“Seasonal variation of health-promoting compounds from Chilean tree species”

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Abstract

Forest bathing, also known as Shinrin-yoku, allows people to reap the benefits of nature, thereby enhancing their overall health and quality of life. This practice has been shown to bolster the immune system, alleviate mental fatigue, and improve cardiovascular health, sleep quality, and other aspects of well-being. The specialized metabolites responsible for these effects are biogenic volatile organic compounds (BVOC), which are primarily composed of terpenes and essential oils. The composition and content of these compound can exhibit seasonal variations, which may influence their biological activities. The objective of this study was to ascertain the impact of seasonal variations on the production of specialized metabolites in the *Cryptocarya alba* and *Peumus boldus* species. Leaves of both species were collected in Nonguén National Park, Chile, during the four seasons. Essential oils were extracted by hydrodistillation and quantified by gas chromatography coupled to mass spectrometry. The phenol content was determined, and antioxidant activity was measured. The extracts of *P. boldus* exhibited a higher proportion of terpenes in the autumn, with α-pinene, o-cymene, and eucalyptol representing the majority. Additionally, the antioxidant capacity was observed to be higher in the autumn and spring. *C. alba* extracts obtained during summer exhibited a higher proportion of terpenes, including β-phellandrene, eucalyptol, and 4-terpineol, and demonstrated the most pronounced antioxidant effect during the winter season. The findings revealed that the composition and antioxidant effect of BVOCs differ between seasons in both species, which could influence the beneficial effects of forest bathing.

Keywords: specialized metabolites, terpenes, Forest bathing, chromatography, essential oils, antioxidant, native species.

1. Introduction

In the contemporary era, the advancement of technology and the process of urbanization have contributed to a reduction in the interaction between humans and the natural environment. This has led to the emergence of sedentary behaviors, psychological issues, and stress (Rodríguez-Redondo et al. 2023). The practice of forest bathing, or Shinrin-yoku, has been demonstrated to have the potential to counteract these effects. These practices originated in Japan as a form of preventative healthcare and also as a healing practice (Hansen et al. 2017). It is currently understood that forest bathing has a number of beneficial effects. These include improvements to the immune system, with increased activity of NK (natural killer) cells and an increase in anti-cancer proteins (Li et al. 2007). Such practices have been demonstrated to enhance cardiovascular health and metabolic function (Lee et al. 2014; Yau and Loke 2020). They markedly enhance parasympathetic nervous system activity and markedly diminish sympathetic activity, thereby fostering positive affect, attenuating negative emotions, and reducing anxiety (Song et al. 2015; Wen et al. 2023).

One of the factors that contribute to the beneficial effects of forest bathing is the presence of biogenic volatile organic compounds (BVOCs) released by (Franco et al. 2017; Antonelli et al. 2020). Trees are the primary source of BVOCs in forests, with leaves representing the most active emitting structures. They possess specific organs that facilitate the synthesis of these compounds, including sebaceous glands and trichomes (Antonelli et al. 2020).

The most abundant terpenes within the BVOCs are isoprene and monoterpenes, the latter of which is one of the primary metabolites involved in the beneficial effects of forest baths on human health (Cho et al. 2017; Kim et al. 2020; Bach et al. 2020). In addition to constituting part of the forest atmosphere, these terpenes are also components of essential oils (EOs) synthesized by trees, specifically in leaf organs designated as trichomes (Antonelli et al. 2020; Salas-Oropeza et al. 2021). The biochemical variations observed in BVOCs occur both along day and seasonally. Peak concentrations are recorded in the morning and late afternoon, with the highest levels occurring in summer. These variations are influenced by meteorological conditions, altitude, light exposure, and also by species type (Meneguzzo et al. 2019; Bach et al. 2020).

One of the benefits of forest bathing is the antioxidant effect produced by inhaling the BVOCs produced by trees, particularly terpenes and terpenoids (Antonelli et al. 2022). It has been demonstrated that inhalation of limonene and α-pinene results in the production of antioxidants and anti-inflammatory agents (Antonelli et al. 2020). Additionally, antioxidant activity has been documented for terpenoids, including linalool, thymol, menthol, α-pinene, and myrcene (Acevedo et al. 2013; Masyita et al. 2022).

The existing research on the seasonal behavior of BVOCs in native Chilean species is limited, particularly with regard to forest bathing. It is therefore important to gain an understanding of how BVOCs behave in native Chilean species, both in terms of their contribution to human health through forest bathing and in order to gain insight into the effects of abiotic factors on the production of specialized metabolites.

