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Essential oils from different *Citrus* species and evaluation of their *in vitro* antibacterial, antiacetylcholinesterase, anti-inflammatory and antifungal activities

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Abstract

Essential oils (EOs) from C*itrus* are not only economic, eco-friendly and natural alternatives to chemical preservatives but also have other biological applications. This study aimed to investigate the chemical composition of *Citrus* species (EOs from *C. limonia, C. latifolia, C. sinensis* and *C. deliciosa* fruit peel) to evaluate their *in vitro* antibacterial activity against *Listeria monocytogenes*, *Yersinia enterocolitica*, *Staphylococcus aureus*, *Clostridium botulinum* and *Bacillus cereus*. The chemotaxis model, which was used for evaluating their anti-inflammatory activity, showed that EOs exhibited effective results when the dose was 100 µg/mL. Regarding all antimicrobial activities, minimum inhibitory concentration (MIC) values of EOs were calculated by the broth microdilution method on 96-well microplates. EOs showed satisfactory antifungal activity against *Malassezia furfur* (MIC values between 32.5 and 62.5 µg/mL). *Citrus deliciosa*, whose MIC = 95.8 µg/mL, inhibited acetylcholinesterase (AChE) more selectively. All EOs from *Citrus* spp. fruit peel showed good antibacterial activity against *Yersinia enterocolitica* (MIC = 62.5 µg/mL) and *Staphylococcus aureus* (MIC = 62.5 µg/mL), mainly the EO from *C. deliciosa* whose MIC values were 50 µg/mL for both. EOs were moderately active against *Clostridium botulinum*, *Bacillus cereus* and *Listeria monocytogenes* since MIC values ranged from 100 µg/mL to 400 µg/mL. Both GC-FID and GC-MS analyses revealed that the single major constituent determined in EOs is the terpene limonene. EOs from *Citrus* may be important active ingredients of several products to prevent bacterial growth in food, to attack the fungus that causes seborrheic dermatitis and to treat inflammatory processes. In short, the promising antiacetylcholinesterase activity of EOs under evaluation was attributed to the high concentration of the monoterpene limonene.

Keywords: foodborne bacteria, spoilage bacteria, *Malassezia furfur*, acetylcholinesterase, limonene.

Introduction

Brazil is one of the four world's largest producers of *Citrus* essential oils (EOs) because they are a by-product of the juice industry (Rezende et al., 2020; Simas et al., 2015). According to Tranchida et al. (2012), in terms of economic and sustainable issues, their production is not only viable, but also profitable, in Brazilian and global markets. The use of EOs in food, fragrance and pharmaceutical industries has great biotechnological potential. The versatility that makes EOs take part in formulations of several types of products as active ingredients brings new perspectives to different research areas, mainly due to their biological activities in medical treatments (Firenzuoli et al., 2014).

Literature in the area of food science and technology has recently focused on the study of the antimicrobial potential of EOs and included them in the so-called food biopreservation systems. Food biopreservation is a universally accepted preservation system since it is considered a natural procedure which is capable of extending shelf life of food and ensuring its satisfactory microbiological safety. EOs from aromatic and medicinal plants and their components have exhibited antibacterial, antifungal and food preservation activities against a wide range of microbial pathogens (Pandey et al., 2017).

The genus *Malassezia* comprises yeast, lipophilic and lipo-dependent fungi which have recently undergone changes in their taxonomic classification, i. e., new species were introduced: *M. globosa*, *M. obtusa*, *M. slooffiae* and *M. restritcta*, besides *M. furfur*, *M. pachydermatis* and *M. sympodialis*, which had been previously described (Rhimi et al., 2020).

Malassezia furfur, the most common species, is part of the normal microbiota of human skin. It grows where there are different concentrations of long-chain fatty acids, such as ricinoleic acid and its derivatives (Maraschin et al., 2008). Its optimal growth temperature is 32°C, although it may also grow at temperatures up to 41°C. Colonies of this species, which have a creamy texture, are friable, convex and opaque white. A microscopic examination shows cells with different sizes and shapes (oval, spherical and cylindrical). *M. furfur* can be found in three forms of superficial infections: pityriasis versicolor, folliculitis and seborrheic dermatitis (Maraschin et al., 2008). However, this microorganism has also been acknowledged as an etiologic agent of severe systemic infections in premature newborns and, less often, in immunocompromised adults (Maraschin et al., 2008).

