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Analyzing essential oils: extraction and characterization from fresh and dry leaves of *Pinus elliottii*

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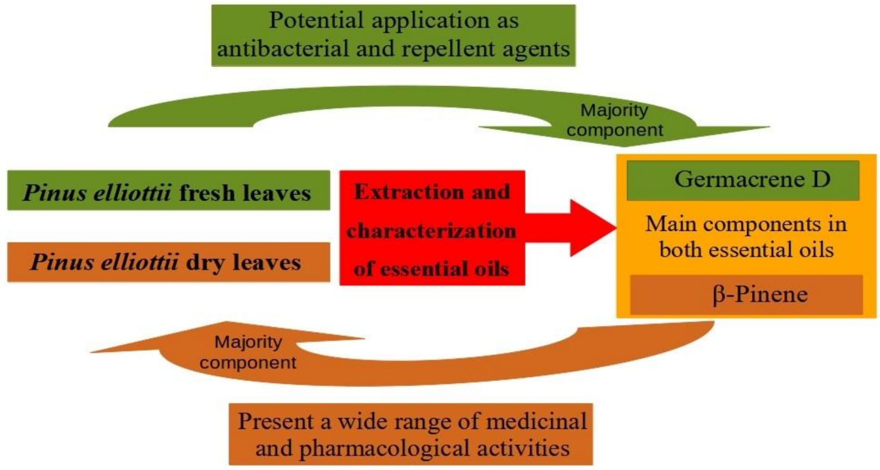
Abstract

Exploration of secondary metabolites, particularly essential oils, reveals diverse properties in antimicrobial, biological, and pharmaceutical contexts, including antibacterial, antifungal, and antiviral attributes, and applications in pest control and insect repellents. In the present work, essential oils were extracted from both fresh and dry leaves of *Pinus elliottii*, using hydrodistillation, followed by meticulous chemical characterization via gas chromatography coupled with a mass spectrometer. The plant leaves, sourced from a reforested area in the southern part of Minas Gerais, Brazil, formed the study's foundation. The main constituents identified in both essential oils were Germacrene D and β-Pinene. Germacrene D dominated in the essential oil from fresh foliage (47.71%), while β-Pinene prevailed in the essential oil from dry foliage (30.06%). Literature indicates that heightened Germacrene D levels may confer antibacterial and repellent properties, while elevated β-Pinene content aligns with various biological, medicinal, and pharmacological activities. Integrating our findings with existing literature, this work highlights potential applications for essential oils derived from both fresh and dry leaves of *Pinus elliottii*.



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5. β-Pinene.

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Highlights

- Germacrene D and β-Pinene are key compounds in *Pinus elliottii* essential oils.
- Fresh leaves of *Pinus elliottii* show high Germacrene D with antibacterial potential.
- Dry leaves of *Pinus elliottii* are rich in β-Pinene, linked to diverse bioactivities.

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1. Introduction

Essential oils have gained renewed significance across various domains. As natural products, they proudly exhibit attractive physicochemical characteristics coupled with environmentally friendly characteristics (El Asbahani *et al.*, 2015). These oils exhibit evident biological activities, finding application in the medical field for their fungicidal, virucidal, and bactericidal properties. Multiple studies underscore their antimicrobial efficacy, even against multi-resistant bacteria (Burt, 2004; Mayaud *et al.*, 2008). Furthermore, essential oils have been deployed in combating nosocomial infections. They are used as aerosols in operating blocks and waiting rooms to purify the air and minimize contamination (El Asbahani *et al.*, 2015). Additionally, these oils serve as cleaning agents for disinfecting medical equipment and surfaces (Warnke *et al.*, 2009). Beyond their medicinal applications, essential oils contribute to a sense of mental comfort for patients, thanks to their pleasant aroma. Notably, these oils have been explored for their potential as food preservatives as well (Burt, 2004; Tiwari *et al.*, 2009).

