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Antimicrobial activity of essential oils from three wild Lamiaceae species in Lebanon - An *in vitro* and *in vivo* study

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Abstract: Many species belonging to the Lamiaceae family are commonly used in traditional Lebanese cuisine and medicine. The essential oils (EOs) obtained from these plants are rich in active compounds with potential applications in various fields including the food sector. Their strong ability to protect foods from pathogenic and decaying microorganisms has been documented. In this context, the effectiveness of EOs obtained from *Lavandula angustifolia* Mill., *Satureja thymbra* L. and *Thymbra capitata* (L.) Cav. (syn. *Coridothymus capitatus* Rchb.f.) collected in Lebanon was evaluated against eight bacteria (*Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus* and *Enterococcus faecalis*) and two yeasts (*Candida albicans* and *C. parapsilosis*) by *in vitro* test. *S. thymbra* showed the highest antibacterial activity followed by *T. capitata* and *L. angustifolia*. *P. aeruginosa* proved to be the most resistant microorganism. All three EOs were able to completely inhibit the *C. albicans* and *C. parapsilosis* growth. *S. thymbra* was further tested *in vivo* against *S. aureus* used to experimentally contaminate chicken breast samples where EO managed to significantly counteract its growth up to 72 (bacterial suspension at 3 log CFU/g) and 48 hours (bacterial suspension at 8 log CFU/g).

Keywords: *antifungal activity*, *aromatic plants*, *biopreservation*, *CFU*, *food pathogens*, *secondary metabolites*

INTRODUCTION

Despite technological development in food production, food safety remains a public health issue of great concern. It is estimated that 600 million people (7.7% of the world population) are affected every year by foodborne diseases causing 420,000 deaths (7.5% of total deaths) [Yu et al., 2022]. New methodologies are therefore necessary to make food products safer and increase the shelf life of the more easily perishable ones. Some plant products including essential oils (EOs) possess antimicrobial properties against a wide range of foodborne pathogens [Angane et al., 2022]. EOs are classified as GRAS (Generally Recognized as Safe) and could be used both as a source of natural preservatives and as additives in active food packaging [Bukvički et al., 2014; da Silva et al., 2021; Sharma et al., 2021; Frederico et al., 2023; Jackson-Davis et al., 2023]. Their role in maintaining the postharvest quality and preservation of various foods (dairy products, fruits, meat, seafood products) has been previously documented by *in vitro* and *in vivo* studies [Alfonzo et al., 2017; Jemaa et al., 2017; Nájera et al., 2021; Cao et al., 2022; Chacha et al., 2022; Vitalini et al., 2023].

EOs contain a wide variety of secondary metabolites such as mono- and sesquiterpenes, along with carbohydrates, phenols, alcohols, ethers, aldehydes, and ketones. Among these, oxygenated terpenoids and some hydrocarbons showed the greatest ability to inhibit or slow the growth of bacteria, yeasts and molds [Semeniuc et al., 2017; Freitas et al., 2018; Jugreet et al., 2020]. In particular, some components have been identified as effective antibacterial agents (e.g., carvacrol, thymol, eugenol, geraniol, terpineol, cinnamaldehyde). They are able to modify or destroy the membrane, the cytoplasm and the cell wall, in some cases completely altering the cell morphology [Nazzaro et al., 2013; Amiri et al., 2020; Angane et al., 2022].

In this work, the antimicrobial activity of EOs obtained from three wild Lebanese plant species [*Lavandula angustifolia* Mill., *Satureja thymbra* L. and *Thymbra capitata* (L.) Cav. (syn. *Coridothymus capitatus* Rchb.f.)], commonly used in local cuisine as a food or condiment as well as in folk medicine [Khoury et al.,

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2016], was firstly investigated against yeasts, Gram-positive and Gram-negative bacteria by in vitro test, then the antibacterial activity of the *S. thymbra* EO, the most active, was tested in vivo against *Staphylococcus aureus* on contaminated experimentally chicken.

MATERIAL AND METHODS

Plant material. The plant samples (flowering tops of *L. angustifolia*, *S. thymbra* and *T. capitata*) were collected in different areas in Lebanon (at 34°05'39"N 35°50'38.99"E, 34°04'24.34"N 35°39'19.03"E and 34°21'38.69"N 35°43'58"E GPS locations, respectively), then exsiccated in the shade at room temperature and stored in a dry place until use. They were determined by Prof. Marc El Beyrouthy (Department of Agriculture and Food Engineering, Holy Spirit University of Kaslik, Lebanon) according to Nouvelle Flore du Liban et de la Syrie [Mouterde 1983]. The herbarium voucher for each species (No. MNC121, MNV173a and MNV190a) was prepared and deposited at the Herbarium of Botany, Medicinal Plants and Malherbology, Faculty of Agronomy of USEK University, Lebanon.

