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Toxicological and pharmacological properties of essential oils of *Calamintha nepeta, Origanum virens* and *Thymus mastichina* of Alentejo (Portugal)

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ABSTRACT

Three autochthonous flavouring herbs from Alentejo (Portugal), *Calamintha nepeta* (syn. *Clinopodium nepeta*), *Origanun virens* and *Thymus mastichina,* were selected to evaluate toxicological, antioxidant, antiproliferative and antimicrobial potential of their essential oils (EOs). *C. nepeta* and *T. mastichina* EOs showed a high content of oxygenated monoterpenes (86–91%) while *O. virens* had similar content of oxygenated and hydrocarbon monoterpenes (45%). Toxicological assessment suggests high activity against *A. salina* ($31.8 < CL_{50} < 128.4$ mg/L) and very low toxicity in *Swiss* mice ($DL_{50} \geq 1500$ mg/kg). EOs showed high antioxidant ability by DPPH radical scavenging assay (0.1–0.6 mg QE/mL EO), total reducing power method (0.2–1.7 mg QE/mL EO) and β-carotene/linoleic acid system (11–501 mg QE/mL EO). An important antiproliferative effect against human breast tumour cell line was observed ($88.9 < EC_{50} < 108.5$ mg/L). Moreover, EOs presented a large antibacterial spectrum. Results point out the low toxicity and high antioxidant, antiproliferative and antimicrobial activities of EOs of these endemic aromatic plants, suggesting their potential use in biotechnological, food and/or pharmaceutical industries.

1. Introduction

The use of medicinal and aromatic plants (MAPs) for treatment and prevention of disorders is an oldest medicinal practice. Nowadays, MAPs are a valuable resource to improve human health and their extracts have been used as alternative therapies, with high efficiency and low adverse effects when compared with conventional drugs (Blowman et al., 2018). The essential oils (EOs) of these plants have been used for several purposes, including flavours, food additives, medicines and aromatherapy products. EOs are natural antioxidants and can be useful to preventing oxidative stress disorders, atherosclerosis, neurodegenerative, autoimmune and cancer diseases (Gulluce et al., 2007). The use of EOs as natural antioxidants may be a promising strategy to prevent stress disorders and to inactivate reactive oxygen species (ROS). An inverse relationship has been reported between the practice of the Mediterranean Diet rich in antioxidants and the incidence of oxidative stress diseases (Del Chierico et al., 2014). EOs are excellent sources of natural preservatives with antioxidant potential useful to detoxify and to promote the human health (Smeriglio et al., 2018). They are also promising antimicrobial agents against pathogenic or opportunistic multi-drug resistant microorganisms (Queiroga et al., 2018). There are several factors such as genus, species, geographical features and harvest season that conditionate the chemical composition of essential oils and consequently their pharmacological properties (Blowman et al., 2018).

Calamintha nepeta (L.) Savi subsp. *nepeta* (syn. *Clinopodium nepeta* (L.) Kuntze) is a perennial and quite aromatic plant, reaching 80 cm in height. It has the appearance of a small shrub of lilac flowers, with small and ovals leaves and it is widely distributed in Mediterranean region (Castroviejo et al., 2010). It is a seasoning and flavouring plant, often applied in traditional medicine as antiseptic, diuretic, spasmolytic and cough suppressant. EOs of *C. nepeta* are rich in terpenoid compounds and have antifungal and antibacterial properties (Marongiu et al., 2010; Negro et al., 2013).

Origanum vulgare L. is described as the only one wild species in Portugal, and it comprises two subspecies, the subsp. *vulgare* (typical one) and the subsp. *virens* (Castroviejo et al., 2010). However, Portuguese Flora consider the subsp. *virens* as a different species: *Origanum virens* Hoffmanns & Link (Vale-Silva et al., 2012). In folk medicine, oregano is used in treatment of some illnesses such as respiratory disorders, dyspepsia, rheumatoid arthritis as well as expectorant,

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carminative, to calm convulsive coughs and to treat urinary tract disorders (Teixeira et al., 2013).

Thymus mastichina L. is an endemic species from the Iberian Peninsula, characterised by simple and opposite leaves and bilabiate flowers grouped in flower heads or capitula. *Thymus mastichina* subsp. *mastichina* is characterised by inflorescences larger than 10 mm in diameter and oblong-ovate or elliptic bracts (Castroviejo et al., 2010). It is widespread from north to south of Portugal and occurs usually in clearings of xerophilic grasses, cultivated fields, pine forests, cork oaks, berms of roads and rocky outcrops (Castroviejo et al., 2010). Commonly known as *mastic thyme* or *spanish marjoram*, this species is recognised by its medicinal properties including antiseptic, digestive, antirheumatic, antispasmodic, expectorant and antitussive (Méndez-Tovar et al., 2015).

Considering that Alentejo is a region very rich in flavouring plants, often used in Mediterranean diet and in traditional medicine, the objective of this study was to evaluate the antioxidant, antiproliferative and antimicrobial activities of essential oils of three autochthonous Lamiaceae flavouring herbs: *Calamintha nepeta* (L.) Savi subsp. *nepeta, Origanum vulgare* subsp. *virens* (Hoffmanns. & Link) Bonnier & Layens and *Thymus mastichina* L. subsp. *mastichina*.

2. Materials and methods

2.1. Chemicals

Analytical standards for chromatography (> 99%) were purchased from Sigma-Aldrich (St. Louis, USA) and Extrasynthese (Genay, France). Quercetin (> 99%), 2,2-diphenyl-1-picryl-hydrazyl (DPPH·) (95%), β-carotene (95%), linoleic acid (99%), tetracycline hydrochloride and Whatman ™ filter paper (Ø = 6 mm) were purchased from Sigma-Aldrich (St.Louis, MO, USA). Muller-Hinton culture medium and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Calbiochem™ were purchased from Merck KGaA (Darmstadt, Germany). Oxoid™ microbiology media and antimicrobial susceptibility disks were purchased from ThermoFisher Scientific (Waltham, USA). All other chemicals and solvents were high purity grade and purchased from Sigma-Aldrich (St. Louis, MO, USA) and Merck KGaA (Darmstadt, Germany).

