$

The results were expressed as IC_{50} values (mg/mL), which is defined as the sample concentration needed to quench 50% of the DPPH free radicals. A lower IC_{50} value is indicative of higher antioxidant activity.

2.5.2. β-Carotene-Linoleic Acid Bleaching Activity (BCBA) Assay

The BCBA of the EOs or EOCs at different concentrations (0.016–2 mg/mL) and gallic acid (positive control) samples was determined according to Miller's [20] method, with slight modifications. In this assay, 25 µL of a chloroform solution of β-carotene (20 mg/mL) was mixed with 20 µL of linoleic acid, 200 mg of Tween 20, and 500 µL of chloroform in a boiling flask. Using a rotary evaporator, the chloroform was evaporated within 38–40 °C for 60 min. Afterwards, 25 mL of distilled water was added to the flask with vigorous stirring, which resulted in the formation of an emulsion (250 µL), which was then combined with 50 µL of each sample. The plate was then incubated at 52 °C for 3 h, with the abs being determined at 450 nm, prior (t = 0) and after incubation, against a blank consisting of an emulsion without β-carotene. The control samples contained 50 µL of water instead. All assays were performed in triplicate. The BCBA of each sample was evaluated as the percentage of the inhibition of β-carotene discoloration using the following Equation (2):

$$\text{BCBA}(\%) = \left[\frac{(S_t - C_t)}{C_0 - C_t} \right] \times 100 \quad (2)$$

where S_t and C_t are the abs of the sample and the control after 3 h of incubation, respectively, and C_0 is the control abs measured at zero minutes (t = 0). The kinetics of this activity allowed for determining the sample's concentration corresponding to a 50% inhibition of β-carotene discoloration (IC_{50} value).

2.6. In Vitro Antimicrobial Activity

2.6.1. Microbial Strains and Culture Media

Among the selected microorganisms were one fungus (*Penicillium italicum* Wehmer) and eight bacteria, including (i) five Gram-positive (Gram+) bacteria, such as *Bacillus subtilis* (Ehrenberg) Cohn (DSM10), *Bacillus licheniformis* (Weigmann) Chester (DSM13), *Micrococcus luteus* (Schroeter) Cohn (DSM20030), *Staphylococcus aureus* Rosenbach (DSM1104), and

Staphylococcus epidermidis (Winslow & Winslow) Evans (DSM20044), and (ii) three Gram-negative (Gram−) bacteria, such as *Serratia marcescens* Bizio (DSM48), *Entereobacter cloacae* (Jordan) Hormaeche & Edwards (DSM30054), and *Escherichia coli* (Migula) Castellani & Chalmers (DSM498).

P. italicum was isolated from an infected citrus fruit (exhibiting the typical blue mold symptoms) and identified by its macro- and micro-morphological characteristics based on mycological keys [21,22], while the bacterial strains were obtained from the collection of the Microbiology Laboratory, Department of Biology, University of the Azores. Among the studied bacteria, both *Bacillus* spp. represent food spoilage Bacillales members [23], the other three selected Gram+ bacteria are potential opportunistic pathogens [24–26], and the Gram− bacteria represent foodborne pathogens [27]. Nutrient agar was employed for the culturing of the bacterial strains, while PDA was employed for the developing of *P. italicum*. Bacterial inocula were prepared through the direct inoculation of colonies in a test tube containing a sterile saline solution, adjusted to the 0.5 standard on the McFarland scale. The fungal spore suspension was prepared by scraping a pure culture of *P. italicum*, using a sterile loop, and adjusting to 5×10^4 spores/mL using a hemocytometer (Hirschmann, Eberstadt, Germany). After the fungus inoculum, the PDA plates were incubated overnight before the loading of the samples.

2.6.2. Disc Diffusion Method (DDM)

The antimicrobial activity of the EO samples and EOCs was evaluated through DDM according to the Kirby–Bauer method [28], with some modifications. Briefly, 5 µL of an undiluted EO or EOC was loaded onto a sterile paper disc, measuring 6 mm in diameter, and placed on target-inoculated MHA or PDA plates. The bacterial cultures were incubated for 24 h at 28 °C (Gram+ bacteria and *S. marcescens*) or 37 °C (*E. cloacae* and *E. coli*), while the fungal cultures were incubated for 48–72 h at 25 °C. After incubation, the diameters of the growth inhibition zones (GIZs) were measured in mm, including the diameter of the disc. Kanamycin (aqueous solution, 50 µg/disc) and Clotrimazole (dissolved in DMSO, 25 µg/disc) were used as positive controls in the antibacterial and antifungal assays, respectively. Inoculated plates without samples were used as negative controls. All assays were performed in triplicate.