The objective of this study was to assess the impact of seasonal variations on the production of BVOCs in Chilean native species, *Peumus boldus* Mol., *both present in Nonguén National Park, located in the province of Concepción, through the analysis of their EOs. P. boldus* has been found to contain a variety of compounds, including α-pinene, β-pinene, limonene, α-terpinene, 1,8-cineole, and linalool, which have been demonstrated to possess a range of beneficial properties for human health (Mariano et al. 2019; Lopes et al. 2020). With regard to *C. alba*, its essential oil contains a

number of volatile compounds, including α-terpineol, linalool, eucalyptol, α-phellandrene, limonene, and others that have been demonstrated to possess beneficial properties for humans (Pinto et al. 2016; Bravo et al. 2017; Touma et al. 2020). Subsequently, the antioxidant capacity of the extracts was quantified through ABTS and DPPH assays, and the total phenol content of the leaves was also determined.

2. Materials and Methods

2.1. Study área

Nonguén National Park is situated in the province of Concepción, Biobío Region, within the Coastal Mountain Range (73°00'W-36°52'S), encompassing an area of 3,056.96 hectares. The climate is classified as warm temperate, with winter rainfall and an average temperature of 13.1°C. The park exhibits pronounced seasonality, with a dry season occurring in January and February. The park is home to one of the last remaining relicts of Concepción's deciduous forest, comprising species such as *Nothofagus dombeyi* (Mirb.) Oerst., *Nothofagus obliqua* (Mirb.) Oerst., *Cryptocaria alba* (Mol.) Looser, *Peumus boldus* Mol., and *Persea lingue*, among others (Bocaz-Torres et al. 2013).

2.2. Plant material

The leaves of *P. boldus* and *C. alba* were obtained from within Nonguén National Park. Five adult trees of each of the two species were selected of the trails of the park, with the same amount of material collected for each individual. The plant material was collected during the early morning hours and across the four seasons: summer, fall, winter, and spring. In order to obtain the leaves, the methodology proposed by Vogel et al. (1997) was followed, whereby the foliage was collected at a constant height of the tree and from the four cardinal points.

2.3. EO’s extraction

The EOs of *P. boldus* and *C. alba* were obtained by hydrodistillation with steam entrainment in a Clevenger-type apparatus (Biobase, HM-I). Three hundred grams of fresh leaves were charged with 2.6 L of distilled water and distilled for four hours. Residual water in the EOs was removed with sodium sulfate anhydride. The extracts were stored in amber glass bottles at room temperature (Ortiz et al. 2017).

2.4. EOs chromatographic analysis

The EOs obtained were characterized using a gas chromatograph coupled to a mass spectrometer (GC-MS) (Agilent 7890A) equipped with an Agilent automatic injector and an HP-5 capillary column (30 m x 0.25 mm id, with a layer thickness of 0.25 μm). The temperature of the injection block was 250°C. The GC oven temperature was programmed as follows: initial temperature 50°C, with an increase of 2°C min-1 for 2 min, then increased to 8°C min-1 to reach 200°C for 5 min, and finally increased to 10°C min-1 to reach 250°C for 10 min, for a total run time of 40.75 min. The relative percentages of EO components were obtained for each species using helium as a carrier gas at a flow rate of 20 ml min-1 at constant volume in a mass detector (Agilent 5975C).

2.5. Determination of the Antioxidant Activity

2.5.1. ABTS radical scavenging assay

For the ABTS assay proposed by Miller et al. (1993), the procedure modified by Crisóstomo-Ayala et al. (2021) was followed. The EO samples of *P. boldus* and *C. alba* were diluted in chloroform as solvent. The radical solution contained 3.5 mM ABTS (2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonate) and 1.22 mmol potassium persulfate. This solution was prepared by mixing the two previous solutions in equal parts and keeping them in the dark for 16 hours at room temperature. After this time, a solution was prepared by diluting 1 ml of ABTS*+ solution with 14 ml of distilled water to give an absorbance of 0.70 ± 0.01 at 734 nm. Fresh ABTS*+ solution was prepared for each of the assays at each station. Each reaction contained 180 µl of the ABTS*+ radical and 20 µl of Trolox at different concentrations (from 0.01 to 0.2 mg ml-1) or the samples (from 25 to 100 mg ml-1), which were allowed to react for 60 min in the dark. Absorbance was measured at 734 nm with an Epoch™ Microplate Spectrophotometer from BioTek, and all determinations were performed separately.

2.5.2. DPPH radical scavenging assay

The antioxidant activity of the EO, together with the preparation of the Trolox standard solution, was analyzed using the DPPH radical scavenging method described by Brand-Williams et al. (1995), with modifications described by Crisóstomo-Ayala et al. (2021). For each sample, a liquid-liquid extraction was performed with equal parts of methanol. The mixture for each assay contained 270 µl of 0.06 mmol DPPH radical solution prepared in methanol and 30 µl of Trolox at different concentrations (from 0.01 to 0.2 mg ml-1) or the samples (from 25 to 100 mg ml-1). Each mixture was incubated for 20 min at 37°C in the dark. The absorbance of the assays was measured at 517 nm using a BioTek Epoch™ microplate spectrophotometer. Trolox was used as a positive control and methanol was used as a blank for free radical solutions. All determinations were performed in triplicate (n=3).

