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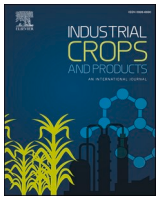

Azorean *Cryptomeria japonica* immature female cones essential oil: Effect of hydrodistillation fractionation on the chemical composition and in vitro antifungal activity against *Thielaviopsis paradoxa*

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ABSTRACT

Cryptomeria japonica's wood production in Azores generates large amounts of underutilized biomass residues, such as immature female cones (Az–CJIFC), that can be used to produce essential oils (EOs). Hydrodistillation (HD) can be used both to obtain and to fractionate EOs. In this study, EOs from Az–CJIFC, grinded (GR) and non-grinded (NGr) fresh samples, were obtained via HD over 4 h, yielding 1.0 % and 0.5 %, *w/w*, respectively. Thus, GR Az–CJIFC was chosen to obtain six EO fractions (Frs. 1–6), collected at sequential HD timeframes (HDTs: 0–2, 2–10, 10–30, 30–60, 60–120, and 120–240 min). The obtained EO samples (crude EOs and fractions) were evaluated for their chemical composition (GC–FID/GC–MS analyses) and antifungal activity (micro-atmosphere method) against phytopathogenic fungi (*Penicillium italicum*, *P. digitatum* and *Thielaviopsis paradoxa*). Results indicated that all samples were active only towards *T. paradoxa*, however, with a differential efficacy, due to their specific composition. Fraction 4 vapor treatment displayed the strongest activity, but lower than that of (–)-terpinen-4-ol, a key oxygen-containing monoterpene (OCM) of Az–CJIFC EO, peaking its concentration in Fr3 (14.5 %) and Fr4 (13.8 %). This latter fraction was the richest in the OCM α-terpineol (a minor Az–CJIFC EOs' component). On the other hand, Frs.1–3 and EOs were dominated by monoterpene hydrocarbons (65.0–96.5 %), mainly α-pinene (19.0–28.4 %) and sabinene (19.9–50.5 %), while Frs.5 and 6 were the richest in oxygen-containing sesquiterpenes (47.1–70.8 %; chiefly elemol plus α-, β- and γ-eudesmol) and diterpene hydrocarbons (5.2–6.4 %; mostly phyllocladene). In conclusion, new high value-added products can now be targeted in Az–CJIFC EO by adjusting the HDT, with potential importance in pineapple fruit black rot disease management caused by *T. paradoxa* on *Ananas comosus* in the Azores, and also contributing for the local *C. japonica*'s EO industry development and sustainable circular bio-economy.

Abbreviations: Az–CJIFC, Azorean *Cryptomeria japonica* immature female cones; Bp, boiling point; CJBR, *Cryptomeria japonica* biomass residues; CJFC, *Cryptomeria japonica* female cones; DH, diterpene hydrocarbons; D.w., dry weight; EO, essential oil; EOC, essential oil component; FC, female cones; Fr, fraction; F.w., fresh weight; GC–FID, gas chromatography with flame ionization detection; GC–MS, Gas chromatography/mass spectrometry; GR, grinded; HD, hydrodistillation; HDT, hydrodistillation timeframe; IFC, immature female cones; MFC, mature female cones; IPM, integrated pest management; MGI, mycelium growth inhibition; MH, monoterpene hydrocarbons; NGr, non-grinded; OCD, oxygen-containing diterpenes; OCM, oxygen-containing monoterpenes; OCS, oxygen-containing sesquiterpenes; PCA, principal component analysis; PDA, potato dextrose agar; PDO, Protected Designation of Origin; RI, retention indices; SH, sesquiterpene hydrocarbons; TTO, tea tree oil.

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1. Introduction

According to the International Standardization Organization (ISO, 9235, 2021) and the European Directorate for the Quality of Medicines and HealthCare EDQM Council of Europe, (2021), an essential oil (EO) is defined as the product obtained from vegetable raw material through distillation (hydro-, steam- or dry distillation), or by a suitable mechanical process without heating, in the case of the *Citrus* sp. fruits' epicarp. The EOs can be constituted of only a few to up to more than 100 individual components (EOCs), mainly terpene compounds, which together contribute to the properties of the complete mixture, through synergistic, additive, antagonistic or even suppressive effects that can occur between the EOCs (Bakkali et al., 2008; de Sousa et al., 2023). Nevertheless, despite the great interest from the scientific community in the discovery of novel drugs and/or botanical pesticides from these natural products, their bioactive potential is still under-scrutinized (Feyaerts et al., 2020; Ngegba et al., 2022), particularly as promising botanical pesticide agents with impacts in future green integrated pest management (IPM) systems, if formulated through encapsulation to enhance their effectiveness (Cruz et al., 2018; Ngegba et al., 2022; Raveau et al., 2020). It is also important to highlight that the antifungal properties of the EO vapor phase render the EOs attractive as possible fumigants to control postharvest diseases in some fruits, constituting, thus, an alternative to spraying or dipping application methods (Laird and Phillips, 2012; Sivakumar and Bautista-Baños, 2014; Tyagi et al., 2012). However, the biological effects of EOs depend on their sources and composition (Barra, 2009; Figueiredo et al., 2008; Figueiredo, 2017).

Cryptomeria japonica (Thunb. ex L. f.) D. Don (Cupressaceae), the target species in this study, is a large evergreen conifer native to Japan, constituting a valuable source of EO and organic extracts, as highlighted in Lima et al.ś critical reviews (Lima et al., 2021; Lima et al., 2023a). *C. japonica* EO can find multiple potential uses in various fields (e.g., food industry, cosmetic, agriculture and complementary medicine), with manifold approaches. In fact, as documented in several reports over the last eight decades (Lima et al., 2021; Nakagawa et al., 2016; Shieh et al., 1981 and the references therein), despite their low nematotoxic activity (Faria et al., 2013), *C. japonica* EO exhibit a broad-spectrum of relevant biological properties, including antibacterial, antifungal (Moiteiro et al., 2013; Lima et al., 2021), termiticidal (Cheng et al., 2007), mosquitocidal (Cheng et al., 2003; Mdoe et al., 2014), antioxidant (Kim et al., 2013; Ruas et al., 2022), anti-inflammatory (Yoon et al., 2009), anticancer (Cha and Kim, 2012) and neuropharmacological (Cheng et al., 2009).

Like most conifer trees, *C. japonica* is a monoecious species, i.e., its reproductive organs or strobili (male and female cones) occur on the same tree. Female cones (FC), the plant part used in this study (Fig. 1), are globular, with a terminal distribution on downcurved branchlets with normal leaves. The young or immature FC (IFC) emerge from a rosette of leaves and are nearly 5 mm in diameter. During the pollination period, which occurs from February to April, IFC possess a flat top, becoming almost globular within one month, while mature FC (MFC) have a tapering apex and a 1–2 cm diameter, containing 20–30 spirally arranged megasporophylls (Hosoo, 2007; Farjon, 1999).

Cryptomeria japonica has been widely introduced into other temperate areas outside Japan, such as in the Azores archipelago (Portugal), where it is, currently, the major plantation tree, representing nearly 60 % of the total wood producing forest area (Dias et al., 2007). Thus, timber production and forest operations produce significant amounts of underutilized Azorean *C. japonica* biomass residues (CJBR), such as bark, sapwood, heartwood, leaves, cones, roots, sawdust and cutter shavings (Figueiredo et al., 2021; Lima et al., 2023a). However, the remarkably increasing demand for EOs in the global market could bring new opportunities for the valorization of these CJBR as abundant renewable EOs-rich resources (Figueiredo et al., 2021; Lima et al., 2021).

Fig. 1. Fresh immature female cone of Azorean *Cryptomeria japonica* used in this study. Bar = 1 cm.

Besides forests, which represent approximately a third of the Azores territory, agriculture is a determinant component of the Azorean economy, occupying about half of the territory, being vineyards, followed by banana, oranges and pineapple, the most representative of the permanent cultures (de Almeida et al., 2021). Currently, pineapple (*Ananas comosus* L. Merr.) is one of the most important tropical fruits worldwide, reaching a production around 30 million tons in 2021 (Chaves et al., 2024). Particularly, Azorean pineapple is renowned as a unique and valued variety, distinguished by its concentrated aroma, setting it apart from other commercially available pineapple (Bastos, 2024; Chaves et al., 2024).

