Seasonal phytochemical variation of *Peumus boldus* and *Cryptocarya alba a*nd its impact on forest therapy.

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Abstract

Plants, in their interaction with the environment, synthesize organic compounds that are considered beneficial for human health in the practice of forest therapy. This study describes seasonal changes in the production and activity of volatile organic compounds in two emblematic southern Chilean trees. Phytochemical profiles were obtained by gas chromatography-mass spectrometry, total phenolic content was determined by the Folin–Ciocalteu method, and antioxidant activity was measured by ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate)) and DPPH (2,2-diphenyl-1-picrylhydrazyl) assays, in *Peumus boldus* and *Cryptocarya alba* leaves obtained from Nonguén National Park (Chile). Climatic data were recorded. The results show that the phytochemical richness of *P. boldus* reached the highest value in autumn, while *C. alba* showed a slight decrease in summer. The four seasons presented a broad core of constitutive compounds including monoterpenes and monoterpenoids, in both species. ABTS assay showed that antioxidant activity in *P. boldus* was significantly higher in autumn, while in *C. alba*, the highest value was reached in winter. DPPH assay showed highest antioxidant activity in spring in *P. boldus*, and in *C. alba* it occurred in winter. The highest phenolic accumulation was scored in spring for *P. boldus,* and in autumn for *C. alba*. Here we provide the first glimpse of the dynamics of phytochemical variation in response to the seasonal changes occurring in two trees of the southern forest. Further investigation is required to fully understand the emission dynamics in response to seasonal changes, and its contribution to human health through the practice of forest therapy.

Keywords: phytochemical seasonality, antioxidant activity, forest therapy, terpenes

Introduction

Because they are sessile organisms, plants have evolved an extremely diverse repertoire of chemical compounds that allow them to adapt to biotic and abiotic stresses imposed by their environment. Of these phytochemicals, those called biogenic volatile organic compounds (BVOCs), such as terpenes, perform essential ecological roles ranging from direct defense to chemical plant-to-plant communication and between plants and animals [1,2]. In forest ecosystems, trees represent the main source of BVOC emissions, with leaves acting as the most active structures in this function due to specialized organs such as oil glands and trichomes [3]. The biochemical and molecular control of BVOC emission in response to

short-term environmental drivers such as temperature, light, CO_2, and O_2, and long-term factors such as circadian, seasonal, and developmental effects has been discussed [4]. Regarding biosynthesis, BVOCs in plants originate from three principal biosynthetic routes: the methylerythritol phosphate (MEP) pathway located in plastids, the mevalonate (MVA) pathway operating in cytoplasmic compartments, and the lipoxygenase (LOX) pathway associated with peroxisomal membranes [5]. Biosynthesis of VOCs depends on the availability of carbon, nitrogen, and sulfur, as well as energy provided by primary metabolism. Based on their biosynthetic origin, all VOCs are divided into several classes, including terpenoids [6].

The biosynthesis of terpenes is strongly regulated by environmental conditions that affect the expression of key genes, such as those encoding the enzyme terpene synthase (TPS). High temperature and high radiation-common during summer-induce TPS expression and the emission of monoterpenes [7–9]. Also, water and salt stress can significantly affect terpene production [10]. Although the influence of each of these factors has been well characterized for some species [11], little is known about the dynamics of BVOC production and activity in response to the seasonal changes in trees growing in southern Chilean forests.

In parallel, these same species produce a wide array of non-volatile secondary metabolites such as phenolic compounds, which contribute to cellular protection against oxidative stress and account for many of their pharmacological and antioxidant properties [12]. Quantifying total phenolic content and antioxidant capacity through ABTS and DPPH [13,14] assays complements BVOC characterization, offering a holistic view of the seasonal metabolic adjustments and bioactive potential of forest species.

Beyond their ecological and biochemical importance, BVOCs have gained attention for their contribution to human well-being. Forest therapy-which involves immersive exposure to forest environments-has shown significant psychological and physiological benefits, largely attributed to inhalation of phytoncides and multisensory stimulation by the forest atmosphere. Several studies have demonstrated their positive effects on health, including stress reduction, cardiovascular improvement, and immune system strengthening [3,15,16]. In this context, the temperate Deciduous Forest of Concepción represents an ideal natural setting for studying the interplay between forest chemistry and human health. This ecosystem is characterized by marked seasonality, with warm, dry summers and cold, rainy winters, and hosts two emblematic endemic species, *Peumus boldus* Mol. (boldo) and *Cryptocarya alba* (Mol.)

Looser (peumo) [17]. Both species are structurally and functionally central to the forest community and are culturally recognized for their distinctive aroma and therapeutic properties.

In this context, the present study aimed to evaluate the influence of seasonality on the production and composition of essential oils, total phenolic content, and antioxidant activity in the leaves of *P. boldus* and *C. alba*. Such knowledge not only deepens our understanding of the ecophysiological resilience of *P. boldus* and *C. alba* under changing climatic regimes but also provides valuable insights for optimizing forest therapy practices based on science-driven evidence.

Materials and Methods

2.1. Environmental conditions

Nonguén National Park is in the peri-urban area of the province of Concepción, Biobío Region, Chile. Covering an area of 3,056.96 ha of the Cordillera de la Costa (73°00′W–36°52′S), it protects the last remnant of the "Concepción deciduous forest", hosting species such as *Nothofagus dombeyi* (Mirb.) Oerst., *Nothofagus obliqua* (Mirb.) Oerst., *Cryptocarya alba* (Mol.) Looser, *Peumus boldus* Mol., *Persea lingue*, among others [17].

The area exhibits a Mediterranean temperate climate, characterized by marked seasons, with winter rainfall and a dry summer, and an average annual temperature of 13.1 °C, and it is increasingly used for health practices such as forest bathing.

The meteorological data used in this study were obtained from the Dirección Meteorológica de Chile (DMC) (Carriel Sur station, code 3600019) and correspond to the year 2022. The following parameters were considered: monthly mean temperature (°C), mean maximum temperature (°C), mean minimum temperature (°C), total monthly precipitation (mm), and UVB radiation index. The representative temperature for each season was obtained by taking an average of the data from the three corresponding months: summer (January-March), autumn (April-June), winter (July-September) and spring (October-December).

115 **2.2. Plant material**

116 To obtain phytochemical profiles, for both *P. boldus* and *C. alba*, leaves were collected each
117 season as follows: Five mature trees, accessible via authorized trails, were identified for each species and
118 used throughout the study to obtain equal amounts of samples. Sampling was conducted in the early
119 morning during each of the four seasons, following the criteria described by [18], considering the four
120 cardinal directions. To obtain a representative sample of each season, material collected was pooled and
121 subjected to phytochemical analysis.