Due to their intricate chemical composition, which frequently comprises over 100 distinct terpenic compounds, essential oils exhibit an extensive spectrum of antimicrobial and biological activities. In the pharmaceutical field, the use of products with essential oils is constantly growing and usually is addressed mainly by local applications and inhalation. They are incorporated into the formulation of numerous pharmaceutical products, including capsules, ointments, creams, syrups, suppositories, aerosols, and sprays (El Asbahani *et al.*, 2015). In nature, essential oils are synthesized by over 17,500 aromatic plant species and are stored in various plant organs. These include flowers (e.g., *Citrus bergamia*, bergamot orange), leaves (e.g., *Cymbopogon citratus*, lemon grass), wood (e.g., *Santalum acuminatum*, sandalwood), roots (e.g., *Chrysopogon zizanioides*, vetiver), rhizomes (e.g., *Zingiber officinale*, ginger; *Curcuma longa*, turmeric), fruits (e.g., *Pimpinella anisum*, anise), and seeds (e.g., *Myristica fragrans*, nutmeg) (Baptista-Silva *et al.*, 2020). In special, the essential chemical composition of oil leaves (also called needles) for more than 40 pine species (genus *Pinus*) is known (Ioannou *et al.*, 2014). *Pinus* and *Eucalyptus* species are the most common trees used for reforestation.

In contemporary society, deforestation has become prevalent to meet the demand for various structural components in civil engineering, furniture, charcoal production, and other applications. To equilibrate this trend, plantation forestry is widely employed to expedite reforestation, mitigate greenhouse gas emissions, and alleviate the pressure on timber resources from native forests. Exotic tree species, particularly fast-growing and high-yielding ones like *Pinus* and *Eucalyptus*, are extensively cultivated in monocultures, often beyond their native habitats (Ning *et al.*, 2019). Pine, the largest genus in the *Pinaceae* family, encompasses over 110 species globally (Richardson *et al.*, 2007). Although primarily found in the Northern Hemisphere, this species adapts to various forest types, including temperate, sub-tropical, tropical, and boreal regions (Imanuddin *et al.*, 2020).

In the early 20th century, Brazil initiated its first economic reforestation efforts, primarily involving the introduction of *Eucalyptus* species. By the late 1940s and early 1950s, coniferous species, particularly those of the genus *Pinus*, were introduced. The introduction of conifers was largely driven by the substantial decline in the natural population of *Araucaria angustifolia* (Parana pine). Reforestation operations gained significant momentum in the 1960s, reaching full operational levels during that decade. Starting in 1966, government-led fiscal incentives led to a substantial increase in reforestation activities in Brazil. Reforestation projects covered approximately 6.2 million hectares, with 52 percent dedicated to Eucalyptus, 30 percent to Pinus, and 18 percent to other species (Jesus, 1990). In 2021, the reforestation area reached around 9.5 million hectares, with 7.3 million hectares of Eucalyptus and 1.8 million hectares of *Pinus*. 45.4% percent of *Eucalyptus* area is located in the Brazilian southeast region, while 83.9 percent of *Pinus* forest is established in the Brazilian south region (IBGE, 2021).

Given the abundant presence of *Pinus elliottii* in our region, this study aims to extract essential oils from both fresh and dried leaves of the mentioned *Pinus* species and analyze their chemical compositions. This characterization together with literature data give some support in order to clear up potential applications to the analyzed essential oils.

2. Experimental

2.1. Plant material

Gathering fresh and dried leaves of *Pinus elliottii* took place in a public area situated in *Bueno Brandão*, a city in the southern region of *Minas Gerais*, Brazil, at an elevation of 1,184 meters above sea level. The precise coordinates for the collection site were latitude 22°25'56.91"S and longitude 46°21'15.03"W. The collection occurred in the morning on September 6th, 2021, under mild temperature conditions with no precipitation. Fresh leaves were directly collected from the trees, while dry leaves were gathered from the ground. Post-collection, both fresh and dry leaves underwent careful selection based on imperfections, dirt, and foreign bodies. Subsequently, they were cut into smaller portions in preparation for the subsequent extraction of essential oils.