2.7. Brine Shrimp Lethality Activity (BSLA) Assay

The toxicity of the EO samples and EOCs was assessed by an in vivo assay employing nauplii of brine shrimp (*Artemia salina* Leach). Cysts of *A. salina* were purchased locally and hatched in artificial seawater for 48 h.

The BSLA assay was performed according to a slightly modified Meyer et al. [29] method [15]. A stock solution of each EO or EOC was prepared by dissolving 150 mg of the sample in ethanol to a final volume of 0.5 mL. Then, the stock solution was diluted to 1 mg/mL in water and sonicated, which was then diluted to 100 µg/mL in artificial seawater. Control samples of artificial seawater and ethanol (<0.1%, *v*/*v*) were also prepared to correct values with the natural mortality rate. In each well of a microplate, ten to fifteen nauplii were brought into contact with the EO or EOC, as well as with the control samples [15]. All assays were performed in triplicate. After 24 h of contact, the mortality rate of the nauplii was calculated according to Abbott's [30] formula (Equation (3)):

$$\mathrm{M}(\%\mathrm{vs.control}) = \left[\frac{(\mathrm{L_C} - \mathrm{L_T})}{\mathrm{L_C}}\right] \times 100 \quad (3)$$

where M is mortality; $\mathrm{L_C}$ is the living nauplii in the control; and $\mathrm{L_T}$ is the living nauplii in the treated sample.

2.8. Statistical Analysis

All analyses were performed by using SPSS version 27.0 software (SPSS Inc., Chicago, IL, USA). All samples were subjected to the Shapiro–Wilk test to determine if the data

followed a normal distribution. All data that did not meet this condition were transformed. One-way analysis of variance (ANOVA) and the least significant difference (LSD) test were then performed to verify statistical significances between groups. The statistical significance of differences among mean values was established at $p < 0.05$. All experiments were performed in triplicate, and the data are expressed as mean ± standard deviation (SD).

3. Results and Discussion

3.1. Essential Oils' Isolation and Chemical Composition of Immature and Mature Female Cones (IFC and MFC) from Azorean C. japonica

The bioactive properties of EOs, for example, their antimicrobial or antioxidant properties [31], depend on their chemical composition, as well as on the possible synergistic, antagonistic, and additive interactions among their bioactive components [32].

The IFC and MFC EO samples under study, obtained by the HD method, were constituted by a diversity of terpene and terpenoid compounds, as reported in our earlier work [16]. However, it was observed [16] that the FC's maturation process remarkably influenced the composition of the FC EOs.

Figure 3A,B show the IFC and MFC EOCs grouped according to chemical class, namely, monoterpene hydrocarbons (MH), oxygen-containing monoterpenes (OCM), sesquiterpene hydrocarbons (SH), oxygen-containing sesquiterpenes (OCS), diterpene hydrocarbons (DH), and oxygen-containing diterpenes (OCD). Thus, among both the IFC and MFC EO samples, the IFC EO was richest in total DH content (8.91% vs. 2.00%), as well as in total terpenoid content (52.56% vs. 34.00%), and presented a remarkable lowest terpenes/terpenoids ratio value (0.9 vs. 1.89) (Figure 3A,B).