2.2. Plant material

Wild-growing *Calamintha nepeta* subsp. *nepeta* (*C. nepeta*), *Origanum vulgare* subsp. *virens* (*O. virens*) and *Thymus mastichina* subsp. *mastichina* (*T. mastichina*) were collected in Alentejo region (Herdade da Mitra, Évora, 38° 31′ 40″ N; 8° 01′ 15″ W). Aerial parts of *C. nepeta* and *T. mastichina* were collected in the flowering stage, while *O. virens* was collected in the vegetative stage. Voucher specimens were identified (Professor Marízia Menezes, University of Évora) and deposited at the herbarium of Aromatic Plants of the University of Évora, with the accession numbers of HPAM_UE 0000020, HPAM_UE 000014, HPAMT_UE 000018, respectively.

2.3. Isolation and analysis of essential oils

Essential oils were obtained by hydrodistillation of air-dried plant material of *C. nepeta* (leaves and flowers), *O. virens* (leaves) and *T. mastichina* (leaves and flowers), using a Clevenger type apparatus during 3 h, according to the European Pharmacopoeia (COE, 2007) and preserved at 4 °C. Relative density of EOs was determined by filling a small bottle of fixed volume at a known temperature and weighed accurately, at least 6 replicates, using the distilled water as reference. The refractive index of EOs was measured with a refractometer (Leica Abbe Mark II, Model 10481).

EOs analyses were achieved by gas chromatography with an ionization flame detector (GC-FID) and gas chromatography-mass spectrometry (GC-MS). GC-FID analyses were performed on a Shimadzu Nexis GC-2030 gas chromatograph equipped with an autoinjector AOC-20i plus and a fused-silica apolar capillary column (30 m × 0.25 mm i.d., film thickness 0.50 μm) Zebron ZB-5HT Inferno™ (Phenomenex, USA) and a LabSolutions software 5.92 (Shimadzu Corporation). The experiments were conducted under the following conditions: oven temperature programme at 40–110 °C (2 °C/min), 110–220 °C (3 °C/min) and 220–280 °C (10 °C/min), injector temperature 250 °C, detector temperature 310 °C, carrier gas flow rate 1.6 mL/min He and split ratio 25:1. GC-MS analyses were performed with a GC-MS-QP2010 Series (Shimadzu) gas chromatograph, fitted with Zebron ZB-5HT Inferno™ fused-silica apolar (30 m × 0.25 mm i.d., film thickness 0.50 μm), interfaced with a detector model Polaris Q (E. I. quadrupole).

Compounds were identified through their retention indices (RI) and their mass spectra when compared from the database NIST 11 (National Institute of Standards and Technology) library. Retention indices were determined by interpolation relatively to the C8–C22 *n*-alkanes retention times and compared with those of authentic samples, from the database of laboratory and with literature data (Cavaleiro et al., 2004; Marongiu et al., 2010; Vale-Silva et al., 2012; Zuzarte et al., 2012). Quantitative data was determined using relative amounts of individual components that were calculated based on GC peak area normalization without response factor correction. Percentage values are the mean at least of three injections per sample.

2.4. Brine shrimp toxicity assay

Screening of EOs toxicity was performed using brine shrimp larvae lethality bioassay (*Artemia salina* Leach). The procedure was performed in accordance with the Artoxkit M protocol (MicroBioTests Inc) (Arantes et al., 2016). Standard and sample solutions of EO (5–1000 mg/L) were prepared in synthetic sea water containing DMSO (10%). A control sample with solvent was used. In a multi-well test plate, 100 μL of each solution was added to 900 μL of synthetic sea water and *A. salina* larvae were added. Assays were repeated nine times (n = 9) for each experiment. Plates were observed after 24 h of incubation using a research stereomicroscope system (Olympus SZX9), and LC_{50} is defined as the lethal concentration that corresponds to 50% dead larvae (Deciga-Campos et al., 2007).

2.5. Acute toxicity mammal evaluation

2.5.1. Animals

Studies were carried out using 8 weeks old male *Swiss* albino mice (*Mus musculus*) weighting 28 ± 2 g. Animals were acclimatized to the laboratory conditions for a week before experimental sessions. Light was maintained over a 12 h light/dark cycle. They were fed with standard diet and water *ad libitum*. Food was suspended 16 h before the experiments. Procedures were conducted in accordance with National Institute of Health Guide for Care and Use of Laboratory Animals guidelines and European Community Guidelines (EEC Directive of 1986). All procedures involving animals were approved by Ethics Committee (Hau and Schapiro, 2003) and supervised by a research coordinator qualified by FELASA (n° 020/08) and Direção Geral de Veterinária from Portugal (DL 1005/92 of 23 October).

2.5.2. Acute oral toxicity

Acute toxicity of EOs was assessed in *Swiss* albino mice according to OECD Guideline 423, that was based on a stepwise procedure. Each step using three animals of a single sex, dosing of three additional animals at the next higher or the next lower dose level (OECD, 2001). EOs were administrated in female mice by oral gavage, with vehicle (saline solution) in doses ranging from 500 mg/kg to 2000 mg/kg. The animals were kept in observation for 14 days, during which they were housed in a controlled environment (12 h light/dark cycles, 23 ± 1 °C) with food and water *ad libitum*. The behaviour (passive, aggressive, fear) and

physical appearance (catalepsy, postural reflexes, sensitivity tail test, pineal reflex, motor activity) were monitored during the first 24 h after administration and daily to evaluate any additional behavioural or clinical signs of toxicity or changes in body weight (Vogel et al., 2002).