122 **2.3. Essential oil extraction**

123 Essential oils from *P. boldus* and *C. alba* were obtained by steam-assisted hydrodistillation using
124 a Clevenger-type apparatus (Biobase, HM-I). A total of 300 g of fresh leaves were distilled with 2.6 L of
125 distilled water for four hours. Residual water in the essential oil was removed using anhydrous sodium
126 sulfate. Extracts were stored in amber glass bottles at room temperature [19].

127 **2.4. Characterization of essential oils**

128 The essential oils were analyzed using gas chromatography-mass spectrometry (GC-MS)
129 (Agilent 7890A) equipped with an automatic injector (Agilent) and an HP-5MS capillary column (30 m ×
130 0.25 mm i.d., 0.25 μm film thickness). The injection port temperature was set at 250°C. The GC oven
131 temperature program was as follows: initial temperature of 50°C (held for 2 min), increased at 8 °C min^{-1}
132 to 200°C (held for 5 min), and finally at 10 °C min^{-1} to 250 °C (held for 10 min). Helium was used as the
133 carrier gas at a constant flow rate of 1.0 mL min^{-1}. The mass detector (Agilent 5975C) was operated in
134 electron ionization mode at 70 eV, with a scan range of 35-500 m/z. Component identification was
135 achieved by comparing their mass spectra with those in the NIST library and confirming identities
136 through retention index (RI) comparisons with a homologous series of n-alkanes (C8-C20), based on
137 literature values reported for similar non-polar columns. Relative quantification was performed by peak
138 area normalization without correction factors.

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142 **2.5. Antioxidant activity assays of essential oils**

143 **2.5.1. ABTS assay**

144 The ABTS assay described by [13] and modified by [20] was employed. Essential oil samples
145 were diluted in chloroform as solvent. The free radical solution contained 3.5 mM ABTS (2,2'-azino-
146 bis(3-ethylbenzothiazoline-6-sulfonate)) and 1.22 mmol potassium persulfate. Both solutions were mixed
147 in equal volumes and kept in the dark for 16 h at room temperature. Subsequently, 1 mL of the $ABTS^{+}$
148 stock solution was diluted with 14 mL of distilled water to reach an absorbance of 0.70 ± 0.01 at 734 nm.
149 For each seasonal assay, a fresh $ABTS^{+}$ solution was prepared. Reaction mixtures contained 180 µL of
150 $ABTS^{+}$ radical solution and 20 µL of either Trolox at concentrations ranging from 0.01 to 0.2 mg mL^{-1} or
151 the oil samples (25–100 mg mL^{-1}), incubated for 60 min in the dark. Absorbance was read at 734 nm
152 using a BioTek Epoch™ microplate spectrophotometer. All determinations were performed separately.

153 **2.5.2. DPPH assay**

154 Antioxidant activity was evaluated according to the method proposed by [14], with modifications
155 by [20]. A liquid-liquid extraction in methanol was performed for each sample. The reaction mixture
156 contained 270 µL of a 0.06 mmol DPPH (2,2-diphenyl-1-picrylhydrazyl) radical solution prepared in
157 methanol and 30 µL of either Trolox (0.01–0.2 mg mL^{-1}) or the oil samples (25–100 mg mL^{-1}). Each
158 mixture was incubated in the dark at 37 °C for 20 min. Absorbance was measured at 517 nm using a
159 BioTek Epoch™ microplate spectrophotometer. Trolox served as a positive control, and methanol was
160 used as a blank. All tests were performed in triplicate (technical replicates).

161 The percentage of inhibition, corresponding to the amount of radicals (DPPH and ABTS)
162 neutralized by the extract at a given concentration, was calculated using the equation:

163 $$\%_{Inhibition} = \left(A_c - A_s\right) / A_c \cdot 100\,\%$$

164 Where A_c is the absorbance of the control and A_s is the absorbance of the sample. Antioxidant
165 activity for both ABTS and DPPH assays was expressed as IC_{50}, representing the sample concentration
166 required to inhibit 50% of the free radicals [21,22].

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2.6. Total Phenolic Content (TPC)

Total phenolic content in each sample was determined using the Folin–Ciocalteu method [23], with modifications by [20]. A liquid-liquid extraction in methanol was performed, using sample concentrations of 100 μg mL^{-1}. The calibration curve was prepared using gallic acid (0.01–0.2 mg mL^{-1}). Each reaction mixture contained 400 μL of distilled water, 20 μL of sample or gallic acid standard solution, 40 μL of Folin-Ciocalteu reagent, and 200 μL of 15% (w/v) sodium carbonate. The mixture was incubated at room temperature for 60 min in darkness, with agitation. All measurements were performed in triplicate (technical replicates). Absorbance was recorded at 765 nm using a BioTek Epoch™ microplate spectrophotometer. TPC was expressed as μg g^{-1} gallic acid equivalents (GAE) on a dry weight basis.

2.7. Statistical analysis

Quantitative data from antioxidant activity and TPC assays are presented as mean ± standard deviation (SD) derived from three technical replicates of composite seasonal samples. Given the pooled sampling design, univariate analysis focused on descriptive trends and the magnitude of change (percentage difference) rather than inferential hypothesis testing, avoiding pseudoreplication while highlighting biological variations. To identify seasonal patterns and compound associations in the essential oils, Hierarchical Cluster Analysis (HCA) was performed on Z-score standardized data using Euclidean distances and Ward's D2 method. All analyses were conducted in R software (v.4.5) using the *tidyverse* suite for data manipulation, *ComplexHeatmap* for visualization, and *patchwork* for figure integration.

3. Results

3.1 Environmental Conditions

Consistent with a warm temperate climate, the area under study exhibited pronounced seasonal variation during the sampling period. Figure 1a shows the variations in temperature, precipitation and radiation that occurred throughout the year. Summer was the warmest season, with a mean maximum temperature of 21.5 °C, an average temperature of 16.5 °C, and a mean minimum of 12.0 °C. In contrast, winter was the coldest season, with a mean maximum temperature of 14.0 °C, an average temperature of

9.5 °C, and a mean minimum of 6.0 °C, while the transitional seasons, autumn and spring, exhibited intermediate values.

In terms of precipitation, the rainiest season was autumn, with total rainfall reaching around 420 mm. This was followed by winter, with around 360 mm of rainfall. Summer was the driest season, with only 25 mm of precipitation. Spring showed slightly higher values, at around 50 mm, as shown in Figure 1b.

In accordance with seasonal sun exposure, the highest UV radiation levels were recorded in summer and spring, and the lowest in autumn (Figure 1c).

