Chemical composition and bioactivity of essential oils from flower and fruit of *Thymbra capitata* and *Thymus* species

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Abstract *Thymbra capitata* and *Thymus* species are commonly known as thyme in Spain and they are currently used as culinary herbs, as well as for ornamental, aromatizing and traditional medicinal purposes. Given the economic importance of thyme oils, many thyme species have been studied, and their essential oils (EOs) and other volatile-containing extracts have been chemically characterized. The aim of this study was to evaluate the antioxidant, antimicrobial and cytotoxic activities as well as the phytochemical composition of the EOs from fruits and flowers of *T. capitata* and *Thymus* species. High antioxidant capacity has been observed and related to the relative amounts of terpenoid and phenolic with good antioxidant properties. The antioxidant activities decreased as follows: TAC > TZ > TC > TM. The results of antimicrobial activity showed that all tested EOs were active against all tested microbial species, including Gram-positive and negative bacteria. The minimum inhibition concentration values obtained ranged from 0.1 to 0.01 $\mu\text{L/mL}$. Furthermore, all EOs assayed induced cell death in both human epitheloid cervix carcinoma and histiocytic leukemia cell lines. Overall, the results of this study indicates that the EOs from fruits and flowers of *T. capitata* and *Thymus* species posses interesting antioxidant properties and

represent a potential source of medicine for the treatment of infectious diseases and cancer.

Keywords Essential oils · Antioxidant · Antimicrobial · Cytotoxicity · *Thymus* · *Thymbra*

Introduction

Bioactive substances derived from foods and plants have recently attracted much attention with regard to human health, which is due to their low toxicity, limited costs, and broad availability. Moreover, extraction is one of the most widely used unit operations in the food industry. It is mainly used for obtaining certain desired bioactive components initially retained in a food matrix (Pinelo et al. 2005). In this context, essential oils (EOs) obtained from plants contain a large and complex variety of compounds, mostly hydrocarbons and oxygenated-types, with potential antioxidant, anticancer and antimicrobial properties that may be utilized in pharmaceutical, food, cosmetic, and other industries (Miguel 2010b). EOs are generally derived from one or more plant parts, such as flowers, leaves, stems, bark, wood, roots, seeds, fruits, rhizomes and gums or oleoresin exudations.

Thyme is the common name of many taxa belonging to the *Thymbra* and *Thymus* genera. *Thymbra* and *Thymus* species are frequent in the west Mediterranean region, which is considered to be the centre of origin of the genus *Thymus*. These species have extended further westwards from the Iberian Peninsula and northwest Africa to the Macaronesian region in the Atlantic Ocean (Figueiredo et al. 2010).

EOs can be defined as volatile products of complex nature, produced by certain vegetables to confer their pleasant characteristic aroma. Officially, EOs are known

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products that can be obtained by dragging current of steam or by expression of pericarps in certain fruits. Usually, they are known as essences, although this denomination is broader because it encompasses other substances obtained by diverse extraction methods (Bruneton 2001). Chemical compounds of EOs depend on several factors. Firstly, the botanic origin, because every species has different chemical compounds, and even in the same species it could be found several chemical races (chemotypes). Quantitative and qualitative characteristics of species vary according to vegetative cycle. Lastly, it is important to note that extraction can alter EO composition with respect to vegetal origin (Bruneton 2001).

EOs are used in a wide variety of consumer goods such as detergents, soaps, toilet products, cosmetics, pharmaceuticals, perfumes, confectionery food products, soft drinks, distilled alcoholic beverages (hard drinks) and insecticides. World production and consumption of EOs and perfumes are increasing very fast. Production technology is an essential element to improve the overall yield and quality of EO. The traditional technologies pertaining to EO processing are of great significance and are still being used in many parts of the globe. Water distillation, water and steam distillation, cohabitation and maceration are the most traditional and commonly used methods. Thus, hydrodistillation is one of the less drastic processes for EOs obtaining. It could induce esters hydrolysis and reunification, isomerizations, razemisations, or oxidations, which are caused by water, acids or temperature of this process. Furthermore, the state of extraction of prime material could influence composition, highlighting the importance of storage and preparation of vegetal material.

