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REVIEW

Thymus × citriodorus: an Emerging Aromatic and Medicinal Hybrid Plant with Relevant Bioactive Potential

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Abstract

Thymus × citriodorus (Pers.) Schreb., Lamiaceae, is an emerging aromatic and medicinal hybrid plant with a lemon-like aroma and a mild and sweet-floral odor, distinct from other species from the *Thymus* genus, a characteristic that makes it appealing for food-related industries. Still, its different chemical profile also makes it particularly interesting for industries in other sectors. This review aimed to clarify the value of *Thymus × citriodorus* for health or well-being applications, by systematizing information on its different bioactive properties, which are relevant for health-related applications. Information on the chemical composition of its volatile oil and different extracts and the factors that contributed to the variation of their major components were also addressed. Geraniol was reported as the major compound in volatile oils from this plant, with relative abundances varying widely. Extracts (water or hydroalcoholic) were chiefly characterized by the presence of flavonoids, phenolic acids, and triterpenic acids. A wide variety of biologically significant activities were reported for the volatile oil and extracts of *Thymus × citriodorus*. This included antioxidant, antiproliferative, anti-inflammatory, and antimicrobial activities and cytoprotective effects on hepatic cells. Still, few studies address the hazardous/toxic effects on non-target organisms. Based on the reported bioactivities, the interest in *Thymus × citriodorus* goes beyond food-related applications. Further studies are still necessary to characterize this emerging hybrid plant for efficacy and safety of its preparations, thus promoting its value as raw material for industries in other fields, such as the cosmetic, perfumery, and pharmaceutical ones.

Keywords Essential Oils · Phytochemistry · Phytotherapy · Plant Extracts · Thyme

Introduction

The use of aromatic and medicinal plants goes back to ancient times. In modern days, their use is widely spread among different fields, from food industries to medicinal applications, the last typically associated with traditional or folk medicine (Pinto et al. 2006; Kodanovi et al. 2020).

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Aromatic and medicinal plants are usually valorized due to their high volatile oil (VO) content, a product with major and distinctive applications (Edris 2007; Bakkali et al. 2008; Bayala et al. 2014).

Thymus L., Lamiaceae, comprises a high number of different species of perennial, aromatic, and medicinal plants and subshrubs distributed across North Africa, Asia, and Europe, particularly in the Mediterranean region (Li et al. 2019; el Yaagoubi et al. 2021; POWO 2023). Species of this genus are typically demanded for their VOs (and/or their main chemical components) by the pharmaceutical, perfumery, and cosmetic industries (Stahl-Biskup and Holthuijzen 1995; Jurevičiūtė et al. 2019; Oliveira et al. 2023). They are also popular among food industries, acting as food flavors or spices and natural antioxidants (Lorenzo et al. 2019). Some *Thymus* species are also recognized as sources of herbal medicinal ingredients, and their use is harmonized by the European Union (EU) through an EU monograph issued by the European Medicines Agency (EMA) while being the object of attentive reports of the European Scientific

Cooperative on Phytotherapy and World Health Organization (WHO 1999; ESCOP 2003; EMA 2007). The demand for these species is so high that the ingathering of plants of this genus from their natural habitats is far from filling their high commercial request, leading to an increase in cultivation (Ložienė et al. 2021). *Thymus* species have been widely studied as promoters of several health benefits and their anti-inflammatory, antiproliferative, antimicrobial, and antioxidant potentials have been reported and reviewed.

Among the several species that comprise the *Thymus* genus, one in particular has gained interest and increased its value among aromatic and medicinal plant producers in the last years, namely *Thymus* × *citriodorus* (Pers.) Schreb. or lemon-thyme (Fig. 1) (Lundgren and Stenhagen 1982; Rita et al. 2018; Ntalli et al. 2020a; Taghouti et al. 2020; Lilia et al. 2022).

Thymus × *citriodorus* (Pers.) Schreb. is characterized by the presence of a pleasant lemon-like aroma and good flavor, making it particularly attractive for food-related industries, as lemon-scented plants are universally perceived as having a very pleasing fragrance and stimulating aroma (Lundgren and Stenhagen 1982; Stahl-Biskup and Holthuijzen 1995; Ložienė et al. 2021).

Apart from its potential to be applied in different industries, this hybrid has also caught the attention of the scientific community, specifically regarding its distinct chemical profile, with several studies being published addressing the composition of its VO (Toncer et al. 2017; Aprotosoaie et al. 2019; Malankina et al. 2019; Jurevičiūtė et al. 2019; Pasławska et al. 2020; Ložienė et al. 2021).

Differently from the most market-valued thyme VOs, typically rich in thymol and/or carvacrol, geraniol is the most relevant chemical component of *T. citriodorus* VO, decisively defining its mild and sweet floral odor, quite different from those of the phenolic types (Mewes et al. 2008; Teixeira and Rodrigues 2014; Li et al. 2019). Geraniol is a

Fig. 1 *Thymus* x *citriodorus* (Pers.) Schreb. or lemon-thyme (source: Ervitas Catitas®, Orada, Portugal)

commercially important open-chain monoterpene alcohol used as a fragrance ingredient in several cosmetic products, scenting a characteristic rose-lemon-like odor. This compound has also been reported as a modulator of signaling pathways associated with inflammation and as active against several pathogenic microorganisms (Su et al. 2010; De Cássia Da Silveira E Sá et al. 2013; Guimarães et al. 2019; Ložienė et al. 2021).

Despite the recognized and increasing interest for *T. citriodorus*, no systematized information is yet available about the bioactivity of its extracts and preparations or about their chemical composition.

Consequently, this review aimed to clarify about the interest of *T. citriodorus* for health or well-being applications, by systematizing information on the bioactive properties that are relevant for these specific purposes, ranging from antimicrobial, antioxidant, anti-inflammatory, antiproliferative to cytoprotective properties. Environmental-related activities such as nematicidal properties, biofertilizer potential, and also information about the environmental toxicity of its preparations are also addressed. We also intended to elucidate about the distinct chemical composition of the VO and different extracts from this hybrid, which differentiates it from other species of the *Thymus* genus, and on the factors that contribute to the variation on their composition, extremely relevant from the application perspective.

Search Strategy

The data search was performed on PubMed, Web of Science, and Scopus from their inception to March 2023. No language restrictions were applied. The search was performed using the terms "*Thymus citriodorus*" or "*Thymus* × *citriodorus*" or "Lemon thyme." From database search, a total of 114 articles were retrieved, specifically 17 from PubMed, 44 from Web of Knowledge, and 53 from Scopus. Additionally, the bibliography from retrieved studies was scrutinized for additional relevant publications. After duplicates removal, a total of 59 studies were left for analysis. Articles were considered relevant to this review, and thus included, if they addressed botanical aspects, traditional uses, phytochemistry, or biological relevant activities. Articles that only address *T. citriodorus* when present in herbal mixtures or blends were not considered for the purpose of this review.

The selected publications from the literature search were analyzed with the VOSviewer software tool for constructing and visualizing bibliometric networks, version 1.6.19, and a network map was constructed (Fig. 2) (van Eck and Waltman 2010). Relevant terms in the titles and abstracts of the selected studies were included, considering terms that appeared in 3 or more papers. Using the VOSviewer analyzing techniques, the 59 most relevant terms were

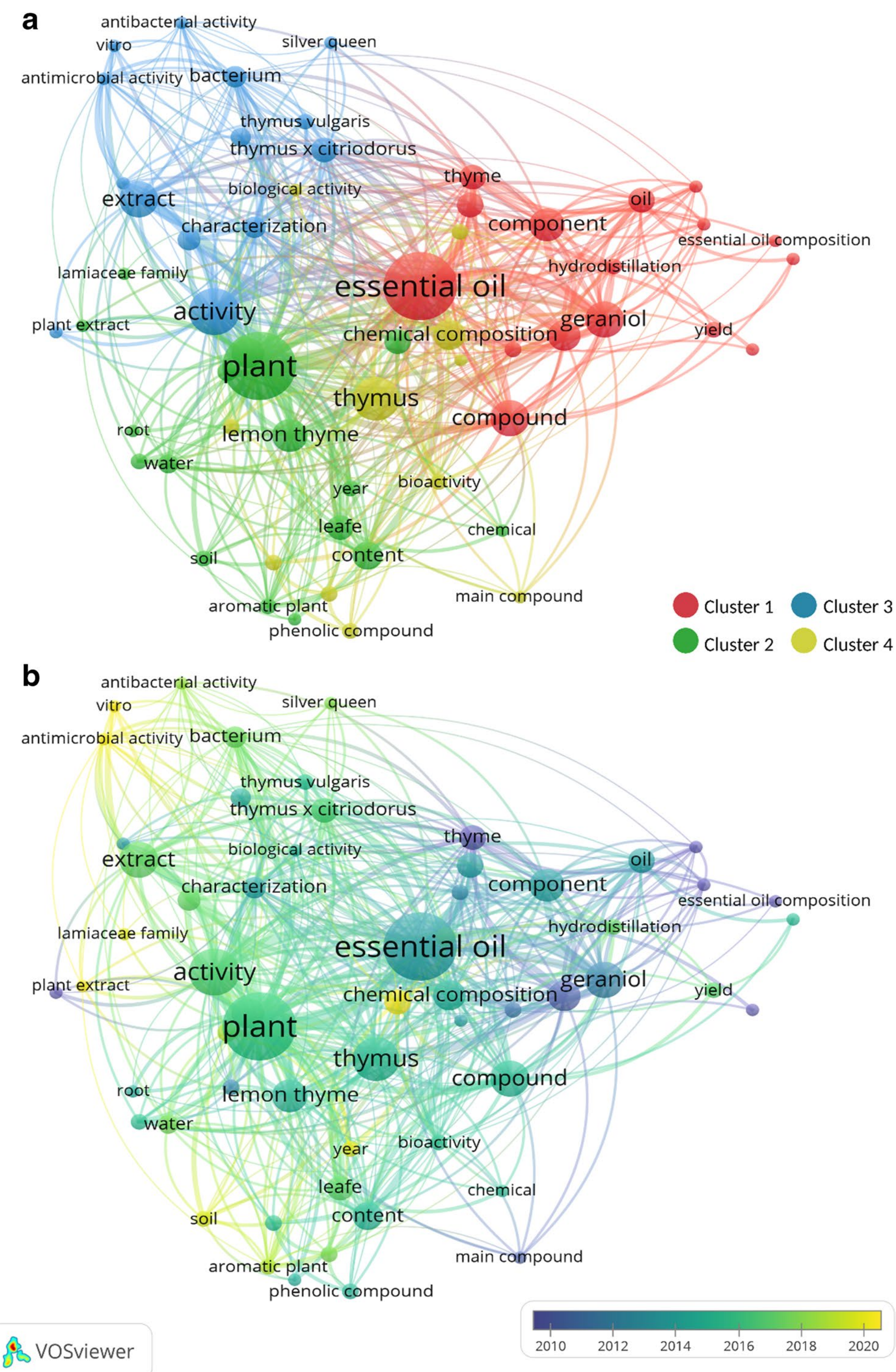


Fig. 2 Network maps of the included studies from the literature search on *Thymus × citriodorus* analyzed by VOSviewer version 1.6.19. **a** Network map showing the co-occurrence of the 59 most relevant terms in the titles and abstracts grouped in four different clusters referring to chemical composition of VOs (cluster 1), *T. citriodorus* growth conditions and production aspects (cluster 2), study of *in vitro* bioactive properties (cluster 3) and other extracts, apart from VO, and their biological activities (cluster 4). The size of the circles represents the frequency of each term and the lines between the circles represent the publication links between two terms. **b** The same network map showing of the trends in annual publications with specific terms from early publications (blue) to more recent publications (yellow)