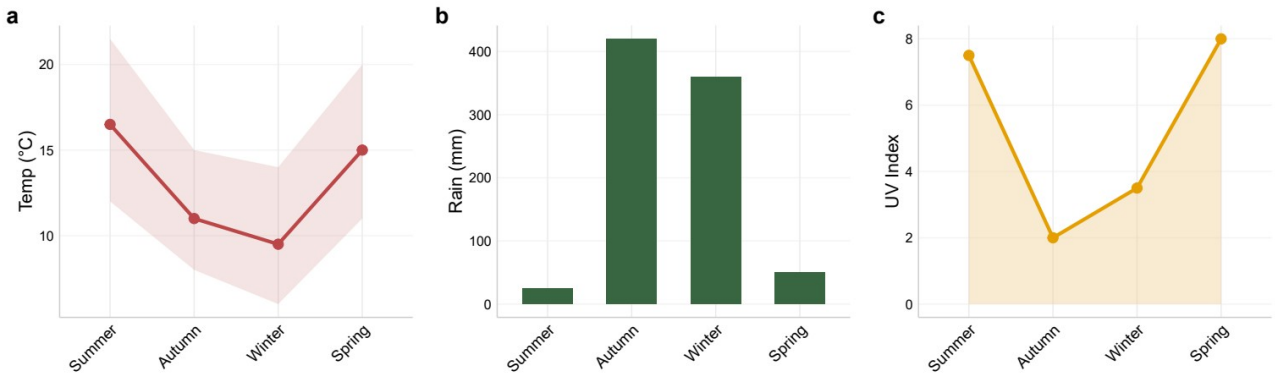


Figure 1. Seasonal environmental dynamics in Nonguén National Park during the study period (2022). **(a)** Average seasonal temperature (°C) represented by the red line, with the pink shaded region indicating the range between maximum and minimum recorded temperatures. **(b)** Total accumulated precipitation (mm) shown as dark green bars. **(c)** Mean UV Radiation Index displayed by the yellow line. Data were retrieved from the Carriel Sur meteorological station (Dirección Meteorológica de Chile).

3.2. Phytochemical profiles

3.2.1. *P. boldus*

In terms of phytochemical richness, the highest values (47 distinct compounds) were observed in autumn (Figure 2), exhibiting also the highest diversity of monoterpenes (MTs; n=14), monoterpenoids (MTDs; n=22), and the largest variety of sesquiterpenoids (STDs; n=5) throughout the year. By contrast, summer showed the lowest metabolic diversity (28 distinct compounds) and a strong dominance of

monoterpenes (MTs; n=9) and monoterpenoids (MTDs; n=13) in the phytochemical profile. Similar intermediate values of chemical richness (n=41) were obtained in spring and winter (n=40). The dynamic behavior of individual compounds (Table 1) can explain seasonal variation in chemical diversity. A core set of constitutive metabolites was present throughout the year, including the monoterpenes α-pinene, p-cymene, and α-terpinene, as well as the monoterpenoids eucalyptol and 4-terpineol.

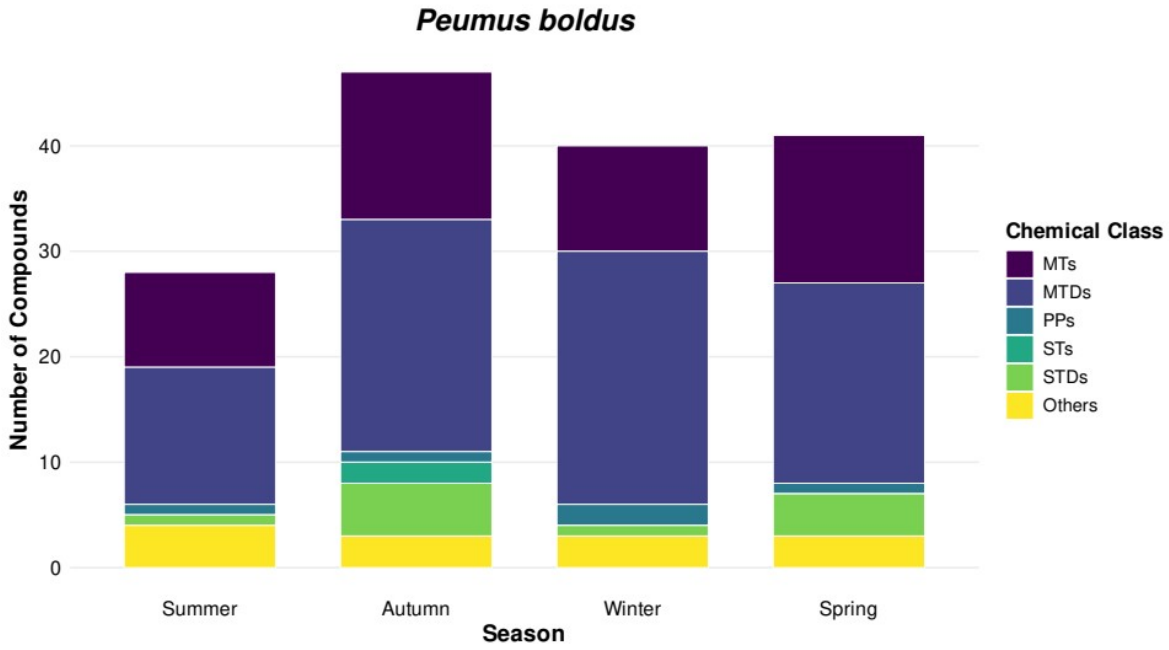


Figure 2. Characterization of the number of compounds in the essential oil of *P. boldus* across four seasons using gas chromatography–mass spectrometry (GC–MS). Compound categories: MTs (monoterpenes), MTDs (monoterpenoids), PPs (phenylpropanoids), STs (sesquiterpenes), STDs (sesquiterpenoids), and Others.

The shift in dominance between eucalyptol and p-cymene was the most distinctive aspect of the chemical profile (Table 1). In summer, the season with the lowest chemical diversity, eucalyptol was the predominant compound, accounting for 56.38%, and it remained dominant in winter, accounting for 23.23% of the oil. In seasons showing the greatest chemical diversity, dominance shifted toward p-cymene, which reached its highest levels in spring (19.71%) and remained relatively high in autumn (15.21%). The presence of significant amounts of other compounds such as cis-geraniol in spring

233 (16.88%), ascaridole in autumn (5.66%), and α-terpinene in summer (5.08%) complemented this
234 dynamic.

235

236 **Table 1.** Composition of the essential oil of *P. boldus* analyzed by gas chromatography-mass
237 spectrometry (GC-MS), where 1 corresponds to summer, 2 to autumn, 3 to winter, and 4 to spring.