Based on the properties of EO, different uses have been developed in several fields. Food industry has applied EOs in preservation of products due to their antiseptic properties (butter, fruits, meat, etc.) or as dressing (liquors, olives, meat, fish, and natural antioxidant for olive oil). Cosmetic industry also includes the use of these products in perfumes and creams. Knowledge acquired along the time on EO has been applied for seeking pharmacological utilities. Among them, it is important to underline its antiseptic, burnt-healing, spasmolytic, anti-inflammatory and antimutagenic activities. For that reason, the aim of this work has been to characterize the chemical composition, antioxidant activity, cytotoxic activity and antimicrobial activity of four species of *Thymbra* and *Thymus* spp.

Materials and methods

Biological material

Samplings of fruits and flowers of various species of *Thymus* and *Thymbra* (TC: *Thymus caespititius*; TAC:

Thymbra capitata; TM: *Thymus mastichina*; TZ: *Thymus zygis*) were picked up from Badajozs province during 2012 season. The UTM coordinates of the central area were $x = 702,192.29$, $y = 4,303,406.71$. The climate of the area is Mediterranean; the average annual rainfall was 404 mm, mostly distributed outside of a 4-months summer drought period. The samplings were carried out in the morning, taking samples randomly, in different parts of the plants with three repetitions. After harvesting, all samples were immediately transported to the Technological Institute of Food and Agriculture of Extremadura (INTAEX) in ventilated storage trays to avoid compositional changes. The plant materials were dried in a dark room and, after that, they were stored in paper bags until study. Herbarium sheets of each studied populations were also stored. Dried fruits and flowers were ground in a knife mill. Then, powdered samples were sieved to select particles between 0.50 and 3.0 mm, and they were stored at room temperature in vacuum conditions until further use.

Bacterial cultures were obtained from the Spanish type culture collection (CECT) of Valencia University. The following bacterial strains were used in the screening of the antimicrobial spectrum of the essential oil: *Listeria innocua* 910, *Staphylococcus aureus* subsp. *aureus* 976, *Salmonella enterica* subsp. *enteric* 409, *Escherichia coli* 45 and *Bacillus cereus* 495.

Human epitheloid cervix carcinoma (HeLa) cell line (ECACC No. 93021013; Dorset, UK) was grown in Dulbecco's Modified Eagle medium (DMEM; Lonza, Basel, Switzerland) supplemented with 2 mM L-glutamine (Lonza), 1 mM sodium pyruvate (Lonza), 10% heat-inactivated fetal bovine serum (Lonza), 100 U/mL penicillin, and 10 µg/mL streptomycin. Human histiocytic leukemia (U937) cell line (ECACC No. 85011440) was grown in RPMI 1640 medium (Lonza) supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were cultured in a humidified atmosphere containing 5% CO₂ at 37 °C.

Essential oils extraction

The EOs were produced separately from fruits and flowers by distillation according to the European Pharmacopoeia (Council of Europe. 1997). In all of them, water is heated to produce steam, which carries the most volatile chemicals of the aromatic material with it. The steam is then chilled (in a condenser) and the resulting distillate is collected. The EOs were floated on top of the hydrosol (the distilled water component) and were separated off. The oils were stored away from the light in amber-coloured glass bottles at 4 °C until analysis (within 1 month).

Chemicals

For antioxidant activity determination, the standard antioxidant used was 6-hidroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) from Sigma–Aldrich (Steinheim, Germany) and 2,2'-azobis-(3-ethylbenzothiazoline-6-sulfonic acid; ABTS) was from Fluka Chemicals (Madrid, Spain).

Chemical composition determination

The analysis of the volatile components of the EOs of *T. capitata* and *Thymus* species were performed with a Hewlett Packard 6890 gas chromatograph equipped with a flame ionization detector (FID) and a 30 m × 0.32 mm × 0.25 μm (film thickness) of 5% phenyl-methyl-silicone column. The injection volume was 3 μL of essential oil, in split mode (12:9) and the injector temperature was kept at 250 °C. The oven temperature was programmed at 3 °C/min from 70 to 240 °C and then held it for 2 min. Carrier gas flow is nitrogen at 0.4 mL/min with 2.5 psi of pressure. The identification of individual constituents was accomplished by comparing their retention indices with those of authentic standards.