2.2. Extraction and yield of essential oils

The Organic Chemistry-Essential Oils Laboratory at the Chemistry Department of the Federal University of Lavras conducted the extraction of essential oils from both fresh and dry leaves of *Pinus elliottii*. The extraction process employed hydrodistillation and utilized a modified Clevenger apparatus connected to a 5 L flask, following the method outlined by Teixeira *et al.* (2014). Each batch of approximately 200 g of plant material underwent hydrodistillation for a duration of two hours. Post-extraction, the essential oils were separated from the hydrolytic solution through centrifugation, employing a benchtop centrifuge with a horizontal crosspiece (FANEM BabyRI Model 206 BL) at 965 g and room temperature for 15 minutes. Subsequently, the essential oils were carefully transferred to amber bottles using a Pasteur pipette and stored at a temperature of 5 °C. The yields of the essential oils were 0.82 ± 0.06% and 1.60 ± 0.08% on a fresh and on a dry-weight basis respectively for the species *Pinus elliottii*. The extractions were performed in triplicate.

2.3. Chemical characterization of the essential oils

The identification of the compounds in the essential oil was performed according to those described in the literature (Adams, 2007; NIST, 2010) by gas chromatography coupled to a mass spectrometer, employing a Shimadzu QP model 5050A GC/MS equipped with a J&W Scientific fused-silica capillary column (5% phenyl 95% dimethylpolysiloxane;

30 m × 0.25 mm id, 0.25 µm film). Helium served as the carrier gas with a flow rate of 1.0 mL min^{-1}. The sample injection volume was 0.5 µL, diluted in hexane (1%). The split ratio was 1:20. The column temperature program initiated at 60 °C for 1 minute, followed by a gradual increase to 246 °C at a rate of 3 °C min^{-1}. Subsequently, there was a further increase of 10 °C min^{-1} to 300 °C, where the temperature was maintained for 7 minutes. The injector and detector temperatures were set at 220 and 240 °C, respectively.

Quantitative analysis was conducted through gas chromatography with a flame ionization detector (GC-FID), utilizing a Shimadzu model GC–2010 chromatograph (Shimadzu Corporation, Kyoto, Japan). The experimental conditions mirrored those employed in qualitative analysis, except for the detector temperature set at 300 °C. Relative percentages of each constituent were determined using the normalization of area method. To establish the retention indices of the constituents, a comparison was made with those reported in the literature (Lunguinho *et al.*, 2021). These retention indices were calculated using the Van Den Dool and Dec Kratz (1963) relative to a homologous series of n-alkanes (C9-C18). For comparison of mass spectra, two libraries, NIST107 and NIST21, were employed. The chemical characterization was performed in triplicate.

3. Results and discussion

Table 1 presents the chemical composition average related to the triplicate chemical characterization and the standard deviation of essential oils derived from fresh and dry leaves of *Pinus elliottii*, highlighting the key components in each kind of essential oil. The chromatograms of the essential oils from fresh and dry leaves of *Pinus elliottii*, along with their indexed substances, are illustrated in **Figs. 1** and **2**, respectively. Only one chromatogram of each kind of essential oil is shown due to their similarity in the triplicate analyses.

Table 1. The essential oil constituents are derived from the fresh and dry leaves of *Pinus elliottii*.

N°. in the Chromatogram from Fig. 1	N°. in the Chromatogram from Fig. 2	RI$_{calc}$^{1}	RI$_{lit}$^{2}	Constituents	Percent of constituent in the fresh leaves	Percent of constituent in the dry leaves
1	1	933	932	α-Pinene	6 ± 1	10 ± 2
-	2	950	964	Camphene	-	0.63 ± 0.09
2	3	979	974	β-Pinene	22 ± 2	30 ± 2
3	-	988	988	Myrcene	1.8 ± 0.9	-
4	4	1196	1186	α-Terpineol	0.22 ± 0.09	2.8 ± 0.2
-	5	1280	1284	Bornyl acetate	-	2.0 ± 0.7
5	6	1420	1417	β-Caryophyllene	7 ± 2	5 ± 2
6	7	1491	1484	Germacrene D	48 ± 3	27 ± 3
7	8	1496	1500	Bicyclogermacrene	2.2 ± 0.8	2.4 ± 0.8
8	9	1493	1500	α-Muurolene	0.47 ± 0.07	0.7 ± 0.1
9	10	1503	1513	γ-Cadinene	2.0 ± 0.7	3.2 ± 0.9
10	11	1518	1522	δ-Cadinene	8 ± 1	9 ± 1
-	12	1577	1577	Spathulenol	-	3 ± 1
11	13	1638	1640	Epi-α-Muurolol	2.8 ± 0.7	4 ± 1
		Total			100.49	99.73