Fraction number	Retention time	Compounds	Concentration (%)			
			1	2	3	4
1	5.790	3-Thujene	-	0.49	-	0.6
2	5.916	α-Pinene	2.46	4.5	2.34	6.38
3	6.211	Camphene	-	0.25	0.28	0.3
4	6.699	β-Phellandrene	3.55	2.73	1.88	2.22
5	6.761	β-Pinene	1.06	2.17	1.46	2.13
6	6.979	2,3-Dehydro-1,8-cineole	-	-	0.34	-
7	7.031	β-Myrcene	0.36	0.7	-	0.34
8	7.301	α-Phellandrene	0.39	1.1	0.3	1.3
9	7.425	3-Carene	-	0.77	0.31	0.69
10	7.545	α-Terpinene	0.3	1.01	0.4	0.73
11	7.732	p-Cymene	6.7	15.21	10.34	19.71
12	7.830	cis-Geraniol	-	8.54	3.23	16.88
13	7.893	Eucalyptol	56.38	13.64	23.23	14.03
14	8.375	γ-Terpinene	0.51	1.79	0.87	1.79
15	8.572	Terpineol, cis-β	-	0.26	-	0.33

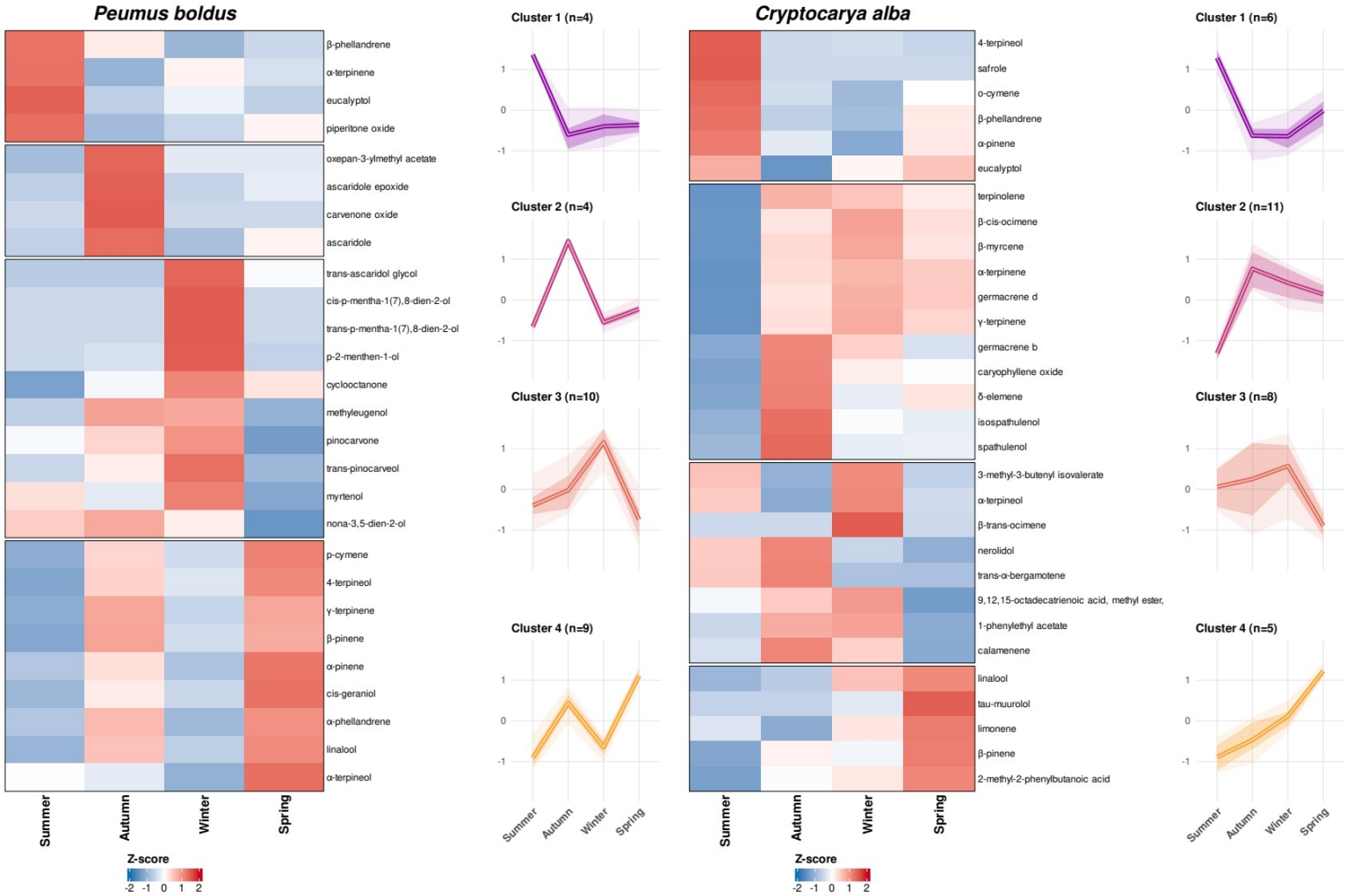
16	8.972	Terpinolene	-	0.24	-	0.23
17	9.008	o-Cymenene	-	0.49	-	-
18	9.174	Linalool	0.81	2.2	1.13	2.66
19	9.433	Fenchol	-	-	0.4	-
20	9.610	p-2-Menthen-1-ol	0.56	0.61	1.45	0.52
21	9.724	Cyclooctanone	0.25	0.65	1.07	0.77
22	9.895	cis-p-Mentha-2,8-dien-1-ol	-	0.24	0.72	0.37
23	9.973	trans-Pinocarveol	0.76	1.24	2.06	0.46
24	10.087	2-Caren-4-ol	-	0.41	0.26	0.38
25	10.321	Sabina ketone	0.24	0.31	0.48	-
26	10.419	Pinocarvone	0.78	1.07	1.46	-
27	10.492	p-Menth-1-en-8-ol	0.38	0.73	0.92	0.34
28	10.689	4-Terpineol	2.27	3.82	3.1	4.45
29	10.814	Benzyl alcohol	0.19	-	-	-
30	10.879	trans-p-Mentha-1(7),8-dien-2-ol	-	-	1.36	-
31	10.938	α-Terpineol	2.48	2.29	1.64	3.57
32	11.052	Myrtenol	0.82	0.52	1.32	-
33	11.110	(-)-trans-Isopiperitenol	-	-	0.69	-
34	11.462	cis-Carveol	-	0.24	0.62	-
35	11.571	cis-p-Mentha-1(7),8-dien-2-ol	-	-	1.28	-
36	11.831	α-Terpinene	5.08	1.13	3.32	2.27