Antioxidant activity assay

For the sample preparation, an aliquot of filtered EOs were weigh (2.5 g) and then, was dissolved in 6 mL of n-hexane. A diol-bonded phase cartridge (Supelco Co., Bellefonte, PA, US) was placed in a vacuum elution apparatus and conditioned by the consecutive addition of 6 mL of methanol and 6 mL of n-hexane. The oil solution was applied to the column, and the solvent was pulled through, leaving the sample on the solid phase. The sample container was washed with 6 mL of n-hexane (2 × 3 mL), which were run out of the cartridge. The sample container was washed again with 4 mL of n-hexane/ethyl acetate (85:15, v/v), which were run out of the cartridge and discarded. The column was eluted with 15 mL of methanol and the solvent was evaporated in a rotary evaporator at room temperature and low speed at reduced pressure until dryness. The phenolic residue was dissolved in 2 mL of methanol (Mateos et al. 2001).

The capacity of radical scavenging of the oil samples were assessed by the ABTS.+ method (Cano et al. 2000). Briefly, 1 mL of the radical cation ABTS (2,2'-azinobis (3-ethylbenzothiazolone 6-sulphonate)) was placed in a spectrometric cuvette and 20 μL of the phenolic extract were added. The initial absorbance value at 730 nm was measured by using an UV–Vis spectrophotometer model HP8453 (Agilent, Madrid, Spain) and then compared with the absorbance obtained after 20 min of reaction. The

results were expressed as a TAC (antioxidant equivalent Trolox) value as g Trolox/L essential oil on a calibration curve using different Trolox concentration.

Antimicrobial activity assay

The antimicrobial activity values were studied for the EOs following the procedure previously established by Delgado Adámez et al. with some minor modifications (Delgado Adámez et al. 2012). The EOs were first dissolved in 10% DMSO and then diluted to the highest concentration (0.1% v/v) to be tested, and were serially diluted from 0.1 to 0.001% (v/v). The target microorganisms were cultured in Mueller–Hinton broth (MHB) at 37 °C for 24 h. To measure the CFU, 180 μL of MHB containing ~10⁵ CFU/mL diluted bacteria (1/1000 ratio in McFarland standard turbidity) plus 20 μL of EO solutions were added per well in a 96-well plate. Then, the microplates were incubated at 37 °C for 24 h under aerophilic conditions. Absorbances at 450 nm were measured at 0 and 24 h in a plate reader. Turbidity readings were related to bacterial growth. The inhibitory effect was calculated using the following formula:

$$\% \text{inhibition} = \frac{\Delta \text{Abs}_{\text{Reference}} - \Delta \text{Abs}_{\text{Assay}}}{\Delta \text{Abs}_{\text{Reference}}} \times 100$$

where:

$\Delta \text{Abs}_{\text{Reference}}$ is the increase in absorbance of control sample.

$\Delta \text{Abs}_{\text{Assay}}$ is the increase in absorbance of test sample.

Cytotoxicity assay

The cytotoxic effects of the different EOs on two human tumor cell lines were assayed by the MTT assay (Mosmann 1983). The cells were seeded at a density of 5 × 10⁴ cells/well. The EOs were serially diluted in 1% dimethyl sulfoxide (DMSO) from 0.1 to 0.001% (v/v), and 5 μL liquid of each concentration was applied to the wells of a 24-well plate containing sub-confluent cell cultures. DMSO was used as a vehicle and the final DMSO concentration did not exceeded 0.1% (v/v). After 24 h of incubation, MTT solution (5 mg/mL) was then added to each well, and the formazan precipitate was dissolved in 200 μL DMSO after 1 h of incubation at 37 °C. The content of the wells was homogenized on a microplate shaker for 5 min. The optical densities (OD) were measured on a microplate reader (Infinite M200; Tecan Austria GmbH, Groedig, Austria) at a test wavelength of 490 nm and a reference wavelength of 650 nm to cancel out the effect of cell debris. All tests and analyses were run in duplicate and mean values were recorded. The cell survival curves were calculated as percentage of control values (untreated samples).