However, fruit production is strongly affected by fungal postharvest diseases worldwide (Sharma et al., 2009), and emerging plant pathogens continue to threaten global food security (Ristaino et al., 2021). For instance, pineapple black rot (Fig. 2) disease, caused by *Thielaviopsis*

Fig. 2. Black rot on Azorean pineapple caused by *Thielaviopsis paradoxa*.

paradoxa (de Seynes) Höhn (Sapak et al., 2021), is a relatively recent issue in Azorean pineapple cultivation, with no prior records of this disease in the region. In contrast, green and blue mold rot diseases of citrus fruits, caused by *Penicillium digitatum* (Person) Saccardo and *P. italicum* Wehmer, respectively, have long been persistent issues in citrus production worldwide (Papoutsis et al., 2019).

The most common practice to protect postharvest agricultural products from phytopathogenic fungi attack is the application of synthetic fungicides (Alhudaib et al., 2022; Sharma et al., 2009), such as azoles, strobilurins or dithiocarbamates (Zubrod et al., 2019). Nonetheless, the global trend for the control of postharvest decay is shifting toward safer and more eco-friendly alternative strategies with greater acceptance by society. Among these options, natural products, mainly EOs, could be used as effective alternatives to synthetic fungicides.

In our continuing strategy to enhance the Azorean CJBR valorization and to support the development of a local EO industry within a sustainable circular bio-economy, we recently evaluated the EO´biological potential from different, and less studied, Azorean *C. japonica* aerial parts, such as FC, who EO was found to be a broad-spectrum antimicrobial agent (Lima et al., 2023b). Earlier, it was demonstrated that, interestingly, IFC EO is a promising source of multi-bioactivities (e.g., antimicrobial and antioxidant), when compared to MFC EO, due to their differential composition (Janeiro et al., 2024a,b). Therefore, in the present study, we focus our attention on the EO fractionation of this Azorean *C. japonica* IFC (Az–CJIFC) sample.

In this context, the objectives of the present study are as follows: (1) to obtain crude EOs (using HD over 4 h) from fresh Az–CJIFC, grinded (GR) and non-grinded (NGr) samples, in order to evaluate the influence of grinding on Az–CJIFC EO extraction efficiency; (2) to obtain six different EO fractions (Frs. 1–6) collected in sequential HD timeframes (HDTs: 0–2, 2–10, 10–30, 30–60, 60–120, and 120–240 min) during HD of the GR Az–CJIFC sample, in order to determine the impact of the fractionation process on several EO parameters; (3) to analyze the chemical composition of the obtained EO samples (crude EOs and EO fractions); (4) to develop regression models to predict EO yield, EO HD rate (HDR) and individual concentration of selected Az–CJIFC EOCs for a given HDT; and (5) to assess the *in vitro* antifungal activity of the obtained EO samples and some of their representative EOCs, namely, (–)-α-pinene and (–)-terpinen-4-ol, against some postharvest phytopathogenic fungi of global interest (*P. italicum*, *P. digitatum* and *T. paradoxa*). Overall, the results of this study can contribute to add more value to the *C. japonica* EO industry, due to the production of EO fractions with stronger *in vitro* antifungal activity than the crude EO.

2. Materials and methods

2.1. Chemicals and reagents

Standard mixture of C_8–C_{22} *n*-alkanes, (–)-α-pinene (≥ 97 %), (–)-terpinen-4-ol (≥ 95 %) and anhydrous sodium sulphate (Na_2SO_4) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Potato dextrose agar (PDA) was purchased from Merck (Darmstadt, Germany).

2.2. Plant material

The foliage of *C. japonica* at seed maturation stage was collected in mid July 2023 (summer season) from a tree population located on Lomba da Maia (latitude, 37° 49' 07.7'' N; longitude, 25° 21' 33.2'' W; altitude, 320 m) in the northeast region of São Miguel Island (Azores archipelago, Portugal). The fresh IFC attached to the foliage sample were immediately removed and stored at –20 °C until further HD. A portion of the fresh IFC sample was air-dried in the shade, until achievement of constant weight, for water content determination, which was 68 % (*w/w*).

The plant material was cut off from at least 10 different healthy *C. japonica* adult trees to generate a representative sample of IFC within the population and to minimize potential variations in chemical composition among individual trees. A voucher specimen (AZB 4583) was deposited in the Herbarium AZB – Ruy Telles Palhinha of the University of the Azores.

2.3. Essential oil (EO) extraction and fractionation via hydrodistillation (HD)

The HD process, using a Clevenger-type apparatus, was carried out to extract and fractionate the EO from the fresh Az–CJIFC sample. Each HD was performed in triplicate. In all experiments, (1) the ratio of the sample to water was 1:7.5 g/mL, (2) the start of the HD process was registered after the first distillate drop fell into the collecting unit of the apparatus, and (3) the distillate flow rate was 6 mL/min.

The crude EOs from the GR and NGr plant material were obtained during a period of 4 h. In the experiment with the GR sample, prior to the HD process, the Az–CJIFC (400 g) were blended in 1 L water (to avoid EO losses by volatilization), during approximately 1 min, and then transferred to the round bottom flask, to which two additional liters of water were added. The GR Az–CJIFC EO fractionation was performed according to the method published by Arruda et al. (2023). Briefly, the EO fractions were collected in the following HDTs: 0–2, 2–10, 10–30, 30–60, 60–120 and 120–240 min (Frs. 1–6), being captured in a sequential order without interrupting the HD process.

The obtained EO samples (crude EOs and EO fractions) were dehydrated with anhydrous Na_2SO_4, filtered, weighted on an analytical balance (Metler AE 240, Mettler Toledo, Columbus, USA), and stored in amber vials at 4 °C until further chemical analyses and biological assays. The EO yield (%) was calculated as EO mass (g) per 100 g of fresh weight (f.w.) of Az–CJIFC, and the HDR as the EO mass accumulated per min (EO mg/min).

2.4. Essential oil (EO) and EO fractions analysis

2.4.1. Chemical composition determination

Gas chromatography with flame ionization detection (GC–FID) was used for component quantification of the EO samples (crude EOs and EO fractions) by the normalization method, in compliance with ISO, (7609) (1985). Gas chromatography coupled to mass spectrometry (GC–MS) was used to identify the EOCs by comparison of their mass spectra with those from a custom-made library, created as fully detailed in Póvoa et al. (2024), based upon the analyses of reference EOs, laboratory-synthesized components, and commercially available standards (Sigma-Aldrich; Fluka, Riedel-de Haën; Extrasynthese, Cymit Química, S.L.), and by calculation of their retention indices (RI) relative to a C_8–C_{22} *n*-alkane ladder.

Both GC–FID and GC–MS analyses were carried out with a DB-1 capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) from J & W Scientific Inc. (Rancho Cordova, CA, USA).

2.4.2. Physical properties (density and color) determination

The density (ρ) of the crude EOs and EO fractions was calculated as follows: $\rho\ (g/cm^3) = EO_m / EO_v$, where EO_m and EO_v represent the weight and the volume of the EO sample, respectively. The color of the EO samples was evaluated subjectively, while, additionally, objective color measurements were obtained using the online tool available at PINETOOL website https://pinetools.com/ (accessed on 11 November 2024). A photograph of each EO sample, taken against a white background, was uploaded to retrieve the hexadecimal (HEX) color code and the Red Green Blue (RGB) color values.

2.5. In vitro antifungal activity determination

2.5.1. Fungal strains and culture media

The three selected ascomycetous fungal isolates (filamentous, soilborne and strict wound pathogenic fungi), obtained from the

collection of the Microbiology Laboratory at the University of the Azores, include: (1) *P. italicum* and *P. digitatum* (Trichomaceae), previously isolated from infected citrus fruits exhibiting the typical blue and green mold symptoms, respectively (Arruda et al., 2024), and (2) *T. paradoxa* (Ceratocystidaceae), isolated from an infected pineapple fruit, supplied by the Regional Plant Health Laboratory. The fungal strains were maintained on PDA medium.