2.6. Screening of antioxidant properties

2.6.1. DPPH· radical assay

Free radical scavenging activity was determined by measuring the bleaching of a purple-coloured solution of the stable radical 2,2-diphenyl-1-picryl-hydrazyl (DPPH·). This method evaluates the ability of EOs to act as donor of hydrogen atoms or electrons in transformation of DPPH into its reduced form DPPH-H. Antioxidant activity of EOs (0.4–32.6 mg/mL) and the standard quercetin (0.4–13.1 μg/mL) was performed according to Arantes et al. (2017). Briefly, in 96 wells microplates 30 μL of each EO ethanolic solution was added to 200 μL of DPPH ethanolic solution 0.1 mM. A control using solvent (ethanol) was also performed. Assays were performed in sextuplicate (n = 6). After incubation at room temperature in the dark, the absorbance was measured at 517 nm at 120 min, using a microplate spectrophotometer (Thermo Scientific, Finland).

Inhibition of DPPH free radical in percentage (I %) was calculated using the equation

$$I\ (\%) = [(AC\text{-}AS)/AC] \times 100$$

where AC is the absorbance of the control and AS is the absorbance of the sample.

2.6.2. Total reducing power

Reducing power of EOs (0.4–60.0 mg/mL) was determined according to Arantes et al. (2017). Briefly, 200 μL of EOs ethanolic solutions were added to 200 μL of phosphate buffer (0.2 M, pH 6.6) and 200 μL of potassium ferrocyanide (1%) and incubated at 50 °C for 20 min. After that, 200 μL of trichloroacetic acid (10%) was added and solutions were centrifuged (3000 rpm, 10 min). 50 μL of supernatant was added with 50 μL distilled water and 100 μL $FeCl_3$ (0.1%). Quercetin (0.5–60.0 μg/mL) was used as standard and a control sample using solvent was also used in the same conditions. Assays were performed in sextuplicate (n = 6). The absorbance was read at 700 nm using a microplate spectrophotometer (Thermo Scientific, Finland).

2.6.3. β-Carotene/linoleic acid system

Bleaching β-carotene/linoleic acid activity of EOs (0.04–2.50 mg/mL) and quercetin (0.8–24.6 μg/mL) ethanolic solutions were determined according to Arantes et al. (2017). Each EO solution (35 μL) or standard was added to 250 μL of β-carotene/linoleic acid stock solution. A control with ethanol was also prepared. Samples and standards were assayed in sextuplicate (n = 6). Absorbance values were measured at 490 nm at minute zero and after 2 h incubation in a water bath at 50 °C, using a microplate spectrophotometer (Thermo Scientific).

Lipid peroxidation (LPO) inhibition was calculated using the following equation:

$$LPO\ (\%) = [(\Delta A_C - \Delta A_S) / \Delta A_C] \times 100$$

where ΔA_C is the difference between absorbance at 0 h and 2 h for the control and ΔA_S is the difference between absorbance at 0 h and 2 h for samples or standards.

2.7. Evaluation of the antiproliferative effects on MDA-MB231 cells

2.7.1. Cell culture and treatment

The human breast carcinoma cell-line MDA-MB-231, an estrogenic receptor-negative cell line derived from a metastatic carcinoma, was obtained from the *American Type Culture Collection* (ATCC, Manassas, VA, USA). Cultures were grown and maintained in RPMI-1640 medium supplemented with 2 mM L-glutamine, 4.5 g/L glucose, 2.0 g/L sodium bicarbonate, 10% heat-inactivated fetal bovine serum (FBS), and 1% (v/v) penicillin/streptomycin solution. Cultures were maintained in a humidified atmosphere in a 5% CO_2 at 37 °C (CO_2 Incubator ICOmed, Memmert GmbH, Germany). Before confluence, cultured cells were treated with trypsin solution (0.5 g/L) with EDTA (0.2 g/L), suspended in fresh growth medium and plated at a density of 1×10^6 cells/mL. Procedure for viable cell counting was made by the trypan blue dye exclusion method (Arunasree, 2010; Luo et al., 2012; Mota et al., 2012).

2.7.2. Cell viability

The effect of essential oils on cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. This method is based on the capacity of mitochondrial dehydrogenase enzymes and reducing agents present in living cells to reduce MTT, a yellowish aqueous solution, to a insoluble purple formazan product (Lü et al., 2012; Wang et al., 2011). The lipid soluble formazan product may be extracted with organic solvents and estimated by spectrophotometry. It is currently thought that the amount of MTT formazan is directly proportional to the number of living cells (Lü et al., 2012).

MDA-MB-231 cells were seeded in 96 well culture plates at 2×10^4 cells/well in a final volume of 100 μL and incubated in 5% CO_2 and 37 °C. After 24 h, cells were incubated with solutions of essential oil (5 μL). EO solutions were prepared with dimethyl sulfoxide (DMSO) 20%, to a final concentration between 3.9 and 1000 mg/L in the well. Two negative controls were performed with 5 μL of ultrapure water and EOs solvent solution (DMSO 20%) to estimate 100% of cell viability. A positive control with DMSO 100% in the same conditions was performed to estimate 100% mortality (Mota et al., 2012). After 44 h of incubation at 37 °C and 5% CO_2, 20 μL of MTT solution (2.5 mg/mL in PBS) was added to each well, followed by an incubation period of 4 h. Medium was then aspirated, and formazan crystals were dissolved with 100 μL of a solution DMSO/ethanol (1:1 v/v) (Luo et al., 2012; Mota et al., 2012; Sun et al., 2012).