algorithmically grouped by the software in four clusters (Fig. 2a). Cluster 1 (red) consisted of 17 terms related with *T. citriodorus* VO yield and composition, focusing on its main chemical compounds. Cluster 2 (green) is composed of 17 terms, referring to *T. citriodorus* growth conditions and production factors influencing its chemical content. In cluster 3 (blue), the 14 terms are related to bioactivity studies with most terms referring to antimicrobial or antioxidant activities. Finally, cluster 4 (yellow) is a smaller cluster composed of 11 terms that are related to other extracts of *T. citriodorus* and their chemical composition and biological activities. The lines between 2 terms represent the linkage of these terms in the publications. The same network was also generated but considering year of publication of the included studies, from early publications in blue, to more recent publications in yellow (Fig. 2b). This network shows a trend shift from studies addressing only the chemical composition of *T. citriodorus* VOs, to studies focusing on *T. citriodorus* production factors and on the relevant biological activities of *T. citriodorus* preparations.

Discussion

Botanical Aspects

Thymus × citriodorus (Pers.) Schreb., Lamiaceae is a perennial, evergreen, pubescent, erect shrub up to 30 cm tall with a characteristic lemon scent. The leaves are narrow, oval to lanceolate, and typically green, although some patterns of yellow and white can appear in some cultivars. The flowers are white or slightly pink and the flowering period occurs during summer months. *Thymus × citriodorus* grows in well drained soils, preferably calcareous and with plenty of sun. Propagation by herbaceous stem cuttings is preferred to propagation by seed, minimizing heterogeneous characteristics associated with its hybrid status (Tavares et al. 2010; Mourão 2012).

Thymus × citriodorus is considered a hybrid between *T. pulegioides* L., geraniol chemotype, and *T. vulgaris* L., although some controversy is present regarding its characterization (Stahl-Biskup and Holthuijzen 1995). Margaret Easter (2021), in her article in *Plant Heritage*, referred to *T. citriodorus* as a species, not as a hybrid. *Thymus × citriodorus* was first described as a species in 1811, but later in the 1970s it was regarded as a hybrid of European garden origin, between *T. pulegioides* and *T. vulgaris*, and consequently perpetuated by the nursery trade and became gradually accepted as such (original reference not available—accessed in (Easter), on March 2023). In the same article, the author discussed about a previous study conducted by herself and colleagues, where Random Amplified Polymorphic DNA analysis was carried out to identify genetic relationships between *Thymus* plants. The study showed that *T. citriodorus* was not included in the cluster formed by the species *T. vulgaris* and *T. pulegioides*, its supposed hybrid parents. Based on these findings, the author proposed that it would be acceptable to revert *T. citriodorus* to its original species status. Still, in a different study conducted by Kerem et al. (2023), the authors analyzed different *Thymus* species using molecular characterization with inter-simple sequence repeat markers and clustered together *T. vulgaris* and *T. citriodorus*. The hybrid status is also repeatedly presented by the scientific community, accepted by the nursery trade, and by the Royal Botanic Kew Gardens, London UK, that accepts and adopts the hybrid status (POWO 2023).

There is also no unanimity regarding its origin, as some authors refer that *T. citriodorus* has no natural distribution, while others, along with the Royal Botanic Kew Gardens, refer to it as native to Southern Europe and Vaičiulytė widely cultivated in the Mediterranean region (Omidbaigi et al. 2005, 2009; Bagdat et al. 2011; Duman and Özcan 2017; Toncer et al. 2017; POWO 2023). Several varieties (or cultivars) of this hybrid are also mentioned in the literature, although most of the conducted scientific studies refers only to *T. citriodorus* (Trindade et al. 2018). These different cultivars are distinguishable by the appearance and color of the leaves and include varieties such as *T. citriodorus* "aureus," *T. citriodorus* "archer's gold," *T. citriodorus* "golden dwarf," and *T. citriodorus* "silver queen" (Horváth et al. 2006; Aprotosoaie et al. 2019; Steshenko et al. 2021; Steshenko and Mazulin 2022).

Traditional Uses

Overall, several species of the genus *Thymus* are associated with a vast list of pharmacological effects, and thyme-derived products (herbal substances and preparations) are applied in traditional medicine for several conditions (Horváth et al. 2004, 2006; Pina-Vaz et al. 2004; Hajimehdipoor et al. 2009; Park 2011).

In the specific case of *T. citriodorus*, its major application is for culinary purposes due to its pleasant lemon flavor (Garibaldi et al. 2007; Ntalli et al. 2020a). *Thymus × citriodorus* is used as an ingredient for confection of several recipes of starters, snacks, sauces, and different meat and vegetarian dishes and soups and is also consumed in salads as well as in marinades. It is also used in jellies and desserts (Li et al. 2019). Besides its direct raw culinary use, *T. citriodorus* is also vastly consumed in the form of infusion or tisane (Omidbaigi et al. 2009; Pereira et al. 2013b). In fact, due to its high consumption and interesting profile as a source of bioactive compounds with potential health effects, it has been recently studied as a potential functional food, specifically as a nutraceutical (Taghouti et al. 2020). The by-products that remain after harvesting for culinary purposes, freely available as waste, are also used for animal feed, contributing to a circular economy (Ntalli et al. 2020a).

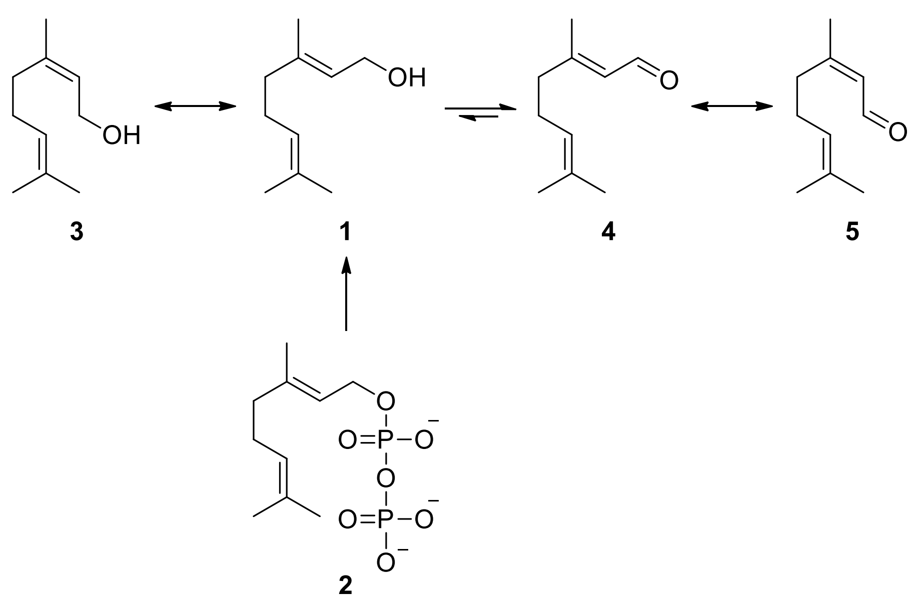
Apart from its value in the culinary field, some medicinal uses are also described for this plant, despite there are no ethnopharmacological studies available. Most of its described medicinal/pharmacological uses are for respiratory tract conditions. Its VO is used in aromatherapy for symptomatic relief of asthma and of other respiratory disorders, especially in children, as it is considered better tolerated than other *Thymus* VOs (Horváth et al. 2006; Pereira et al. 2013a). Tinctures prepared from this species are used for alleviating respiratory symptoms in cases of pneumonia and bronchitis (Steshenko and Mazulin 2022). The dried leaves of the plant prepared in potpourri and herbal-filled pillows are also used for symptomatic relief of respiratory disorders, as bronchitis (Horváth et al. 2006; Omidbaigi et al. 2009; Rita et al. 2018). In addition to the traditional uses for respiratory tract affections, compresses and baths prepared from the stems and leaves of the plant are used as analgesic, to reduce pain in arthritis (Steshenko and Mazulin 2022). Its VO is also used due to its antiseptic and deodorant properties (Pereira et al. 2013a). Other preparations of this plant, such as hydrolates (by-products of VO production), have also been valorized, and their properties as regulators of oily skin and hair, anti-dandruff agents, and adjuvants in the treatment of pediculosis and as hair tonics are often advertised by sellers of these products (Oliveira et al. 2022).

Chemical Composition

Volatile Oil

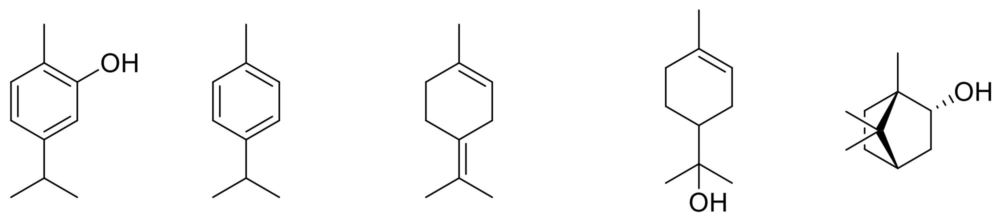
Since some of the beneficial activities associated with *T. citriodorus* are presumably related to its volatile metabolites, the composition of the VO obtained from this plant has been the focus of many investigations (Toncer et al. 2017; Aprotosoaie et al. 2019; Malankina et al. 2019; Jurevičiūtė et al. 2019; Pasławska et al. 2020; Ložienė et al. 2021; Oliveira et al. 2022). Table S1 abridges the source, processing features, and chemical characterization of the *T. citriodorus* VOs analyzed by gas chromatography–mass spectrometry (GC–MS), along with information regarding the major compounds in each VO and their relative abundance. In most studies, the VO was obtained from hydrodistillation of air-dried plant material using a Clevenger-type apparatus. Hydrodistillation duration varied across studies but was majorly between 2 and 4 h. The use of steam distillation was also described in some studies (Horváth et al. 2004, 2006; Sacchetti et al. 2005; Wu et al. 2013; Checcucci et al. 2017; Oliveira et al. 2022).