Many studies have been carried out in search for compounds that may be effectively applied as anti-inflammatory agents or with antinociceptive activity and which bring benefits with the fewest possible adverse effects caused by their use (Junior, 2019). Inflammatory processes usually occur when pathogenic microorganisms (viruses, fungi and bacteria) invade the body and live in specific tissues or circulate in the bloodstream. Another form of inflammation may take place as the result of tissue lesion, cell death, cancer, ischemia and degeneration. Two types of immunological systems – innate and adaptive – are responsible for protecting the body against invasive species (Azab et al., 2016). In the search for new anti-inflammatory compounds, plants have stood out as rich sources of compounds that have promising potential. Some natural products that exhibit anti-inflammatory activity are plant extracts, EOs, juices and powders (Azab et al., 2016).

Throughout an inflammatory process, neutrophils that circulate in the peripheral blood get close to vessel walls due to stimulus of inflammatory mediators released in lesion regions; thus, they occupy a more peripheral position. Afterwards, they adhere to the endothelium temporarily and cross vessel walls. After diapedesis, they keep migrating towards the inflammatory focus by the chemotaxis process so as to eliminate the agent and restore the tissue that was damaged (Presibella et al., 2003).

The Alzheimer's Disease (AD), which is a progressive neurodegenerative disorder, affects mainly older adults and accounts for most cases of dementia in people who are 65 or older. Acetylcholine (ACh) deficit is a neurochemical characteristic of patients who have been diagnosed with the AD. The use of acetylcholinesterase (AChE) inhibitors to delay catabolic hydrolysis of ACh in order to compensate its deficit mainly in synaptic terminals, is a drug alternative to treat the AD (Souza et al., 2012).

EOs have shown inhibitory activity against AChE, which has been attributed to monoterpenoids, such as geranial, neral and linalool. Both sesquiterpenoids caryophyllene oxide and tumerone and the phenylpropanoid eugenol have already shown their antiacetylcholinesterase activity (Souza et al., 2012). Both components of EOs from *Citrus* (Rutaceae) limonene and perillyl alcohol improved memory impairment induced by scopolamine, the inhibitory result of AChE (*in vitro* activity). In addition, some studies have shown that molecules that have oxygen-containing functional groups lead to decrease in the inhibitory potential of AChE (Souza et al., 2012).

In order to deepen studies of EOs from *Citrus* spp. that have been carried out by our research group (Dias et al., 2020; Lemes et al., 2018), this study aimed at investigating *in vitro* antibacterial, antiacetylcholinesterase, antifungal and anti-inflammatory activities and chemical composition of EOs from *Citrus limonia, C. latifolia, C. sinensis* and *C. deliciosa* fruit peel.

Results and Discussion

Yields of EOs, identified chemical constituents and correlation with the literature

EOs were extracted from *Citrus limonia, C. latifolia, C. sinensis* and *C. deliciosa* fruit peel, whose yields were 1.0%, 1.2%, 1.5% and 1.7% (w/w), respectively. Volatile compounds were identified by gas chromatography-flame ionization detection (GC-FID) and gas chromatography-mass spectrometry (GC-MS). Table 1 shows that the major component of EOs were monoterpene hydrocarbons. One of the major components identified in EOs from fruit peel was limonene (relative abundance between 64.7% and 79.0%) (Table 1). Three components were identified in EOs from *C. limonia* (99.8% of total oil composition), four components in EOs from *C. latifolia* (97.5% of total oil composition), three components in EOs from *C. sinensis* (99.2% of total oil composition) and eight components in EOs from *C. deliciosa* (96.8% of total oil composition). All chemical structures of constituents identified in EOs from *Citrus limonia, C. latifolia,* and *C. sinensis* are shown in Figure 1.