Essential oils. EOs were previously obtained by hydrodistillation using a Clevenger-type apparatus and their constituents chemically characterized by GC-MS analysis [Iriti et al., 2014; Khoury et al., 2016].

Antimicrobial activity

Microorganism strains. EOs were tested against wild strains previously isolated from different food and non-food matrices and kept in Microbanks™ Freezer Storage Box at -20 °C belonging to the collection of the Department of Veterinary Medicine and Animal Sciences, (Milan State University), in particular four Gram-negative bacteria (*Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Proteus mirabilis*), four Gram-positive bacteria (*Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*, *Enterococcus faecalis*) and two yeasts (*Candida albicans*, *Candida parapsilosis*).

In vitro test. All eight bacterial samples were inoculated to brain-heart infusion (BHI Medium Oxoid, Milan, Italy) and incubated at 37 °C for 24 hours while the two yeasts were transferred to Sabouraud dextrose broth (SDB) (Merck, Darmstadt, Germany) and incubated at 30 °C for 5 days. The increase in bacterial growth within the broth was verified by visually estimating its degree of turbidity. Subsequently, BHI and SDB agar plates were prepared and inoculated by both streaking and pour plate method, with 0.1 ml of each strain suspension serially diluted. Finally, 1 µL of each EO was added. All plates were incubated in a thermostat at 37 °C for 24 hours (bacteria) and at 30 °C for 5 days (yeasts). This procedure was performed in duplicate for each microorganism with all EOs. Control plates were prepared without EO.

In vivo test. In this case, only *S. thymbra* EO was tested against a single microorganism (*S. aureus*). It was inoculated to BHI broth and incubated at 37 °C for 24 hours. The bacterial growth within the broth was verified by visually estimating its degree of turbidity. The diluted test solution was then inoculated to make the final level of 3 log CFU/g (representing a normal contamination) and 8 log CFU/g (simulating overstated contamination) in chicken breast purchased from the GDO and stored in the refrigerator until use. The test was set up in sanitized and hermetically sealed food containers. In detail, the samples were prepared in duplicate as follows: i) 3 x 25 g chicken breast (control; *S. aureus* absent) (A); ii) 3 x 25 g chicken breast experimentally contaminated with *S. aureus* at 3 log CFU/g (B); iii) 3 x 25 g chicken breast experimentally inoculated with *S. aureus* at 8 log CFU/g (C); iv) 3 x 25 g chicken breast experimentally contaminated with *S. aureus* at 3 log CFU/g + 10 µL of *S. thymbra* EO dropped on a sterile paper disc (6 mm Ø) (D); v) 3 x 25 g chicken breast experimentally contaminated with *S. aureus* at 8 log CFU/g + 10 µL of *S. thymbra* EO dropped on a sterile paper disc (6 mm Ø) (E). The containers were kept at 4°C to simulate the domestic conservation. Then, the *S. aureus* development was verified at 24 (T1), 48 (T2) and 72 (T3) hours on Baird Parker medium incubated at 37°C for 48 hours.

RESULTS

In vitro test. The ability of the three EOs to counteract the target microorganisms, all relevant for food safety and human health, is detailed in Table 1. EOs showed some variability in inhibiting microbial growth. *S. thymbra* demonstrated the highest antibacterial activity followed by *T. capitata* and *L. angustifolia*. It was the only one to succeed in reducing, even if partially, the development of *P. aeruginosa*, which proved to be the microorganism least sensitive to the action of EOs. *S. thymbra* EO, together with *T. capitata* EO, was also very effective against six other bacterial strains such as *E. coli*, *S. typhimurium*, *L. monocytogenes*, *S. aureus*, *P. mirabilis* and *B. cereus*, completely stopping their growth, regardless of the inoculation method (streak plate and pour plate). The method instead affected the activity of the two EOs against *E. faecalis*, which partially grew under the effect of *T. capitata* when streaked and *S. thymbra* when pour-

Table 2. *In vitro* inhibition of microbial growth by *Thymbra capitata*, *Lavandula angustifolia* and *Satureja thymbra* EOs.