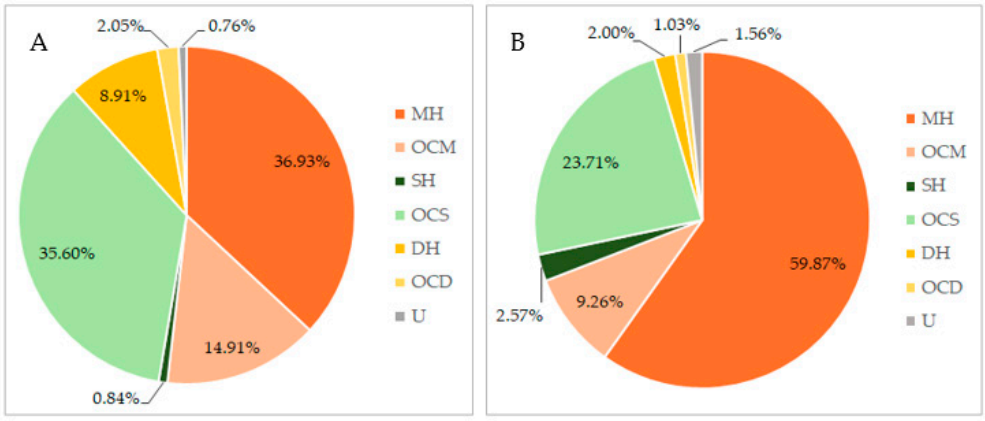

Figure 3. Percentage of grouped components of essential oils isolated by hydrodistillation from Azorean *Cryptomeria japonica* female cones: (**A**) immature female cones and (**B**) mature female cones (data from Janeiro et al. [16]). Legend: MH—monoterpene hydrocarbons; OCM—oxygen-containing monoterpenes; SH—sesquiterpene hydrocarbons; OCS—oxygen-containing sesquiterpenes; DH—diterpene hydrocarbons; OCD—oxygen-containing diterpenes; and U—unidentified components.

Table 1 shows some major EOCs from the IFC and MFC EO samples. Thus, concerning the individual EOCs, the FC's maturation process revealed a notable increase in α-pinene content, but remarkable decreases in the amounts of other important bioactive terpenes/terpenoids (e.g., terpinen-4-ol, bornyl acetate, elemol, γ-eudesmol, phyllocladene and nezukol) (Table 1), as detailed in our earlier work [16]. It should also be noted that all the selected EOCs (Table 1) have already demonstrated valuable biological properties, namely: anticancer, anti-inflammatory, antimicrobial, antioxidant, neuroprotective, and pesticidal (**1**–**5**) [9]; antioxidant (**6** and **11**) [33]; anticancer and pesticidal (**7**) [9]; antiangiogenic, anticancer, and antimicrobial (**8** and **9**) [9,34], and antimicrobial (**10**) [9].

Table 1. Composition of some major components of essential oils (EO) isolated by hydrodistillation from Azorean *Cryptomeria japonica* immature and mature female cones (IFC and MFC) (data from Janeiro et al. [16]).

No.	Class	Component	RT (min)	RI	Relative Content (%)	
					IFC EO	MFC EO
1	MH	α-Pinene	12.63	929	19.53 ± 0.45	41.32 ± 1.31
2	MH	Sabinene	14.78	967	3.86 ± 0.04	5.38 ± 0.51
3	MH	Limonene	18.22	1024	1.54 ± 0.17	1.11 ± 0.03
4	MH	γ-Terpinene	20.11	1052	2.69 ± 0.25	1.02 ± 0.08
5	OCM	Terpinen-4-ol	28.49	1175	11.21 ± 1.08	5.97 ± 0.51
6	OCM	Bornyl acetate	35.55	1278	1.55 ± 0.09	0.43 ± 0.02
7	OCS	Elemol	52.32	1540	11.01 ± 0.05	7.00 ± 0.02
8	OCS	γ-Eudesmol	57.19	1623	11.41 ± 0.21	2.82 ± 0.03
9	OCS	α+β-Eudesmol	58.52	1646	10.32 ± 1.00	11.81 ± 0.29
10	DH	Phyllocladene	77.34	2009	6.54 ± 0.09	1.32 ± 0.01
11	OCD	Nezukol	82.41	2119	1.77 ± 0.03	0.45 ± 0.02
Total identified components			-	-	99.24 ± 0.89	98.44 ± 1.29

Values are mean ± SD (n = 3). Legend: MH—monoterpene hydrocarbons; OCM—oxygen-containing monoterpenes; OCS–oxygen-containing sesquiterpenes; DH—diterpene hydrocarbons; OCD—oxygen-containing diterpenes; and RT and RI—retention time (min) and retention index on ZB—5MSPlus capillary column, respectively.

3.2. Biological Activities of Immature and Mature Female Cones (IFC and MFC) from Azorean C. japonica

3.2.1. In Vitro Antioxidant Activities Evaluated by Radical Scavenging and Lipid Peroxidation Assays

Radical scavenging activity is very important due to the deleterious role of free radicals in foods and biological systems [35]. Over time, diverse methods have been proposed to assess the antioxidant activity of natural products, such as extracts, EOs, or pure natural compounds. They include the chemical-based assays group that, in turn, are sub-classified as assays based on single-electron transfer (SET) reactions and hydrogen transfer atom (HAT) reactions [36].