The identification and quantitative analysis of VO components was typically performed by GC–MS. Most of the studies reported compositions characterized by high percentages of oxygen-containing monoterpenes followed by moderate percentages of monoterpene hydrocarbons. The major compound usually reported in *T. citriodorus* VOs was geraniol (**1**), an open-chain monoterpenic alcohol derived from geranyl pyrophosphate (**2**) (Stahl-Biskup and Holthuijzen 1995; Horváth et al. 2004, 2006; Omidbaigi et al. 2005, 2009; Bertoli et al. 2010; Keyhani and Keyhani 2010; De Lisi et al. 2011; Teixeira and Rodrigues 2014; Toncer et al. 2017; Malankina et al. 2019; Jurevičiūtė et al. 2019; Ntalli et al. 2020a; Ložienė et al. 2021; Vaičiulytė et al. 2022; Oliveira et al. 2022). Relative amounts of this component ranged widely across different studies varying from 4.3 to 83.3% (Tátrai et al. 2016; Malankina et al. 2019). Details of how intrinsic or extrinsic factors influenced the major components of *T. citriodorus* will be addressed in detail in the section "External Influences on the Chemical Variation of *Thymus* × *citriodorus* VO and Extracts." Other relevant components described in *T. citriodorus* VO plants were nerol (**3**)—the *cis*-isomer of geraniol—and the structurally and biogenetically related aldehydes, geranial (**4**) and neral (**5**), together known as citral (Chen and Viljoen 2010). These two aldehydes are responsible for the lemon-like odor and flavor as studies show that citral flavor is much more associated to lemon by consumers than even *d*-limonene, despite the last being the primary component of lemon (Hirai et al. 2022). Altogether, these oxygen-containing monoterpenes assign to *T. citriodorus* its characteristic citrus-sweet rose flavor and aroma (Pasławska et al. 2020).

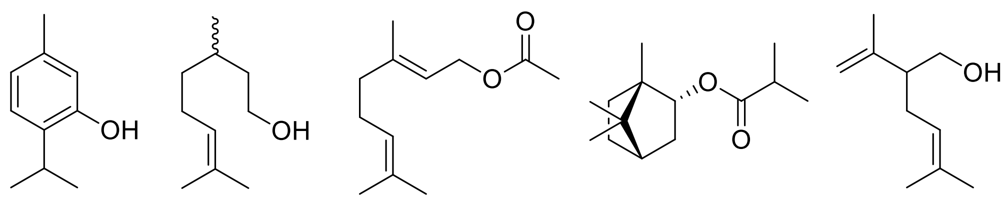


In additon to the most common geraniol chemotype, until 2018, other three were proposed for this hybrid, specifically, the carvacrol, borneol, and terpinolene chemotypes (Trindade et al. 2018). Horváth et al. (2006) reported carvacrol (**6**) and *p*-cymene (**7**) in considerable relative amounts (43.5% for carvacrol; 21.1% for *p*-cymene) in a *T. citriodorus* VO obtained from plants cultivated in Hungary, being carvacrol (**6**) the major component. This VO was obtained from the "archer's gold" cultivar. Terpinolene (**8**) and α-terpineol (**9**) were reported as the major compounds in a VO from *T. citriodorus* cultivated in south-eastern Anatolia, Turkey (Toncer et al. 2017). Also, Wu et al. (2013) reported borneol (**10**) and thymol (**11**) (28.82 and 14.43%, respectively) as the major compounds in a *T. citriodorus* VO. There was also a report of citronellol (40.1%) (**12**), followed by geranyl acetate (27.5%) (**13**) as the major compounds of an VO distilled from fresh plants grown in Italy (Checcucci et al. 2017).

Recently, other major compounds were reported in relative amounts that could account for new pure chemotypes, where the dominant component represented over 50% of the total composition. Malankina et al. (2019) reported thymol (**11**) (53.46%), followed by isobornyl propionate (**14**) (12.92%), as the main components of VO from *T. citriodorus* var. *variegata*, a hybrid with different parental forms. Also in 2019, Aprotosoaie et al. (2019) reported (*R*)-(-)-lavandulol (**15**) (54.27%) as the major constituent in a Moldavian *T. citriodorus* VO.



6 **7** **8** **9** **10**

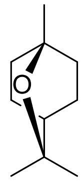


11 **12** **13** **14** **15**

Extracts

Other extracts have also been studied for their chemical composition, specifically regarding phenolic compounds (Park 2011; Pereira et al. 2013a, b; Villanueva Bermejo et al. 2015; Rita et al. 2018; Taghouti et al. 2020). Table S2 compiles information of the source, processing, and extraction features (when disclosed in the original paper) and chemical characterization of *T. citriodorus* extracts. From these, the majority consisted of aqueous extracts, prepared either as an infusion or as a decoction (Rita et al. 2018; Ntalli et al. 2020a, b; Taghouti et al. 2020). Ethanol extracts and methanol extracts were also prepared and studied by several authors (Pereira et al. 2013a, b; Kolniak-Ostek and Oszmiański 2015; Raudone et al. 2017; Abramovič et al. 2018; Taghouti et al. 2020; Steshenko and Mazulin 2022).

The composition of two hydrolates, by-products of VO production, was also reported (Ntalli et al. 2020a; Oliveira et al. 2022). Hydrolates, also known as hydrosols or aromatic waters, are obtained from the condensation of the steam used in the distillation to obtain the VO and consist of very diluted aqueous solutions of the most hydrophilic volatile components isolated during the distillation process. Ntalli et al. (2020a) reported geraniol (**1**) as the major component of *T. citriodorus* hydrosol, with a relative amount of 44.06%, corresponding to the percentage present in the volatile part of the preparation. Oliveira et al. (2022) reported 1,8-cineole (**16**), linalool (**17**), geraniol (**1**), and α-terpineol (**9**) as the major components in *T. citriodorus* hydrolate with relative amounts of 0.0145, 0.0134, 0.0077, and 0.0062% respectively, in the whole hydrolate, and of 26.3, 24.3, 13.9, and 11.2%, when considering the volatile part of the preparation. Attention must be drawn to the results from the chemical analysis of hydrolates obtained by CG-MS, as they typically refer to relative amounts present in the volatile part of the hydrolate.



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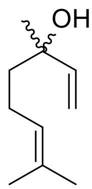

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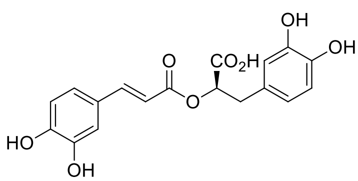
Similarly, one study reported the use of supercritical fluid extraction with CO_2, aiming to obtain an aromatic extract (Teixeira and Rodrigues 2014). In this study, the authors compared and discussed the advantages of using supercritical fluid extraction over methods typically used to obtain VOs. One additional study also used solvent extraction (with diethyl ether) to obtain an aromatic extract (Hirai et al. 2022). Nevertheless, the classic hydrodistillation process remains the most commonly used method (Teixeira and Rodrigues 2014). Rosmarinic acid (**18**), ubiquitous in the Lamiaceae family and characteristic of different *Thymus* species, was the most frequently described phenolic acid of *T. citriodorus* (Park 2011; Raudone et al. 2017; Rita et al. 2018; Taghouti et al. 2020). Salvianolic acids (**19–21**), lithospermic acid A (**22**), and caffeic acid (**23**) have also been reported.



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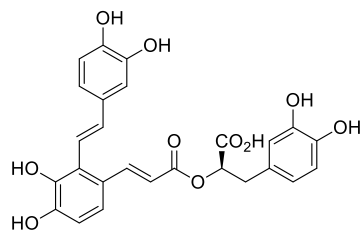

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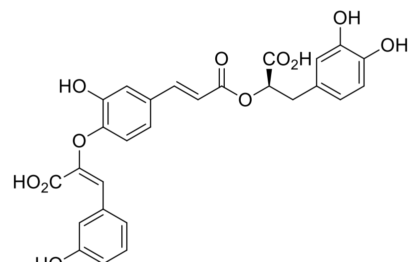

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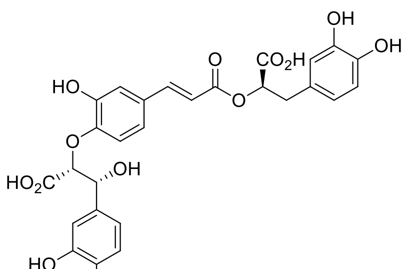

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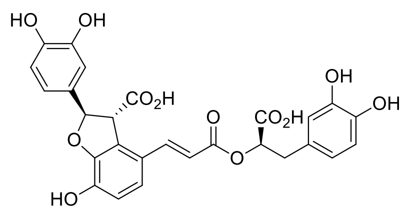

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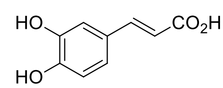

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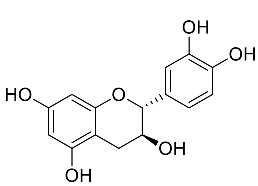
Regarding the content of flavonoids, the presence of catechin (**24**), different derivates of luteolin (**25–27**) (luteolin-7-*O*-glucuronide, luteolin-7-rutinoside, luteolin-7-glucoside), and apigenin-7-β-*O*-glucuronide (**28**) were described (Rita et al. 2018). The 7-α-*O*-glucuronide derivative of luteolin (**25**) and apigenin-7-β-*O*-glucuronide (**28**) were reported as being non-common flavonoids among *Thymus* species, thus being specific for *T. citriodorus* (Pereira et al. 2013b). Other novel glycosylated flavonoids described for *T. citriodorus* ethanolic extracts comprised eriodictyol dihexoside with *O*-glycosidic linkages (**29**), eriodictyol-*O*-monohexosides (**30**), quercetagetin dimethyl ether-*O*-hexoside (**31**), naringenin-*O*-hexoside (**32**), and chrysoeriol-7-β-*O*-glucoside (**33**) (Pereira et al. 2013b).