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

$$\%\text{inhibition} = ([A(\text{blank}) - A(\text{sample})]/A(\text{blank})) \times 100$$

Where Ac is the absorbance of the control and As is the absorbance of the samples. The antioxidant activity for the ABTS and DPPH assays was expressed as IC50, which corresponds to the sample concentration required for 50% free radical inhibition (Mkaddem et al. 2009; Rubilar et al. 2011).

2.6. Determination of total phenolic content (TPC)

The total phenolic compound (TPC) content of each sample was determined using the method described by (Crisóstomo-Ayala et al. 2021), with certain modifications. A liquid-liquid extraction with methanol was conducted for each sample, which was then used for the assay at a concentration of 100 µg/ml of sample. A calibration curve was constructed using gallic acid (0.01 to 0.2 mg/ml). For each reaction, a mixture was prepared containing the following components: distilled water (400 µl), sample or gallic acid solution for the standard curve (20 µl), Folin-Ciocalteu reagent (40 µl), and 15% (m/v) sodium carbonate (200 µl). The mixture was then incubated at room temperature for 60 minutes in the dark, with stirring to facilitate the reaction. All determinations were conducted in triplicate (n=3). The absorbance was determined at 765 nm with an Epoch™ Microplate Spectrophotometer from BioTek. The total phenolic content (TPC) was expressed as micrograms per gram of dry mass in gallic acid equivalents (GAE).

2.7. Statistical analysis

All analyses were conducted in triplicate, and the resulting data were expressed as mean ± standard deviation (SD). The statistical analysis was conducted using RStudio 4.3.1 software, employing a one-way ANOVA followed by Tukey's test at a 95% confidence level to ascertain the existence of individual differences in means. Prior to conducting the ANOVA analysis, the assumptions of normality and homoscedasticity were evaluated using the Shapiro-Wilk W test and Levene test, respectively.

3. Results

3.1. Chemical composition

3.1.1. *P. boldus*

The analysis of the EO of *P. boldus* revealed that the components of these compounds exhibit seasonal variations, with a total of 61 different compounds identified across the four seasons. In the summer season, the diversity of metabolites was observed to be lower, with a total of 28 identified. Of these, 22 were classified as monoterpenes, one as sesquiterpenes, one as phenylpropanoids, and four remained unidentified. Similarly, in the winter and spring seasons, the same number of compounds were identified (41), yet the proportions differed. In the winter season, 33 monoterpenes, one sesquiterpene, two phenylpropanoids, and five unidentified compounds were detected. In the spring season, the number of monoterpenes decreased (29) while the variety of sesquiterpenes increased (5). Additionally, one phenylpropanoid was identified, and six compounds remained unidentified. The highest number of compounds was identified in the autumn season, with a total of 47, comprising 34 monoterpenes, eight sesquiterpenes, one phenylpropanoid, and four unidentified compounds (Figure 1).

The compounds that demonstrated consistent levels across all samples were as follows: for monoterpenes, α-pinene, β-phellandrene, α-phellandrene, α-terpinene, o-cymene, eucalyptol, and linalool. With regard to sesquiterpenes, spatulenol was the sole compound that was maintained in all four seasons. With regard to phenylpropanoids, only methyleugenol was identified in all four measurements (see Table 2).

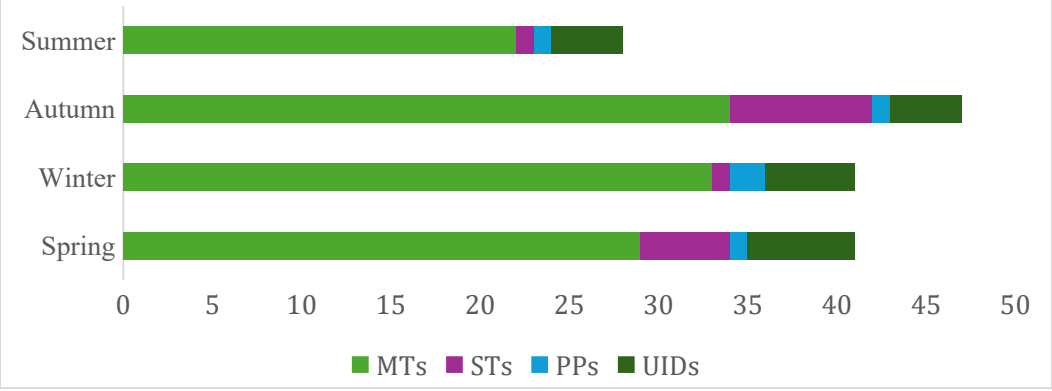


Figure 1. Characterization of the number of compounds in the EO of *P. boldus* at four season by gas chromatography-mass spectrometry (MTs: monoterpenoids; STs: sesquiterpenoids; PPs: phenylpropanoids; UIDs: unidentified).