The absorbance was read at 570 nm in a microplate spectrophotometer (Multiskan GO, Thermo Fisher Scientific). The relative cell viability was determined by the amount of MTT converted to the insoluble formazan salt. Viability (%) was determined by absorbance decrease of different EOs concentrations (Mota et al., 2012; Yoo et al., 2008). EC_{50} value was defined as the effective concentration that cause 50% of cell viability inhibition and was calculated by plotting the cell viability (%) against the different EO concentrations and compared with the control. All experiments were performed in triplicate for each of the three independent samples (n = 9) and the data are presented as mean ± standard deviation (SD).

2.8. Antimicrobial properties of essential oils

2.8.1. Microorganisms

The antimicrobial activity was assayed against a set of microorganisms provided by *American Type Culture Collection* (ATCC) and clinical isolates of the Medical Diagnostic Laboratory Flaviano Gusmão (SYNLAB, Évora, Portugal): three Gram-positive strains, *Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 29212, and six Gram-negative strains, *Escherichia coli* ATCC 25922, *Morganella morganii* LFG 1008, *Proteus mirabilis* LFG 1004, *Salmonella enteritidis* LFG 1005, *Salmonella typhimurium* LFG 1006, *Pseudomonas aeruginosa* ATCC 9027. Strains were maintained on Nutrient Agar slants at 4 °C. All bacterial strains were activated in Nutrient Agar at 37 °C for 24 h before testing.

2.8.2. Agar disk diffusion assay

The agar disc diffusion assay was performed in accordance with the Committee on Clinical Laboratory Standard Guidelines (CLSI, 2012a). A suspension of each tested microorganism was prepared with sterilized

physiological saline solution (0.9% w/v) and adjusted at 0.5 *Mc*Farland standard turbidity. Mueller-Hinton agar (Oxoid CM0337) plates were inoculated with bacterial suspension using a sterile swab in three different directions. Sterile filter paper discs (Ø = 6 mm) were placed on the previous inoculated media by pressing slightly and then impregnated with 5 μL of each essential oil. The assay was performed in triplicate for each concentration level (n = 3). Plates were kept at 4 °C for 2 h to avoid oil volatilization and incubated at 37 °C for 24 h. Tetracycline (30 μg) was used as standard. The diameters of the inhibition zones were measured in mm using a 'Fisher-Lilly Antibiotic Zone Reader' (Fisher Scientific Co. USA).

2.8.3. Determination of minimum inhibitory concentration (MIC)

The minimal inhibitory concentrations (MICs) were evaluated by broth microdilution assay according to with the Committee on Clinical Laboratory Standard Guidelines (CLSI, 2012b). EOs were diluted in Mueller-Hinton broth (MHB) containing 5% v/v DMSO in concentrations ranging from 31.3 to 2000 μL/mL. Standardized suspension of each tested organism (10^6 CFU/mL) was prepared on culture media Mueller-Hinton broth. Seventy-five microlitres of each EO dilution were placed into 96-well plates and 75 μL of a bacterial suspension were added to assess the inhibitory activity. MHB containing 5% DMSO was tested for negative control. Tetracycline hydrochloride (0.05–40 mg/mL) was used as standard. All experiments were performed in triplicate. The plates were incubated at 37 °C for 18 h. MIC value was defined as the lowest concentration of EO that inhibits microbial growth.

2.9. Data analysis

All data were expressed as mean ± standard deviation of different measurements. Statistical analysis of data was performed using ANOVA *one way* to determine statistically significant differences at the 95% confidence level ($p < 0.05$). Multiple comparisons of means were analysed by using the Tukey test. Analyses were performed using SPSS® 24.0 Windows *Copyright®*, IBM. The 50% lethal concentration (LC_{50}) and 50% antiproliferative effective concentration (EC_{50}) were determined in dose response curves using the *OriginLab* software (Microsoft Corporation).

3. Results and discussion

3.1. Chemical characterisation of essential oils

The extraction yield of essential oils of *C. nepeta* (EOCn), *O. virens* (EOOv) and *T. mastichina* (EOTm) was 0.61 ± 0.18, 0.16 ± 0.05 and 1.06 ± 0.03% (volume/dry weight), respectively. The density of EOs was 0.990 ± 0.001, 0.900 ± 0.001, 0.920 ± 0.002 and the refractive index was 1.466 ± 0.001, 1.496 ± 0.001, 1.465 ± 0.001, for *C. nepeta*, *O. virens* and *T. mastichina*, respectively.

Results of chromatographical analysis of EOs were summarised in Table 1. Twenty-nine compounds of *C. nepeta* EO were identified, representing 91% of oxygenated monoterpenes, 7% of hydrocarbon monoterpenes and 1% of sesquiterpenes. The major components of *C. nepeta* essential oil were 1,8-cineole (28%), menthone (22%), menthol (16%) and pulegone (5%). Twenty-eight compounds were identified in *O. virens* leaves EO, representing 46% of oxygenated monoterpenes, 45% of hydrocarbon monoterpenes and 8% of sesquiterpenes, with γ-terpinene (20%), thymol (19%), thymol methyl ether (13%) and *m*-cymene (12%), as major constituents. Twenty-six compounds were identified for *T. mastichina* EO, representing 86% of oxygenated monoterpenes, 7% of hydrocarbon monoterpenes and 5% of sesquiterpenes, with 1,8-cineole (71%) and α-terpinol (10%) as main components.

Chemical composition of EOs can be different according to the genus, species and the part of the plant used for extraction as well as to geographical, climatic and seasonal conditions. Previous studies performed with *C. nepeta* from the Alentejo region, harvested in different years, reported EOs very rich in oxygenated monoterpenes, with isopulegol, isopulegone and 1,8-cineole as major components (Arantes et al., 2017;Queiroga et al., 2018). Some works with essential oils of *C. nepeta* reported two chemotypes: one characterised by the predominance of pulegone and menthone, menthol and/or its isomers,

Table 1
Chemical composition of *C. nepeta* (EOCn), *O. vulgare* (EOOv) and *T. mastichina* (EOTm) essential oils.