Since EOs contain a variety of bioactive components, their antioxidant potential cannot be achieved by a single mechanism of action. Thus, in the present study, we chose (i) the DPPH–FRSA, a simple, rapid, sensitive, and reproducible SET-based method [36], and (ii) another antioxidant assessment method based on the HAT mechanism, the BCBA assay [36], using ascorbic acid and gallic acid as positive control samples, respectively. The results of the antioxidant properties of the EO samples and some of their components, as well as of the positive controls, are shown in Table 2.

Table 2. Antioxidant activity of the essential oils (EO) isolated by hydrodistillation from Azorean *Cryptomeria japonica* immature and mature female cones (IFC and MFC) and some of their components.

EO and Compound	IC_{50}, mg/mL	
	DPPH–FRSA	BCBA
IFC EO	0.67 ± 0.24 [a]	0.097 ± 0.09 [a]
MFC EO	2.04 ± 0.54 [b]	0.204 ± 0.04 [b]
α-Pinene	NA	NA
Terpinen-4-ol	NA	NA
Bornyl acetate	NA	NA
Ascorbic acid	0.004 ± 0.0002	-
Gallic acid	-	0.02 ± 0.003

Values are mean ± SD (n = 3). Different superscript letters in the same column indicate statistically significant differences at $p < 0.05$. Legend: NA—no activity; DPPH—2,2-diphenyl-1-picrylhydrazyl; FRSA—free-radical-scavenging activity; and BCBA—β-carotene-linoleic acid bleaching activity.

Both the IFC and MFC EO samples exhibited moderate activity in the DPPH–FRSA assay, with IC_{50} values of 0.67 and 2.04 mg/mL, respectively, when compared with the ascorbic acid positive control (Table 2). However, the antioxidant activity of the IFC EO was three times higher than that of the MFC EO ($p = 0.01$), probably due to its inferior MH and α-pinene contents (Figure 3A,B and Table 1), since it has already been seen that this MH is inactive in DPPH–FRSA assay [37]. In fact, in the present study, none of the studied EOCs, i.e., (–)-α-pinene, (–)-terpinen-4-ol, and (–)-bornyl acetate, presented DPPH–FRSA. In addition, Ruas et al. [38] reported a weak antioxidant activity of the Azorean CJF EO (23.1 mg/mL) in DPPH–FRSA assay, which was clearly dominated by MH (66%) in its composition. Hence, it appears that EOs derived from *C. japonica* parts with a higher polar content (e.g., EOCs bearing hydroxyl groups), such as IFC (Figure 3A), are likely to exhibit more robust antioxidant properties. In fact, some EOCs, such as elemol, α-eudesmol, and mainly γ-eudesmol [39], are SET-based agents, explaining why the IFC EO (Table 1) was a good free radical scavenger.

A BCBA assay was also performed to ascertain whether the presence of different antioxidants in the EOs under study could hinder the bleaching of β–carotene by quenching free radicals in the system. The inhibition percentages of different concentrations of IFC and MFC EOs on β-carotene bleaching were dose-dependent for all samples, and their IC_{50} values are displayed in Table 2. The IFC EO exhibited, again, the most pronounced antioxidant activity, as evidenced by its IC_{50} value of 0.097 mg/mL. It is important to highlight, however, that this value, while indicative of substantial antioxidant potential, was approximately five times higher than that of the gallic acid positive control (Table 2). Concerning the studied EOCs, none of them presented BCBA.

Overall, this study confirms the influence of the developmental stage of *C. japonica* FC on the antioxidant activity of their EOs, with IFC EO being the most active sample due to its unique chemical profile, as reported above (Section 3.1). Interestingly, a study on FC organic extracts of *C. japonica* from other origins revealed similar results, i.e., IFC presented a higher antioxidant potential than mature and opened FC extracts (DPPH IC_{50} values of 10.13, 10.55, and 17.51 μg/mL, respectively) [40]. However, these extracts presented a higher DPPH–FRSA than the FC EOs under study, as expected. The same trend was reported for a CJF EO that showed a lower DPPH–FRSA as compared to CJF organic and aqueous extracts that are rich in polyphenols [13,41]. Hence, ongoing research should delve into the exploration of extracts from these Azorean *C. japonica* FC samples, due to (i) their higher yields compared to EOs (0.72–1.12% *w*/*w*, dry weight basis) [16], and (ii) the knowledge that FC organic extracts of *C. japonica* from other origins present a unique chemical composition that includes six uncommon triterpenes, namely, chamaecydin, isochamaecydin, cryptoquinonemethide D, sugikurojin C, cryptoquinonemethide E, and 6α-hydroxychamaecydin. Interestingly, these C30-terpene quinone methides are structurally related to the sesquarterpene cryptotrione, isolated from *C. japonica* bark, that displays notable cytotoxic activity against human oral epidermoid carcinoma KB cells' proliferation, with an inhibitory concentration 50% (IC_{50}) value of 6.44 μM, only slightly weaker than that of the clinically used anticancer drug etoposide (VP-16, $IC_{50} = 2.0$ μM) [13,41].