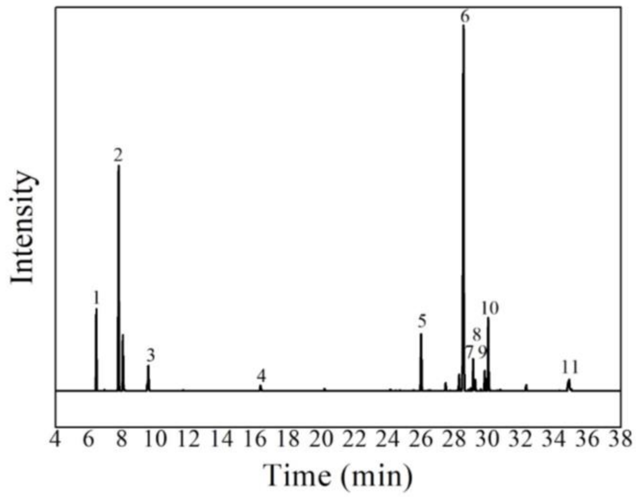

Figure 1. Chromatogram of the essential oil of the fresh leaves of *Pinus elliottii*.

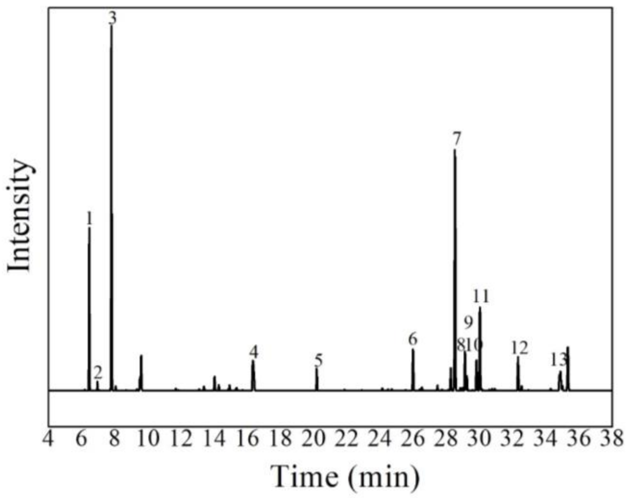

Figure 2. Chromatogram of the essential oil of the dry leaves of *Pinus elliottii*.

The data presented in **Table 1** and **Fig. 1** reveal that Germacrene D (47.71%) predominated as the primary component in the essential oil extracted from fresh *Pinus elliottii* foliage, followed by β-Pinene (21.80%), δ-Cadinene (8.09%), (E)-Caryophyllene (6.86%), and α-Pinene (6.01%). Conversely, in the essential oil derived from dry foliage, the key constituents were β-Pinene (30.06%), Germacrene D (26.77%), α-Pinene (10.26%), δ-Cadinene (9.32%), and (E)-Caryophyllene (5.03%) (**Fig. 3**). Limited literature data exists on the chemical composition of *Pinus elliottii* leaf oil. Ioannou *et al.* (2014) provided the initial report on this type of essential oil. According to their findings, the essential oil from fresh *Pinus elliottii* foliage contained four compounds, with Germacrene D (24.5%), β-Pinene (12.9%), α-Pinene (10.6%), and β-Caryophyllene (6.6%) identified as the main constituents. Notably, there is currently no available literature data on the chemical composition of the essential oil from dry foliage of the analyzed plant.