Components	RI[a]	RI ref	Area (%)[f]		
			EOCn	EOOv	EOTm
α-Thujene	919	928[N]	–	0.9	–
α-Pinene	930	930[b]	0.7	0.5	1.7
Camphene	942	943[b]	0.1	0.3	0.1
β-Pinene	971	970[b]	1.8	0.3	4.1
β-Myrcene	990	981[b]	0.8	1.6	0.8
α-Phellandrene	1002	998[b]	0.3	0.2	–
α-Terpinene	1014	1009[b]	tr	2.4	0.2
p-Cymene	1023	1012[b]	–	12.2	–
Limonene	1026	1020[b]	2.6	–	–
1,8-Cineole	1028	1023[c]	27.9	4.9	71.2
Z-β-Ocimene	1038	1025[b]	tr	0.5	0.1
E-β-Ocimene	1037	1035[b]	0.2	6.2	tr
γ-terpinene	1042	1047[b]	tr	20.2	0.3
Sabinene hydrate	1064	1051[b]	tr	–	0.1
Fenchone	1076	1066[d]	1.9	0.1	–
Linalool	1095	1082[b]	0.5	0.3	1.4
trans-Pinocarveol	1134	1121[d]	0.1	–	0.1
Isopulegol	1144	1144 [Inj]	3.4	–	–
Isopulegone	1147	1149[c]	2.7	–	–
Menthone	1150	1130[c]	22.0	–	–
Isomenthone	1160	1139[c]	2.6	0.4	–
Borneol	1162	1145[b]	–	0.6	0.1
neo-Menthol	1143	1151[c]	2.2	–	–
δ-Terpineol	1140	1151[c]	–	–	2.3
4-Terpineol	1174	1158[b]	–	0.7	0.8
Menthol	1170	1156[c]	16.3	–	–
α-Terpineol	1178	1170[b]	0.3	–	9.7
Estragole	1196	1196 [Inj]	1.0	0.1	–
Pulegone	1225	1216[c]	5.0	1.8	–
Thymol methyl ether	1238	1231[N]	–	13.1	–
Isothymol methyl ether	1245	1244[N]	–	3.5	–
Carvone	1242	1212[b]	0.4	–	0.2
Menthyl acetate	1280	1294[N]	0.3	–	–
Anethol	1282	1282 [Inj]	4.4	0.6	–
Thymol	1294	1268[b]	–	19.4	–
β-Caryophyllene	1417	1411[b]	0.7	1.8	0.4
γ - Elemene	1450	1432[N]	0.1	1.6	–
D-Germacrene	1462	1469[b]	–	1.4	tr
β-Bisabolene	1500	1497[b]	–	2.5	–
Elemol	1523	1525[e]	–	–	0.9
Ledol	1588	1583[d]	–	–	0.2
Spathulenol	1558	1551[e]	–	0.5	0.3
Caryophyllene oxide	1582	1560[b]	0.2	–	tr
γ-Eudesmol	1596	1606[e]	–	–	0.3
β-Eudesmol	1607	1628[e]	–	–	0.6
α-Eudesmol	1610	1622[e]	–	–	2.3
Total identified compounds (%)			98.5	98.6	98.2
Hydrocarbon monoterpenes			6.5	45.3	7.3
Oxygenated monoterpenes			91.0	45.5	85.9
Hydrocarbon sesquiterpenes			0.8	7.3	0.4
Oxygenated sesquiterpenes			0.2	0.5	4.6

Values are mean ± standard deviation of triplicate analyses.
[Inj] RI determined by authors from authentic product.
[N] RI obtained from NIST 11 library.
[a] Retention indices relative to C8 – C22 n-alkanes on apolar Zebron ZB 5HT Inferno™ column.
[b] Retention indices reported by Vale-Silva et al. (2012).
[c] Retention indices reported by Marongiu et al. (2010).
[d] Retention indices reported by Zuzarte et al. (2012).
[e] Retention indices reported by Cavaleiro et al. (2004).
[f] Relative quantitative data by GC-FID analysis.

piperitenone, piperitone and its oxides (Cozzolino et al., 2000; Flamini et al., 1999; Kitic et al., 2005). Another chemotype characterised by predominance of piperitenone oxide and/or piperitone oxide is described by Fraternale et al. (1998) and Kitic et al. (2005). Marongiu et al. (2010), in a study comparing the chemical profile of Portuguese and Italian *C. nepeta*, reported isomenthone, 1,8-cineole and isopulegone as major components of EO of *C. nepeta* from north of Portugal. The variation in *O. vulgare* essential oil chemical composition is a constant dependent on the oregano subspecies, altitude and other agronomic features (Pande et al., 2012). Based on this difference, some authors define two subspecies, the subsp. *vulgare* (typical one) and the subsp. *virens,* recently considered as a different species, *O. virens* (Vale-Silva et al., 2012). Teixeira et al. (2013) reported that *O. vulgare* EO from Portugal contains carvacrol, terpinene and thymol as major compounds. Other studies referred two wild forms of *O. vulgare* in Portugal, one with generalised growth and rich in linalool and the other wild grown in southern Portugal, rich in thymol (Carmo et al., 1989; Faleiro et al., 2005). Results obtained in this study suggested that *O. virens* is a thymol chemotype, presenting thymol and γ-terpinene in similar percentages to those described by Faleiro et al. (2005). Chemical composition of *T. mastichina* EO is in agreement with that reported for Portuguese *T. mastichina*, with 1,8-cineole as main constituent (Salgueiro et al., 1997). A previous study with *T. mastichina* from Alentejo region, collected in other year reported a high oxygenated monoterpenes content (91%), with 1,8-cineole (72%) and α-terpineol (9%) as major compounds (Arantes et al., 2017). Another study with essential oils of *T. mastichina* cultivated in northwest of Portugal reported 1,8-cineole (64%) as major component followed by α-terpineol (6%) and β-pinene (5%) (Moldão-Martins et al., 2004).