In addition, by comparing the antioxidant activities (IC_{50} values) of the studied EO samples, it can be concluded that their BCBA was far stronger than the DPPH–FRSA, which indicates that the EOs showed better antioxidant activity in a lipid system.

3.2.2. In Vitro Antimicrobial Activities

EOs can also play important roles in inhibiting microbial growth [42,43] and, therefore, could be an effective alternative in the fight against human and other animals' infectious diseases or in green plant protection and food preservation.

The results of the antimicrobial properties (growth inhibitory activities) of the EO samples and some of their components, as well as of the positive controls, are shown in Table 3.

Table 3. In vitro antimicrobial activity of the essential oils (EO) isolated by hydrodistillation from Azorean *Cryptomeria japonica* immature and mature female cones (IFC and MFC) and some of their components.

Microorganism	Growth Inhibition Zone (mm)						
	EO Sample		EO Compound			Reference Compound	
	IFC	MFC	α-Pinene	Terpinen-4-ol	Bornyl acetate	Kanamycin	Clotrimazole
Bacillus subtilis	12 ± 1^{b}	10 ± 1^{c}	8 ± 1^{c}	13 ± 1^{b}	21 ± 1^{a}	39 ± 2	-
Bacillus licheniformis	9 ± 1^{b}	8 ± 1^{b}	9 ± 1^{b}	16 ± 1^{a}	17 ± 3^{a}	34 ± 2	-
Micrococcus luteus	10 ± 1^{c}	12 ± 2^{b}	18 ± 1^{a}	7 ± 1^{d}	NA	30 ± 0	-
Staphylococcus aureus	20 ± 2^{a}	9 ± 2^{bc}	17 ± 1^{a}	10 ± 1^{b}	8 ± 0^{c}	29 ± 2	-
Staphylococcus epidermidis	13 ± 3^{a}	10 ± 1^{ab}	10 ± 2^{ab}	8 ± 2^{b}	NA	36 ± 3	-
Serratia marcescens	NA	NA	8 ± 1^{b}	21 ± 2^{a}	NA	24 ± 0	-
Enterobacter cloacae	NA	NA	7 ± 1^{b}	31 ± 3^{a}	NA	21 ± 1	-
Escherichia coli	NA	NA	8 ± 1^{b}	18 ± 1^{a}	NA	24 ± 1	-
Penicillium italicum	9 ± 1^{b}	NA	7 ± 0^{b}	13 ± 0^{a}	NA	-	20 ± 1

Values are mean ± SD (n = 3). Different superscript letters in the same row of the same strain indicate statistically significant differences ($p < 0.05$). Legend: NA—no activity; 7–10 mm (weak activity); 10–15 mm (moderate activity); and >15 mm (strong activity).

Concerning the bacterial properties, it is clear from the data that both EO samples exhibited activity against Gram+ bacteria (GIZs ranging from 9 to 20 mm), but not against Gram− ones. Similar results have already been observed for other Azorean *C. japonica* plant parts [15]. It is also clear that Gram− bacteria were more sensitive to terpinen-4-ol than to α-pinene, while bornyl acetate proved ineffective.