37	12.095	Piperitone oxide	1.84	-	0.3	0.82
38	12.142	Ascaridole	1.56	5.66	1.03	3.04
39	12.178	Carvenone oxide	-	5.66	-	-
40	12.513	α-Limonene diepoxide	-	-	-	0.34
41	12.619	Bornyl acetate	-	0.34	0.38	0.32
42	12.645	Safrole	-	-	0.71	-
43	12.713	trans-Ascaridol glycol	-	-	1.21	0.36
44	12.811	Thymol	-	0.82	-	-
45	12.931	Ascaridole epoxide	0.4	1.32	0.43	0.58
46	13.045	Nona-3,5-dien-2-ol	9.38	9.91	8.93	6.52
47	14.591	Methyleugenol	0.84	2.43	2.38	0.38
48	14.892	Caryophyllene	-	0.35	-	-
49	15.540	Alloaromadendrene	-	0.23	-	-
50	17.004	±-trans-Nerolidol	-	0.69	-	0.62
51	17.341	Spathulenol	0.26	0.97	0.47	0.66
52	17.429	Caryophyllene oxide	-	0.36	-	0.34
53	17.787	Oxepan-3-ylmethyl acetate	0.37	1.09	0.56	0.56
54	17.938	Limonene oxide, cis-	-	0.32	-	-
55	18.14	Humulenol-II	-	0.37	-	-
56	18.192	Isoaromadendrene epoxide	-	0.51	-	0.34
57	18.389	β-Eudesmol	-	0.22	-	-
58	18.422	α-Cadinol	-	-	-	0.27

59	18.665	p-Menth-8-ene, 3-methylene-	-	-	-	0.3
60	18.889	Ledene oxide-(II)	-	-	-	0.6

238

239 To further elucidate the dynamic behavior of individual compounds and explain the seasonal
240 variation in diversity, a hierarchical clustering analysis based on normalized relative abundances (Z-
241 scores) was performed (Figure 3). This analysis categorized the volatile constituents into four distinct
242 clusters based on their temporal patterns. Cluster 1, the "Summer Phenotype," includes the major
243 compound eucalyptol alongside β-phellandrene and α-terpinene, exhibiting maximal relative abundance
244 exclusively during the summer, followed by a sharp decline in subsequent seasons. This pattern aligns
245 with the summer dominance of specific monoterpenoids. Cluster 2, the "Autumn Phenotype," is
246 characterized by a peak in autumn and contains key oxygenated compounds such as ascaridole, ascaridole
247 epoxide, and carvenone oxide, correlating with the high phytochemical richness and the diversification of
248 oxidative products observed post-summer. Cluster 3, the "Winter Accumulation" group, represents the
249 largest group (n=10), with compounds including methyleugenol, trans-pinocarveol, and nona-3,5-dien-2-
250 ol showing a progressive accumulation tendency towards winter, suggesting a specific metabolic response
251 to colder conditions. Finally, Cluster 4, the "Spring Resurgence," contrasts with the summer and autumn
252 profiles, maintaining low levels during the dormant seasons but experiencing a significant surge in spring.
253 This group comprises constitutive monoterpenes such as α-pinene, β-pinene, linalool, and γ-terpinene,
254 with their upregulation in spring explaining the recovery of monoterpene diversity and aligning with the
255 increased levels of p-cymene observed at the onset of the growing season.



256 Figure 3. Seasonal variation and clustering analysis of the volatile profiles of *P.boldus* and *C. alba* essential oils. The heatmaps display the normalized relative abundance (Z-score)
257 of volatile compounds identified by GC-MS across four seasons (Summer, Autumn, Winter, and Spring). The color gradient ranges from dark blue (lowest abundance, -2) to dark red
258 (highest abundance, +2). The trend plots to the right of each heatmap represent the aggregated seasonal profiles for each cluster. The central solid line indicates the mean
259 standardized abundance. The shaded ribbons represent the distributional variability: the darker inner band denotes the interquartile range (25th - 75th percentiles), while the lighter
260 outer band captures the 90% distribution interval (5th -95th percentiles). The number of 15constituents (n) per cluster is indicated in each subplot.

3.2.2. *C. alba*

This species showed only a slight decrease in phytochemical richness during the summer (n=39), with the rest of the year showing values around n=48 (Figure 4). The overall chemical profile was characterized by a balance between monoterpenes (MTs and MTDs) and a substantial proportion of sesquiterpenes (STs and STDs), which exhibited high diversity throughout most of the year. All four seasons presented a broad core of constitutive compounds which included monoterpenes α-pinene, β-phellandrene, β-pinene, and γ-terpinene, as well as the monoterpenoids eucalyptol and 4-terpineol, forming a complex and multifunctional chemical base (Table 2).

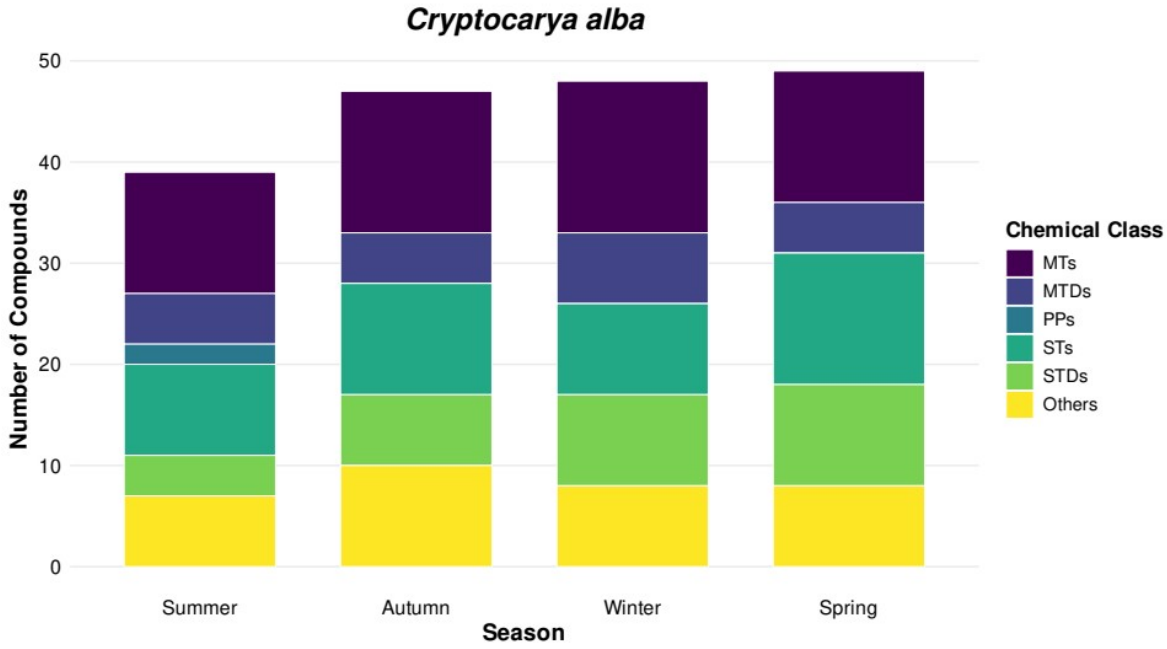


Figure 4. Characterization of the number of compounds in the essential oil of *C. alba* across four seasons by gas chromatography–mass spectrometry (GC-MS). Compound categories: MTs (monoterpenes), MTDs (monoterpenoids), PPs (phenylpropanoids), STs (sesquiterpenes), STDs (sesquiterpenoids), and Others.