Statistical analysis

For statistical studies, SPSS 17.0 software was used (SPSS Inc. Chicago, IL, USA). Data are expressed as mean $\pm$ standard error of the mean (S.E.M.) and were analyzed using a one-way analysis of variance (ANOVA) followed by Tukey's multiple range test. To evaluate the effects of phenological stage on chemical composition and anti-cancer, antioxidant and antimicrobial activities of the essential oils, paired student t-test was used. $P < 0.05$ was considered to indicate a statistically significant difference.

Results and discussion

Evaluation of the chemical composition of the EOs

Determination of compounds was higher than 70% in all the samples, reaching determination rates higher than 98% for *T. capitata*, 96% for *T. mastichina* and 91% for *T. zygis* (Table 1).

The main compounds vary depending mainly on species, being the most abundant molecules: carvacrol in the case of *T. capitata* (with values around 75%); α -terpineol for *T. caespitius* (with values of $42.44 \pm 1.55\%$ and $53.20 \pm 0.84\%$ for Eos from flower and fruit respectively); limonene + 1-8 cineole for *T. mastichina* (reaching $78.37 \pm 1.26\%$); and thymol and p-cymene for *T. zygis* (Table 1).

In general, chemical composition of EOs depends on the species, however, some intergenus differences may be suggested based on the fact that β -caryophyllene oxide, α -pinene, limonene + 1-8 cineole and α -terpineol percentage determined in *T. capitata* were statistically different to those in the rest of samples (all belonging to *Thymus* Genus). Whereas the differences due to phenological stage also vary depending on species, γ -terpinene determination varies in all the species between flower and fruit, being the influence of phenological stage specially marked in *T. zygis*.

Carvacrol can be denominated as a functionalized terpene, whose function is phenolic. Due to observed properties in EOs and its structure, carvacrol has been tested in several experiments, confirming its antimicrobial (Klein et al. 2013), antifungal (Abbaszadeh et al. 2014), and antioxidant activities (Kumar and Rawat 2013). In some studies carried out in plants from Iberian Peninsula α -terpinene has been determined as the main compound for *T. caespitius* (Miguel et al. 2004). Moreover, 1,8-cineole, also known as eucalyptol, is characterized as an oxygenated monoterpene with ether function. A long list of properties has been recognized for this compound. Among them, antimicrobial (de Sousa et al. 2015) and antifungal

capacities (Vilela et al. 2009) have been identified. It has been also tested as insecticide (Kumar et al. 2013), and possesses anti-inflammatory capacity (Ehrnhöfer-Ressler et al. 2013). Despite the fact that elevated values of thymol are uncommon in literature, Gonçalves and co-workers found high values of thymol also in *T. zygis*, mainly the chemotypes thymol, linalool/1,8-cineole and carvacrol (Gonçalves et al. 2010). Thymol has antimicrobial, anti-fungal, antioxidant, and anti-inflammatory capacities, and also can activate or inactivate proteins. Regarding the stage of the vegetative cycle, Miguel and colleagues reported that there are not main differences in the chemical composition between May and December (Miguel et al. 1999).