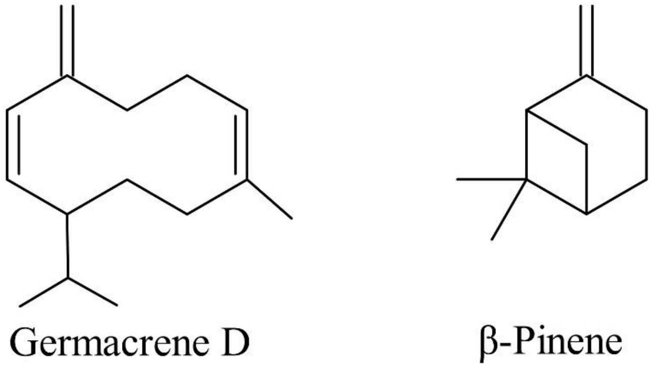

Figure 3. The majority of constituents of essential oils are from fresh and dry leaves of *Pinus elliottii.*

Some discrepancies were evidenced between Ioannou *et al.* (2014) results and those found in the present work, mainly related to the compound amounts in the analyzed fresh foliage essential oil. The variations in the chemical compositions of essential oils derived from plants of identical species can be elucidated through various factors such as geographical location and time of harvest, the nature of the soil and its nutrients. This is because the secondary metabolites are prone to alterations depending on the season during which the collection takes place. Furthermore, factors such as the plant's age, seasonal patterns, temperature, and others exert an influence on virtually all categories of secondary metabolites, including essential oils. Monoterpenes (whether oxygenated or not) and sesquiterpenes (whether oxygenated or not) were found to exhibit a higher concentration in the essential oils (Gobbo-Neto and Lopes, 2007). The discussion below is concerned to the majority of substances found in each analyzed essential oil.

The primary component discovered in the essential oil derived from the fresh leaves of *Pinus elliottii* is Germacrene D, constituting 47.71% of the composition. In the essential oil extracted from dry leaves, Germacrene D is the second most prevalent substance, constituting 26.77% of the composition. The Germacrene D percentage decrease in the essential oil from dry leaves may be explained by the following reasons: Germacrene D, like many other compounds in essential oils, is volatile. During the extraction process, especially if heat is involved, some of the volatile compounds can evaporate or degrade, leading to a decrease in their concentration. Some compounds may undergo chemical reactions during the extraction process, leading to the formation of different compounds or degradation products. This could result in a decrease in the percentage of Germacrene D in the extracted oil derived from the dry leaves compared to the essential oil extracted from fresh leaves. In addition, different compounds have different solubilities in the extraction solvent. The extraction process may selectively extract certain compounds more efficiently than others, leading to a change in the relative concentrations of compounds in the extracted oil compared to the fresh plant material (Chakravarty *et al.*, 2023; Kurti *et al.*, 2019).

Germacrene D falls under the category of sesquiterpenoids or sesquiterpenes, characterized by a group of 15-carbon compounds formed from three isoprene units. These sesquiterpenoids are predominantly found in higher plants and manifest in various acyclic, mono-, bi-, tri-, and tetracyclic systems (Modzelewska *et al.*, 2005). They exist in nature as hydrocarbons or in oxygenated forms like lactones, alcohols, acids, aldehydes, and ketones. Sesquiterpenes present in essential oils and aromatic elements of plants exhibit diverse fundamental structures with distinct nomenclatures (Awouafack *et al.*, 2013). Many of these sesquiterpenes showcase biological activities, including antimicrobial, antitumor, and cytotoxic properties. Within plants, they play crucial ecological roles in interactions with insects and microbes, functioning as attractants, deterrents, antifeedants, and phytoalexins (Modzelewska *et al.*, 2005).