3.2. Toxicological assessment

Toxicity tests against *Artemia salina* showed LC_{50} values of 32, 74 and 128 mg/L for EOs of *T. mastichina*, *O. virens and C. nepeta,* respectively. In addition, the standard potassium dichromate ($K_2Cr_2O_7$) presented a LC_{50} value of 50 mg/L (Table 2). According to the bibliography, values of LC_{50} lower than 500 μg/mL indicate a high toxicity level for brine shrimp (Bastos et al., 2009; Deciga-Campos et al., 2007). The lethal toxicity evaluation of *A. salina* is an important biological screening widely used to evaluate the presence of biological active compounds from medicinal plants, as well as an excellent method for preliminary investigations of toxicity (Bastos et al., 2009; Devaraj et al., 2013; Parra et al., 2001).

Oral lethal toxicity doses (LD_{50}) of EOs of *C. nepeta*, *T. mastichina* and *O. virens* ranged from 1000 to > 2000 mg/kg (Table 2). Results indicated low toxicity of EOs in accordance with OECD 423 (2001). Moreover, pharmacological screening in *Swiss* mice revealed a normal behavioural with no perceptible changes in sleeping pattern, salivation, diarrhoea, postural and pineal reflexes, sensitivity tail test, catalepsy and motor activity, when compared with control group. Besides, at the end of the experiment (14 days), the tissues did not showed differences in macroscopically-observed organs, with no toxicant-induced changes.

Some studies reported the toxicological effects of essential oils of these flavouring herbs. A study with *Calamintha officinalis* Moench reported low toxicity of essential oil administered intraperitoneally, with LD_{50} of 100 mg/kg (Monforte et al., 2011). Llana-Ruiz-Cabello et al. (2017) reported no observed-adverse-effect level (NOAEL) for *O. virens* EO in *Wistar* rats, after oral administration of 200 mg/kg during 90-day. Dires et al. (2018) reported similar toxicological results for *Thymus schimperi* EO to this one obtained for *Thymus mastichina* EO.

Table 2
Toxicological assessment of essential oils of *C. nepeta* (EOCn), *O. virens* (EOOv) and *T. mastichina* (EOTm).

Sample	Brine shrimp lethality	Acute oral toxicity
	CL_{50} (mg/L)	DL_{50} (mg/kg)
EOCn	128.4 ± 7.3	1500
EOOv	31.8 ± 3.5	> 2000
EOTm	74.1 ± 13.8	2000
$K_2Cr_2O_7$	50.0 ± 1.0	–

Values are mean ± standard deviation of three separate experiments.

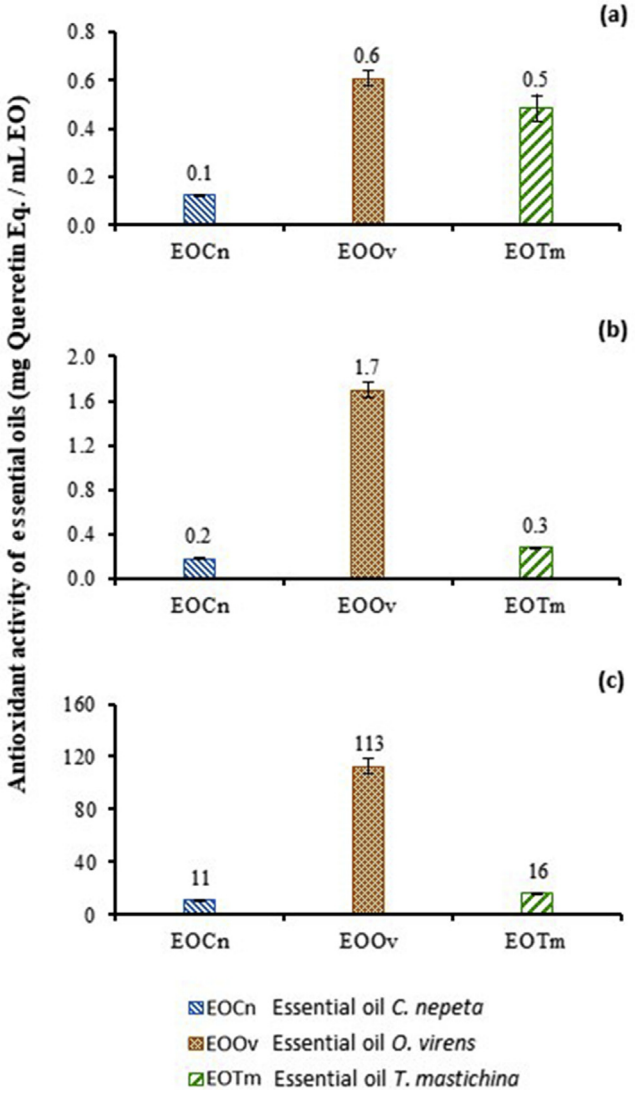

Fig. 1. Antioxidant activity of essential oils of *C. nepeta* (EOCn), *O. virens* (EOOv) and *T. mastichina* (EOTm) evaluated by DPPH radical scavenging method (a), Reducing power (b) and β-carotene/linoleic acid system (c). Values are mean ± standard deviation of six replicates (n = 6).

3.3. Antioxidant activity

In-vitro antioxidant activity of EOs was accessed by three different mechanisms and results were expressed in quercetin equivalents (mg QE/mL EO) (Fig. 1). EOs exhibited high antioxidant potential and *O. virens* (EOOv) was the most effective with 0.5, 1.7 and 113 mg QE/mL, for DPPH radical method, reducing power assay and β-carotene/linoleic acid system respectively.