Chemical composition of EOs from *C. sinensis* and *C. deliciosa* fruit peel agrees with a recent study carried out by Dias et al., 2020. The study reported by this paper reinforces that limonene is actually the major constituent of EOs from *C. sinensis* and *C. deliciosa*. Gomes et al. (2020) and their collaborators have recently reported the larvicidal potential of *C. limonia* fruit peel; results found by their study and the ones of the study reported by this paper show subtle similarity between its chemical composition. Limonene, beta-pinene, meta-cymene, beta-phellandrene and alpha-pinene were the major constituents identified in the oil in Maranhão state, Brazil (Gomes et al., 2020). Twenty chemical constituents were identified in EOs from *C. latifolia* green fruit and the major ones were limonene (47.5%), β-pinene (12.4%) and γ-terpinene (12.3%) (Atti-Santos et al., 2005). Besides the high concentration of limonene, which is always found in different *Citrus* species, other constituents are often identified. The variety of compounds depends on stages of fruit ripening, storage conditions and extraction methods (Kademi & Garba, 2017).

Antibacterial activity of EOs from Citrus

In vitro antibacterial activity of EOs from *Citrus* spp. against microorganisms under study and their potential activities were quantitatively evaluated by minimum inhibitory concentration (MIC) values. Data on MIC values of EOs were calculated by the broth microdilution method on 96-well microplates. MIC results are shown in Table 2. Data indicated that EOs exhibited different levels of antibacterial activity against microorganisms under investigation. Inhibitory properties of EOs were observed within a range of concentrations, from 50 to 400 µg/mL. Maximum activity was observed against *Yersinia enterocolitica* (MIC = 62.5 µg/mL) and *Staphylococcus aureus* (MIC = 62.5 µg/mL). However, the best result (MIC = 50 µg/mL) was the one of EO from *C. deliciosa* against both types of bacteria (Table 2). All EOs under evaluation were moderately active against *Clostridium botulinum*, *Listeria monocytogenes* and *Bacillus*

Table 1. Chemical composition of EOs from *Citrus* spp. fruit peel.

Compound	RT (min)	RI_{exp}	RI_{lit}	% RA			
				1	2	3	4
α-Tricyclene	9.25	922	926	—	—	—	2.6
Sabinene	12.48	974	976	—	—	—	2.9
p-Cymene	15.45	1022	1026	6.9	4.9	7.7	5.3
Limonene	15.82	1028	1031	79.0	74.2	77.0	64.7
γ-Terpinene	17.92	1062	1062	13.9	15.1	14.5	16.0
α-Terpineol	26.30	1188	1189	—	3.3	—	2.3
Thymol	30.98	1288	1290	—	—	—	0.8
Caryophyllene oxide	42.83	1581	1581	—	—	—	2.2
Monoterpene hydrocarbons				99.8	94.2	99.2	91.5
Oxygenated monterpenes				—	3.3	—	3.1
Oxygenated sesquiterpenes				—	—	—	2.2
Total				99.8	97.5	99.2	96.8

RT: retention time; RI_{exp}: Retention index relative to *n*-alkanes (C_8–C_{20}) in the Rtx-5MS column; RI_{lit}: Retention index found in the literature [Adams, 2007]. %RA: relative area. (–) = not detected. **1** – *Citrus limonia*; **2** – *C. latifolia*; **3** – *C. sinensis* and **4** – *C. deliciosa*.