2.5.2. Micro-atmosphere method

The micro-atmosphere method, also known as inverse Petri dish method, allows the evaluation of the volatile fraction of an EO or an EOC as an antimicrobial agent (Cardiet et al., 2012). This method was selected to evaluate the antifungal potential of the EO samples because pineapple cultivation in the Azores occurs in greenhouses, making the micro-atmosphere method a closer representation of real-world conditions in which these crops are inserted. In this assay, a Petri dish (9 cm diameter × 1.5 cm height; 90 mL volume) was uniformly filled with 20 mL of PDA medium, and then inoculated in the center with a mycelium fragment (2–3 mm diameter) taken from the periphery of an actively growing fungal culture. Afterwards, a sterile paper disc was placed in the inner center of the lid of the Petri dish and loaded with 20 μL (286 μL/L of air) of undiluted EO sample (NGr, GR and Frs.1–6) or EOC, namely, (–)-α-pinene and (–)-terpinen-4-ol. The inverted (lid down) Petri dishes were immediately sealed with 2 layers of Parafilm© (to prevent the loss of volatile EOCs) and then incubated for 5–8 days at 25 ± 1 °C. A negative control was prepared in the same way, being the paper disc loaded with distilled water. The mycelium diameter (mm) was measured daily as the mean diameter of two opposite sides. Each experiment was performed in triplicate.

Terpinen-4-ol, a component of Az–CJIFC EO, was also considered as the positive control, specifically its (–)-terpinen-4-ol isomer, due to its recognized potential as a fungicide alternative (Terzi et al., 2007), with effectiveness against several phytopathogenic fungi (Li et al., 2016; Morcia et al., 2012; Terzi et al., 2007; Yu et al., 2015), along with its low levels of phytotoxicity (Morcia et al., 2012).

2.6. Statistical analyses

Statistical analyses were conducted using IBM SPSS Statistics 28.0.1.0 version (SPSS Inc., Chicago, IL, USA). All determinations were performed in triplicate, and the results are expressed as mean ± standard deviations (SD). The effects of (1) HDTs (0–2, 2–10, 10–30, 30–60, 60–120, 120–240, and 0–240 min) and (2) sample pre-treatment (i.e., grinding) on the Az–CJIFC EO properties, namely, yield, HDR, density, chemical composition and antifungal activity, were determined using a one-way analysis of variance (ANOVA). For each response variable, the validity of model assumptions was checked by using the residuals (Montgomery, 2017). When the HDTs or sample pre-treatment effect proved to be significant ($p < 0.05$), Duncan's multiple-range test was conducted at the 5 % level of significance for multiple means comparison and subsequent letter grouping generation. Furthermore, principal component analysis (PCA), based on the contents of representative EOCs of each terpene class (namely, α-pinene, sabinene, β-myrcene, α-terpinene, γ-terpinene, δ-terpinene, terpinen-4-ol, α-terpineol, germacrene D, δ-cadinene, elemol, γ-eudesmol, β-eudesmol, α-eudesmol and phyllocladene), was performed to classify the EO samples under study (crude EOs and EO fractions) into chemical groups.

The relationships between the HDTs (excluding the 0–240 min) and (i) the EO yield, (ii) the EO HDR and (iii) the individual concentration of nine of the selected EOCs (namely, α-pinene, sabinene, β-myrcene, δ-cadinene, elemol, γ-eudesmol, β-eudesmol, α-eudesmol and phyllocladene) were adequately modelled by the power, exponential decay, logarithmic or linear models, as shown in Eqs. 1–4, respectively. While Eq. 4 is a linear regression model, the other three models (Eqs. 1–3) are nonlinear (NLIN) and their parameters were estimated iteratively through the NLIN Regression Procedure of IBM SPSS Statistics (software version as referred above), meeting the fitted models all the adequacy requirements of NLIN models (Bates and Watts, 2007).

$$Y = \theta_1 X^{\theta_2} + \varepsilon \quad (1)$$

$$Y = \theta_1 Exp(\theta_2 X) + \varepsilon \quad (2)$$

$$Y = \theta_1 Log(X) + \theta_2 + \varepsilon \quad (3)$$

$$Y = \beta_0 + \beta_1 X + \varepsilon \quad (4)$$

In the four regression equations, Y is the dependent (response) variable, X is the independent (HDT, excluding the 0–240 min) variable, and ε is the error term assumed to have normal distribution with constant variance. Validity of the normality, constant variance, and independence assumptions on the ε were verified by the examination of residuals (Bates and Watts, 2007).

3. Results and discussion

3.1. Yield, hydrodistillation rate (HDR) and physical properties of the Azorean Cryptomeria japonica immature female cones (Az–CJIFC) essential oil (EO) and EO fractions

As shown in Table 1, the EO yield value of the GR sample doubled in relation to that of the NGr sample (1.0 vs. 0.5 %, *w/w*, f.w.), indicating that the grinding step prior to HD is critical to disrupt the secretory structures in the Az–CJIFC, besides increasing the plant material surface area. This interestingly finding on the improvement of Azorean *C. japonica* EO content is reported here for the first time. A similar trend was observed by Zheljazkov et al. (2017), with respect to the EO extraction efficiency of *Juniperus* spp. galbuli samples. Concerning the yield of NGr Az–CJIFC samples, our prior investigation (Janeiro et al., 2024a) reported that it exhibited a value of 0.72 % (*w/w*) on a dry weight (d.w.) basis. However, in the current study, we found a higher yield (1.61 %, *w/w*, d.w.). This result may be explained by the different raw material used (e.g., growing location, plant developmental stage, and sampling time), which influences the EO yield. As also illustrated in Table 1, the sequential separation of GR Az–CJIFC sample at specific HDT resulted in EO fractions with significant differences ($p < 0.05$) in their yield and HDR, being the relationship between HDTs and EO yield (Fig. 3a), and between HDTs and EO HDR (Fig. 3b), adequately modelled by the logarithmic (Eq. 3) and power (Eq. 1) models, respectively. A great variability is also observed in other EO fractions' parameters, namely, some physical properties, such as density and color (Table 1).

Relatively to the yield, the GR Az–CJIFC EO fractions presented values ranging from 0.06 % to 0.28 % (∑ 0.91 %), revealing no significant losses in the EO fractionation by HD method. Regarding the HDR (accumulation of EO mass over time), the EO fractions showed values ranging from 2.1 to 558.5 mg/min, decreasing as follows: Fr1 > Fr2 > Fr3 > Fr4 > Fr5 > Fr6. Concerning the density, the EO fractions under study exhibited a narrow range, spanning from 0.843 to 0.907 g/cm^3, being all these values lower than that of water. Moreover, a discernible color spectrum was evident, transitioning from colorless in Fr1 to yellow in Fr6.

It should be highlighted that the fraction with maximum EO yield (0.28 %) was Fr1 (0–2 min HDT), which, despite being the fraction with the shortest collection time (only 2 min), presented the maximum HDR (558.5 mg/min), due to the simultaneous distillation of monoterpene hydrocarbons (MH), the most abundant terpene class in the studied crude Az–CJIFC EOs (see 3.2.), and of the EOCs with the lowest boiling points.

Overall, in the beginning of the HD of the GR Az–CJIFC sample, the EO was extracted at a very high rate, but which rapidly decreased as HD progressed. In fact, during the first half-hour, 77 % of the EO had already been extracted (31 % in Fr1, 23 % in Fr2 and 23 % in Fr3), with

Table 1
Yield, hydrodistillation rate (HDR), density and color (subjective, HEX code and RGB values) of the Azorean *Cryptomeria japonica* immature female cones essential oil (EO) and EO fractions (Frs. 1–6).

HDTs (min)	Essential oil						
	Samples	Yield* (%, w/w, f.w.)	HDR* (mg/min)	Density* (g/cm^3)	Color		
					Subjective	HEX codes	RGB values
0–2	Fr1	0.279 ± 0.023^{c}	558.5 ± 47.0^{a}	0.843 ± 0.008^{c}	colorless	#8f8480	143,132,128
2–10	Fr2	0.211 ± 0.011^{d}	105.7 ± 5.3^{b}	0.853 ± 0.011^{bc}	pale yellow	#a6987b	166,152,123
10–30	Fr3	0.207 ± 0.018^{d}	41.4 ± 3.5^{c}	0.869 ± 0.013^{abc}	pale yellow	#a89867	168, 152, 103
30–60	Fr4	0.081 ± 0.003^{e}	10.8 ± 0.4^{e}	0.886 ± 0.014^{ab}	yellowish	#917c39	145, 124, 57
60–120	Fr5	0.066 ± 0.003^{ef}	4.4 ± 0.2 ^{g}	0.907 ± 0.015^{a}	yellow	#8a7026	138, 112, 38
120–240	Fr6	0.064 ± 0.010 ^{f}	2.1 ± 0.3 ^{h}	0.901 ± 0.015^{a}	yellow	#856822	133, 104, 34
0–240	GR	0.998 ± 0.050^{a}	16.6 ± 0.8^{d}	0.890 ± 0.004^{ab}	yellow	#b59b3c	181, 155, 60
0–240	NGr	0.516 ± 0.012^{b}	8.6 ± 0.2 ^{f}	0.904 ± 0.003^{a}	yellow	#b49a3d	180, 154, 61

* Values are mean ± SD (n = 3). Within each column, means sharing the same superscript letter are not significantly different (p < 0.05). HDTs: hydrodistillation timeframes. f.w.: fresh weight. GR and NG: grinded and non-grinded plant material, respectively; HEX: hexadecimal; RGB: Red Green Blue.