The EO of *P. boldus* exhibited notable variations in the relative abundance of the predominant compounds. The most abundant metabolites were observed to be the monoterpene o-cymene and the monoterpenoid eucalyptol during all four seasons. O-Cymene was the most abundant compound in spring (19.71%) and in autumn (15.21%). Although it was not the most abundant in the other two seasons, high contents were recorded: 10.34% in winter and 6.7% in summer. Eucalyptol was the predominant metabolite in the summer (56.38%) and winter (22.99%) samples, representing the majority of the extracted oil composition. In the spring and autumn, the abundance was 14.03% and 13.64%, respectively. In addition, the content of other monoterpenes, including α-pinene (6.38% abundance in spring), α-terpineol (3.57% in spring), and α-terpinene (5.08% in summer), was also considerable. Furthermore, a notable proportion of nona-3,5-dien-2-ol was identified, with a percentage abundance of 9.38% in summer, 9.91% in autumn, 8.93% in winter, and 6.52% in spring (Table 1).

Table 1. Composition of *P. boldus* EO's analyzed by gas chromatography-mass spectrometry (GC-MS), 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	5,786	3-Thujene	-	0,49	-	0,60
2	5,916	α-Pinene	2,46	4,50	2,34	6,38
3	6,211	Camphene	-	0,25	0,28	0,30
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	0,70	-	0,34
8	7,301	α-Phellandrene	0,39	1,10	0,30	1,30
9	7,425	3-Carene	-	0,77	0,31	0,69
10	7,545	α-Terpinen	0,30	1,01	0,40	0,73
11	7,732	o-Cymene	6,70	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	22,99	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	Terpinolen	-	0,24	-	0,23
17	9,008	p-Cymenene	-	0,49	-	-
18	9,174	Linalol	0,81	2,20	1,13	2,66
19	9,433	Fenchol	-	-	0,40	-
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	3,10	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-Terpinen	5,08	1,13	3,32	2,27
37	12,095	Piperitone oxide	1,84	-	0,30	0,82
38	12,142	Ascaridiol	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	Safrol	-	-	0,71	-
43	12,713	trans-Ascaridol glycol	-	-	1,21	0,36
44	12,811	Thymol	-	0,82	-	-
45	12,931	Ascaridole epoxide	0,40	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	Acetic acid, 6,6-dimethyl-2-methylene-7-(3-oxobutylidene)oxepan-3-ylmethyl	0,37	1,09	0,56	0,56
54	17,938	Limonene oxide, cis-	-	0,32	-	-
55	18,135	Humulenol-II	-	0,37	-	-
56	18,192	Isoaromadendrene epoxide	-	0,51	-	0,34
57	18,316	Cyclooctasiloxane, hexadecamethyl-	-	-	1,03	-
58	18,389	β-Eudesmol	-	0,22	-	-
59	18,422	α-Cadinol	-	-	-	0,27
60	18,665	p-Menth-8-ene, 3-methylene-	-	-	-	0,30
61	18,889	Ledene oxide-(II)	-	-	-	0,60

3.1.2. *C. alba*

In the autumn, winter, and spring, 49 compounds were identified, while in the summer only 40 were found, wich included 17 monoterpenes, 13 sesquiterpenes, two phenylpropanoids, three fatty acid esters, one carboxylic acid ester, and four compounds that could not be identified. In the autumn, a total of 19 monoterpenes, 17 sesquiterpenes, three fatty acid esters, one carboxylic acid ester, one aldehyde, and eight unidentified compounds were identified. In winter, the proportion of monoterpenes increased, while the proportion of sesquiterpenes synthesized in autumn was maintained, with 22 monoterpenes, 17 sesquiterpenes, three fatty acid esters, one carboxylic acid ester, and six compounds that could not be identified. In spring, the variety of monoterpenes decreased, while the number of sesquiterpenes identified increased. The latter group included 18 monoterpenes, 22 sesquiterpenes, two fatty acid esters, one carboxylic acid ester, one aldehyde, and five compounds that could not be identified (Figure 2).