Evaluation of the antioxidant activity of the EOs

Antioxidant activity of the EOs from different parts of *T. capitata* and *T. species* is summarized in Fig. 1. The EOs exhibited varying degrees of scavenging ability, regardless of the phenological stage of the plant. The free radical scavenging activity of EO ranged from 0.75 ± 0.06 to 845.53 ± 6.77 g Trolox/L EO (Fig. 1). It was found that two out of four species (*T. zygis* and *T. capitata*) contained more than 650 g Trolox/L EO, while the other two species (*T. caespitius* and *T. mastichina*) were below 4 g Trolox/L EO. Among the eight EOs examined in this study, *T. capitata* fruit had the highest reducing power, while *T. mastichina* fruit had the lowest. Overall, the specific chemical composition of the EOs is presumably responsible for the variations in their antioxidant properties. Since there are a large number of different types of antioxidant compounds that might contribute to the total antioxidant capacity, it is not clear which components are responsible for the observed antioxidant capacity. The structure–activity relationships of compounds present in these species require further investigation. Thus, the terpenoid and phenolic compounds present in the EOs are closely associated with their antioxidant function, mainly due to their redox properties. Such properties may be exerted by various possible mechanisms: free radical-scavenging activity, hydrogen donors, transition metal chelating activity, and/or singlet oxygen quenching capacity (Miguel 2010b). However, the underlying action mechanism is still not clearly understood. On one hand, Amensour and colleagues have suggested that the antioxidant activity of EOs is mainly due to their redox properties, which play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Amensour et al. 2009). On the other hand, some works have also shown that the main components have less activity than the EO and suggested that the constituents of EO may act synergistically to scavenge free radicals (Amorati et al. 2013).

Table 1 Chemical composition of essential oils from *Thymbra capitata* and *Thymus* species at different phenological stages

	<i>Thymbra capitata</i>		<i>Thymus caespitius</i>		<i>Thymus mastichina</i>		<i>Thymus zygis</i>	
	Flower	Fruit	Flower	Fruit	Flower	Fruit	Flower	Fruit
α -pinene	0.82 $\pm$ 0.15 ²	0.99 $\pm$ 0.11 ^b	2.25 $\pm$ 0.18 ¹	2.32 $\pm$ 0.25 ^a	0.27 $\pm$ 0.08 ³	0.27 $\pm$ 0.07 ^c	0.45 $\pm$ 0.13 ³	0.43 $\pm$ 0.06 ^c
Camphene	0.87 $\pm$ 0.11 ³	1.00 $\pm$ 0.07 ^{bc}	0.94 $\pm$ 0.11 ³	0.78 $\pm$ 0.10 ^c	5.15 $\pm$ 0.28 ^{1A}	3.39 $\pm$ 0.19 ^{ab}	1.38 $\pm$ 0.10 ²	1.18 $\pm$ 0.14 ^c
β -myrcene	1.61 $\pm$ 0.37 ³	1.87 $\pm$ 0.12 ^b	3.17 $\pm$ 0.24 ^{2A}	2.25 $\pm$ 0.09 ^{bb}	9.81 $\pm$ 0.70 ^{1A}	5.69 $\pm$ 0.33 ^{ab}	2.87 $\pm$ 0.08 ^{2A}	1.36 $\pm$ 0.13 ^{cb}
Limonene + 1-8 cineole	nd ³	nd ^c	1.33 $\pm$ 0.14 ²	0.88 $\pm$ 0.25 ^b	71.82 $\pm$ 1.22 ¹	78.37 $\pm$ 1.26 ^a	1.50 $\pm$ 0.24 ^{2A}	0.90 $\pm$ 0.10 ^{bb}
α -terpinene	1.53 $\pm$ 0.10 ^{1A}	1.09 $\pm$ 0.10 ^{ab}	nd ²	nd ^b	nd ²	nd ^b	nd ²	nd ^b
p-cymene	7.29 $\pm$ 0.31 ^{3B}	9.10 $\pm$ 0.05 ^{bA}	10.66 $\pm$ 0.52 ²	10.02 $\pm$ 1.82 ^b	nd ⁴	nd ^c	22.56 $\pm$ 1.35 ^{1B}	29.68 $\pm$ 1.10 ^{aA}
γ -terpinene	4.83 $\pm$ 0.25 ^{3A}	4.14 $\pm$ 0.31 ^{abb}	6.15 $\pm$ 0.52 ^{2A}	3.54 $\pm$ 0.21 ^{bb}	0.53 $\pm$ 0.04 ^{4B}	0.66 $\pm$ 0.04 ^{cA}	11.31 $\pm$ 0.64 ^{1A}	4.53 $\pm$ 0.35 ^{ab}
Linalool	3.38 $\pm$ 0.47 ¹	3.25 $\pm$ 0.17 ^a	0.74 $\pm$ 0.06 ²	0.91 $\pm$ 0.22 ^b	3.37 $\pm$ 0.21 ^{1A}	1.30 $\pm$ 0.31 ^{bb}	1.50 $\pm$ 0.40 ²	1.37 $\pm$ 0.14 ^b
Terpinen-4-ol	0.39 $\pm$ 0.20 ³	0.21 $\pm$ 0.08 ^d	1.34 $\pm$ 0.12 ²	1.39 $\pm$ 0.06 ^b	2.34 $\pm$ 0.26 ¹	2.15 $\pm$ 0.18 ^a	0.91 $\pm$ 0.12 ²³	0.88 $\pm$ 0.12 ^c
α -terpineol	0.88 $\pm$ 0.25 ³	0.82 $\pm$ 0.09 ^c	42.44 $\pm$ 1.55 ^{1B}	53.20 $\pm$ 0.84 ^{aa}	5.32 $\pm$ 0.56 ²	5.05 $\pm$ 0.16 ^b	tr ⁴	tr ^d
Carvacrol	75.51 $\pm$ 1.58 ¹	74.27 $\pm$ 2.00 ^a	0.32 $\pm$ 0.10 ³	0.63 $\pm$ 0.22 ^b	nd ⁴	nd ^c	4.29 $\pm$ 0.20 ^{2A}	1.08 $\pm$ 0.14 ^{bb}
β -caryophyllene oxide	1.29 $\pm$ 0.15 ¹	1.36 $\pm$ 0.10 ^a	nd ²	nd ^b	nd ²	nd ^b	nd ²	nd ^b
Thymol	nd ³	nd ^c	0.82 $\pm$ 0.12 ²	0.92 $\pm$ 0.09 ^b	nd ³	nd ^c	45.09 $\pm$ 3.92 ^{1B}	52.70 $\pm$ 2.32 ^{ab}
% total	98.41	98.09	70.16	76.84	98.59	96.89	91.87	94.11