Microorganisms	Strains	Essential oils	Streaking method	Pour plate method
Gram-negative bacteria	*Escherichia coli*	La	-	+/-
		St	-	-
		Tc	-	-
	Salmonella typhimurium	La	+/-	+/-
		St	-	-
		Tc	-	-
	Pseudomonas aeruginosa	La	+	+
		St	+/-	+
		Tc	+	+
	Listeria monocytogenes	La	-	+
		St	-	-
		Tc	-	-
Gram-positive bacteria	*Staphylococcus aureus*	La	+/-	-
		St	-	-
		Tc	-	-
	Proteus mirabilis	La	-	+/-
		St	-	-
		Tc	-	-
	Bacillus cereus	La	-	+/-
		St	-	-
		Tc	-	-
	Enterococcus faecalis	La	+/-	+
		St	-	+/-
		Tc	+/-	-
Yeasts	*Candida albicans*	La	-	-
		St	-	-
		Tc	-	-
	Candida parapsilosis	La	-	-
		St	-	-
		Tc	-	-

Note: La, *Lavandula angustifolia*; St, *Satureja thymbra*; Tc, *Coridothymus capitatus*; - = total inhibition of microbial growth; + = no inhibition of microbial growth; +/- = partial inhibition of microbial growth.

plated. The activity of *L. angustifolia* EO was irrelevant against *L. monocytogenes* and *E. faecalis* and partially incisive against pour-plated *E. coli*, *S. typhimurium*, *P. mirabilis*, *B. cereus*. Otherwise, it inhibited streaked *E. coli*, *L. monocytogenes*, *P. mirabilis*, *B. cereus* and pour-plated *S. aureus*. Lastly, all three EOs managed to be effective against *C. albicans* and *C. parapsilosis*, whose proliferation was totally stopped both when streaked and pour-plated.

In vivo test. The efficacy of *S. thymbra* EO was also assessed on chicken breast experimentally contaminated with *S. aureus* at 3 and 8 log CFU/g (samples D and E, respectively). After 24 hours (T1), in both cases, EO influenced bacterial growth, inhibiting it more evidently in sample E, whose concentration decreased to 3 log CFU/g (Fig. 1). After 48 hours (T2), *S. aureus* grew up to 5 log CFU/g, however still significantly lower than the values of the EO-free C sample (> 8 log CFU/g), while at 72 hours (T3), EO was totally ineffective (*S. aureus* > 8 log CFU/g) (Fig. 1).

As regards sample D, EO, after lowering at T1 the concentration of *S. aureus* (from 3 to 2 log CFU/g), reduced its effect, allowing the bacterium to return to the initial 3 log CFU/g, which remained constant until