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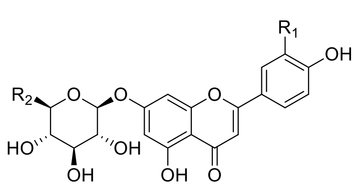
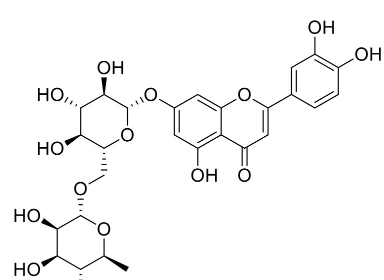


25 R_1=OH; R_2=CO_2H
27 R_1=OH; R_2=CH_2OH
28 R_1=H; R_2=CO_2H
33 R_1=OCH_3; R_2=CH_2OH



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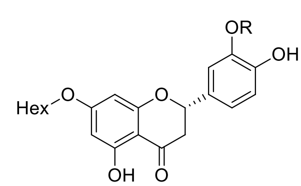
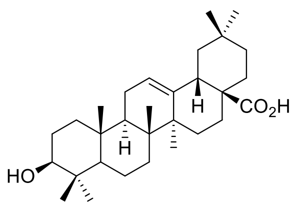


29 R=Hex
30 R=OH
31 R=OCH_3
32 R=H

Triterpenic acids such as oleanolic (**34**), ursolic (**35**), corosolic (**36**), and betulinic (**37**) acids were identified in a methanolic extract described by Raudone et al. (2017). Ursolic acid was found to be the predominant compound in this extract.



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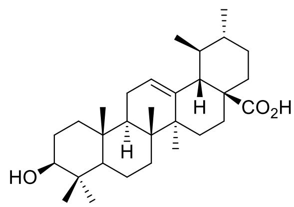

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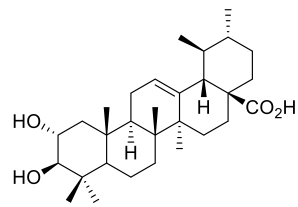

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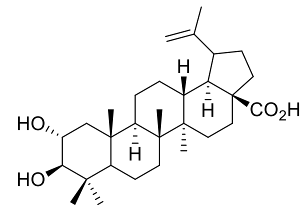

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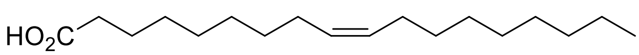
The chemical composition of an alcoholic extract from the variety "silver queen" by application of GC–MS for the analysis of this cultivar has been described, which reported oleic acid (**38**) (10.35 ± 1.42%), caryophyllene oxide (**39**) (9.62 ± 0.97%), matricarin (**40**) (7.59 ± 0.86%), geranial (**4**) (6.35 ± 0.75%), β-bisabolene (**41**) (6.15 ± 0.75%), and thymol (**11**) (5.46 ± 0.70%), as the major components (Steshenko and Mazulin 2022).



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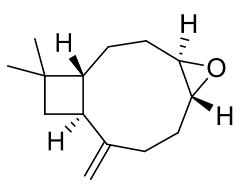

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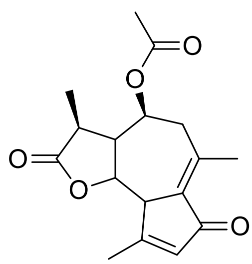

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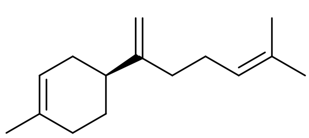

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External Influences on the Chemical Composition

Different factors can play a role in modulating and influencing the characteristics for the chemotypes of specific species. These factors include both intrinsic factors, such as the plant's genetic origin, but also external factors that go from the geographic origin and extraction methods to even storage conditions. Typically, a plant's chemotype, despite being determined genetically, is also majorly influenced by environmental conditions (Ložienė et al. 2021). Different factors that modulated the chemical composition of the obtained VOs and extracts are revised in the following subsections.

Ontogeny

Species of the genus *Thymus* are described to accumulate the highest amounts of secondary metabolites (as VOs), and consequently to be harvested, at flowering stages (Ložienė et al. 2021). However, variations can be present among different species. Some authors have studied how factors such as plant ontogeny can affect the production of *T. citriodorus* VO, not only in its yield but also in the chemical composition. Omidbaigi et al. (2009) aimed to clarify what was the most suitable phenological stage to harvest *T. citriodorus* in Tehran (Iran), in order to achieve the maximum quantity and quality of its VO. For this specific location, the authors reported that the most suitable time for harvesting *T. citriodorus* in order to improve VO yield was at the beginning of the flowering stage, reaching a yield of 2.21% (*m*/*m*) when compared with before flowering stage (1.8% (*m*/*m*)) and with full flowering stage (1.45% (*m*/*m*)). Toncer et al. (2017) also evaluated the effect of plant development, in this case of plants grown in Southeastern Anatolia Region of Turkey, and obtained similar results, obtaining a higher yield of 2% (*m*/*m*) at the pre-flowering stage when compared with the flowering stage (1.9% (*m*/*m*)) and post-flowering stage (1.3% (*m*/*m*)).

In addition to affecting VO accumulation, plant development can also influence its specific chemical composition. The same authors also evaluated variations in the main compounds of *T. citriodorus* VO among different flowering stages. Omidbaigi et al. (2009) reported that the highest amount of geraniol (**1**) was identified at the pre-flowering stage (72.5%) and the lowest amount at the fruit set stage (54.2%). Contrarily, the amount of geranial (**4**) increased from before flowering to the fruit set stage, varying from 3.2 to 11.9% between these two periods. Toncer et al. (2017) despite describing a different chemical profile for *T. citriodorus* with terpinolene (**8**) being reported as a major compound, also found that its relative percentage varied among flowering stages, being the highest (71%) at the flowering stage and the lowest (59%) at the post-flowering stage. α-Terpineol (**9**), another relevant component of the VO described by this team, also suffered variations during the different plant developing stages, being the highest rate (29.56%) at the post-flowering stage and the lowest rate (20.03%) at the flowering stage. This variation among different stages was also verified for other components such as linalool (**17**) and borneol (**10**). However, both authors also concluded that the best phenological periods for harvesting *T. citriodorus* to achieve the highest quantity and quality of VO should be recommended according to each location and environmental conditions.

Soil Fertilization

The richness of the soil where plants are cultivated can have a major impact not only in plant development but in the yield and composition of secondary metabolites (Nurzyńska-Wierdak 2015). To counteract the nutrient deficiency in soils, fertilization is often used, and organic fertilizers are valuable sources of naturally available minerals (Vaičiulytė et al. 2022). To clarify if organic fertilization would enhance *T. citriodorus* development and yield, Vaičiulytė et al. (2022) evaluated how the fertilization with liquid cattle dung and humus affected the quantitative and qualitative composition of VO, plant biomass and some morphometrical and anatomical parameters of plants grown in open ground in Lithuania for two years. Regarding VO yield, the authors described that fertilization with liquid cattle dung increased VO yield, and humus fertilization decreased the yield, specifically in the second year of experiment (which was rainier and cooler), with a decrease in 37.04% relatively to the yield obtained in control plants. In relation to the VO's composition, geraniol (**1**) was the major compound in all tested conditions, with minor variations across harvesting years in control plants (24.48 ± 1.13% to 23.91 ± 1.35%) and in liquid cattle dung treated plants (23.78 ± 0.44% to 23.60 ± 2.58%), but with a reduction in humus treated plants (26.27 ± 2.73% to 20.55 ± 2.78%) in the second year. Nerol (**3**), geranial (**4**), and neral (**5**) were also present in relevant amounts and suffered variations according to the different treatments in both years. The authors ultimately concluded that the fertilization with liquid cattle dung was useful, and that the fertilization with humus or cultivation in soils with large amounts of humus was not economically useful, as it decreased plant biomass and VO yield. Nevertheless, the effect of different climatic conditions in different years overpowered the variations caused by fertilizers, as they influenced chemical, anatomical, and morphometrical parameters in plants exposed to the same fertilizer.

Climatic and Seasonal Influences

Apart from specific plant development, other factors that are associated with changes in VO content and chemical composition are climatic and seasonal changes occurring in the plant growth location.

Considering the significance of *T. citriodorus* in pharmaceutical and fragrance industries as a source of lemon flavor and fragrance due to its natural biologically active compounds, Ložienė et al. (2021) studied how temperature, rainfall, sunshine duration, and photosynthetically active solar radiation influence both VO yield and composition, by collecting plant material for three sequenced years at the same location. The authors found that VO yield in one of the studied years differed significantly from the other two. The lower-yield-producing-year was distinguishable by the highest values of temperature, sunshine duration, and photosynthetically active solar radiation at harvesting seasons. Interestingly, the author also found that geraniol (**1**) content was affected in the same manner as the VO yield. The authors concluded, based on the positive relationship of VO yield with rainfall and on the negative relation with temperature, sunshine duration, and photosynthetically active solar radiation, that cool weather, little sunny, and moisture could be the most suitable conditions for a higher accumulation of VO in *T. citriodorus*, also leading to a higher relative amount of geraniol (**1**). The authors also conclude that, when aiming to maximize a specific component, cultivation of different clones of a specific chemotype was better than one clone monoculture, overcoming a possible negative effect of climatic conditions on VO yield, while maintaining the desirable chemical composition.

Similar results were found by Tátrai et al. (2016) when evaluating how limited moisture availability affected the growth and VO content, as water supply is one of the most determinative cultivation conditions that can affect yield and VO content (de Abreu and Mazzafera 2005; Taherkhani et al. 2011). By inducing drought stress using different polyethylene glycol (PEG-6000) levels (0, 2, and 4%), the authors found that, in general, VO yield was limited and independent of provoked drought stress (≈10 μl/g of dry weight). A significant reduction in VO content was recorded with a higher level of PEG-treated plants (6 μl/g of dry weight), suggesting that higher drought stress levels contributed to the reduction of VO yield. Contrary to the previous study, the authors also found that geraniol (**1**) content increased significantly upon drought stresses (28.4 and 21.9% *versus* 4.2% in the control plants). Variations in carvacrol (**6**) and thymol (**11**) were also found, as carvacrol content significantly increased in 4% PEG-stressed plants by 31.7%, compared to its negligible amount in control and mildly stressed plants. The authors ultimately concluded that the main VO components were influenced by drought stress and that their relative abundance may be maximized, thus increasing plant value, by manipulating the water supply (Tátrai et al. 2016).