Values were expressed as mean $\pm$ SEM. nd Not detected. tr. trace (<0.01). Within each column, values followed by the same capital letter in a column did not share significant differences at 5% (Student's t-test). Within each row, values followed by the same lowercase letter or number did not share significant differences at 5% (Tukey's test)

Fig. 1 Total antioxidant capacity determined by ABTS+ method of the essential oils from different *Thymbra capitata* and *Thymus* species parts according to organs and phenological stages. *TC* *Thymus caespititius*, *TAC* *Thymbra capitata*, *TM* *Thymus mastichina*, *TZ* *Thymus zygis*. Values expressed are mean $\pm$ SEM of six experiments. Values with the same lowercase letter or number are not significantly different (ANOVA, $P < 0.05$); values with the same capital letter are not significantly different (Student's t-test, $P < 0.05$)

The major components identified in these eight EOs (Table 1) have reported antioxidant properties. Carvacrol and thymol were the main compounds found in EOs from *T. capitata* and *T. zygis*, respectively. Different studies evaluated the antioxidant activity of the volatile components present in the EOs assayed in the present study (Dorman et al. 2000). Thymol (1702.04 mg Trolox/100 mL) and carvacrol (1451.74 mg Trolox/100 mL) showed the highest antioxidant activity. The inhibition of oxidation by the EOs from plants of species was highly dependent on the content of carvacrol + thymol (Quiroga et al. 2015). In contrast to these two compounds, linalool (357.67 mg Trolox/100 mL), terpinen-4-ol (190.72 mg Trolox/100 mL) and sabinene (369.44 mg Trolox/100 mL), the main compounds in EOs from *T. mastichina* and *T. caespititius*, are compounds that exhibit lower antioxidant capacity compared to carvacrol and thymol (Dorman et al. 2000; Ruberto and Baratta 2000). Limonene, the major component of *T. mastichina* EOs, is a monocyclic terpene consumed by humans, e.g., in citrus fruit, carrots, coffee, orange, and nutmeg. Despite the fact that it is an isomer of terpinolene, α - and γ -terpinene (position of a double-bond), limonene curiously showed a moderate-to-low antioxidant activity in Ruberto's study (Ruberto and Baratta 2000; Fig. 1). By comparison with the structure of germacrene-d, it appears that conjugation of the extracyclic methylene could be required for efficient radical scavenging; however, terpinolene and γ -terpinene are effective antioxidants despite non-conjugated double-bonds. Antioxidant properties have indeed been shown for limonene (Limem-Ben Amor et al. 2008). EOs of *T.*

caespititius with α -terpineol as the major compound also showed radical-scavenging activity (Miguel 2010a).