Numerous investigations have delved into the antibacterial and repellent potentials of plant essential oils abundant in Germacrene D, aiming to combat microbial resistance to antibiotics and traditional drugs. The objective is to circumvent the use of expensive synthetic crop protection chemicals by promoting the commercialization of insecticides based on these essential oils (El Mokni *et al.*, 2019). The essential oil, enriched with a significant amount of the aforementioned sesquiterpene, demonstrates moderate antimicrobial activity, particularly against Gram-positive bacteria such as *S. aureus*, *B. subtilis*, and *C. albicans*. In contrast, Gram-negative bacteria like *E. coli*, *P. aeruginosa*, and *S. enterica* inherently exhibit resistance to the antimicrobial effects of essential oils. The efficacy of this type of essential oil is more pronounced against Gram-positive bacteria, attributed to the inherent characteristics of the bacterial cell membrane (Kilani *et al.*, 2005). The limited antibacterial effectiveness against Gram-negative bacteria is associated with the presence of a hydrophilic outer membrane, hindering the penetration of hydrophobic compounds into the target cell membrane (Inouye *et al.*, 2001). In contrast, Gram-positive bacteria boast proteins, mucopolysaccharides, and a lower quantity of phospholipids in their cell membrane. This composition facilitates the permeability, entry, and reaction of most antibiotics and/or antimicrobial agents through the cell envelope (Al-Saimary *et al.*, 2006).

In addition, the insecticidal potential of some essential oils is also related to the presence of high amounts of terpenes, such as Germacrene D and its volatility acts as chemical messengers for insects (Al-Ghanim *et al.*, 2023; Chaieb *et al.*, 2018). Earlier research has also suggested that Germacrene D exhibits impact on herbivores and insecticidal properties, notably against mosquitoes. Additionally, it demonstrates repellent activity against aphids, as highlighted in prior studies by Noge *et al.* (2009). The biological effects of numerous essential oils are linked to their primary biologically active components, thus from our results, the fresh foliage oil from *Pinus elliottii* is a natural source of Germacrene D, a sesquiterpene with high application potential due to its biological activities as aforementioned, such as antimicrobial and insecticidal potentials. Additional biological effects of essential oils abundant in sesquiterpenes are also delineated, encompassing activities like an anti-arthritic, anti-inflammatory, antiviral, antimutagenic, local anesthetic, and anticarcinogenic properties (Oliveira-Tintino *et al.*, 2018). It is crucial to acknowledge that essential oils constitute phytocomplexes, emphasizing the significance of interactions between both minor and major constituents. Each individual compound operates synergistically with others, a fact that shouldn't be overlooked (El Mokni *et al.*, 2019).

β-pinene stands out as the predominant component identified in the essential oil extracted from dry leaves, constituting 30.06% as depicted in **Table 1** and **Fig. 2**. It also holds the position of the second most abundant substance in the essential oil derived from fresh leaves, accounting for 21.80%, as illustrated in **Table 1** and **Fig. 1**, of *Pinus elliottii*. Pinene ($C_{10}H_{16}$) is characterized as a bicyclic, double-bonded terpenoid hydrocarbon, as outlined by Winnacker (2018). α- and β-pinene represent a pair of isomers present in the coniferous trees (pines) essential oils and they belong to the vast family of monoterpenes (Salehi *et al.*, 2019). α-pinene can be detected in more than 40 different essential oils (Vespermann *et al.*, 2017) and β-pinene is commercially acquired through distillation or the conversion of α-pinene (Salehi *et al.*, 2019). Both α- and β-pinene showcase a range of biological activities, some like the biological effects of Germacrene D. These shared activities include fungicidal, antiviral, and antimicrobial properties. Moreover, pinenes find applications in the production of components contributing to aroma, flavor, and fragrance, as discussed in studies by Silva *et al.* (2012), Salehi *et al.* (2019), Van Der Werf *et al.* (1997) and Arya *et al.* (2022). α- and β-pinenes are integral elements in renal and hepatic medications. Their utilization as antibacterials stems from their detrimental impact on membranes (Alma *et al.*, 2004). Additionally, research has uncovered their inhibitory effects on breast cancer and leukemia (Zhou *et al.*, 2004). Some polymers can be synthesized from pinenes (Thomsett *et al.*, 2019) and these materials can present better quality than other conventional polymers (Satoh *et al.*, 2014).