Contrary to these results, in a different study, where the authors studied the influence of fertilization in VO yield and composition, the VO yield of control plants (without fertilization) was higher in the warmer and drier year (~0.5–0.6% of dry weight) compared with the rainier and cooler year (~0.2–0.35% of dry weight) (Vaičiulytė et al. 2022). This impact of climatic conditions on the chemical composition of plant extracts also affects other extracts apart from VO, and studies have shown that environmental conditions during plant growth can account for differences in the levels of total and individual phenolics (Singh et al. 1997). Considering that phenolic compounds are strongly associated with preservative (antioxidant and antimicrobial) potential of extracts, Abramovič et al. analyzed the influence of environmental conditions in the phenolic content of ethanol extracts from *T. citriodorus* harvested in two different years (Abramovič et al. 2018). The authors reported that one of the extracts had almost a third of the total phenolic content when compared to the second extract. Since the plants were grown in the same experimental field with the same soil composition, the authors ultimately concluded that the difference in total phenolics might have been originated by other factors, like temperature, photoperiod, and precipitation between the two growing seasons. The much lower precipitation in one of the years (194 mm compared to 297 mm) provoked a more pronounced accumulation of total phenolics.

Isolation Processes

The plant processing methods and storage conditions can also play an important role in the chemical composition of different *T. citriodorus* preparations (Pasławska et al. 2020). It is well established in the literature that the duration of the distillation process to obtain VOs can influence their yield and chemical composition (Miguel et al. 2011; Cannon et al. 2013). In order to understand if the optimization of the hydrodistillation process could modulate the relative amounts of its major compounds, specifically the ones with higher commercial and biological value, such as geraniol **(1)** and citral [geranial **(4)** + neral **(5)**], Jurevičiūtė et al. (2019) evaluated how the duration of distillation influenced yield and/or qualitative composition of *T. citriodorus* VO. The authors found differences in VO yield obtained at different distillation times, being the lowest (0.28 ± 0.04%) established at 5 min and the highest (0.75 ± 0.01%) at 150 min of hydrodistillation. In a more relevant perspective, the authors also found that the increase in VO yield was very slow after 60 min, showing an increment of only 0.01% every 10 min. Therefore, they concluded that hydrodistillation times longer than 60 min proved non-relevant in terms of VO yield (Jurevičiūtė et al. 2019). Regarding chemical composition, the authors found that extension of hydrodistillation time decreased percentages of monoterpenes due to the increase of sesquiterpenes' recovery. Interestingly, the highest percentage of neral (**5**) (18.4%) was observed at 5 min of distillation time, and it was 1.5–2.5 higher in comparison with other distillation times (Jurevičiūtė et al. 2019). Overall, they established a negative correlation between nerol (**3**), geranial (**4**), and neral (**5**) percentages and the increment of hydrodistillation time. Only geraniol (**1**) did not present a significant correlation between percentage and hydrodistillation time. The authors ultimately concluded that it was possible to obtain a VO richer in economically valuable components, as citral, by adjusting hydrodistillation duration.

Plant Preservation Techniques

Although much of the available research concerns different extracts or VOs obtained from this hybrid plant, fresh and/or dried plant materials are also susceptible to alterations due to external factors. In fact, and despite fresh *T. citriodorus* has been widely consumed in culinary, its tendency to perish can make the dehydrated variety more popular and convenient. Preservation by drying is typically carried out to stabilize the biologically active substances during storage periods. Nevertheless, this drying process entails negative changes in the structural properties of the plant tissues and in their sensorial characteristics, as well as a significant reduction in their content of bioactive components (Pasławska et al. 2020).

There are different techniques commonly used for plant preservation, one of the most popular procedures is convective drying. However, due to the relatively high temperatures and long dehydration process, degradation of valuable nutritional components may occur. Pasławska et al. (2020) evaluated how variations of the drying method (or combinations) could be applied to enhance the chemical properties of the end product. To do so, these authors compared two conventional hot-air drying methods (convective drying and fluidized bed method), with two innovative drying methods specifically, the fluidized bed method and the vacuum method, both combined with microwave heating. The endpoints evaluated were color changes, chemical composition, and antioxidant capacity of dried plants. The drying process caused a significant change in the color of *T. citriodorus*, depending on the drying method and the process parameters. All drying methods led to the darkening of leaves, except for convective drying at 80 °C, for which the level stayed the same as that of the fresh material. The authors also found that all drying methods caused significant reductions in polyphenol, chlorophyll, and carotenoid contents, but that the level of chemical destruction was dependent on the conditions and parameters of dehydration. The authors concluded that vacuum method combined with microwave heating was the best for preserving *T. citriodorus* nutritional properties, as it undertook the shortest process time and resulted in the highest retention of polyphenols (78.90–82.14%) and chlorophylls (51.54–52.68%) and resulted in the minor color change (color change (ΔE) = 25.57–28.32). However, if carotenoid content should be preserved, the most protective method was the fluidized bed method combined with microwave heating, ensuring a retention of 68.46–70.61% (Pasławska et al. 2020). In relation to the polyphenols, the authors found that all drying methods were destructive for polyphenols in *T. citriodorus*, with the highest polyphenol loss observed with convective drying. Drying processes caused the reduction, conversion, disintegration, or deletion of phenolic components, depending on the method and parameters used, with time of processing (time of contact with oxygen), affecting polyphenol retention in a stronger manner than temperature. On the basis of phenolic profiling of *T. citriodorus*, the authors concluded that microwave-assisted methods were more protective than hot-air methods; and those harder conditions (such as higher microwave power or temperature levels) which caused a shortening of the drying process, provided a higher retention of chemical components (Pasławska et al. 2020).

Health-Related Properties

Apart from studies focusing on *T. citriodorus* chemistry, typically associated with production concerns, most recent studies focused in the pharmaceutical/medicinal applications of *T. citriodorus*. Several relevant bioactivities have been reported across different studies, including antioxidant, anti-inflammatory, antimicrobial, and cytoprotective potential, as disclosed in Table S3, that presents the relevant activities of *T. citriodorus* VO and extracts and the major compounds described for each preparation. In the following subsections, the results obtained from different authors will be addressed. Detailed results regarding antimicrobial activity can be consulted in Table S5. Antiproliferative/cytotoxic activity and anti-inflammatory activity can be consulted in Table S5, respectively. Results from antioxidant potential, evaluated by different techniques, are summarized in Table S6.

Antiproliferative and Cytotoxic Effects

Plant-driven drugs compose around 60% of the available anticancer agents, thus making the search for new antiproliferative plant extracts an attractive research topic (Gordaliza 2008). Regarding the antiproliferative potential of *T. citriodorus*, few data was available in the literature. Taghouti et al. (2020) in a recent study, reported the antiproliferative activity of a *T. citriodorus* aqueous decoction and a hydroethanolic extract against two cancer cell lines, specifically human colon adenocarcinoma (Caco-2) and human hepatocellular carcinoma (HepG2). The authors found that the effect of both extracts was more pronounced on Caco-2 cells than on HepG2 cells, as indicated by statistically significant lower IC_{50} values after 24 or 48 h of exposure to both extracts. The hydroethanolic extract presented a higher antiproliferative activity by presenting time-dependent lower IC_{50} values (24 h: 128.2 ± 5.75 µg/ml; 48 h: 114.6 ± 4.38 µg/ml) when compared with aqueous decoction (24 h: 223.70 ± 8.38 µg/ml; 48 h: 159.40 ± 16.85 µg/ml) for the Caco-2 cell line. Concerning HepG2 cells, at both exposure times, the IC_{50} values obtained for *T. citriodorus* aqueous decoction and hydroethanolic extract were higher than the experimental concentrations tested (> 500 µg/ml), denoting very low cytotoxicity for this cell line. The higher activity of the hydroethanolic extract was correlated with the higher concentration in phenolic compounds of this extract when compared with the aqueous decoction.

Wu et al. (2013) also used HepG2 cells to study the antiproliferative activity of *T. citriodorus*, in this particular case of its VO, and reported an IC_{50} of 0.34% for HepG2 cells. The authors further related the induced apoptosis mechanism behind this antiproliferative effect with the expression of nuclear factor kappa B (NF-κB), which is often deregulated in cancer cells (Bayala et al. 2014).

Cellular cytotoxicity of *T. citriodorus* preparations has also been studied using other cell lines, apart from cancer cell lines, focusing more on a safety perspective. Oliveira et al. (2022) studied the cellular toxicity of *T. citriodorus* VO and the correspondent hydrolate on a murine macrophage cell line (RAW 264.7), a model used to evaluate basal toxicity on macrophages when aiming to study the anti-inflammatory potential of an extract or ingredient. The authors reported that both preparations impair cellular viability in a dose-dependent manner, with VO presenting higher cytotoxicity when compared with hydrolate, with an IC_{50} value of 0.01 (v/v) *versus* 16.90% (v/v), respectively.

Cytoprotective and Anti-aging Properties

In addition to the cytotoxic and antiproliferative activity, plant extracts can also be evaluated regarding their ability to confer cellular protection to induced toxicity. Pereira et al. (2013a) studied the ability of purified *T. citriodorus* ethanolic extracts to protect HepG2 cells from induced toxicity. This cell line, despite being a cancerigenous cell line derived from human hepatoblastoma, retains many of the specialized functions of normal hepatocytes, thus being extensively used as an *in vitro* model to study the benefits of plant extracts with respect to hepatic injury conditions. The purified ethanolic extract from *T. citriodorus* exhibited protection against the potassium dichromate-induced toxicity on HepG2 cells, in the acute exposition model (200 µM for 6 h), by increasing cellular viability by around 30% when compared with the control with the chemical inducer but failed to protect cells in the long term exposure model (2 µM for 72 h). The authors tried to associate this protective effect with the polyphenolic compounds present in the extract by mixing individual compounds to simulate the content determined in 50 µg/ml of *T. citriodorus* purified extract. The mixture protected the cells in a similar manner to the extract, increasing cellular viability by around 32% when compared with the control with potassium dichromate, in the acute toxicity model; but conferred no protection in the long-term exposure model. As the extract presented a poor phenolic compound composition (149 mg/g), this could justify the lack of ability to protect HepG2 cells from potassium dichromate toxicity in longer incubation times. The authors concluded that the cytoprotective effects of *T. citriodorus* ethanolic extract might be conferred by its major phenolic compounds, the luteolin derivates (Pereira et al. 2013a).

In addition to the reported cytoprotective properties, *T. citriodorus* has also been described as relevant to anti-aging applications. Fitoussi et al. (2011) reported the effect of a *T. citriodorus* extract (details of type of extract were not disclosed) on the expression of emilin-1, a glycoprotein of the network of elastic fibers, which participates in the adhesion of the elastin to the cells. The authors reported an increase in the expression of emilin-1 by 34 and 54% after 10 and 14 days of treatment of normal human skin fibroblasts with 1% of *T. citriodorus* extract, respectively. The authors ultimately concluded that the *T. citriodorus* extract exerted a stimulating effect against the morphological changes of elastic fibers induced by aging and may have potential to be used as an active ingredient for cosmetic purposes (Fitoussi et al. 2011).