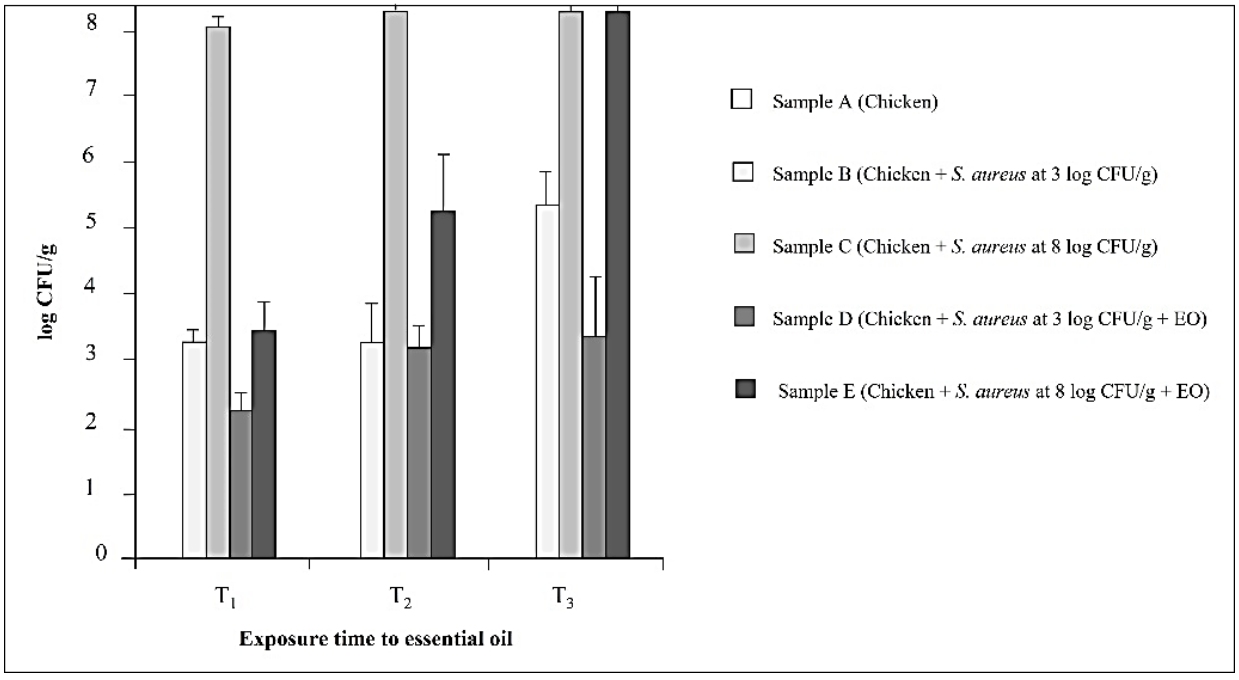

Figure 1. Antibacterial effect of *Satureja thymbra* essential oil on chicken breast experimentally contaminated with *Staphylococcus aureus*.

72 hours (T3) unlike sample B (without EO) where *S. aureus* proliferated reaching 5 log CFU/g (Fig. 1).

In general, EOs from *L. angustifolia*, *S. thymbra* and *T. capitata* demonstrated interesting antimicrobial potential in both *in vitro* and *in vivo* tests. All three EOs were predominantly composed of oxygenated monoterpenes (51.7% to 83.9%) [Iriti et al., 2014; Khoury et al., 2016] whose antibacterial and antifungal effects were previously reported [Kotan et al., 2007; Badawy et al., 2019; Guimarães et al., 2019]. The higher activity of *S. thymbra* EO was probably related to the presence of thymol (44.5%) and carvacrol (5.3%) with hydroxyl groups as main components [Guimarães et al., 2019]. These compounds are predicted to cause structural and functional damage in the cytoplasmic membrane and interact with membrane proteins and intracellular targets [Chouhan et al., 2017]. In *T. capitata* EO, piperitone (47.0%) was the most abundant constituent. It, together with thymol (19.9%), could be responsible for EO ability to inhibit microbial growth [Abdolpour et al., 2008; Rondón et al., 2016]. The different chemical composition of *L. angustifolia* EO may have resulted in its lower effectiveness, even if linalool, present in a high percentage (45.8%), is recognized as a strong antibacterial agent either alone or in combination. It is in fact capable of damaging the structure of the cell membrane and causing the leakage of cytoplasmic contents including alkaline phosphatase and macromolecules, such as DNA, RNA and proteins [Guo et al., 2021].

As expected, in *the vitro* test, Gram-negative bacteria were more resistant to EO activity than Gram-positive ones due to the different structure of their wall. In fact, the former have hydrophilic cell walls made up of peptidoglycan and lipopolysaccharides which prevent the penetration of hydrophobic substances while the latter are surrounded by much thicker layers of peptidoglycan forming a structure favorable for lipophilic compounds [Silhavy et al., 2010; Nazzaro et al., 2013; Amiri et al., 2020].

The lower activity of *S. thymbra* EO in the *in vivo* test compared to its bactericidal activity recorded in the *in vitro* test was in agreement with literature data on the interactions of EOs and their components with food matrices including meat and meat products highly susceptible to microbial spoilage. The presence of fats, carbohydrates, proteins, and salts in such food systems may be responsible for decreasing the effectiveness of EOs [Jayasena and Cheorun, 2013]. Their antimicrobial potency also depends on pH, temperature and level of contamination [Hyldgaard et al., 2012].

To conclude, although plant-derived EOs are known for their antimicrobial properties, commercial-scale application in protecting foods from pathogenic and decaying microorganisms is still minimal due to their

intense aroma that can change the flavor of foods [Calo et al., 2015; Falleh et al., 2020; Al-Maqtari et al., 2022; Angane et al., 2022]. However, these problems can be solved, in particular through hurdle technology which involves, for example, the incorporation of EO volatile compounds into edible films or coatings and the encapsulation of EOs in nanoemulsions [Aslam et al., 2021; Maurya et al., 2021; Pandey et al., 2022]. Otherwise, a synergistic effect between EO and other antimicrobial compounds or emerging preservative technologies may decrease the EO concentrations while still improving food safety [de Souza Pedrosa et al., 2021].