Essential oils showed ability to act as radical scavengers and ferric reducers and with high activity to protect the lipid substrate. Their antioxidant properties could be related with the high content in oxygenated monoterpenes (46–91%). Previous studies carried out with *C. nepeta* and *T. mastichina* from southwest of Portugal reported the *in-vitro*

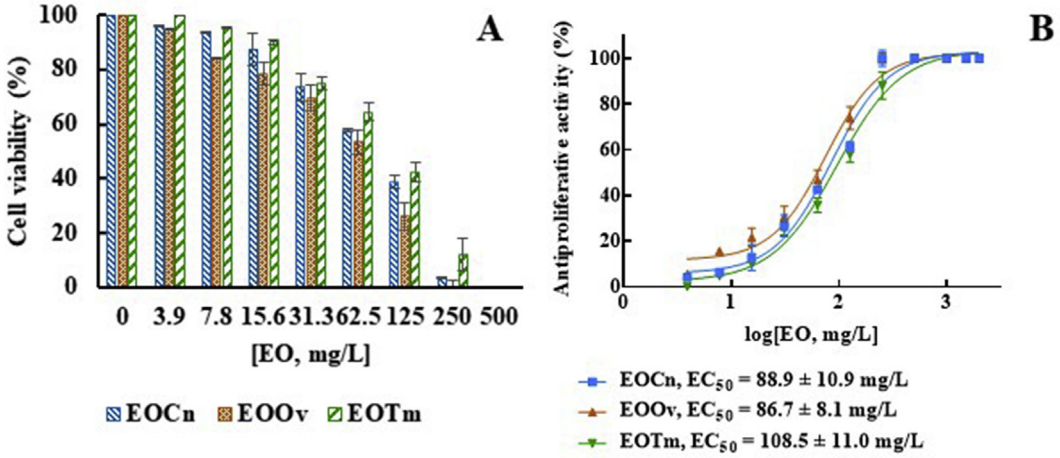

Fig. 2. Antiproliferative activity of *C. nepeta* (EOCn), *O. virens* (EOOv) and *T. mastichina* (EOTm). (A) Cytotoxicity of EOs on breast cancer (MDA-MB-231) cells; (B) dose - response curve (mortality, %). Values are mean ± standard deviation of triplicates for each of the three separate experiments (n = 9).

antioxidant properties of their EOs and extracts (Arantes et al., 2017). Several studies related the antioxidant potential of *C. nepeta* EOs with its main constituent pulegone (Bozovic and Ragno, 2017). Some authors correlated the high antioxidant scavenging ability of *Origanum* EOs with the high content in thymol or carvacrol (Puertas-Mejía et al., 2002; Teixeira et al., 2013). The highest antioxidant activity observed in EO of *O. virens*, can be also justified by the high amount of hydrocarbon monoterpenes (45%) and the presence of hydrocarbon sesquiterpenes content (7%). This synergetic antioxidant effect of monoterpenes and sesquiterpenes was reported in other studies (Prasad, 2017).

3.4. Antiproliferative effect of EOs against MDA-MB-231 cell line

Essential oils of *C. nepeta*, *O. virens and T. mastichina* induced a decrease in MDA-MB-231 cell viability (Fig. 2 A) for concentrations higher than 125 mg/L. From the fitted curve, it was observed that EOs were able to obtain 50% effective cell inhibition grow (EC_{50}) suggesting that these essential oils have antiproliferative activity on MDA-MB-231 cells (Fig. 2 B). EOs of *O. virens, C. nepeta*, and *T. mastichina* showed dose-dependent cell death on MDA-MB-231 cells after 24 h of treatment, with EC_{50} values of 86.74 ± 8.1 mg/L, 88.9 ± 10.9 mg/L and 108.5 ± 11.0 mg/L, respectively. Furthermore, MDA-MB-231 cells lost their ability adherence, presenting morphological alterations similar to necrotic cells, based on their size and shape after 24 h of treatment with different concentrations of EOs. Hakkim et al. (2016) observe that Doxorubicin (DOX) at 100 mg/L presented cell viability lower than 20% when tested in MDA-MB-231 cells with similar conditions. This standard, commercialised as trade name Adriamycin, is an anthracycline with antineoplasic activity, often used as a chemotherapy medication to treat cancer, including breast cancer.

Literature related the anti-proliferative effects with the presence of sesquiterpenoid compounds and phenylpropanoids (Carvalho et al., 2015; Zhong et al., 2017). Thymol is a phenolic monoterpene compound with anticancer activity, acting on cell growth suppressing, inducing apoptosis or producing intracellular reactive oxygen species (Aydin et al., 2017).

This study revealed that EO of *O. virens*, containing high content of thymol (20%) and sesquiterpenes (8%) presented the highest antiproliferative activity against MDA-MB-231 cells. A similar experiment with methanolic extract of *O. vulgare* that evaluated their cytotoxicity in two cell lines (HCT-116 and MDA-MB-231) showed cytotoxic activity with EC_{50} higher than 140 mg/L (Grbovic et al., 2013). Other studies indicated that *Thymus* EOs have an high activity against several tumour cell lines, very likely depending on the monoterpenes content and on their ability to affect the oxidative stress (Miguel et al., 2015; Nikolić et al., 2014). *Thymus vulgaris* EO with high content in thymol (33%), *p*-cymene (24%) and carvacrol (9%) showed anti-proliferative effect against both human osteosarcoma U2OS and pancreatic cancer PANC-1 cell lines (Catauro et al., 2017). The anti-proliferative activity was also reported for *C. nepeta* and *T. mastichina* EOs, that containing 1,8-cineole as major component (Cha et al., 2010). Several studies reported that EOs have anticancer properties, including cancer preventive mechanisms, as well as acting on the established tumour cell itself and interaction with the microenvironment. The preventive effect of EOs on cancer disorders is promoted through cell cycle arrest, stimulating cell apoptosis and DNA repair mechanisms, inhibiting cancer cell proliferation, metastasis formation and multidrug resistance (MDR) which make them potential candidates toward adjuvant anticancer therapeutic agents (Aras et al., 2014; Bayala et al., 2014; Blowman et al., 2018; Gautam et al., 2014; Lesgards et al., 2014).