Anti-inflammatory Activity

The anti-inflammatory activity of *Thymus* species, particularly of their VOs, has been studied across different studies for species such as *T. vulgaris*, *T. albicans*, *T. zygis*, and *T. carnosus*, among others (Zuzarte et al. 2018; Roxo et al. 2020). Regarding *T. citriodorus*, the anti-inflammatory potential of its preparations was studied, using cellular models, specifically by evaluating their ability to inhibit NO production in lipopolysaccharide (LPS)-stimulated macrophages. Oliveira et al. (2022) aimed to evaluate, *in vitro*, the potential of *T. citriodorus* preparations to act as active ingredients for anti-acne applications, targeting several aspects related to *acne vulgaris* disease, including inflammation. The authors reported the ability of *T. citriodorus* VO and hydrolate to inhibit NO production, in LPS-stimulated macrophages, in a dose-dependent manner and even at much lower concentrations than the ones required to induce cytotoxicity. Interestingly, VO presented comparable reductions with the drug dexamethasone (10 µM) even at very low concentrations, reducing NO production by 50%. In an attempt to clarify whether the reduction of NO was not a direct scavenging effect of plant preparations, the authors also performed a reaction between an external NO donor (SNAP) and different concentrations of the plant preparations. They concluded that the reduction in NO levels resulted from a true reduction in its production, as preparations failed to scavenge NO from the exogenous donor (Oliveira et al. 2022).

Antimicrobial Activity

Antimicrobial activity of VOs or other plant extracts is a topic majorly researched in plant-based studies, as there is a growing interest in researching and developing new antimicrobial agents from natural sources to combat microbial resistance (Chouhan et al. 2017). Typically, antimicrobial activity (specifically antibacterial and antifungal activities) is determined using disk diffusion methods or broth dilution (either in a microdilution or macrodilution scale), the last enabling the study of several concentrations at once, thus allowing the determination of minimal inhibitory concentration (MIC). Minimal lethal concentrations are sometimes used, along with MIC determinations, to estimate the bactericidal or fungicidal effect of plant extracts.

Thymus species have been majorly studied regarding their antimicrobial activity (Lorenzo et al. 2019). For *T. citriodorus*, most of the studies focused on microorganisms related to food spoilage. A summary of the results reported among different studies is presented in Table S4. Aprotosoaie et al. (2019) reported the antifungal activity of VO from *T. citriodorus* and from *T. citriodorus* "aureus" cultivar, against *Aspergillus flavus*, a fungus responsible for the contamination of food and human invasive aspergillosis and superficial skin infections. *Thymus citriodorus* "aureus" VO, presented a relevant antifungal activity, with a MIC value of 0.50 µl/ml, being fungistatic at this concentration. The authors attributed this activity to the percentage of aromatic compounds alongside the high level of geraniol (**1**) (60.31%). *Thymus citriodorus* VO that presented lavandulol (**15**) as the major compound also presented antifungal activity being fungicidal at effective concentrations, as MIC and MFC values were the same (1 µl/ml).

Sacchetti et al. (2005) evaluated the antimicrobial properties of VOs from different plants with the potential to be classified as functional foods, against five food-spoilage yeasts (*Candida albicans*, *Rhodotorula glutinis*, *Schizosaccharomyces pombe*, *Saccharomyces cerevisiae*, and *Yarrowia lypolitica*), and reported a high antifungal activity for *T. citriodorus* VO. The VO showed good effectiveness and broad-spectrum activity, with MIC values ranging from 0.03 mg/ml for *Y. lypolitica* to 0.09 mg/ml for *R. glutinis*, being 0.06 mg/ml for the remaining species. The obtained results were comparable to, or even better, than those provided by the used reference VO, *T. vulgaris*. The authors also related the higher activity of *T. citriodorus* VO to its higher composition in geraniol (**1**) and citral isomers. The antimicrobial activity of geraniol (**1**) has been well reported in the literature against several foodborne pathogens such as *Escherichia coli*, *Salmonella typhimurium*, *Salmonella enterica*, and *Listeria monocytogenes* (Friedman et al. 2002; Kim et al. 2002).

Although the major focus has been given to antimicrobial activity against microorganisms related to food spoilage, the activity of the VO against other pathogens has also been determined. Steshenko et al. (2021) reported the antibacterial and antifungal activity of a VO obtained from the "silver queen" cultivar against relevant pathogens associated with mucocutaneus infections. The authors tested a representative panel that include both Gram-positive (*S. aureus*) and Gram-negative bacteria (*E. coli* and *Pseudomonas aeruginosa*), and the yeast *Candida albicans*. From the tested microorganisms, only the non-fermenting Gram-negative bacteria *P. aeruginosa* was resistant to the VO activity. For the remaining microorganisms, the VO was more active against *C. albicans*, followed by *E. coli* and *S. aureus*, with inhibition zones ranging from 29.30 ± 2.8 mm, to 19.60 ± 1.8 mm, and 14.60 ± 1.5 mm, respectively.

The VO and its by-product (hydrolate) from *T. citriodorus* have also been studied for their activity against skin-related pathogens. In a recent study, Oliveira et al. (2022) reported the activity of both preparations on acne-related pathogens, specifically *S. aureus*, *S. epidermidis*, and *Cutibacterium acnes*, the last typically associated with *acne vulgaris* pathogenesis. The authors reported that *T. citriodorus* VO was more potent than the hydrolate since for the last, visual MIC was only obtained for *C. acnes* at very high concentrations. For the VO, the authors reported a high susceptibility of *C. acnes* and *S. epidermidis*, presenting lower visual MIC values of 0.06% (v/v) when compared to *S. aureus* (MIC: 0.125% (v/v)). Minimum lethal concentration values were higher than the obtained MIC values for all tested bacteria, thus presenting a bacteriostatic effect. The authors also related the antimicrobial activity of the VO to the presence of geraniol (**1**) as a major compound. The lack of activity from the hydrolate was discussed as being related to the low concentrations of volatile organic compounds combined with the fact that the main component, 1,8-cineole (**16**), was not associated with demarked antibacterial properties in the literature (Oliveira et al. 2022).

Thymus × *citriodorus* extracts were also studied against different food-related pathogens and other relevant bacteria by different authors. Abramovič et al. (2018) studied the antibacterial activity of ethanolic extracts from frozen and dried *T. citriodorus* against four food-related bacteria, specifically *S. aureus*, *Listeria monocytogenes*, *Campylobacter jejuni*, and verotoxin *E. coli* (VTEC). The authors expressed MIC values as µg of CAE/ml. Overall, the obtained MICs were relatively high, but greater activities were found against *S. aureus* and *C. jejuni*. Neither of the ethanolic extracts was particularly efficient against the Gram-negative VTEC strains. Despite not being clear how the different chemical compositions of the extracts affected their antimicrobial activities, the authors determined Pearson correlation coefficients in an attempt to correlate the antimicrobial results with total flavonoids, flavones, flavonols, flavanones, or dihydroflavonols (Abramovič et al. 2018). The highest correlation was obtained between the MIC for *S. aureus* with the total content of phenolics and flavonoids.

Kerem et al. (2023) evaluated the antibacterial effect of three different extracts from *T. citriodorus*, namely ethanolic, hexane, and chloroformic on growth inhibition of *Enterococcus faecalis*, *S. aureus*, *P. aeruginosa* and *E. coli* using the Kirby-Bauer Disk Diffusion method. Overall, the Gram-positive bacteria *S. aureus* was the most susceptible one. Ultimately, the authors concluded that among the extracts tested, ethanolic extract was the most effective in inhibiting bacterial growth, followed by the chloroformic extract and the last was hexane extract, and inferred that ethanol and chloroform could enhance the extraction of the secondary metabolites with more effective antimicrobial activities (Kerem et al. 2023).

Finally, in a study performed by Orłowska et al. (2015), a different technique was applied to evaluate several *Thymus* species' extracts obtained by methanol–water extraction from leaves and rhizomes, specifically the dot-blot test with direct bioautography. Using this technique, a developed chromatographic plate was first immersed in a suspension of *Bacillus subtilis*, followed by an incubation period at optimum growth conditions. Microorganism growth took place directly on the developed chromatogram, and the microbial growth inhibition zones were evaluated with the aid of tetrazolium salt. The authors reported that all investigated extracts exhibited antibacterial activity, yet distinct results were perceived in the size of the bacterial inhibition zones among the compared thyme species, with *T. citriodorus* "golden dwarf" gaining relevance due to higher inhibition zones. Also, the leaves showed a higher inhibition zone compared with the rhizomes, possibly due to the higher accumulation of bioactive compounds in these structures. The authors ultimately selected *T. citriodorus*, among the studied *Thymus* species, as a promising target for further investigations.

Apart from the reported antimicrobial activity, typically evaluated through methods addressing the planktonic growth forms of bacteria and fungi, the study of the effect of plant preparations against biofilm growth forms is often addressed. Biofilms are, by definition, a sessile multicellular community of microbial cells with a modified transcriptome and phenotype that adhere to a surface and are protected by an auto-secreted extracellular polymeric matrix (Nuță et al. 2021). As biofilm growth forms are more resistant to antimicrobials and disinfectant processes, they present major challenges not only to the medical field but also to the food manufacturing industry (Nuță et al. 2021). Plant extracts or preparations, by presenting a complex composition, are being studied as alternatives to inhibit bacterial and fungal adhesion and further biofilm development on different surfaces from the medical, industrial, and natural environments.

Regarding *T. citriodorus*, only one study reported the activity of VO and hydrolate against biofilm formation and as disruptors of preformed bacterial biofilms, specifically the ones formed by *C. acnes* (Oliveira et al. 2022). The presence of biofilms from these bacteria in *acne vulgaris* is associated with reduced effectiveness of antimicrobial agents, thus leading to longer treatment periods with low success rates (Linfante et al. 2017). The authors reported that *T. citriodorus* VO was effective in reducing biofilm formation, even at planktonic sub-inhibitory concentrations (35% reduction at ¼ of the MIC; 90% reductions for MIC and 2MIC). *Thymus* × *citriodorus* hydrolate was not as effective, with significant reduction present only at MIC value. Both preparations were also effective in disrupting preformed biofilms (Oliveira et al. 2022).