Still related to pinenes, numerous pharmacological effects have been documented, spanning antibiotic resistance modulation, anticoagulant properties, antitumor activity, antimicrobial action, antimalarial effects, antioxidant capabilities, anti-inflammatory responses, anti-Leishmania effects, and analgesic effects. Other prominent effects are also reported, such as cytogenetic, gastroprotective, anxiolytic, cytoprotective, anticonvulsant, and neuroprotective properties have been observed. Additionally, these substances exhibit efficacy against oxidative stress induced by H_2O_2, pancreatitis, hyperthermia triggered by stress, and pulpal pain. Although pinenes showcase various biological activities, their swift metabolism and elimination from the body, attributed to their volatile nature, result in their brief presence at low concentrations within organisms. Despite the acknowledged positive properties of α-pinene and β-pinene, the bioavailability of these terpenes in the human body remains understudied in most investigations. While some in vivo and clinical studies have linked the biological effects of pinenes, additional endeavors are essential to deepening our understanding in this domain, as emphasized by Salehi *et al.* (2019).

Therefore, from our results, the essential oils from fresh and dry foliage from *Pinus elliottii* are natural sources of Germacrene D and β-Pinene. According to the literature data, as mentioned before, Germacrene D has more potential in applications such as antimicrobial and insecticidal activities, while β-Pinene has more potential in applications related to pharmacological activities, although some biological activities are coincident for both substances, such as antimicrobial and antitumor properties. Our results also point that essential oil from fresh leaves of *Pinus elliottii* may be applied mainly as antimicrobial and insecticide, due its majority component Germacrene D, while the essential oil from dry leaves may be applied mainly in a wide range of pharmacological activities due to its majority component β-pinene. However further studies are needed to support these hypotheses. In addition, many other *Pinus elliottii* essential oils' possibilities of application can be investigated, due to the chemical composition complexity of each essential oil, once there are chemical interactions between minor and major constituents, as well as, each compound works together synergistically with the others, creating a combined effect that enhances their overall impact.

4. Conclusions

The findings show that the majority of compounds from fresh foliage essential oil are Germacrene D (47.71%), followed by β-Pinene (21.80%), in addition to minor compounds, such as δ-Cadinene (8.09%), (E)-Caryophyllene (6.86%) and α-Pinene (6.01%). The results also show that the majority of compounds from dry foliage essential oil are β-Pinene (30.06%), followed by Germacrene D (26.77%), in addition to minor compounds, such as α-Pinene (10.26%), δ-Cadinene (9.32%) and (E)-Caryophyllene (5.03%).

Due to the essential oils' chemical compositions found, the present investigation opens the possibility of finding potential applications to essential oils extracted from the fresh and dry leaves of *Pinus elliottii*. The literature points to Germacrene D with excellent antimicrobial and insecticidal properties and β-Pinene with several pharmacological activities. Many other possibilities of application also can be explored because the characterized compounds can interact with each other.

Authors' contributions

Conceptualization: Leonardo Pratavieira Deo; Kassy Jhones Garcia; Maria das Graças Cardoso; **Data curation**: Kassy Jhones Garcia; Maria das Graças Cardoso; Gabriela Aguiar Campolina; Cassia Duarte Oliveira; **Formal Analysis**: Maria das Graças Cardoso; Gabriela Aguiar Campolina; Cassia Duarte Oliveira; **Funding acquisition**: Maria das Graças Cardoso; **Investigation**: Kassy Jhones Garcia; Maria das Graças Cardoso; Gabriela Aguiar Campolina; Cassia Duarte Oliveira; **Methodology**: Kassy Jhones Garcia; Maria das Graças Cardoso; Gabriela Aguiar Campolina; Cassia Duarte Oliveira; **Project administration**: Leonardo Pratavieira Deo; Maria das Graças Cardoso; **Resources**: Maria das Graças Cardoso; **Software**: Maria das Graças Cardoso; **Supervision**: Leonardo Pratavieira Deo; Maria das Graças Cardoso; **Validation**: Maria das Graças Cardoso; Gabriela Aguiar Campolina; Cassia Duarte Oliveira; **Visualization**: Leonardo Pratavieira Deo; Maria das Graças Cardoso; **Writing – original draft**: Leonardo Pratavieira Deo; **Writing – review & editing**: Maria das Graças Cardoso; Gabriela Aguiar Campolina; Cassia Duarte Oliveira;

Data availability statement

All data sets were generated or analyzed in the current study.

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