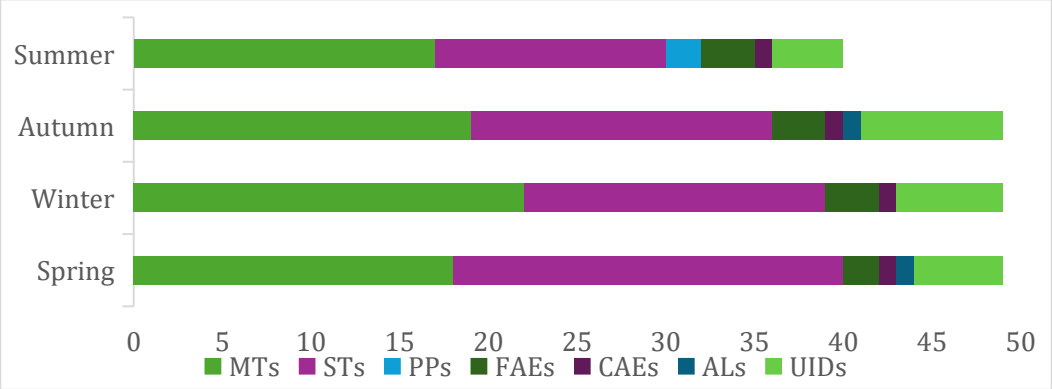


Figure 2. Characterization of the number of EO compounds of *C. alba* in four stations analyzed by gas chromatography-mass spectrometry (MTs: monoterpens; STs: sesquiterpens; PPs: phenylpropanoids; FAEs: fatty acid esters; CAEs: carboxylic acid esters; ALs aldehydes; UIDs: unidentified).

Table 2 illustrates the composition of the *C. alba* EOs obtained throughout the course of the year. The monoterpenes α-pinene, β-pinene, β-myrcene, limonene, linalool, and 4-terpineol demonstrated consistent presence throughout the four seasons. With regard to sesquiterpenes, the presence of δ-elemene, caryophyllene, D-germacrene, calamenene, and spatulenol was consistently observed. Two additional fatty acid esters, hexanoic acid, 4-pentenyl ester and β-phenylethyl butyrate, were also consistently present at all four stations (Table 2).

Table 2. Composition of *C. alba* EOs analyzed by gas chromatography-mass spectrometry (GC-MS), 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	5,210	Heptanal	-	-	-	0,17
2	5,781	3-Thujene	0,67	0,95	0,76	0,96
3	5,916	α-Pinene	6,62	5,60	4,99	5,93
4	6,206	Camphene	0,28	0,79	0,45	0,86
5	6,709	β-Phellandrene	12,21	7,01	6,33	9,23

6	6,772	β-Pinene	5,22	6,20	6,02	7,01
7	7,026	β-Myrcene	0,69	1,17	1,30	1,14
8	7,306	α-Phellandrene	-	0,45	0,38	0,45
9	7,425	3-Carene	-	0,32	0,34	-
10	7,550	α-Terpinen	1,49	3,45	3,83	3,62
11	7,716	o-Cymene	4,35	2,72	2,28	3,12
12	7,799	Limonene	2,21	1,74	2,55	3,15
13	7,856	Eucalyptol	9,38	5,47	8,19	9,03
14	7,955	β-trans-Ocimene	-	-	1,38	-
15	8,157	β-cis-Ocimene	1,39	1,94	2,15	1,96
16	8,380	γ-Terpinene	3,37	6,08	6,84	6,24
17	8,961	Terpinolen	1,13	2,31	2,22	2,02
18	9,174	Linalol	0,48	0,57	0,95	1,11
19	9,418	Butanoic acid, 3-methyl-, 3-methyl-3-butenyl ester	3,06	2,76	3,15	2,84
20	9,968	Terpineol, cis-β	0,27	0,53	0,37	0,49
21	9,976	p-menth-2-en-1-ol	-	-	0,51	-
22	10,321	2-Nonenal	-	0,33	-	-
23	10,746	4-Terpineol	26,39	15,71	16,14	15,51
24	10,938	α-Terpineol	1,76	1,42	1,89	1,56
25	11,063	Myrtenol	-	-	0,28	-
26	12,116	Hexanoic acid, 4-pentenyl ester	0,46	0,76	0,46	0,25
27	12,412	4-Chloro-3-n-hexyltetrahydropyran	-	0,53	0,47	0,17
28	12,702	Safrol	1,16	-	-	-
29	12,998	Trichloroacetic acid, pentadecyl ester	-	0,46	-	-
30	13,486	δ-Elemene	0,33	1,15	0,67	0,83
31	14,046	Copaene	-	-	-	0,19
32	14,399	β-Elemen	0,47	0,80	0,54	0,79
33	14,441	β-Phenylethyl butyrate	0,37	0,53	0,59	0,42
34	14,591	Methyleugenol	0,36	-	-	-
35	14,887	Caryophyllene	0,52	0,89	0,58	0,63
36	15,079	trans-α-Bergamotene	1,93	2,16	1,48	1,47
37	15,359	β-Farnesene	0,37	0,83	0,48	0,30
38	15,432	α-Caryophyllene	0,51	0,71	0,57	0,66
39	15,468	β-Cubebene	-	-	-	0,16
40	15,753	gamma-Muurolene	-	0,40	-	0,34
41	15,852	Germacrene D	1,18	1,66	1,80	1,73
42	15,904	Ácido butanoico, 2-metil-, 2-fenilo	1,31	2,21	2,40	3,04
43	15,950	bencenometanol, 2-metil-, acetato	1,09	1,56	1,59	0,87
44	16,096	Germacrene B	0,69	1,23	1,07	0,60
45	16,474	Calamenene	0,93	1,61	1,30	0,33
46	16,713	Spiro[tricyclo[4.4.0.0(5,9)]decane-10,2'-oxirane], 1-methyl-4-isopropyl	-	0,71	0,32	0,27
47	17,004	Nerolidol	0,79	1,01	0,45	1,42