The most distinctive feature in the chemical profile was the dominance of the monoterpenoid 4-terpineol, reaching its peak (26.48%) in summer (Table 2). Although it declined in the other seasons, it remained the most abundant compound throughout the year (15.94% in autumn, 16.14% in winter, and 15.51% in spring). Furthermore, an increase in the levels of two other monoterpenes was observed,

particularly during times of high temperatures and radiation. β-Phellandrene reached its maximum levels in summer (12.21%) and remained high in spring (9.23%). Similarly, eucalyptol was more abundant in summer (9.38%) and spring (9.03%).

To further characterize the seasonal dynamics of these metabolites, hierarchical clustering defined four distinct groups based on normalized relative abundance (Z-scores) (Figure 3). Cluster 1 (n=6) closely mirrored the behavior of the major compounds, grouping the dominant 4-terpineol with other key volatiles such as safrole, eucalyptol, and β-phellandrene; these compounds exhibited their maximum expression exclusively during the summer, followed by a decline. As the season shifted, Cluster 2 (n=11) emerged with a peak in autumn, characterized by an increased abundance of compounds like terpinolene, spathulenol, and germacrene D. A distinct accumulation trend was observed for Cluster 3 (n=8), which included oxygenated sesquiterpenes such as nerolidol; these compounds showed a progressive increase from summer to reach their maximum levels in winter. Finally, Cluster 4 (n=5) displayed a sharp resurgence in spring after remaining at minimal levels (negative Z-scores) during summer and autumn. This cluster was driven by the significant upregulation of specific monoterpenes like linalool, limonene, and β-pinene, contributing to the maintenance of the high chemical diversity observed at the beginning of the vegetative cycle.

Table 2. Composition of the essential oil of *C. alba* analyzed by gas chromatography-mass spectrometry (GC-MS), where 1 corresponds to summer, 2 to autumn, 3 to winter, and 4 to spring.

Fraction number	Retention time	Compounds	Concentration (%)			
			1	2	3	4
1	5.21	Heptanal	-	-	-	0.17
2	5.78	3-Thujene	0.67	0.95	0.76	0.96
3	5.92	α-Pinene	6.62	5.6	4.99	5.93
4	6.21	Camphene	0.28	0.79	0.45	0.86
5	6.71	β-Phellandrene	12.21	7.01	6.33	9.23

6	6.77	β-Pinene	5.22	6.2	6.02	7.01
7	7.03	β-Myrcene	0.69	1.17	1.3	1.14
8	7.31	α-Phellandrene	-	0.45	0.38	0.45
9	7.43	3-Carene	-	0.32	0.34	-
10	7.55	α-Terpinene	1.49	3.45	3.83	3.62
11	7.72	o-Cymene	4.35	2.72	2.28	3.12
12	7.8	Limonene	2.21	1.74	2.55	3.15
13	7.86	Eucalyptol	9.38	5.47	8.19	9.03
14	7.96	β-trans-Ocimene	-	-	1.38	-
15	8.16	β-cis-Ocimene	1.39	1.94	2.15	1.96
16	8.38	γ-Terpinene	3.37	6.08	6.84	6.24
17	8.96	Terpinolene	1.13	2.31	2.22	2.02
18	9.17	Linalool	0.48	0.57	0.95	1.11
19	9.42	3-Methyl-3-butenyl isovalerate	3.06	2.76	3.15	2.84
20	9.97	Terpineol, cis-β	0.27	0.53	0.37	0.49
21	9.98	p-Menth-2-en-1-ol	-	-	0.51	-
22	10.32	2-Nonenal	-	0.33	-	-
23	10.75	4-Terpineol	26.48	15.94	16.14	15.51
24	10.94	α-Terpineol	1.76	1.42	1.89	1.56
25	11.06	Myrtenol	-	-	0.28	-
26	12.12	Hexanoic acid, 4-pentenyl ester	0.46	0.76	0.46	0.25

27	12.41	4-Chloro-3-n-hexyltetrahydropyran	-	0.53	0.47	0.17
28	12.7	Safrole	1.16	-	-	-
29	13.49	δ-Elemene	0.33	1.15	0.67	0.83
30	14.05	Copaene	-	-	-	0.19
31	14.4	β-Elemene	0.47	0.8	0.54	0.79
32	14.44	β-Phenylethyl butyrate	0.37	0.53	0.59	0.42
33	14.59	Methyleugenol	0.36	-	-	-
34	14.89	Caryophyllene	0.52	0.89	0.58	0.63
35	15.08	trans-α-Bergamotene	1.93	2.16	1.48	1.47
36	15.36	β-Farnesene	0.37	0.83	0.48	0.3
37	15.43	α-Caryophyllene	0.51	0.71	0.57	0.66
38	15.47	β-Cubebene	-	-	-	0.16
39	15.75	gamma-Muurolene	-	0.4	-	0.34
40	15.85	Germacrene D	1.18	1.66	1.8	1.73
41	15.9	2-methyl-2-phenylbutanoic acid	1.31	2.21	2.4	3.04
42	15.95	1-Phenylethyl acetate	1.09	1.56	1.59	0.87
43	16.1	Germacrene B	0.69	1.23	1.07	0.6
44	16.47	Calamenene	0.93	1.61	1.3	0.33
45	16.71	Spiro[tricyclo[4,4,0,0(5,9)]decane-10,2'-oxirane], 1-methyl-4-isopropyl	-	0.71	0.32	0.27
46	17	Nerolidol	0.79	1.01	0.45	1.42

47	17.35	Spathulenol	1.2	1.87	1.41	2.47
48	17.44	Caryophyllene oxide	1.63	3.24	2.58	0.28
49	17.56	Viridiflorol	-	-	0.29	0.17
50	17.61	Aromadendrene oxide	-	-	-	0.18
51	17.73	Isoaromadendrene epoxide	-	0.41	0.32	0.17
52	18.05	Isospathulenol	0.75	1.41	1.03	0.98
53	18.2	1.8-Cyclopentadecadiyne	0.33	0.72	0.47	0.18
54	18.24	tau-Muurolol	-	-	0.32	2.22
55	18.3	β-Guaiene	-	0.32	-	-
56	18.39	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	2.32	3.45	4.33	-
57	18.89	Ledene oxide-(II)	-	0.54	0.44	0.51
58	19.75	1-Hexadecanol, 2-methyl-	-	0.35	-	-