The IFC EO was more active against *B. subtilis*, *S. epidermidis*, and *S. aureus* than the MFC EO (12 vs. 10 mm, 13 vs. 10 mm, and 20 vs. 9 mm, respectively) (Table 3). The prominent activity of the IFC EO against *S. aureus* could be attributed to its particular chemical composition, i.e., a higher polar content than the MFC EO (Figure 3A,B), and should be further investigated, since this bacterium quickly becomes drug-resistant and causes a wide variety of clinical diseases [44]. Unfortunately, the antibacterial activities of (+)-terpinen-4-ol enantiomer and OCS were not evaluated, which could have better explained these results. In fact, it was observed that γ-eudesmol, a component present in a higher content in the IFC EO over the MFC EO (11.41 vs. 2.82%) (Table 1), was highly effective against *S. aureus* [39]. As also observed in Table 3, bornyl acetate exhibited strong activity against both *Bacillus* strains, followed by terpinen-4-ol and α-pinene. On the contrary, *M. luteus* and *Staphylococcus* strains were found to be more susceptible to (–)-α-pinene, followed by (–)-terpinen-4-ol. Among these bacteria, only *S. aureus* was weakly susceptible to bornyl acetate.

Beyond pathogenic bacteria, various fungal species actively contribute to food deterioration through sporulation and the production of mycotoxins. Mycotoxins, characterized as low molecular weight SMs, are primarily synthesized by different fungal genera like *Penicillium*, *Aspergillus*, and *Fusarium*, capable of contaminating various stored food items, inducing variable toxic effects [45]. Concerning the antifungal properties of the EOs under study, only the IFC sample exhibited slight activity against *P. italicum* (Table 3). Among the tested EOCs, it was observed that terpinen-4-ol exhibited superior efficacy against *P. italicum* (Table 3). Again, it seems that hydroxylated EOCs are responsible for the biological properties observed.

Overall, this study also confirms the influence of the *C. japonica* FC's developmental stage on the antimicrobial activities of their EOs, highlighting the importance of the IFC EO as a promising source of multi-bioactivities, at least antioxidant, antibacterial, and antifungal, as demonstrated herein.

3.2.3. Brine Shrimp Lethality Activity (BSLA)

The BSLA, an in vivo assay, has been performed as a simple, economical, and rapid screening method for the cytotoxic effects of several multi-component matrices, such as extracts and EOs. Moreover, the results of this assay exhibit a positive correlation with toxicity data from both rodents and humans, displaying a notable alignment with cytotoxicity tests. Consequently, these measurements serve as valuable preliminary toxicity screening for further experiments on mammalian animal models [46,47].

The results of the BSLA of the EO samples, as well as of some their components, are shown in Figure 4. The IFC EO was found to be more toxic than the FMC EO, with 28.3 ± 7.6% and 25.8 ± 7.5% mortality rates (at 100 µg/mL), respectively, although there were no significant differences between them. Regarding the EOCs tested, terpinen-4-ol, one of the major components in both EO samples (Table 1), also displayed some toxic activity (17.42 ± 3.94%), followed by α-pinene (15.2 ± 1.7%), the most abundant component in both EO samples (Table 1), and bornyl acetate (10.6 ± 3.4%), which did not seem to be involved in toxicity against the *A. salina* nauplii. The preliminary results of the BSLA assay show that both the IFC and MFC EOs may contain some bioactive components, which should be considered for further investigations of their potential pharmacological properties. These results are in accordance with previous findings [15].

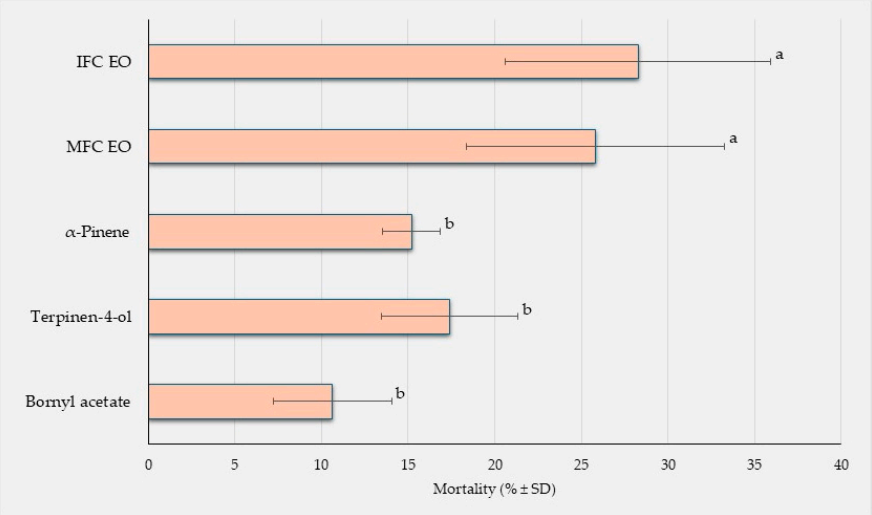

Figure 4. Toxicity of the essential oils (EO) isolated by hydrodistillation from Azorean *Cryptomeria japonica* immature and mature cones (IFC and MFC) and some of their components against *Artemia salina* nauplii at 100 µg/mL. Within each bar, means followed by the same letter are not significantly different at $p < 0.05$.