The present results are in accordance with those of other authors who demonstrated that the antioxidant effect of aromatic plants is due to the presence of hydroxyl groups in their phenolic compounds, and that the number and pattern of hydroxyl groups are associated with the highest antioxidant activity (Heim et al. 2002).

Evaluation of the antimicrobial activity of the EOs

The antibacterial activity of EOs was assayed in vitro by a micro-dilution method against both Gram-positive and Gram-negative bacteria in order to determine the MIC, which is the lowest concentration inhibiting the growth of at least 90% of microbial strain (Fig. 2). All EOs analyzed showed antimicrobial activity against all bacteria. *T. zygis* EO showed the strongest antibacterial activity, while *T. mastichina* exhibited the lowest antibacterial potential. The oils have low activity on the growth of *S. enterica* subsp. *enterica*, which was only inhibited at high concentration, whereas the strongest inhibitory effect was achieved against *L. innocua*. The results were in concordance with the ones already published that showed that *T. capitata* and *Thymus* species has antibacterial activity against both Gram-negative and Gram-positive bacteria (Hosseini Behbahani et al. 2013).

Figure 2 presents the variation of the inhibition capacity with the concentration of EO in the medium. It can be seen that the inhibition percentage increases with EO concentration. For *L. innocua*, *S. aureus* subsp. *aureus*, *B. cereus* and *E. coli* the inhibitory effect of thyme oils were concentration-dependent. Statistically significant differences ($P < 0.05$) existed between all three doses used. In the case of *S. enterica*, differences were not significantly different ($P > 0.05$) between 0.01 and 0.001 μ L/mL, but were substantially distinct ($P < 0.05$) between them and 0.1 μ L/mL doses.

Antimicrobial activity of thyme oils can be justified by their main components, which are summarized in Table 1. Jaberian and co-workers reported that alkaloid and phenolic compounds in plants could be responsible for antibacterial and antioxidant activities (Jaberian et al. 2013). Since the EOs are complex and impure mixtures, their biological activities vary in accordance with the compounds that they include; the amount of small compounds should not be neglected. Interestingly, biological activity could be more powerful as a result of the synergistic effect that is formed by the compounds coming together. However, sometimes the antagonistic effect leading to lower activity can also be observed. The high percentage of thymol in *T. zygis* and carvacrol in *T. capitata* could be responsible for the antimicrobial activity of these plants. Several authors have claimed that the major

Fig. 2 Minimal inhibitory concentration (MIC) of *Thymbra capitata* and *Thymus* species essential oils at flowering and fruit maturation stage. TC *Thymus caespititius*, TAC *Thymbra capitata*, TM *Thymus mastichina*, TZ *Thymus zygis*. Values expressed are mean $\pm$ SEM of

six experiments. Values with the same lowercase letter or number are not significantly different (ANOVA, $P < 0.05$); values with the same capital letter are not significantly different (Student's t-test, $P < 0.05$)

component of thyme EO is thymol (Viuda-Martos et al. 2011), and the antimicrobial activity of this compound has been confirmed on bacteria such as *E. coli*, *Pseudomonas* spp., *Shigella flexneri* and *Bacillus cereus* (Marchese et al. 2016). Thymol has been shown to cause disruption of the cellular membrane, inhibition of ATPase activity, and release of intracellular ATP and other constituents (Marchese et al. 2016). Similarly, carvacrol is indicated as a high antibacterial and antifungal compound.