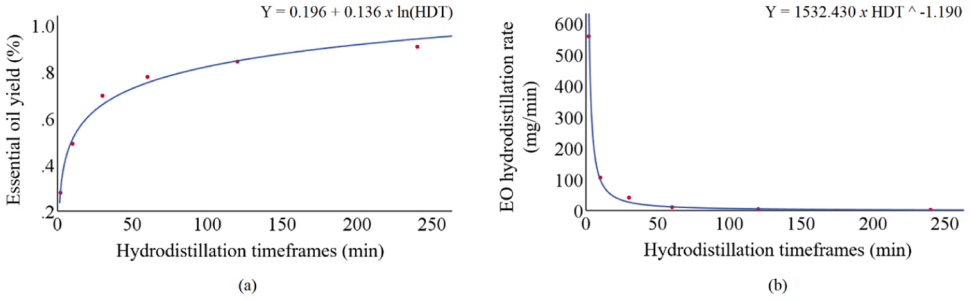

Fig. 3. Plot of hydrodistillation timeframes (HDTs) vs. (a) essential oil (EO) cumulative yield and (b) EO hydrodistillation rate, along with the fitted logarithmic and power regression models, respectively.

Table 2
Representative components of each terpene class in the Azorean *Cryptomeria japonica* immature female cones essential oil (EO) and EO fractions (Frs. 1–6).

Selected components						Relative content in percentage							
						EO samples (hydrodistillation timeframes, in min)							
N.	Name	Class	IP	RI	bp (°C)	NGr (0–240)	GR (0–240)	Fr1 (0–2)	Fr2 (2–10)	Fr3 (10–30)	Fr4 (30–60)	Fr5 (60–120)	Fr6 (120–240)
1	α-Pinene	MH	a,b	930	156	19.0 ^{cd}	21.1^{bc}	28.4^{a}	22.8^{abc}	26.2^{ab}	24.2^{abc}	13.3^{d}	3.5^{e}
2	Sabinene	MH	a,b	958	164	27.2^{c}	32.6^{b}	50.5^{a}	36.6^{b}	19.9^{d}	7.5^{e}	3.8^{ef}	1.8 ^{f}
3	β-Myrcene	MH	a,b	975	167	3.9^{d}	4.3^{c}	5.5^{a}	5.0^{b}	4.9^{b}	3.5^{e}	1.7^{d}	0.4 ^{f}
4	α-Terpinene	MH	a,b	1002	173–175	2.8^{b}	2.0^{c}	1.3^{d}	2.6^{b}	3.9^{a}	3.5^{a}	2.1^{c}	0.8^{e}
5	γ-Terpinene	MH	a,b	1035	181–183	3.9^{bc}	3.3^{c}	2.2^{d}	4.5^{b}	6.5^{a}	5.8^{a}	3.4^{c}	1.3^{e}
6	δ-Terpinene	MH	a	1064	184	1.5^{c}	1.4^{c}	1.3^{d}	1.9^{b}	2.3^{a}	1.9^{b}	1.1^{e}	0.4 ^{f}
7	Terpinen−4-ol	OCM	a,b	1148	209	12.1^{b}	6.8^{d}	1.9^{e}	10.0^{c}	14.5^{a}	13.8^{a}	7.3^{d}	2.9^{e}
8	α-Terpineol	OCM	a,b	1159	218–221	0.6^{c}	0.3^{d}	t	0.3^{d}	0.6^{c}	1.0^{a}	0.8^{b}	0.3^{d}
9	Germacrene D	SH	a,b	1474	279	0.4^{e}	0.6^{d}	0.3^{e}	0.7 ^{cd}	0.6^{d}	1.0^{b}	1.4^{a}	0.9^{bc}
10	δ-Cadinene	SH	b	1505	279	0.2^{d}	0.6^{c}	0.1^{e}	0.3^{d}	0.6^{c}	1.0^{b}	1.8^{a}	1.8^{a}
11	Elemol	OCS	b	1530	289	6.8^{c}	6.0 ^{cd}	0.5^{e}	2.9^{de}	4.4 ^{cd}	10.2^{b}	14.9^{a}	13.2^{ab}
12	γ-Eudesmol	OCS	b	1609	301–302	2.9^{c}	3.4^{c}	0.1 ^{f}	0.3^{e}	0.9^{d}	3.7^{c}	11.5^{b}	22.8^{a}
13	β-Eudesmol	OCS	b	1620	301	1.7^{d}	2.1 ^{cd}	t	0.3 ^{f}	0.7^{e}	2.8^{c}	7.5^{b}	13.3^{a}
14	α-Eudesmol	OCS	b	1634	299–302	2.2^{d}	2.9 ^{cd}	0.1 ^{g}	0.4 ^{f}	1.0^{e}	3.7^{c}	9.9^{b}	17.5^{a}
15	Phyllocladene	DH	a,b	2006	> 300	3.2^{b}	1.6^{c}	0.1^{e}	0.8^{d}	1.1 ^{cd}	2.5^{b}	4.6^{a}	5.8^{a}
16	Nezukol	OCD	c	2176	> 300	0.2^{b}	0.1^{b}	t	t	t	t	0.1^{b}	0.4^{a}
Total grouped components (%)													
Monoterpene hydrocarbons (MH)						65.0^{c}	71.2^{c}	96.5^{a}	80.4^{b}	71.7^{c}	53.1^{d}	29.7^{e}	9.7 ^{f}
Oxygen-containing monoterpenes (OCM)						14.6^{b}	8.7^{d}	2.3^{e}	12.7^{c}	17.8^{a}	17.3^{a}	9.1^{d}	3.5^{e}
Sesquiterpenes hydrocarbons (SH)						0.8^{ef}	1.6^{d}	0.3 ^{f}	1.4^{de}	1.7^{d}	3.0^{c}	4.8^{a}	3.6^{b}
Oxygen-containing sesquiterpenes (OCS)						14.4^{d}	15.5^{d}	0.7 ^{f}	4.4^{e}	7.2^{e}	21.9^{c}	47.1^{b}	70.8^{a}
Diterpene hydrocarbons (DH)						3.6^{c}	1.8^{d}	0.1 ^{f}	0.8^{ef}	1.3^{de}	2.8^{c}	5.2^{b}	6.4^{a}
Oxygen-containing diterpenes (OCD)						0.2	0.1	t	t	t	0.1	0.3^{b}	0.8^{a}
Total identified components (%)						98.6	98.9	99.9	99.7	99.7	98.2	96.2	94.8
Total terpenes (%)						69.4	74.6	96.9	82.6	74.7	58.9	39.7	19.7
Total terpenoids (%)						29.2	24.3	3.0	17.1	25.0	39.3	56.5	75.1
Ratio terpenes/terpenoids						2.4	3.1	32.3	4.8	3.0	1.5	0.7	0.3

Values are mean ± SD (n = 3). Within each row, means sharing the same superscript letter are not significantly different (p < 0.05). IP (identification procedure): a = commercial standards; b = reference EOs; c = mass spectra (Póvoa et al., 2024). RI: retention indices calculated on a DB-1 column. bp: boiling point. t: trace (<0.05 %). GR and NGr: grinded and non-grinded plant material, respectively.

the remaining 23 % being extracted over the next 3.5 h (9 % in Fr4, 7 % in Fr5 and 7 % in Fr6).

In our earlier investigation on the influence of HDTs on the EO yield of other Azorean *C. japonica* parts, namely foliage (Arruda et al., 2023), a significant higher EO yield was also observed during the first minutes of the HD fractionation process, namely, 53 % of the total EO had been extracted during the first hour (Frs. 1–4). However, in the current study we obtained a higher yield (86 %) for GR Az–CJIFC sample during the same HDT period (0–60 min), because of differences in the endogenous (e.g., plant part) and exogenous (e.g., growing location, sampling time and extraction protocol) factors between the two studies, which influence the EO yield.