297

298 **3.3. Antioxidant Activity from ABTS and DPPH Assays**

299 **3.3.1. Antioxidant Activity of *P. boldus***

300 ABTS assay showed that antioxidant activity was considerably higher during autumn (IC_{50} =
301 10.17 ± 2.77 μg mL^{-1}) and spring (IC_{50} = 20.34 ± 0.68 μg mL^{-1}), with similar IC_{50} values observed
302 between these two seasons. Antioxidant capacity was considerably lower during winter (IC_{50} = 45.28 ±
303 4.35 μg mL^{-1}) and summer (IC_{50} = 49.52 ± 8.55 μg mL^{-1}). On the other hand, DPPH assay showed the
304 highest antioxidant activity in spring (IC_{50} = 20.56 ± 0.39 μg mL^{-1}). Autumn and summer exhibited
305 comparable intermediate activities (IC_{50} = 45.64 ± 6.05 and 49.14 ± 5.98 μg mL^{-1}), respectively. Winter
306 displayed the lowest DPPH radical-scavenging capacity, with the highest IC_{50} value (63.38 ± 4.42 μg
307 mL^{-1}) (Table 3, Figure 5).

3.3.2. Antioxidant Activity of *C. alba*

As with the previous species, variations in antioxidant activity were observed in accordance with seasonal changes in *C. alba*, showing marked differences between ABTS and DPPH assays (Figure 5). ABTS assay scored the highest antioxidant activity in winter (IC_{50} = 81.10 ± 1.86 µg mL^{-1}). This activity decreased markedly in the other seasons in the following order: summer (IC_{50} = 126.39 ± 4.90 µg mL^{-1}), spring (IC_{50} = 165.82 ± 10.01 µg mL^{-1}), and autumn (IC_{50} = 183.50 ± 3.78 µg mL^{-1}). DPPH assay, in turn, revealed that the highest antioxidant activity occurred in winter (IC_{50} = 19.13 ± 0.49 µg mL^{-1}) and spring (IC_{50} = 20.80 ± 0.65 µg mL^{-1}), with comparable values between them. Antioxidant activity decreased in summer (IC_{50} = 37.07 ± 4.43 µg mL^{-1}) and dropped sharply in autumn, scoring the lowest levels with an IC_{50} of 128.19 ± 3.09 µg mL^{-1} (Table 3).

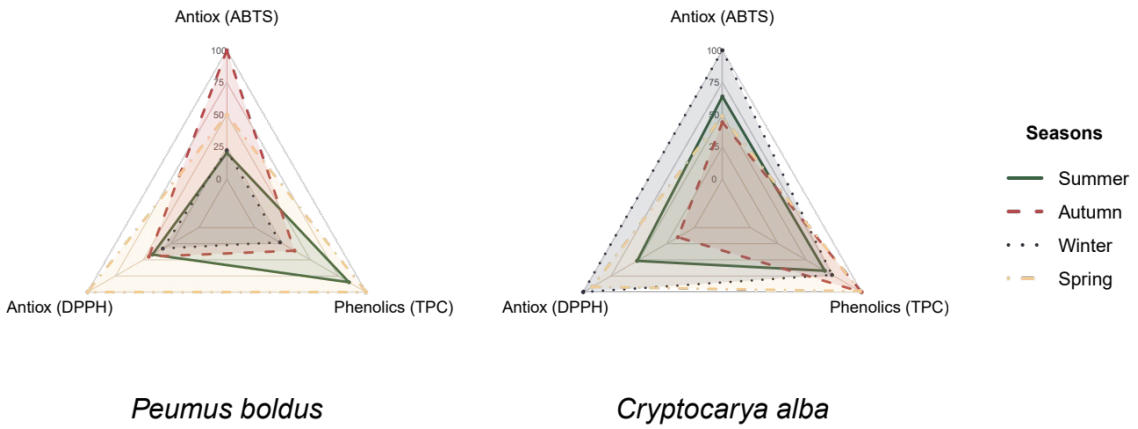


Figure 5. Radar charts illustrating the seasonal profiles of antioxidant capacity and phenolic content in leaves of *P. boldus* and *C. alba*. The three axes represent the normalized values (scaled 0-100) for Total Phenolic Content (TPC) and antioxidant activity measured by ABTS and DPPH assays. Data points plotted further from the center indicate higher phenolic concentration and higher antioxidant potency (corresponding to lower IC_{50} values). The seasonal trends are distinguished by line color and type: Summer (green solid), Autumn (red dashed), Winter (dark dotted), and Spring (yellow dot-dash).

Table 3. Antioxidant activity of *P. boldus* and *C. alba* across the four seasons of the year, expressed as IC_{50}, determined using the ABTS and DPPH free radical-scavenging assays. Each value represents the mean ± S.D. (technical replicates).

Species	Assay	Summer	Autumn	Winter	Spring
P. boldus	ABTS	49.52±8.55	10.17±2.77	45.28±4.35	20.34±0.68
	DPPH	49.14±5.98	45.64±6.05	63.38±4.42	20.56±0.39
C. alba	ABTS	126.39±4.90	183.50±3.78	81.10±1.86	165.82±10.01
	DPPH	37.07±4.43	128.19±3.09	19.13±0.49	20.80±0.65

3.4. Total Phenolic Content (TPC)

3.4.1. Phenolic Content in *P. boldus*

Pronounced variations in the phenolic content of *P. boldus* were detected throughout the year (Figure 5). The highest accumulation was scored in spring, with a value of 166.67 ± 16.40 μg g^{-1} GAE. The second-highest level was observed in summer, at 141.31 ± 0.96 μg g^{-1} GAE. Subsequently, phenolic content decreased sharply during the colder seasons. In autumn, the value dropped to 59.68 ± 1.01 μg g^{-1} GAE and reached its minimum in winter, with only 37.94 ± 0.77 μg g^{-1} GAE (Table 4). Regarding phenolic accumulation, all four seasons showed distinct concentration levels.

3.4.2. Phenolic Content in *C. alba*

In contrast to the previous species, the phenolic content of *C. alba* remained considerably lower and more stable throughout the year (Figure 5). Results indicated that that the highest levels occurred in autumn (9.48 ± 0.21 μg g^{-1} GAE) and spring (9.36 ± 1.01 μg g^{-1} GAE), with similar concentrations among them, while the lowest values were scored in summer (6.35 ± 0.23 μg g^{-1} GAE) and winter (6.96 ± 0.13 μg g^{-1} GAE) (Table 4).

Table 4. Total phenolic compound content of *P. boldus* and *C. alba* across the four seasons of the year, expressed as μg g^{-1} of phenolics in dry mass equivalent to gallic acid (GAE). Each value represents the mean ± S.D. (technical replicates).