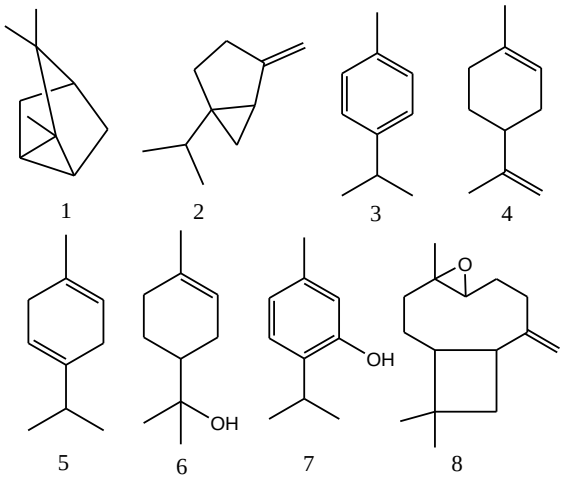

Figure 1. Chemical structures of constituents identified in EOs from *Citrus limonia, C. latifolia, C. sinensis*. 1- α-Tricyclene; 2- Sabinene; 3- *p*-Cymene; 4- Limonene; 5- γ-Terpinene; 6- α-Terpineol; 7- Thymol and 8- Caryophyllene oxide.

Table 2. Determination of MIC (µg/mL) of EOs from *Citrus* spp. fruit peel against foodborne pathogens and food spoilage bacteria.

Bacteria	1	2	3	4	Penicillin*
Clostridium botulinum[a]	150	150	150	100	1.25
Yersinia enterocolítica[b]	62.5	62.5	200	50	5.9
Bacillus cereus[a]	250	200	200	100	5.9
Staphylococcus aureus[a]	62.5	62.5	250	50	1.25
Listeria monocytogenes[a]	400	400	400	250	1.25

Minimum Inhibitory Concentration (MIC); **1** – *Citrus limonia*; **2** – *C. latifolia*; **3** – *C. sinensis* and **4** – *C. deliciosa*. *Positive control. [a]Gram-positive bacteria; [b]Gram-negative bacteria.

cereus, since MIC values ranged between 100 and 400 µg/mL (Table 2).

Based on the criteria established by Machado et al. (2005), natural products with MIC values between 10 and 100 µg/mL were considered good, while values between 100 and 500 µg/mL were considered moderate. Other studies have advocated that natural products exhibit adequate antimicrobial activity when their inhibitory concentration is below 100 mg/mL (Freire et al., 2014).

It was previously reported that resistance of Gram-negative bacteria could result from the fact that their impermeable outer membranes provide hydrophilic properties to lipophilic compounds, such as EOs. On the other hand, when this barrier does not exist in Gram-positive bacteria, it favors the contact of EOs with the phospholipid bilayer of the cell membrane. Thus, it might cause increase in ion permeability and leakage of intracellular constituents or impairment of bacterium enzyme systems. In addition, inhibitory effects of phenols could be explained by interactions with the cell membrane of microorganisms and are often correlated with compound hydrophobicity (Barrera-Ruiz et al., 2020).

Antifungal activity of EOs from Citrus

Regarding antifungal activity, EOs from *Citrus* spp. were promising against *Malassezia furfur*. *Citrus limonia*, *C. latifolia*, *C. sinensis* and *C. deliciosa* exhibited the following MIC values: 50, 62.5, 50 and 32.5 µg/mL, respectively. Fluconazole (IC_{50} = 6.25 µg/mL) was used as positive control. EOs from *Citrus limonia*, *C. latifolia*, *C. sinensis* and *C. deliciosa* fruit peel were more active against *M. furfur* than other EOs, such as oils extracted from *Origanum vulgare* and *Thymus vulgaris*, whose MIC values were much higher,

between 450 and 900 µg/mL (Vinciguerra et al., 2018). Specifically, EO from *C. deliciosa* fruit peel also exhibited lower MIC values than oils from *Zataria multiflora*, whose MIC was 35 µg/mL (Khosravi et al., 2016). However, EO from *C. deliciosa* fruit peel had almost the same activity as the extract from *Stellaria scordifolia* (MIC = 32 µg/mL) (Giordani et al., 2019). Excellent anti-*Malassezia furfur* activity shown by EOs in the study reported by this paper may result from the activity of their major chemical constituents since their strong antifungal activity has been reported by the literature. For instance, it is the case of the terpene limonene (Chee et al., 2009).