3.5. Antimicrobial activity

Antimicrobial activity of EOs was evaluated according to the determination of inhibition zones (Table 3). All studied EOs showed a broad spectrum of antibacterial activity and *Gram*-negative strains, presenting higher inhibition zone than the commercial antibacterial standard, tetracycline. *O. virens* EO revealed greater effectiveness, with total inhibition of the bacterial growth of all *Gram*-positive strains and inhibition zones above 15 mm against *Gram*-negative strains. *O. vulgare* EO has an antimicrobial action on the level of pH homeostasis, with alteration of the ionic balance of cell (Lambert et al., 2001).

EOs showed high ability to inhibit the growth of several selected bacterial strains in liquid media, with determination of MIC (Table 3). *Gram*-negative bacteria exhibited higher susceptibility to EOs than Gram-positive bacteria. This effect may be correlated to the presence of monoterpene and phenolic compounds capable of disintegrating the outer membrane of *Gram*-negative strains (Calo et al., 2015). EOs of *C. nepeta* and *T. mastichina* presented higher activity against Gram-negative bacteria than *O. virens* EO, with MICs ranging from 0.8 to > 2 mg/mL. Results may be related with the high content of 1,8-cineole in *C. nepeta* and *T. mastichina* EOs, which is reported to have high antibacterial activity (Bozovic and Ragno, 2017; Vieira et al., 2017). EOs present unique antibacterial potential due to the high number of chemical compounds presented in their constitution, which act simultaneously, preventing resistance mechanisms in bacteria. Furthermore, synergistic interactions between EOs compound can potentiate their natural antimicrobial effect. Thus, the antimicrobial potential cannot be associated to only one component or mechanism of action (Candy et al.,

Table 3
Antibacterial activity of essential oils of *C. nepeta* (EOCn), *O. virens* (EOOv) and *T. mastichina* (EOTm).

Microorganisms	Inhibition growth zone (mm)				Minimal Inhibition Concentration (MIC)			
	EOCn	EOOv	EOTm	TE	EOCn	EOOv	EOTm	TE-Cl (μg/mL)
					(μL/mL)			
S. aureus ATCC 6538	8.5 ± 0.4	t.i.	19.0 ± 2.0	29.0 ± 1.2	> 2.0	> 2.0	> 2.0	0.1
S. epidermidis ATCC 12228	8.2 ± 0.2	t.i.	21.0 ± 6.0	11.5 ± 0.7	> 2.0	1.0	> 2.0	60.0
E. faecalis ATCC 29212	10.2 ± 0.8	t.i.	21.0 ± 1.0	15.5 ± 0.8	> 2.0	> 2.0	> 2.0	10.0
E. coli ATCC 25922	18.3 ± 2.1	25.0 ± 1.0	11.0 ± 1.0	26.5 ± 1.9	1.0	1.1	> 2.0	0.3
M. morganii LFG 1008	14.5 ± 1.3	34.0 ± 2.0	17.0 ± 0.5	w.i.	0.7	1.2	1.1	250.0
P. mirabilis LFG 1004	12.2 ± 1.3	16.0 ± 1.0	9.0 ± 1.0	8.3 ± 0.5	1.0	0.6	0.5	40.0
Salm. enteritidis LFG 1005	9.2 ± 0.3	33.0 ± 1.0	11.0 ± 2.0	28.7 ± 1.6	0.7	> 2.0	0.1	0.3
Salm. typhimurium LFG 1006	8.2 ± 0.3	19.0 ± 1.0	8.0 ± 0.5	8.5 ± 0.5	> 2.0	> 2.0	> 2.0	100.0
P. aeruginosa ATCC 9027	7.8 ± 0.8	t.i.	17.0 ± 5.0	24.0 ± 1.5	1.0	> 2.0	1.1	10.0

w.i. – without inhibition; t.i. – total inhibition.
EOCn – *C. nepeta* EO (5 μL); EOOv – *O. virens* EO (5 μL); EOTm – *T. mastichina* EO (5 μL); TE - tetracycline (30 μg); TE-Cl – tetracycline hydrochloride.
Values are mean ± standard deviation of three separate experiments.

2018; Lv et al., 2011). Nevertheless, due to the lipophilic character of EOs, several researchers accept that the mechanism of action goes through the alteration of the cell membrane properties (Calo et al., 2015; Cox et al., 2000).

4. Conclusions

Essential oils of autochthonous aromatic plants from Alentejo are rich in monoterpene compounds. *C. nepeta* and *T. mastichina* EOs presented higher content in oxygenated monoterpenes, with 1,8-cineole as main component, while *O. virens* EOs showed similar content in hydrocarbons and oxygenated monoterpenes, with γ-terpinene and thymol as major compounds. Toxicological approach of EOs revealed high toxicity against *A. salina* ($31.8 < LC_{50} < 128.4$ mg/L) and low toxicity in *Swiss* mice ($DL_{50} > 1000$ mg/kg). EOs showed high antioxidant activity with ability to act as radical scavengers, as ferric reducers and with high ability to protect lipid substrate, although *O. virens* EO was the most effective. Moreover, EOs presented high antiproliferative ability against breast cancer MDA-MB-231 cells ($EC_{50} < 110$ mg/L) and a broad spectrum of antibacterial activity against the selected Gram-positive and Gram-negative bacteria.

Results highlighted the low toxicity and high antioxidant, antiproliferative and antimicrobial properties of essential oils of these selected flavouring herbs suggesting their potential use in food and pharmaceutical industries.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2019.110747.

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