4. Conclusions

Owing to *C. japonica*'s importance in the wood industry, the phytochemistry and bioactivities of its residues have been intensively studied for many years and still remain the focus of attention of many researchers, especially in light of the recent interest in the use of forestry and wood industry residues in a sustainable world economy. Nevertheless, some *C. japonica* forestry waste, such as the strobili (cones), remain a relatively underutilized sustainable resource, considering their bioactive potential, as demonstrated in our previous research. In the present study, we evaluated and compared, for the first time, the antioxidant and antimicrobial activities, as well as the cytotoxicity for *A. salina* nauplii, of EOs from Azorean *C. japonica* immature and mature female cones (IFC and MFC).

The antioxidant activity of the studied EOs samples was evaluated using DPPH–FRSA and BCBA assays, and the results demonstrated that their BCBA was far stronger than the DPPH–FRSA.

Comparing both EO samples, the IFC EO exhibited the best antioxidant and antimicrobial results, posing it as a potential alternative raw material for food preservation and medicinal products, namely, in action against the pathogenic bacterium *S. aureus*. The existence of this bacterium in food presents a potential public health risk due to its capability to generate enterotoxins, posing a subsequent threat of food poisoning. On the other hand, the use of artificial chemicals as food preservatives is a pressing concern in the current context due to their lack of compatibility with the environment, inability to biodegrade, and unsustainability [45]. Although the antioxidant and antimicrobial assays used in this study were in vitro and, thus, do not reflect the cellular physiological conditions, the comparison of the antioxidant/antimicrobial properties of EOs from Azorean *C. japonica* FC with the activities of standards (ascorbic acid or gallic acid) indicates their effectiveness as antioxidant/antimicrobial agents. Moreover, the cytotoxicity of the studied EOs, measured via an *A. salina* test, exhibited interesting results, with the IFC EO being more toxic than the MFC EO, although there were no significant differences between them. According to the literature [48], the BSLA assay is an adequate method for the preliminary toxicity testing of EOs in tumor cell lines. In fact, a plethora of bioactivities, including anticancer properties, have been found in the major components of Azorean IFC and MFC EOs, such as the monoterpenes α-pinene and sabinene, the oxygenated monoterpene terpinen-4-ol, and the oxygenated sesquiterpenes elemol and eudesmol isomers (α, β, and γ).

However, more studies are warranted with respect to the potential application of Azorean *C. japonica* FC EOs for the development of natural health-related products, such as an in-depth in vivo and toxicology evaluation. Additionally, given the scarcity of studies with FC EO samples, further research will be necessary to verify the reproducibility of the results obtained in the present work.

Finally, it should also be highlighted that both the IFC and MFC samples were harvested from the same batch of Azorean *C. japonica* in the same seasonal period, thus, the observed variations in their EO bioactivities are attributable to differences in their chemical compositions, related to the FC's maturation process that, as demonstrated in this study, decreased their EOs' bioactivities.

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Abbreviations

Abs, absorbance; BCBA, β-carotene-linoleic acid bleaching activity; BSLA, brine shrimp lethality activity; CJBR, *Cryptomeria japonica* biomass residues; CJF, *Cryptomeria japonica* foliage; DDM, disc diffusion method; DH, diterpene hydrocarbons; DPPH, 2,2-diphenyl-1-picrylhydrazyl; EO, essential oil; EOC, essential oil component; FC, female cones; FRSA, free radical-scavenging activity; GC–MS, gas chromatography–mass spectroscopy; GIZ,

growth inhibition zones; HAT, hydrogen transfer atom; HD, hydrodistillation; IFC, immature female cones; IPMP, integrated pest management programs; MFC, mature female cones; MH, monoterpene hydrocarbons; MHA, Muller–Hinton Agar; OCD, oxygen-containing diterpenes; OCM, oxygen-containing monoterpenes; OCS, oxygen-containing sesquiterpenes; PDA, potato dextrose agar; RT, retention time; RI, retention indices; SET, single electron transfer; SH, sesquiterpene hydrocarbons; SMs, secondary metabolites.

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