Considering similar available literature on the EO yield of other important conifer genera, such as *Juniperus* (Cupressaceae) (Semerdjieva et al., 2019; Zheljazkov et al., 2017) and *Pinus* (Pinaceae) (Semerdjieva et al., 2022), as a function of HDT, the authors also found a significantly higher yield value during the first minutes of distillation. Nevertheless, the maximum EO release of the different studied conifer species occurred at different timeframes. Besides both the plant species and the extraction processes used, Semerdjieva et al. (2022) also reported that the EO yield depends on the studied plant parts (leaves, twigs, twigs with leaves, cones).

3.2. Chemical composition of the Azorean Cryptomeria japonica immature female cones (Az–CJIFC) essential oil (EO) and EO fractions

The chemical composition of the obtained EO samples (crude EOs and EO fractions), determined through GC–FID and GC–MS, are detailed in Supplementary Table S1. A total of 81 EOCs were identified in the NGr and GR Az–CJIFC EO samples, accounting for 98.6 % and 98.9 % of the total EO, respectively, and grouped into six terpene classes (Supplementary Table S1 and Table 2), namely: MH, oxygen-containing monoterpenes (OCM), sesquiterpene hydrocarbons (SH), oxygen-containing sesquiterpenes (OCS), diterpene hydrocarbons (DH), and oxygen-containing diterpenes (OCD). Among the identified EOCs (Supplementary Table S1), sixteen are reported in Table 2, selected as representative compounds of each terpene class identified in the EO samples under study (crude EOs and EO fractions).

The EOs of NGr and GR Az–CJIFC samples were primarily comprised of MH (65 vs. 71.2 %), followed by OCS (14.4 vs. 15.5 %) and OCM (14.6 vs. 8.7 %), while DH (3.6 vs. 1.8 %), SH (0.8 vs. 1.6 %) and OCD (0.2 vs. 0.1 %) were found in a lesser extent. The results indicate that the qualitative composition of both NGr and GR Az–CJIFC EOs was similar, whereas the relative contents of certain EOCs / terpene classes varied (Supplementary Table S1 and Table 2). Nevertheless, both NGr and GR EOs display similar terpenes/terpenoids ratio values (2.4 and 3.1, respectively) (Table 2).

As illustrated in Table 2, the major EOCs (≥4 %) in the GR Az–CJIFC EO sample were, in decreasing order, sabinene (MH; 32.6 %), α-pinene (MH; 21.1 %), terpinen-4-ol (OCM; 6.8 %), elemol (OCS; 6.0 %) and β-myrcene (MH; 4.3 %). Besides these, other EOCs were selected (compounds ns. 4–6, 8–10 and 12–16, Table 2) for analysis of their content (%) variation throughout the HD process, as detailed below.

The MH class was the highest at the beginning of the HD, accounting for 96.5 % of Fr1 EO content. Concerning the six selected MH compounds, the concentration of α-pinene, sabinene and β-myrcene was highest at Fr1, whereas that of α-terpinene, γ-terpinene and δ-terpinene reached its peak only in Fr3. In general, a decrease in the MH content as HDT increased was observed, as expected, reaching its lowest in Fr6. The same trend was reported in our prior study (Arruda et al., 2023) on the fractionation of Azorean *C. japonica* foliage EO during HD. Furthermore, the OCM started being extracted also relatively early in the HD process, reaching its maximum concentration at mid-distillation, in Fr3 and Fr4, for terpinen-4-ol and α-terpineol, respectively. However, there were no statistically significant differences between the terpinen-4-ol content in both fractions (Fr3 and Fr4). The relationships between HDTs and the concentrations of the MH α-pinene, sabinene and β-myrcene (Fig. 4) were adequately modelled by the linear (Eq. 4), power (Eq. 1) and exponential (Eq. 2) models, respectively. On the other hand, for the other selected MH (α-terpinene, γ-terpinene and δ-terpinene), as well as for the OCM (terpinen-4-ol and α-terpineol), there was no linear or NLIN regression model that could describe the relationship between their concentrations and the HDTs.

The SH class was present at low amounts, as already mentioned, with both germacrene D and δ-cadinene peaking their concentrations in Fr5. Conversely, OCS were found in a greater extent and mostly extracted towards the end of the HD process, with elemol reaching its maximum concentration in Fr5, and γ-eudesmol, β-eudesmol and α-eudesmol in Fr6. Among the SH compounds, only the relationship between HDTs and δ-cadinene concentration could be described, namely, by the logarithmic (Eq. 3) model (Fig. 5). In terms of the OCS, the model that best described the relationship between the elemol concentration and HDTs was the logarithmic, whereas the relationships between eudesmol isomers (α, β and γ) concentrations and HDTs (Fig. 6) were adequately described by a linear regression model.

The DH class was also found in low amounts, with phyllocladene reaching its maximum concentration in Fr6. Following the same pattern, OCD compounds were present in very low amounts, with nezukol reaching its maximum at Fr6. The relationship between HDTs and the DT phyllocladene concentration (Fig. 7) was adequately described by the power model.

Overall, the different HDTs significantly influenced the chemical composition through all terpene classes. MH class dominated in Frs. 1–4 (97 %–53 %), as well as in the crude EOs, with sabinene as the major MH in Frs. 1 and 2 and crude EOs, and α-pinene in Fr3 and Fr4. On the other hand, OCS dominated in Frs. 5 (47 %) and 6 (71 %), with the combination of elemol plus eudesmol isomers (α, β and γ) as major OCS. Thus, the results clearly validated our hypothesis that the fractionation of Az–CJIFC EO during HD process is a valuable tool for obtaining EO fractions with differential compositions, due to its complex chemical profile, comprising mono-, sesqui- and diterpenes, and their oxygenated derivatives. This diverse array of compounds, with different physicochemical properties, namely, vapor-pressure, boiling point, molecular weight and chemical structure, enables the extraction of EO fractions characterized by diverse terpene profiles. Similar results to those found in the present study have been described in the literature for other conifer species, i.e., the HDTs influenced the chemical composition of EOs from *Juniperus* spp. (Cupressaceae), namely *J. communis* L., *J. excelsa* M. Bieb., *J. sabina* L. and *J. virginiana* L. (Semerdjieva et al., 2019; Zheljazkov et al., 2017), with MHs (e.g. α-pinene, sabinene, β-myrcene and limonene) generally eluted in higher amounts early in the HD process, followed by their oxygenated derivatives, and then sesquiterpene compounds at the latter stages of HD. Contrarywise, Semerdjieva et al. (2022) reported that the EO from conifer *Pinus* genera (Pineceae), namely *P. heldreichii* Christ., *P. peuce* Griseb. and *P. mugo* Turra, had relatively constant compositional profile at the different HDTs.

To further assess the quantitative chemical variations within the studied EO samples (Table 2), PCA was used. The results (Fig. 8) allowed the separation of the EO samples into three chemical groups, as follows: (i) G1 (Frs.1–3 and crude EOs), characterized by higher MH content (mainly α-pinene and sabinene); (ii) G2 (Frs.5 and 6), having higher OCS (mainly elemol, and α-, β- and γ-eudesmol) and DH contents; and (iii) G3, constituted by only one EO sample (Fr4), which presented intermediate concentrations of MH, OCS and DH, but had the highest levels of α-terpineol.