48	17,346	Spathulenol	1,20	1,87	1,41	2,47
49	17,440	Caryophyllene oxide	1,63	3,24	2,58	0,28
50	17,559	Viridiflorol	-	-	0,29	0,17
51	17,611	Aromadendrene oxide	-	-	-	0,18
52	17,730	Isoaromadendrene epoxide	-	0,41	0,32	0,17
53	17,803	Cyclohexene, 3,5,5-trimethyl-	0,34	0,97	0,73	0,19
54	18,052	Isospathulenol	0,75	1,41	1,03	0,98
55	18,202	1,8-Cyclopentadecadiyne	0,33	0,72	0,47	0,18
56	18,244	tau.-Muurolol	-	-	0,32	2,22
57	18,301	β-Guaiene	-	0,32	-	-
58	18,389	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	2,32	3,45	4,33	-
59	18,887	Ledene oxide-(II)	-	0,54	0,44	0,51
60	19,754	1-Hexadecanol, 2-methyl-	-	0,35	-	-

In the seasonal analysis of components, the EO of *C. alba* demonstrated fluctuations in the abundance percentages of specific compounds. One such compound was the monoterpene 4-terpineol, which exhibited the highest percentage of abundance. In the summer months, the compound constituted 26.39% of the essential oil (EO), a figure that declined to 15.71% in the autumn, 16.14% in the winter, and 15.51% in the spring. The next most prominent components of the oil were the monoterpenes β-phellandrene and eucalyptol. The highest abundance of β-phellandrene was recorded in summer (12.21%), followed by spring (9.23%), autumn (7.01%), and the lowest proportion in winter (6.33%). Additionally, eucalyptol exhibited the highest abundance in the summer season (9.38%), followed by spring (9.03%), winter (8.19%), and autumn (5.47%). The monoterpenes α-pinene and β-pinene exhibited high abundance percentages, with the highest percentages of α-pinene occurring in summer (6.62%) and of β-pinene occurring in spring (7.01%) (Table 1).

3.2. Antioxidant Activity of ABTS and DPPH Assays

The antioxidant activity of *P. boldus* EO demonstrated seasonal variations. The ABTS assay demonstrated statistically significant differences across the four seasons. The lowest IC50 value was observed in summer and fall, indicating enhanced antioxidant activity with reduced sample requirements for radical inhibition. Conversely, the winter and summer samples exhibited the least pronounced antioxidant activity. With regard to the DPPH assay, the antioxidant activity of the spring sample exhibited a similar trend to that observed in the ABTS assay, demonstrating a superior antioxidant effect compared to the other seasons. Similarly, the results for winter and summer exhibited a comparable trend to that observed in the ABTS assay, with higher IC50 values. The autumn season yielded disparate outcomes in both assays. In ABTS, it exhibited the most robust antioxidant activity among the four seasons. However, in DPPH, it did not attain the lowest IC50 value.

In the ABTS assay, the samples of *C. alba* exhibited statistically significant differences across all seasons. The winter samples demonstrated the most pronounced antioxidant effect, while the fall samples exhibited the highest IC5O value. In the DPPH assay, winter exhibited the most pronounced antioxidant effect, while fall demonstrated the least pronounced effect.

Species	Assay	Summer	Autumn	Winter	Spring	F
P. boldus	ABTS	49.52±8.55a	10.17±2.77b	45.28±4.35a	20.34±0.68b	<0.001***
	DPPH	49.14±5.98b	45.64±6.05b	63.38±4.42a	20.56±0.39c	<0.001***
C. alba	ABTS	126.39±4.90c	183.50±3.78a	81.10±1.86d	165.82±10.01b	<0.001***
	DPPH	37.07±4.43b	128.19±3.09a	19.13±0.49c	20.80±0.65c	<0.001***

Table 3. Antioxidant activity of *P. boldus* and *C. alba* in the four seasons, expressed as IC50, determined by ABTS and DPPH free radical scavenging assays. Each value represents the mean ± S.D. (n=3). Different letters indicate statistically significant differences according to Tukey's test ($p \leq 0.05$). ANOVA *p*-values for the different species and experiments. *** Significant at a 99% confidence level.