Antioxidant Activity

There is a wide variety of available methods to study the antioxidant capacity of plant preparations. In a broad approach, the available methods can be divided into two major groups depending on the mechanism of action by which the applied compounds stop chain-breaking reactions, specifically hydrogen atom transfer reactions and single electron transfer (Chaves et al. 2020). Among the last, the most used are 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, ferric reducing assay, Trolox equivalent antioxidant capacity (TEAC or ABTS), copper reduction (CUPRAC) assay, and reducing power assay. Among hydrogen atom transfer reaction assays, the crocin bleaching assay, the total peroxyl radical-trapping antioxidant parameter (TRAP) assay, and the oxygen radical absorbance capacity assay are the most described in the literature (Pereira et al. 2013a; Chaves et al. 2020). Despite this wide variety of available methods, there is a major variability of experimental conditions, thus hampering the selection of the most convenient method and the comparison of obtained results with those of other authors (Chaves et al. 2020).

Some results can be found in the literature regarding the antioxidant capacity of *T. citriodorus* preparations and extracts. These results are summarized in Table S6.

Taghouti et al. (2020) reported the ability of *T. citriodorus* hydroethanolic and aqueous decoction extracts to scavenge ABTS radical cations. Higher scavenging activity values were found for hydroethanolic extracts (1.52 mmol Trolox eq/g extract) when compared to aqueous decoction extracts (1.21 mmol Trolox eq/g extract). Results from another assay, specifically the hydroxyl radical (·OH) scavenging assay, demonstrated that aqueous decoction extracts from *T. citriodorus* inhibit hydroxyl radicals in the absence and in the presence of EDTA in the test system (30.59 ± 2.08% and 37.97 ± 1.12%, respectively). The authors also found that *T. citriodorus* aqueous decoction extracts were more potent in scavenging ·OH than extracts from other *Thymus* species, specifically from *T. vulgaris*. Contrarily, extracts from *T. citriodorus* performed poorly in the scavenging of ·NO radical (41.15 ± 3.64%) when compared to *T. vulgaris* (57.61 ± 2.76%) (Taghouti et al. 2020). Pereira et al. (2013a) also evaluated the antioxidant capacity of *T. citriodorus* extracts, particularly of an ethanolic extract, using other chemical methods, such as DPPH radical scavenging assay and the reducing power assay. The purified ethanolic extract presented a considerable DPPH scavenging ability (EC_{50} 11.7 ± 1.5 μg/ml), being five times lower than the obtained result for ascorbic acid (positive control). Regarding the reducing power assay, *T. citriodorus* EC_{50} values (88.2 ± 0.8 μg/ml or 1.9 mmol BHA/g extracts) were approximately 3 times lower than the used positive control—butylated hydroxyanisole (BHA) (Pereira et al. 2013a). Studies have also reported the antioxidant capacities of *T. citriodorus* extracts from different lots. Rita et al. (2018) studied the antioxidant activity of infusions prepared from standard and reserve lots from *T. citriodorus* through four different assays: DPPH radical-scavenging activity, reducing power, β-carotene bleaching inhibition, and lipid peroxidation inhibition by TBARS assay. The authors reported that standard and reserve lots gave similar results of antioxidant capacity, except in the reducing power assay, with the standard lot presenting a higher activity (EC_{50} 0.228 and 0.393 mg/ml for standard and reserve lots, respectively), which the authors attributed to a higher concentration of phenolic compounds (Rita et al. 2018).

Regarding the reported antioxidant activity of *T. citriodorus* VO, Aprotosoaie et al. (2019) studied the antioxidant capacity of several varieties of *T. citriodorus* using the free radical scavenging assay (DPPH) and ferric ion reduction assays and compared the obtained results with BHA, used as the positive control. *Thymus* × *citriodorus* VO, containing 54.27% lavandulol (**15**), showed the weakest antioxidant capacity (EC_{50} 34.28 and 0.52 mg/

ml in DPPH and ABTS scavenging assays, respectively). The ferric ion reduction capacity was not assessed due to low activity. Higher antioxidant activity was determined for the VO isolated from *T. citriodorus* "aureus" (containing geraniol (**1**) as the predominant component) (Aprotosoaie et al. 2019). Both the monoterpene alcohols geraniol (**1**) and lavandulol (**15**) are weaker antioxidants when compared with thymol (**11**), typically present in other *Thymus* species. Still, since geraniol (**1**) presents a more potent antioxidant capacity than lavandulol (**15**), this could justify the higher antioxidant capacity of the "aureus" variety. Oliveira et al. (2022) also evaluated the antioxidant potential of *T. citriodorus* VO and of its corresponding hydrolate using the DPPH radical-scavenging assay. The authors reported a poor antioxidant capacity for both preparations (and low antioxidant activity indexes) when compared to ascorbic acid, used as a positive control. The reported EC_{50} values were 0.071 (v/v) ± 0.002% (v/v) and 20.08 (v/v) ± 1.25% (v/v) for *T. citriodorus* VO and hydrolate, respectively. Finally, Sacchetti et al. (2005) also studied the antioxidant capacity of *T. citriodorus* VO using the DPPH radical-scavenging assay and β-carotene bleaching test. *Thymus citriodorus* VO performed poorly when compared with other thyme species, with a free radical-scavenging activity percentage of only 25%, compared with 75% obtained with *T. vulgaris*. When considering the lipid peroxidation inhibitory activity of the VO, results were consistent with data obtained from the DPPH test, with *T. citriodorus* performing poorly when compared with other thyme species (Sacchetti et al. 2005).

Despite the usefulness of the above-reported methods, one of their most important limitations is the difficulty of extrapolating results to an *in vivo* reality. Some *in vitro* models aim to tackle this limitation by using a cellular model to study the ability of different extracts to scavenge reactive oxygen species (ROS), which can cause oxidative stress to the cells. Pereira et al. (2013a) used human hepatoblastoma HepG2 cells, stimulated with potassium dichromate as an inducer of ROS, to evaluate the ability of purified ethanolic extract from *T. citriodorus* to scavenge such radicals. The authors found that the co-incubation of cells with potassium dichromate combined with the purified ethanolic extract partially prevented the increase of intracellular ROS levels. Specifically, 50 µg/ml of *T. citriodorus* extracts decreased ROS by 20% when exposed to 25 µM of potassium dichromate. Also, by using a mixture prepared individual polyphenolic compounds, simulating the phenolic composition of the extract, the authors found a correlation between *in vitro* antioxidant capacity and chemical composition, as this mixture was also able to decrease intracellular ROS production at a similar level when compared with the extract (Pereira et al. 2013a).

DNA Damage and Mutagenic Activity

Despite the inherent perception that plant-based products are safer compared with modern medicine, there have been reports of toxic effects following prolonged consumption or application of these products (Zin et al. 2018). Nevertheless, not much attention is drawn to the toxic profile of plant preparations, as studies typically focus on their efficacy.

Different plant species have been demonstrated to contain large amounts of endogenous substances with mutagenic and/or carcinogenic properties (Zani et al. 1991). Regarding the mutagenic, genotoxic or carcinogenic assessment of *T. citriodorus*, very little data is available in the literature. In fact, only one study reported the assessment of mutagenic activity of *T. citriodorus* VO by two different methods, specifically the *Bacillus subtilis* rec-assay and the Ames test (Zani et al. 1991). The authors combine these two methods since they are based on different genetic endpoints (DNA damage and gene mutation), thus allowing for the detection of genotoxic compounds with different endpoints. In their study, no DNA damaging activity was determined for *T. citriodorus* using the rec assay. Similarly, the VO did not show any mutagenic activity with the standard strains used in the Ames test, in the absence and in the presence of metabolic activation (Zani et al. 1991).

Environmental Applications and Concerns

Apart from studies focusing their methodological approaches on a production or health-related perspective, some environmental-related studies have also been recently published regarding not only the potential of *T. citriodorus* to be applied as an eco-friendly pesticidal and fertilizer but also regarding the environmental safety of its preparations, when considering non-target organisms.

Bio-pesticidal-Related Properties

Although many synthetic pesticides have been used in the past, only a few are still authorized according to European legislation (EMA 2007). Such fact creates an urgent need for discovering less toxic and more environmentally friendly substitutes for commercial use, thus promoting the research on VOs or other plant extracts for this use (Ntalli et al. 2020b).

Ntalli et al. (2020a) studied the potential of *T. citriodorus* extracts to be used as eco-friendly control tools for root-knot nematodes. The authors reported a dose–response nematicidal activity of four *T. citriodorus* preparations, specifically VO, water extract (filtered and unfiltered), and hydrolate against two plant-parasitic nematodes, *Meloidogyne incognita* and *Meloidogyne javanica*, in the *in vitro* paralysis bioassay (Ntalli et al. 2020a, b). The nematicidal

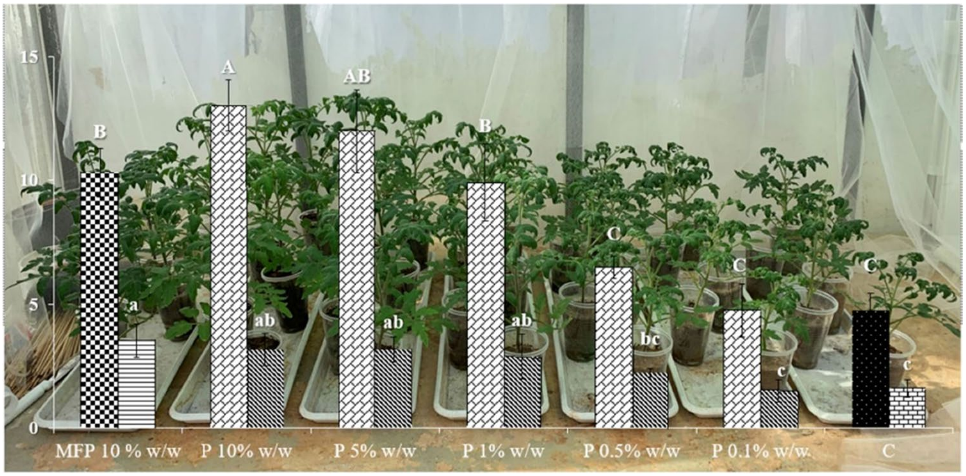

Fig. 3 Weight increase of aerial parts and tomato roots after treatment with lemon thyme powder (P) at 0.1 to 10% against *Melia azedarach* powder 10% (MFP) and water control (C). Data are presented as means of five replicates with standard deviations. Means followed by the same letter are not significantly different according to Tukey's test ($p \leq 0.05$). Upper case letters correspond to statistical differences on fresh aerial parts weight. Lower case letters correspond to statistical differences on fresh roots weight. Reprinted from Ntalli et al. (2020a) under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/). Licensee MDPI, Basel, Switzerland

activity increased over exposure time and was irreversible after 2 days. The most effective extract against *M. incognita* and *M. javanica* was the VO, followed by filtered water extract and finally hydrolate. Detailed results regarding *T. citriodorus* nematicidal activity can be consulted in Table S7. The authors also tested the efficacy of the extracts and compared them with *T. citriodorus* plant powder on a pot bioassay with 7-week-old tomato plants by mixing the different extracts or powder directly into soil incubated with *M. javanica* and evaluating the parasitation of the host roots. The most effective treatment was the plant powder, followed by the water extract, both exhibiting comparable efficacy results with the garlic extract (positive control), namely 95 and 94% *versus* 98%, respectively (Ntalli et al. 2020a). The authors ultimately conclude that specific *T. citriodorus* extracts had the potential to be integrated in a pest-management program.