Species	Summer	Autumn	Winter	Spring
P. boldus	141.31±0.96	59.68±1.01	37.94±0.77	166.67±16.40
C. alba	6.35±0.23	9.48±0.21	6.96±0.13	9.36±1.01

4. Discussion

During summer-the period of highest temperature and radiation, and lowest precipitation-the phytochemical diversity of *P. boldus* almost halved, coinciding with a massive production of the monoterpenoid eucalyptol. Previous studies have identified eucalyptol as one of the main components of *P. boldus* essential oils [19,24]. This compound has been reported to exert a protective effect in plants against high temperatures and radiation by reducing reactive oxygen species (ROS), increasing pigment content, and enhancing photosynthetic capacity [25,26]. Therefore, the higher proportion of this compound in summer is likely to be an adaptive response to heat stress. From the therapeutic perspective, in concordance with forest therapy, it has been reported that eucalyptol provided neuroprotection via glucose-lowering, antioxidant, anti-inflammatory and neurotrophic actions [27,28]. During the intermediate seasons of spring and autumn, the dominant VOC was the monoterpene p-cymene and a greater presence of sesquiterpenes (STs) and sesquiterpenoids (STDs) was also detected. p-Cymene is a naturally occurring monoterpene found in essential oils of various medicinal plants, where it plays an antioxidant role [29]. Interestingly, in vitro studies have demonstrated that p-cymene, exhibits significant anti-inflammatory and anticancer activities, making it a promising candidate for cancer therapy, since preliminary research suggests that it can induce apoptosis in cancer cells, modulating key signaling pathways involved in tumor progression [30]. Similarly to eucalyptol, p-cymene may contribute to health-promoting effects associated with forest-based therapeutic environments. The production of sesquiterpenes has been previously associated with mechanical stress from herbivores and exposure to cold in conifers [31], suggesting that their increased production may result from similar triggers.

The findings in *C. alba* show a very different landscape, as the diversity of compounds remains stable throughout the year. Its ability to adapt to summer stress appears to rely on the overproduction of 4-terpineol rather than a reduction in diversity. Comparisons with other studies confirm that eucalyptol remains a predominant component of *C. alba*'s essential oil [32,33], likely serving a similar protective role in the foliage as in *P. boldus*. In other species, this compound increases in response to drought stress [34] and it has also been reported to possess strong insecticidal activity [35]. From a biomedical perspective, in addition to its antioxidant and anti-inflammatory properties, its anticancer potential has been recently highlighted since this monoterpenoid presented a cytostatic effect against six cell lines, especially for breast adenocarcinoma and chronic myeloid leukemia [36].

A particularly noteworthy finding in this study is the divergence between total phenolic content (TPC) and antioxidant activity, which may indicate that *P. boldus* and *C. alba* have evolved fundamentally different antioxidant defense mechanisms based on seasonal modulation of their terpene profiles.

In *P. boldus*, antioxidant defense relies on the synergistic interactions within a complex mixture of terpenes rather than phenolic compounds, whose accumulation does not directly correlate with peak antioxidant activity observed in autumn and spring [33,37]. The highest diversity and antioxidant capacity in autumn are likely driven by the combined action of monoterpenes such as p-cymene and eucalyptol, whose synergistic effects enhance free radical neutralization [29]. Phenolic content is mainly modulated by precipitation, reaching maxima in summer and spring, which contrasts with the classic drought-stress–phenolic hypothesis and suggests that UV radiation and temperature are more influential in this species [38,39]. UV radiation emerges as a key regulator of secondary metabolism in both *P. boldus* and *C. alba*, driving metabolic specialization in summer with dominance of eucalyptol, a compound with established photoprotective and antioxidative roles [25,26].

When considering TPC individually, precipitation appears to be the dominant climatic factor modulating phenolic content in *P. boldus*, increasing its value. This trend contrasts with the classic "drought-stress–phenolic" hypothesis, which associates water deficit with phenolic accumulation [41]. In the deciduous forest of Concepción, the data suggest that limited water availability is not a major stress factor; instead, reduced precipitation coincides with higher UV radiation and temperature, both of which co-induce phenolic synthesis [39].

In *C. alba*, antioxidant activity appeared to reflect a specialized response to winter stress. This species maintained consistently low TPC levels, confirming that its antioxidant capacity resides primarily in its terpene components. The highest activity was observed during winter in both assays, under temperatures ranging from 6 °C to 14 °C and high rainfall, suggesting adaptation to cold-induced oxidative stress. Low temperatures are known triggers of ROS overproduction in plants [40,41]. In response, plants activate defense mechanisms, including antioxidant secondary metabolite synthesis, to protect cell membranes and proteins from oxidative damage [42]. Importantly, maximum antioxidant activity did not coincide with maximum production of the dominant compound 4-terpineol, indicating that the plant's response likely involves the targeted synthesis of specific minor compounds highly effective in counteracting cold stress.

5. Conclusion

The seasonal phytochemical variation in *P. boldus* and *C. alba* reflects distinct metabolic strategies: *P. boldus* specializes in eucalyptol production during summer, while *C. alba* maintains year-round diversity through 4-terpineol overproduction. Antioxidant defense in both species is primarily terpene-driven and strictly modulated by UV radiation, temperature, and precipitation. Our findings suggest that autumn and spring may provide the most diverse and bioactive phytochemical atmospheres for forest therapy in these ecosystems, characterized by a higher richness of therapeutic compounds such as p-cymene and eucalyptol. This study provides the first comprehensive insight into the dynamics of VOC synthesis in response to seasonal changes in two emblematic trees of the southern Chilean forest. Further investigation is required to fully understand emission dynamics and their specific physiological contributions to human health through the practice of forest therapy.

Author Contributions

Conceptualization, N.R.M.K., M.H.d.l.T., K.C.-A. and C.P.M.; conducting of experiments, N.R.M.K. and K.C.-A.; methodology, N.R.M.K., M.H.d.l.T., K.C.-A. and C.P.M.; formal analysis, N.R.M.K., M.A.-D. and D.B.C.; writing—draft preparation, N.R.M.K.; writing—review and editing,

N.R.M.K., M.H.d.l.T., F.T., C.P.M., D.B.C.; software, N.R.M.K., M.A.-D., F.T. and D.B.C.; supervision, M.H.d.l.T., K.C.-A., and C.P.M.. All authors have read and agreed to the published version of the manuscript.

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Statements and Declarations

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This study complies with all relevant ethical guidelines. All data and samples were collected in accordance with the regulations of Corporación Nacional Forestal of Chile (CONAF) and the authorities of Nonguén National Park (Chile). No experiments involving humans or animals were conducted. The

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