3.3. In vitro antifungal activity of the vapor phases of Azorean Cryptomeria japonica immature female cones (Az–CJIFC) essential oil (EO) and EO fractions

There is increasing evidence that vapor phases of EOs are more

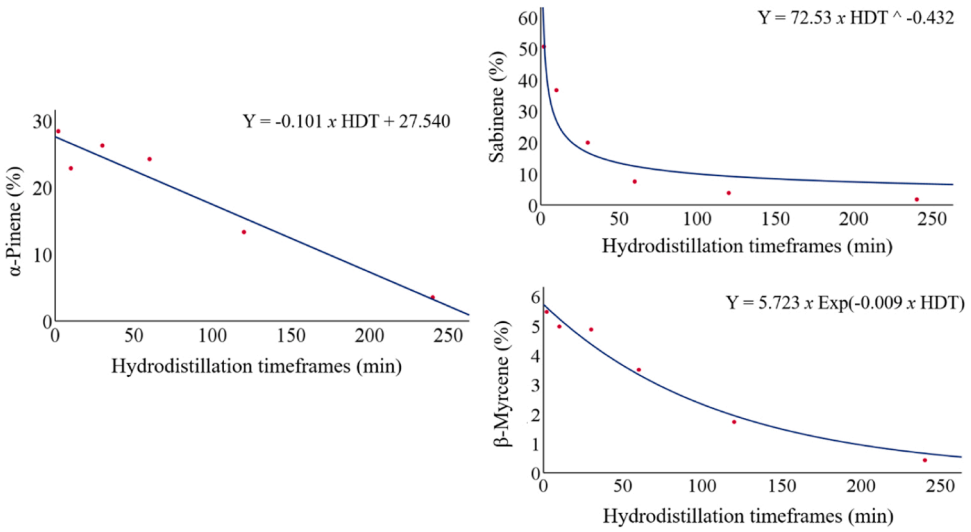

Fig. 4. Plot of hydrodistillation timeframes (HDTs) vs. the concentration of α-pinene, sabinene and β-myrcene (monoterpene hydrocarbons), along with the fitted linear, power and exponential models, respectively.

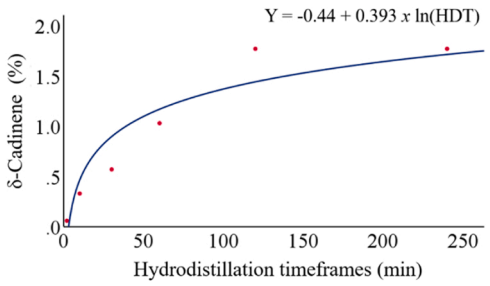

Fig. 5. Plot of hydrodistillation timeframes (HDTs) vs. the concentration of δ-cadinene (sesquiterpene hydrocarbon), along with the fitted logarithmic regression model.

effective against some fungi than the directly applied liquid phase (whole EO) (Abers et al., 2021; Benjilali et al., 1984; Inouye et al., 2006; Reyes-Jurado et al., 2020; Střelková et al., 2024). According to Inouye et al. (2003), this behavior can be explained, at least in part, by the fact that bioactive hydrophobic EOCs might associate in the aqueous phase to form micelles, and thus, suppress their attachment to the organism, whereas the vapor phase allows free attachment.

The antifungal activity of the obtained Az–CJIFC EO samples (crude EOs and EO fractions) was assessed through micro-atmosphere method, for practical application for fumigation. Results indicated that all samples were inactive in the case of *P. digitatum* or *P. italicum* (data not shown), but displayed activity against *T. paradoxa*, indicating, thus, a selective antifungal action relative to the studied filamentous fungi. Previous reports (Arruda et al., 2024; Janeiro et al., 2024b; Lima et al., 2023b) on the activity against *Penicillium* spp. of EOs / EO fractions, obtained from different parts of Azorean *C. japonica*, also revealed no growth inhibition effect or weak effect, as assessed via disc diffusion method. This inefficacy may be due to the high-level intrinsic resistance mechanisms of certain *Penicillium* species toward antifungal agents, through their metabolization or neutralization into less harmful forms (Tao et al., 2014). Furthermore, our results suggest a positive correlation

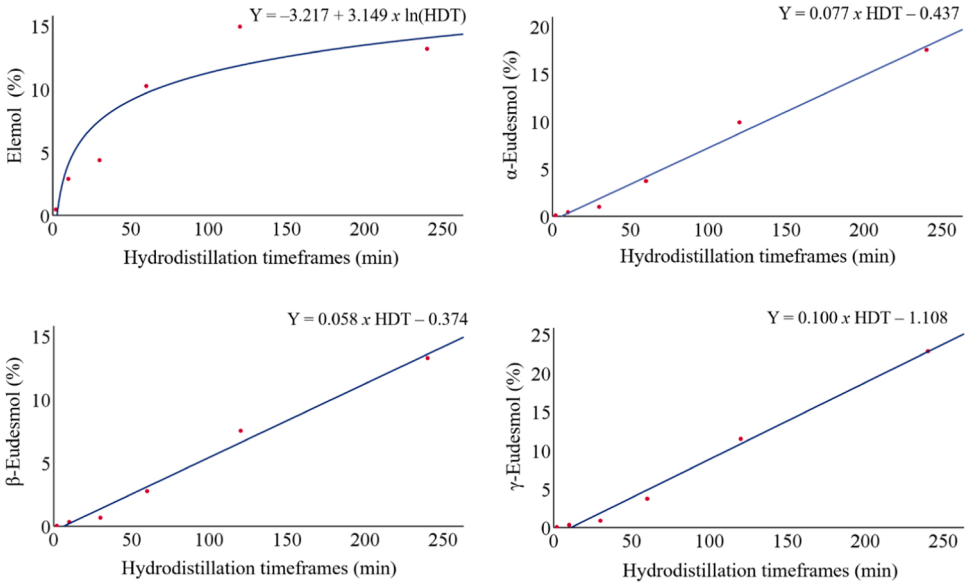

Fig. 6. Plot of hydrodistillation timeframes (HDTs) vs. the concentration of the oxygen-containing sesquiterpenes elemol and eudesmol isomers (α, β and γ), along with the fitted logarithmic and linear regression models, respectively.

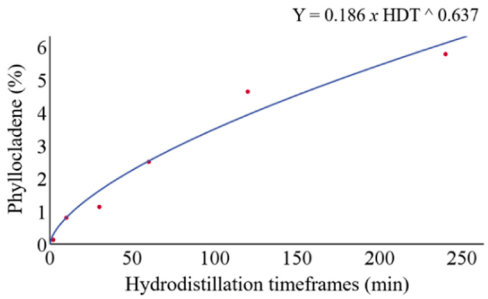

Fig. 7. Plot of hydrodistillation timeframes (HDTs) vs. the concentration of phyllocladene (diterpene hydrocarbon), along with the fitted power regression model.

between the vapor and the contact activities of Azorean *C. japonica* EO against the selected *Penicillium* fungal strains. The same pattern was reported in other similar studies (Inouye et al., 2006).

The activity of the Az–CJIFC EO samples and some of their EOCs, namely (–)-α-pinene and (–)-terpinen-4-ol, against *T. paradoxa*, expressed as mycelium growth inhibition (MGI), is illustrated in Fig. 9 and detailed in Table 3 as well.

The results show that the *T. paradoxa* MGI of the tested samples decreased in the following order: (–)-terpinen-4-ol > Fr4 >> Fr5 > NGr ≈ Fr3 > GR > (–)-α-pinene ≈ Fr2 = Fr6 > Fr1, after 3 days of incubation, which correspond to the incubation time in which the PDA control plate was completely covered by *T. paradoxa* mycelium.

Considering the EO samples (crude EOs and EO fractions), it is noteworthy the strongest effect of Fr4 on *T. paradoxa* MGI, as illustrated in Fig. 10. Specifically, in the presence of this fraction, the *T. paradoxa* mycelium reached only 8 mm in diameter after 3 days of incubation, compared to 90 mm in the PDA control plate, and only covered the PDA plate completely after 8 days of incubation (Table 3). Contrarywise, the other samples presented weaker effect on *T. paradoxa* MGI, being the respective PDA plate completely covered in 3 days (Fr1), 4 days (Fr2, Fr3, Fr6, GR and NGr) and 5 days (Fr5) (Table 3).

Considering now the tested EOCs (Table 3), it is also noteworthy the strong effect of (–)-terpinen-4-ol on *T. paradoxa* MGI. Although the assay was originally designed for an 8-day incubation period, (–)-terpinen-4-ol demonstrated exceptional antifungal efficacy by completely inhibiting *T. paradoxa* mycelial growth for 20 days, establishing it as a potent antifungal agent against this fungus. Contrarywise, (–)-α-pinene presented weaker *T. paradoxa* MGI, being the PDA plate completely covered in 4 days.