3.3. Total Phenolic Compound Content (TPC)

The concentration of phenolic compounds (TPC) in *P. boldus* exhibited notable seasonal variations, with the highest concentration observed in spring (166.67 µg g-1 of phenols) and the lowest in winter (37.94 µg g-1) (Table 4).

In the case of *C. alba*, the statistical analysis yielded two distinct groups. The highest phenol content was observed in the autumn and spring seasons, while the lowest was observed in summer and winter (Table 4).

Species	Summer	Autumn	Winter	Spring	F
P. boldus	141.31±0.96b	59.68±1.01c	37.94±0.77d	166.67±16.40a	<0.001***
C. alba	6.35±0.23b	9.48±0.21a	6.96±0.13b	9.36±1.01a	<0.001***

Table 4. Content of total phenolic compounds of *P. boldus* and *C. alba* in the four seasons of the year, expressed in µg g-1 of phenols in dry mass gallic acid equivalents (GAE). Each value corresponds to the mean ± S.D. (n=3). Means with unequal letters have statistically significant differences (one-factor ANOVA, Tukey, $p \leq 0.05$). ANOVA F-values for the different species and trials. *** significant at the 99.9% probability level.

4. Discussion

The BVOCs present in EO of *P. boldus* and *C. alba* exhibited seasonal fluctuations that could be influenced by meteorological conditions. In both cases, the quantity and diversity of compounds were found to be lower in the summer than in the other seasons. The volatility and emission of BVOCs into the forest atmosphere are dependent on temperature and solar radiation. Consequently, summer is the season with the highest presence of these compounds in the environment (Meneguzzo et al. 2019;

Bach et al. 2020). Therefore, the reduced diversity of these components in the EO during the summer months may be attributed to the fact that the majority of them are being volatilized and released to the exterior.

This could be an explanation for the greater diversity of BVOCs observed in the chromatograms of *P. boldus* in seasons where temperatures and solar exposure decrease. The lowest proportion of BVOCs was observed in the summer months, while the highest variety was noted in the autumn and winter, coinciding with a decrease in temperature and solar exposure. In spring, as these variables increased, the amounts of BVOCs in the oils decreased once more, potentially due to the volatilization of these compounds. A similar pattern was observed in *C. alba*. In seasons where temperatures and solar exposure decreased, a greater diversity of BVOCs was observed in the chromatograms. Subsequently, in the spring season, the presence and quantity of compounds with lower molecular weight, such as monoterpenes, decrease as a result of their potential volatilization due to the increase in temperature and solar radiation. This is reflected in a study conducted in China by Tian et al. (2021), in which the terpene emission of the species *Cinnamomum camphora* at four growth stages during all seasons of the year was analyzed. The results indicated that the highest terpene emission occurred in summer, irrespective of the physiological age of the species.

With regard to the biological properties of the components of *P. boldus* oils, a number of authors have demonstrated the presence of a range of biological activities in the EO, including antimicrobial, anti-inflammatory, antitumor, sedative, and antidepressant properties associated with α-pinene and β-pinene (Zorić et al. 2022). For α-terpineol, a number of additional biological activities have been described, including anti-ulcer, antioxidant, anticancer, anticonvulsant, and antihypertensive effects (Khaleel et al. 2018). The absence of certain compounds, including β-myrcene, cis-geraniol, and thymol, in some measurements indicates that their synthesis is influenced by seasonal changes. The differential release of these compounds in certain seasons also allows for the enhancement of the beneficial effects of forest bathings, as is the case with α-phellandrene and myrtenol in *C. alba*.

With regard to the biological properties of the components of *C. alba* oils, metabolites that are analogous to those synthesized by *P. boldus* have been identified, including α-pinene, β-pinene, α-phellandrene, and 4-terpineol. With regard to α-terpineol, Additionally, it has been documented to possess anti-inflammatory, antioxidant, and neuroprotective properties (Guimarães et al. 2013; Cho et al. 2017). Conversely, 4-terpineol has been demonstrated to possess antitumor and proapoptotic activity (Di Martile et al. 2021), in addition to exhibiting potential antiviral properties (Bose et al. 2020). The aforementioned compounds were identified in the EO obtained in this study across all seasons. Their release into the atmosphere would serve to demonstrate the positive effect of forest bathings on human health.

The presence of different metabolites in the EO, related to seasonal variation, lends further support to the view that undertaking the trial in a specific season can confer benefits. For example, α-phellandrene, which is present in autumn, winter, and spring, has been demonstrated to enhance immune system function by activating natural killer (NK) cells and B cell proliferation. Additionally, this compound has been shown to possess wound-healing and anti-inflammatory effects (Lin et al. 2013; Salas-Oropeza et al. 2021). Additionally, myrtenol, which was only detected in winter, has neuromodulatory and antioxidant properties, in addition to having protective effects on neurodegenerative processes (Dragomanova et al. 2018). In both instances, the seasons in which these compounds are identified present a greater number of beneficial effects for forest bathing than those in which they are absent.