Results presented in Fig. 2 showed that, among the five bacterial strains tested, *B. cereus*, *L. innocua* and *S. aureus* (Gram-positive) seems to be the most sensitive to the EO in terms of inhibition and MIC. The type of bacteria also has an influence on the effectiveness of the EOs. Gram-negative bacteria, in this case *S. enterica* and *E. coli*, were generally less susceptible than Gram-positive. The difference in susceptibility of the bacteria to an EO is thought to arise as a result of the differences in their cell membrane structure (Gilles et al. 2010). The cell envelopes of Gram-negative bacteria are more complex than the cell wall of Gram-positive bacteria. This phenomenon lies in the presence of external membrane structure which is particularly impermeable to EO molecules and the action of efflux mechanisms which results in protecting the bacteria against the action of EOs (Nazzaro et al. 2013).

Evaluation of the cytotoxic activity of the EOs

To investigate the cytotoxic activities, two human tumor cell lines, HeLa (adherent cells) and U937 (free-floating cells), were exposed to increasing concentrations of the various EOs. Cell viability was determined by the MTT assay. As shown in Fig. 3, the EOs revealed different cytotoxic activities towards the two human cancer cell lines investigated. In general, a dose-dependent decrease in the survival of both tumor cell lines was observed after treatment with all the EOs tested. At the highest concentration (0.1%, v/v), all EOs exhibited a substantial, statistically significant ($P < 0.05$) cytotoxic effect. Especially, *T. zygis* flower and fruit EOs caused the strongest cytotoxicity to HeLa (adherent) cells, thus reducing the cell viability by 82 and 75%, respectively. Similarly, *T. capitata* flower and fruit EOs as well as *T. caespititius* flower EO produced the most powerful cytotoxic effect to U937 (free-floating) cells, thereby decreasing the cell viability by 82, 80, and 78%, respectively. The intermediate concentration (0.01%, v/v) was partially efficient in diminishing cell viability. In fact, only four out of eight EOs, namely *T. caespititius* flower, *T. capitata* fruit, and *T. zygis* flower and fruit, showed statistically evident ($P < 0.05$) signs of cytotoxicity against HeLa cells, whereas only three out of eight

Fig. 3 Dose-dependent cytotoxicity of different essential oils extracted from fruits and flowers of various species of *Thymus* and *Thymbra*. After 24 h of exposure to the essential oils, their cytotoxicity towards HeLa (a) and U937 (b) cell lines was determined by the MTT assay. Values are presented as mean $\pm$ SEM of six independent experiments and expressed as percentage of control values (untreated samples). TC *Thymus caespititius*, TAC *Thymbra capitata*, TM *Thymus mastichina*, TZ *Thymus zygis*. * $P < 0.05$ compared to control values

EOs, i.e., *T. caespititius* fruit, and *T. capitata* flower and fruit, provoked a statistically significant ($P < 0.05$) diminution in U937 cell viability. At the lowest concentration tested (0.001%, v/v), EOs displayed poor cytotoxicity. Importantly, our results fit into previous investigations. Thus, EOs from different plants have been reported to present anticancer potential against mouth, breast, lung, prostate, liver, colon, and brain cancers, and even in leukemia. Not only EOs but also their constituents such as carvacrol, d-limonene, thymol and others have also been reported to possess cytotoxic effect on both cancer cell lines and in vivo studies (Deb et al. 2011; Jayakumar et al. 2012). Also, terpene analogues like terpinen-4-ol have been described to possess anticancer properties and induce apoptosis (Döll-Boscardin et al. 2012).

Conclusion

The results demonstrated that the phenological stage of the plant influenced the chemical composition of the EOs of the *T. capitata* and *Thymus species* plants. The EOs from *T. capitata* and *Thymus* spp. exhibited strong antioxidant and antimicrobial activities. Moreover, results clearly showed that these EOs possessed cytotoxic activity in vitro, since they exerted killing actions against tested cell lines, namely, HeLa (adherent cells) and U937 (free-floating cells). However, further studies are needed to elucidate their cytotoxic mechanism. Overall, these findings suggested that the EOs tested could be a potential source of pharmaceuticals.

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Compliance with ethical standards

Conflict of interest There are no conflicts of interest to disclose.

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