According to the published data availability, studies on the integrated reduced use of conventional fungicides against *T. paradoxa* are very limited (Cruz et al., 2018; Riska et al., 2023). The latter study, similar to ours, investigated the activity against *T. paradoxa* of EO vapor phase from *Syzygium aromaticum* (L.) Merr. & L.M. Perry (Myrtaceae) and *Cinnamomum burmanii* (Nees & T. Nees) Blume (Lauraceae), known as clove and cinnamon, respectively. The authors (Riska et al., 2023) reported total MGI up to 7 days incubation at a dose of 4 μL/petri dish, and suggest that the observed antifungal activity of clove and cinnamon EOs is due to their major compounds, namely eugenol and cinnamaldehyde, respectively, since these phenylpropanoids are known antifungal agents with described action mechanisms against fungi. Nevertheless, to our knowledge, the present study is the first report on the antifungal activity of the vapor phase EO from conifers against *T. paradoxa*. Contrary to the reported chemical profiles of the EOs from clove and cinnamon species (Riska et al., 2023), the terpene compounds are the major fraction of EOs from conifer trees. Furthermore, conifers' EOs are now well recognized as one of the promising natural products for EO-based pesticide formulations, highlighting conifers' importance in future green IPM programs (Bhardwaj et al., 2020).

A previous study (Inouye et al., 2006) on the antifungal vapor activity of 72 EOs and their major EOCs against a common dermatophyte fungus reported that EOs rich in monoterpene alcohols had stronger activity than MH-rich EOs, as usually expected, being the same trend observed with the EOCs themselves. The authors (Inouye et al., 2006) also found that monoterpenol-rich EOs showed a more enhanced antifungal vapor activity than that of contact activity, whereas sesquiterpenol-rich EOs showed antifungal contact activity, but no vapor activity. Interestingly, similar findings were found in the present study, despite the use of different fungal strains. Indeed, the weak effect on *T. paradoxa* MGI displayed by the EO samples classified into the chemical group G1 (Frs.1–3 and crude EOs, Fig. 8) appears to be associated with its higher α-pinene content, with Fr1, the highest in α-pinene concentration, presenting the lowest antifungal effect, among the tested EO samples (Table 3). On the other hand, the displayed weak effect of the EO samples from the chemical group G2 (Fr5 and Fr6, Fig. 8), the

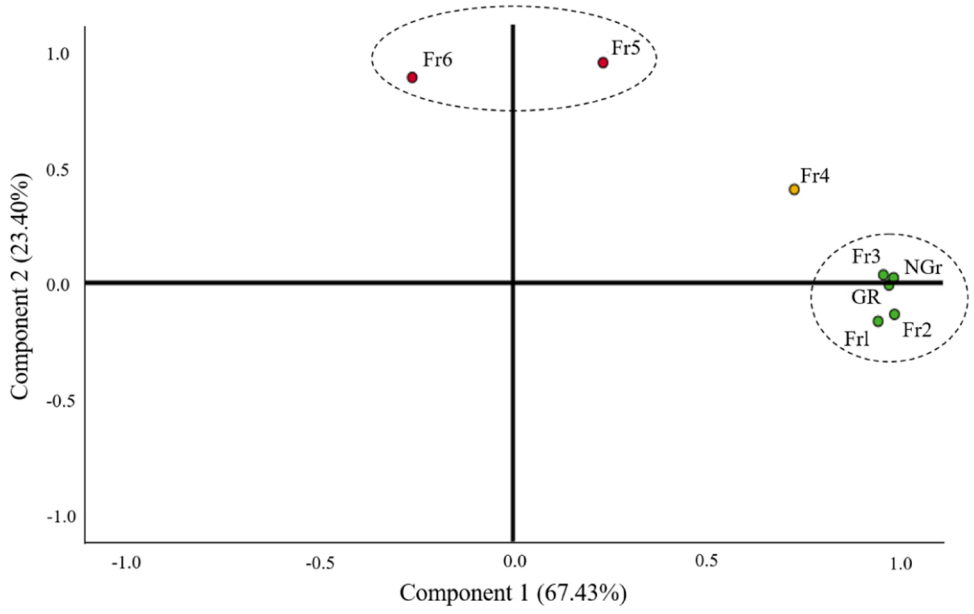

Fig. 8. Loading plot of principal components PC1 and PC2 for the Azorean *Cryptomeria japonica* immature female cones essential oil (EO) and EO fractions (Frs. 1–6). GR and NGr: grinded and non-grinded plant material, respectively.

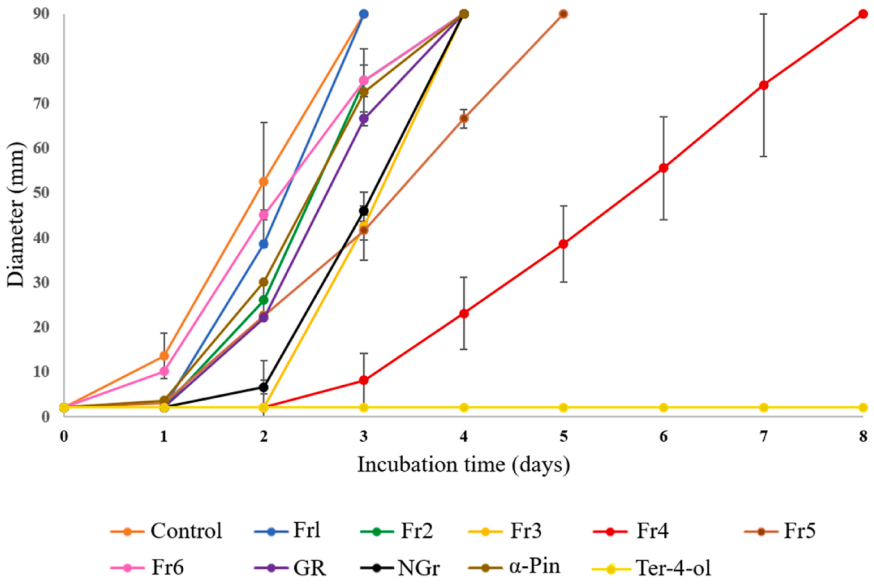

Fig. 9. Micro-atmosphere assay dynamics of *Thielaviopsis paradoxa* mycelium growth in presence of the Azorean *Cryptomeria japonica* immature female cones essential oil (EO), EO fractions (Frs. 1–6), pure EO compounds, and a control sample (water). For the hydrodistillation timeframes (HDTs) values of the EO samples see Table 3. GR and NGr: grinded and non-grinded plant material, respectively. α-pin: (–)-α-pinene. ter-4-ol: (–)-terpinen-4-ol.

Table 3
Thielaviopsis paradoxa mycelium growth-inhibitory activity of the vapor phases of Azorean *Cryptomeria japonica* immature female cones essential oil (EO), EO fractions (Frs. 1–6), and some pure EO compounds.

Samples (HDTs, min)	*T. paradoxa* mycelium growth (mm)								Days to cover plate
	Incubation time (days) at 25 °C								
	1	2	3	4	5	6	7	8	
Fr1 (0–2)	2 ± 0^{a}	39 ± 1^{cd}	90 ± 0^{e}						3
Fr2 (2–10)	2 ± 0^{a}	26 ± 4^{b}	75 ± 7^{cd}	90 ± 0^{d}					4
Fr3 (10–30)	2 ± 0^{a}	8 ± 4^{a}	47 ± 8^{b}	90 ± 0^{d}					4
Fr4 (30–60)	2 ± 0^{a}	2 ± 0^{a}	8 ± 6^{a}	23 ± 8^{b}	39 ± 9^{b}	56 ± 12^{b}	74 ± 16^{b}	90 ± 0^{b}	8
Fr5 (60–120)	2 ± 0^{a}	23 ± 1^{b}	42 ± 6^{b}	67 ± 8^{c}	90 ± 0^{c}				5
Fr6 (120–240)	10 ± 0^{c}	45 ± 1^{d}	75 ± 4^{d}	90 ± 0^{d}					4
GR (0–240)	2 ± 0^{a}	22 ± 0^{b}	67 ± 4^{c}	90 ± 0^{d}					4
NGr (0–240)	2 ± 0^{a}	7 ± 2^{a}	46 ± 3^{b}	90 ± 0^{d}					4
α-Pin	4 ± 1^{b}	30 ± 14^{bc}	73 ± 18^{cd}	90 ± 0^{d}					4
Ter−4-ol	2 ± 0^{a}	2 ± 0^{a}	2 ± 0^{a}	2 ± 0^{a}	2 ± 0^{a}	2 ± 0^{a}	2 ± 0^{a}	2 ± 0^{a}	20
Control	17 ± 5^{d}	64 ± 13^{e}	90 ± 0^{e}						3

Values are the mean ± SD ($n = 3$). Within each column, means sharing the same superscript letter are not significantly different ($p < 0.05$). HDTs: hydrodistillation timeframes. GR and NGr: grinded and non-grinded plant material, respectively. α-pin: (–)-α-pinene. ter-4-ol: (–)-terpinen-4-ol.