Extracts from *T. citriodorus* were also reported to present pesticidal properties against barley and wheat loose smut, caused by the fungi *Ustilago nuda* and *Ustilago tritici*, respectively (Karsou and Samara 2021). Karsou and Samara (2021) reported the activity of a methanolic extract from *T. citriodorus* against these two microorganisms, by reducing their growth by 42 ± 3% and 39 ± 4%, respectively.

In a different perspective, the natural presence of VO in VO-bearing plants can act as an intrinsic defense, protecting them against fungal, bacterial, and viral agents, insects, and even herbivores (Papachristos et al. 2004; Caboni et al. 2013; Chouhan et al. 2017; Ntalli et al. 2020a). Thus, the presence of VO in a specific plant structure can influence the microbial colonization of leaves, roots, and soil, with the protection efficacy depending on the species (Checcucci et al. 2017). Within *T. citriodorus* species, some cultivars have been more associated with different plant diseases, as the "silver queen" and the "aureus". Garibaldi et al. (2007, 2016) reported the first cases of white mold caused by the fungus *Sclerotinia sclerotiorum* and of powdery mildew caused by *Golovinomyces biocellatus* on *T. citriodorus* plants grown in Italy, from the cultivars "silver queen" and "aureus" cultivars, respectively. The activity against specific microorganisms associated with plant diseases must be further investigated to evaluate the higher susceptibility of some cultivars to specific pathogens and how the difference on the chemical profile of these different cultivars can influence this difference.

Bio-fertilizing Applications

Apart from the above-discussed bio-pesticidal activities described for *T. citriodorus*, this species has also been reported to possess other relevant properties, less discussed across different studies, as biofertilization capacity. Ntalli et al. (2020a) studied the biofertilization capacity of *T. citriodorus* plant powder and found a clear dose–response, considering fresh weights of both the aerial parts and the roots of tomatoes treated with increasing amounts of *T. citriodorus* plant powder, as represented in Fig. 3. The biofertilizer effect at the highest tested concentration (10% w/w) was even higher than the positive control used, specifically *Melia azedarach* ripe fruit powder at the same concentration (Ntalli et al. 2020a).

Eco-safety Concerns

Associated with a high demand for plant-based products by the consumers and a spread increase in their use and production, VOs, and their by-products often reach the environment. However, the potentially harmful effects that these products can have on the ecosystems' are mostly unknown, as the study of the possible toxic effects of VOs and plant extracts on non-target organisms has only recently gained the interest of the scientific community (Ferraz et al. 2022).

Ntalli et al. (2020a), when considering the possible biopesticidal properties of different extracts of *T. citriodorus*, also evaluated the possible ecotoxicological effects for non-target organisms, accompanying the biological activity of interest. The authors reported that, despite *T. citriodorus* VO and water extracts caused an increase in bacterial biomass on the soil, compared to the control, the VO also significantly lowered the abundance of bacterial/fungal-feeding nematodes. On the contrary, the treatment with the water extract significantly increased the numbers of the bacterivorous nematodes (Ntalli et al. 2020a). Therefore, *T. citriodorus* VO, despite performing better on paralysis and biological cycle arrest, also lowered the abundance of bacterial and fungal feeding nematodes, thus not being the optimal choice for a biopesticide. Still, extracts as unfiltered water extracts augmented bacterial biomass while also increasing bacterivorous nematodes, thus presenting a safer choice.

The ecotoxicity of *T. citriodorus* VO and hydrolate on aquatic organisms were also studied using freshwater crustacean *Daphnia magna*, in an acute toxicity model, in order to classify them under the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) (Oliveira et al. 2022). Oliveira et al. (2022) reported that *T. citriodorus* VO caused immobilization of *D. magna* in a dose-dependent manner with an EC_{50} at 48 h of 32.05 mg/l. No toxicity was obtained for the hydrolate even at the highest tested concentration of 2000 mg/l. Finally, the authors classified the VO under the acute 3 category and the hydrolate as being not toxic according to GHS of the United Nations.

Perspectives and Future Directions

The overgrowing interest for *T. citriodorus* by different industries has led to an increase in its popularity among aromatic and medicinal plant producers that are actively cultivating it, thus emerging as a hybrid of high economical interest. This value has triggered several investigations on how to maximize its production, ensuring better yields of high-quality added value secondary metabolites, as VOs.

Since plants of genus *Thymus* are cultivated at non controlled (or semi-controlled) environment, it is important to predict how extrinsic factors will influence VO yield, to select the most favorable locations and conditions for cultivation. Also, since the bioactivity of plant extracts is characteristically related to their chemical composition, several studies have directed their focus on how these external conditions truly influence the extracts' composition, in order to adapt and optimize production processes, obtaining high-quality products with increased bioactivities. External factors, as seasonal and climatic variations have also direct influences on plant development, that *per se* influence VO yield and composition, highlighting the importance of selecting optimal ontogenetic stages for obtaining the highest amounts of bioactive compounds. Since these two are interconnected, optimal harvest periods will, ultimately, vary between regions. Therefore, the harvesting time for a specific plant production should be optimized according to its location and environmental conditions (Toncer et al. 2017). More studies addressing the effect of climatic variations on VO yield and main components of *T. citriodorus* VOs are necessary to ultimately conclude on the true effects of these factors and to clarify the differences found on across different studies. In an active attempt to mitigate or, at least, control this high impact of external factors in VO yield and plant development, some authors are also investing in clonal selection from best-performance cultivars, achieving highest productivity of both raw material and its VO and more resistance to harsh conditions, and less vulnerability to the long deficiencies of precipitation, as reported by Lilia et al. (2022) with a new *T. citriodorus* cultivar, the "citronel-pink."

Adjustments in processing and isolation methods are also being investigated with the same purpose of higher yield and higher quality products. Despite the European Pharmacopoeia report that the distillation time of VO from *Thymi herba* (that includes whole leaves and flowers of *T. vulgaris* or *T. zygis* or a mixture of both) is 2 h, as the chemical composition of *T. citriodorus* is different from the mentioned thyme species, the standardized 2 h may not be necessarily the optimum distillation time for all species of the *Thymus* genus (European Directorate for the Quality of Medicines & Healthcare (EDQM) 2023). Some authors have addressed the influence of methodological factors on *T. citriodorus* VO chemical composition and found that geraniol did not present a significant correlation between its percentage and the hydrodistillation time, which was an important finding as geraniol is typically reported as the main and most relevant component of *T. citriodorus* VO. Since the long distillation time is one of the reported disadvantages of the hydrodistillation method, the optimization of distillation times for particular species may aid in the optimization of energy expenditure, in addition to preventing unwanted chemical transformation. Also, as processing methods along with seasonal variations and other environmental factors can highly affect the composition of VOs, it can ultimately lead to the misidentification of chemotypes, so major attention must be drawn to these factors, highlighting the need for more studies.

Apart from the studies focusing on the chemistry, recent research has been focused on the study of the pharmaceutical/medicinal applications of *T. citriodorus*, by evaluating bioactive properties of its extracts or preparations. Despite several relevant activities were already reported that included antioxidant, antifungal, antibacterial, anti-biofilm, anti-inflammatory, anti-proliferative, cytoprotective activities, and anti-aging properties, data remains scarce, specifically when comparing with other species of the *Thymus* genus. There is also an active need for the pursing of different methods, approaching different endpoints more closely related to the *in vivo* application. This will also contribute to the scientific validation of some of the traditional applications described for *T. citriodorus*. Considering the safety assessment of *T. citriodorus* preparations, there is a major lack of information concerning this matter. The general idea that plant-based products are generally safer than their chemical counterparts could be one possible explanation for the small number of studies on this subject. Thus, it becomes essential to characterize the safety profile of these products, not only to the consumer, but also regarding environmental toxicity, as plant extracts can also have unknown effects on the environment and non-target organisms, despite their natural origin.

Conclusions

Despite not much information is currently available about the hybrid *T. citriodorus*, specifically when compared with other species from the *Thymus* genus, it has been gaining relevance among the scientific community with an increasing number of published studies focusing on its bioactive properties and, to a greater extent, on the chemical composition of its VOs and extracts.

The geraniol present in *T. citriodorus* is accountable for its characteristic mild and sweet floral odor, thus differentiating it from other *Thymus* species belonging to the thymol and carvacrol chemotypes. The high content in this specific monoterpene alcohol, which can reach levels as high as 80%, increases the value of this hybrid plant, as geraniol has a high commercial value. As a consequence, the majority of the published information referred to studies addressing its chemical composition and how external factors can influence the relative abundances of its bioactive compounds. Ultimately, by controlling growth conditions such as temperature, sunshine duration, and water supply, producers can ensure high qualitative and chemically homogeneous raw material, increasing its value, particularly for the pharmaceutical industry. Similarly, by optimizing extraction and process techniques for *T. citriodorus*, as hydrodistillation time, the production yield and chemical content can be maximized. Additionally, as collecting raw material from natural habitats significantly increases the risks inherent to chemical polymorphisms (a characteristic of *Thymus* species), the cultivation of *T. citriodorus*, eventually the most adapted cultivars, is recommended as it ensures qualitative and chemical homogeneity. This practice ultimately reduces the vulnerability of this hybrid plant. Finally, apart from the value associated with its geraniol content, studies addressing bioactivities from *T. citriodorus* preparations highlighted their diversified potential, showing promising results as cytoprotective, antioxidant, antiproliferative, anti-inflammatory, antibacterial, and antifungal agents. Additionally, and despite the little attention drawn to other applications as environmental-related ones, some interesting properties as bio-pesticidal and biofertilizer were already reported for *T. citriodorus*.

In conclusion, despite its major application in the food industry, the applications of *T. citriodorus* extend beyond its culinary value. *Thymus citriodorus* VO and/or extracts can grant a major interest to other fields and industries, specifically considering the high interest of pharmaceutical and cosmetic industries for plant sources with attractive lemon scents, contributing to its increasing popularity among medicinal and aromatic plant producers.

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