The antioxidant tests yielded significant differences between seasons for both species. The greater antioxidant effect observed in certain seasons can be attributed to a number of factors. In *P. boldus*, when the antioxidant activity assays were correlated with the phenol content of the extracts, a more pronounced antioxidant effect was observed in spring, where a higher phenol content was detected. These compounds are known to be potent antioxidants (Meyer Torres et al. 2018), and thus, the high antioxidant activity may be specifically linked to the presence of phenolic structures in the oils. This result is not comparable to the autumn measurement, as the antioxidant assays yielded similar outcomes to those observed in spring, yet the phenol content was considerably lower. This indicates that the enhanced antioxidant effect may be attributable to other factors, such as the presence of a specific synergistic interaction between some of the components of the EO's or the greater diversity of components (Touma et al. 2020).

The oils obtained in winter from *C. alba* possess a greater diversity of terpenes and compounds with registered antioxidant activities, including eucalyptol, linalool, α-pinene, 4-terpineol, caryophyllene, and D-germacrene. These compounds may contribute to a greater antioxidant effect for both ABTS and DPPH assays (Granados Conde et al. 2012; Acevedo et al. 2013; Cho et al. 2017; Masyita et al. 2022). However, the autumn samples exhibited a greater number of terpenes than those obtained in summer, yet demonstrated the lowest antioxidant effect of all the measurements. One potential explanation for this phenomenon is that the chemical compositions of EO ultimately determine their biological activities. In this instance, the antioxidant effect may fluctuate due to the formation of synergistic interactions between the constituent components, which may either enhance or diminish this activity, resulting in variations across different seasons (Touma et al. 2020).

In a research article published by Khodaei et al. (2021), the antioxidant potential of 38 EOs corresponding to 18 plant species was evaluated with the objective of developing predictive models to relate the chemical diversity of EO to their antioxidant effect. This involved exploring the relationship between chemical families and antioxidant activity, which was measured using the DPPH and ORAC assays. The research revealed that the presence of non-isoprenoid compounds, including ketones and esters, as well as sesquiterpenes, exhibited low antioxidant activity. Conversely, it was observed that oils with high monoterpene abundance exhibited diminished antioxidant activity in assays. Among the observed synergies, a positive effect was noted between phenols/esters and phenols/alcohols. Increasing the phenol content was found to favor the antioxidant effect, with a significant portion of the antioxidant activity attributed to this combination. The combination of monoterpenes and ketones with phenols did not result in an enhancement of the antioxidant effect, as observed. Moreover, the researchers discovered that some monoterpenes exhibited antioxidant activity, whereas others demonstrated the opposite effect, resulting in an antagonistic impact on this activity.

The aforementioned models provide information that facilitates a more comprehensive understanding of the impact of the compounds in question, thereby elucidating their potential antioxidant activity. However, it is important to note that not all cases allow for a direct correlation. In *P. boldus*, a correlation was observed between the results obtained and those proposed by the models, with an increase in antioxidant effect noted in autumn, coinciding with an increase in monoterpene variety in the oils. In the spring season, there was a notable increase in antioxidant activity, which may be attributed to the presence of phenolic compounds rather than the diversity of monoterpenes synthesized. Conversely, in *C. alba*, an increase in phenol content resulted in a reduction in antioxidant activity during the winter months, while the opposite was observed during the autumn. This may be attributed to the structural differences in the phenol composition of the extracts obtained during each season. Nevertheless, further study and evaluation of the effect of the EO components

synergy and their biological activities is recommended for both species. The demonstrated antioxidant activity of the extracts obtained is dependent on the composition of the synthesized metabolites, which varies as a function of seasonal variations. These findings contribute to the study of forest bathings, which allows for the implementation of differentiated schedules and times to enhance the beneficial effects of nature on human health.

5. Conclusion

It was determined that seasonal variations exert a significant influence on the volatilization of the BVOCs present in the leaves. During the summer months, there is a notable increase in the emission of these compounds into the surrounding environment, which is closely associated with the rise in temperature and radiation levels. Monoterpenes were the primary BVOCs identified of the EO, and their beneficial biological properties for humans are being leveraged in the practice of forest bathings. Moreover, it was demonstrated that essential oils are intricate combinations of specialized metabolites, which presents a challenge in comprehending the mechanisms underlying their antioxidant efficacy. It was observed that these effects are conditioned by synergistic or antagonistic interactions between the components present in the oils. The findings of this study may prove valuable in comprehending these ecosystems and their resilience to changing climatic conditions. Additionally, they may contribute to our understanding of the influence of abiotic factors on the synthesis of specialized metabolites in Chilean species.

6. References

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