highest in the OCS elemol, could be due to the low volatility of elemol. In fact, a previous study (Kusumoto and Shibutani, 2015) on the antifungal activity of *C. japonica* EO reported that elemol is the major responsible compound for its strong activity on the mycelial growth of wood decay fungi. Thus, it appears that EOs containing OCS as the active EOCs are inappropriate for vapor phase application (Inouye et al., 2006), which should be considered for further investigation using agar diffusion assay for evaluation of the efficacy of Fr5 and Fr6 samples against *T. paradoxa* MGI. Finally, the efficacy of Fr4 (G3, Fig. 8) for vapor treatment appears to be associated with its terpinen-4-ol and α-terpineol content. Interestingly, a previous investigation (Kong et al., 2019) on the antifungal mechanisms of terpinen-4-ol and α-terpineol (the most common terpineol isomers in a wide variety of EOs) showed that they are the critical components in *Melaleuca alternifolia* (Maiden & Betche) Cheel (tea tree) oil (TTO) for inhibition of rot disease caused by *Aspergillus ochraceus* Wilh (Aspergillaceae) in postharvest grapes. Thus, it is possible that the best antifungal activity of Fr.4 could be due to synergistic effects between terpinen-4-ol and α-terpineol, which should be considered for further investigation, using the α-terpineol/terpinen-4-ol ratio value presented by Fr4 (0.07, Table 2).

It should also be highlighted that, similarly to the findings in our antifungal study, the research of Yu et al. (2015) showed that terpinen-4-ol had the highest activity against the necrotrophic and polyphagic phytopathogenic *Botrytis cinerea* (Persoon: Fries) fungus, in comparison to the commercial TTO and its other EOCs. In addition, the same authors (Yu et al., 2015) reported that the terpinen-4-ol treatment produced marked changes in mycelial morphology, cellular ultrastructure, and membrane permeability, as well as decreased the ergosterol content in the fungal cell membrane. In this context, another relevant study (Li et al., 2020) showed that terpinen-4-ol vapor treatment enhances disease resistance in strawberry through the activation of the phenylpropanoid pathway, with superior effectiveness than TTO and its other EOCs. In fact, as already compiled by Sivakumar and Bautista-Baños (2014), several EOs are effective to control postharvest decay, prolonging shelf life and improving quality of fruits, mainly due to their direct antifungal action (inhibiting the mycelial growth and spore germination) and the mentioned induced disease resistance in fruits.

Overall, this study contributes to enhance the knowledge of the antifungal potentialities of *C. japonica* strobili EO, obtained from GR and

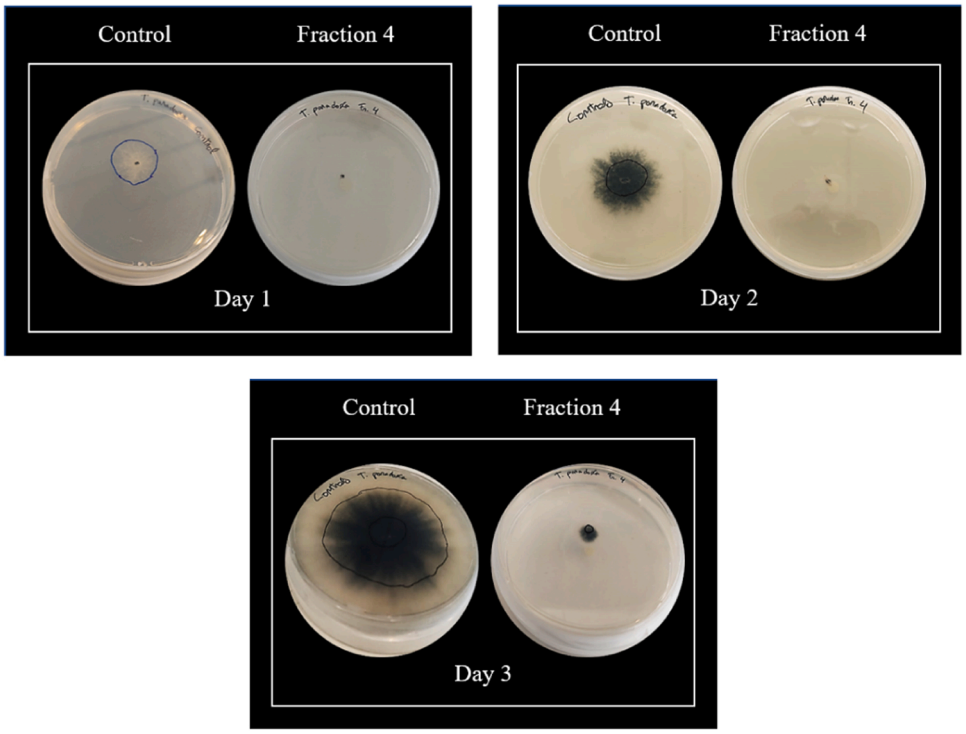

Fig. 10. The effect of EO fraction 4 (30–60 min), obtained via hydrodistillation from grinded Azorean *Cryptomeria japonica* immature female cones, on the mycelium growth of *Thielaviopsis paradoxa* during micro-atmosphere assay, at 25 °C on potato dextrose agar. Control plates (water) on the left and fraction 4 on the right.

NGr plant material, and highlights the role of (–)-terpinen-4-ol, a key component of this EO. These findings contribute significantly to the expanding of the knowledge base and value of the *C. japonica* EO industry, supporting its potential for broader applications. In fact, the fractionation of GR Az–CJIFC EO, during the HD process, allowed the production of some EO fractions (Frs. 3–5) with higher potential for *T. paradoxa* MGI than that of the crude EO, due to their differential volatile compositions. The results also revealed that the grinding step prior to the HD had no significant effect against *T. paradoxa* mycelium growth. Nevertheless, the aforementioned active EO fractions presented lower potency than that of the pure (–)-terpinen-4-ol. Future *in situ* investigation is imperative to evaluate the effectiveness of (–)-terpinen-4-ol and Fr4 as fumigants to control black rot in pineapple postharvest in the Azores.

The present study, however, comprised some limitations, mainly the chemical analysis of the vapor phases of the tested EOs samples, which can be achieved using headspace solid-phase microextraction technique coupled with GC–MS to additionally prove the physical presence of the volatile EOCs.

4. Conclusion

To our knowledge, the present study is the first report on the antifungal activity of the vapor phase EO from conifers against *T. paradoxa*. The results clearly show that Az–CJIFC EO fractions with activity against this fungus could be efficiently generated in different HDTs, especially when combined with the grinding of the plant material, to enhance EO yield. In particular, Fr4 (30–60 min HDT), corresponding to around 10 % of the total extracted EO, showed the strongest effect. Its activity, after 3 days of incubation, was 8.4-fold higher than that of the crude EO, but 4-fold lower when compared to (–)-terpinen-4-ol, a very strong antifungal agent against *T. paradoxa*, as also demonstrated for the first time in the present research work. The great potential of Fr4 in the control of *T. paradoxa*, by inhibiting its mycelial growth, appears to be associated, at least in part, with its terpineol isomers (terpinen-4-ol and α-terpineol) content. Therefore, additional investigations are needed on the antifungal mechanisms of this new EO fraction against *T. paradoxa*, responsible for the development of black rot disease of pineapple.

CRediT authorship contribution statement

Lima Elisabete: Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Lima Ana:** Writing – review & editing, Software, Methodology. **Arruda Filipe:** Writing – review & editing, Writing – original draft, Software, Methodology, Funding acquisition, Conceptualization. **Rosa José S.:** Writing – review & editing, Supervision, Software, Methodology. **Baptista José:** Writing – review & editing, Supervision, Funding acquisition. **Rodrigues Tânia:** Writing – review & editing, Software, Methodology. **Janeiro Alexandre:** Writing – review & editing, Software, Methodology. **Figueiredo Ana Cristina:** Writing – review & editing, Software, Methodology. **Machado Alexandra:** Writing – review & editing, Software, Methodology.

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Supplementary Material

Table S1: GC–FID / GC–MS analyses of the Azorean *Cryptomeria japonica* immature female cones (Az–CJIFC) essential oil (EO) and EO fractions obtained via hydrodistillation (HD) process

Declaration of Competing Interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.indcrop.2025.121182.

Data availability

No data was used for the research described in the article.

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