Lastly, these preliminary data confirm that the use of EOs, including *S. thymbra*, as potential alternatives to conventional preservatives to extend the food shelf life and fight foodborne pathogens, remains a focal area of study in order to meet the growing interest of the food industry and consumer demand for natural and safe products. Specifically, *S. thymbra* will require other studies that further evaluate some variables such as the concentration of contaminating microorganisms and the possible enterotoxin production, the quantity and formulation of EO to be used as well as the application method in an attempt to improve its performance as an agent natural and green additive acceptable from an organoleptic point of view.

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Livanda üç yabanı Lamiaceae növünün efir yağlarının mikrob əleyhinə aktivliyi – *in vitro* və *in vivo* tədqiqat

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Lamiaceae fəsiləsinə aid bir çox növ ənənəvi Livan mətbəxində və təbabətində geniş istifadə olunur. Bu bitkilərdən əldə edilən efir yağları (EY) aktiv birləşmələrlə zəngindir və müxtəlif sahələrdə, o cümlədən qida sənayesində istifadə oluna bilər. Onların qida məhsullarını patogen və çürüyən mikroorqanizmlərdən güclü qorumaq qabiliyyəti sənədləşdirilmişdir. Bu çərçivədə Livanda toplanan *Lavandula angustifolia* Mill., *Satureja thymbra* L. and *Thymbra capitata* (L.) Cav. (syn. *Coridothymus capitatus* Rchb.f.) növlərindən əldə edilən EY-nın effektivliyi səkkiz bakteriyaya (*Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus* and *Enterococcus faecalis*) və iki mayaya (*Candida albicans* və *C. parapsilosis*) qarşı *in vitro* testlə qiymətləndirilmişdir. *S. thymbra* ən yüksək, daha sonra *T. capitata* və *L. angustifolia* antibakterial aktivklik göstərib. *P. aeruginosa* növü isə ən davamlı mikroorqanizm kimi müəyyən edilib. Hər üç EY-ları *C. albicans* və *C. parapsilosis* növlərinin böyüməsinə tamamilə inhibə edib. *S. thymbra*, toyuq döş nümunələrini eksperimental yoluxdurmaq üçün istifadə edilən *S. aureus* növünə qarşı *in vivo* sınaqdan keçirilib, burada EY onun böyüməsini 72 (3 log KƏV/q-da bakterial suspenziya) və 48 saatadək (8 log KƏV/q-da bakterial suspenziya) əhəmiyyətli dərəcədə dayandırıb.

Açar sözlər: *göbələk əleyhinə aktivlik; aromatik bitkilər; biomühafizə; KƏV, qida patogenləri; ikincil metabolitlər*

Антимикробная активность эфирных масел трех дикорастущих видов Lamiaceae в Ливане – исследование *in vitro* и *in vivo*

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Многие виды, принадлежащие к семейству Lamiaceae, широко используются в традиционной ливанской кухне и медицине. Благодаря богатому

компонентному составу эфирные масла (ЭМ), полученные из этих растений, могут применяться в различных областях, в том числе в пищевой промышленности. Документально подтверждена высокая активность ЭМ по отношению к патогенным микроорганизмам, т.е. они способны защищать пищевые продукты от разлагающихся микроорганизмов. В этом контексте методом *in vitro*, эффективность ЭМ растений *Lavandula angustifolia* Mill., *Satureja thymbra* L. и *Thymbra capitata* (L.) Cav. (син. *Coridothymus capitatus* Rchb.f.), собранных в Ливане, оценивали по отношению к 8-ми бактериям (*Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus* и *Enterococcus faecalis*) и к двум дрожжевым грибкам (*Candida albicans* и *C. parapsilosis*). ЭМ *Satureja thymbra* показало самую высокую антибактериальную и антифунгальную активность, далее следуют ЭМ *Thymbra capitata* и *Lavandula angustifolia*. Отметим, что ЭМ трех исследуемых видов полностью ингибировать рост *C. albicans* и *C. parapsilosis*. Однако высокую устойчивость к этим ЭМ проявила тест-культура *Pseudomonas aeruginosa*. ЭМ *Satureja thymbra* была дополнительно протестирована *in vivo* относительно к тест-культуре *Staphylococcus aureus*, которую использовали для экспериментального заражения образцов куриной грудки. В результате ЭМ *S. thymbra* проявило высокую ингибирующую активность, подавляя рост тест-культуры до 72 (бактериальная суспензия при 3 log КОЕ/г) и 48 часов (бактериальная суспензия при 8 log КОЕ/г).
Ключевые слова: *противогрибковая активность*, *ароматические растения*, *биоконсервация*, *КОЕ*, *пищевые патогены*, *вторичные метаболиты*