Anti-inflammatory activity of EOs from Citrus

The evaluation of anti-inflammatory activity was based on the fact that neutrophils are capable of migrating towards fMLP, since cells incubated with the culture medium (assay control) move farther in this case than when they move towards the culture medium (basal). After diapedesis, neutrophils orderly migrate to the focus of the inflammatory lesion in the extravascular matrix and respond to concentration gradients of mediators with chemotactic activities that may be connected to the matrix or not. To test the hypothesis that all four EOs from *Citrus* could interfere with mechanisms involved in the orderly migration of neutrophils, this study used the formyl peptide fMLP, the most potent chemotactic factor that has already been described by the literature (Selvatici et al., 2006; Resende et al., 2020).

Incubation with EOs from *Citrus limonia*, *C. latifolia*, *C. sinensis* and *C. deliciosa* triggered migration of neutrophils towards fMLP at the concentration under evaluation (100 µg/mL). In short, EOs under investigation worked as mediating gradients of chemotactic activities, since they run farther than the control (128 µM). EO from *C. limonia* made neutrophils move in suspension for 145 µM, followed by EO from *C. latifolia* (158 µM), *C. sinensis* (140 µM) and *C. deliciosa* (170 µM). Results of the evaluation of anti-inflammatory activity may be explained by the high concentration of limonene in all oils under investigation. Limonene has scientifically shown its effective anti-inflammatory activity (Santana et al., 2020). The literature describes the evaluation of anti-inflammatory activity of EO from *C. latifolia* and pure limonene by a model of analysis in which both were active (Kummer et al., 2013). Such information reinforces results of the study reported by this paper.

Antiacetylcholinesterase activity of EOs from Citrus

In this study, the role of EOs from four *Citrus* species in the treatment of the AD was evaluated by the AChE assay. The best activity was exerted by *C. deliciosa* and *C. limonia*; they inhibited AChE of EOs with IC_{50} values of 95.8 and 128.7 *µ*g/mL, respectively. Higher IC_{50} values were exhibited by EOs from *C. latifolia* (142.3 *µ*g/mL) and *C. sinensis* (135.6 *µ*g/mL). The positive control physostigmine exhibited IC_{50} = 1.3 *µ*g/mL. Tundis et al. (2012) evaluated EOs from other species of *Citrus* – C. *aurantium*, *C. aurantifolia* and *C. bergamia* – and showed that they have antiacetylcholinesterase activity whose IC_{50} values range from 139.3 and 161.6 *µ*g/mL. The authors have advocated that the high content of monoterpenes and the hydrophobicity of EOs are essential factors of good bioactivity. In fact, the hydrophobic active site of AChE is reported to be susceptible to hydrophobic interactions, a fact that may justify the bioactivity of EOs from *Citrus*.

According to Ademosun et al. (2016), limonene, a major component of EOs under study, is a potent cholinesterase inhibitor. Nevertheless, the synergistic effect of chemicals found in EOs cannot be neglected. The ability of EOs to interact with hydrophobic active sites of cholinesterases, as suggested by Dohi et al. (2009), could be one of the principles behind their effective antiacetylcholinesterase activity.

Material and methods

Plant material and EO extraction

Citrus limonia, C. latifolia, C. sinensis and *C. deliciosa* fruit peel were collected in November 2019 in Rio Verde, Goiás state, Brazil. Plants were identified by the botanist Erika Amaral, M. Sc., and voucher specimens (HJ 7522, HJ7521, #4489 and #4490, respectively) were deposited in the herbarium in Rio Verde, at the Instituto Federal Goiano (IFGOIANO). EOs were extracted from *Citrus* species by hydrodistillation in a Clevenger apparatus for 2 h. Hydrodistillation was performed in triplicate. Plant material was divided into three 500-g samples and 500 mL distilled water was added to each sample. After manual collection of EOs, traces of water remaining in the oils were removed with anhydrous sodium sulfate; this process was followed by filtration. EOs were stored in amber bottles and kept in a refrigerator at 4°C until analysis. Calculation of their yields was based on the weight of the fruit peel and expressed as the average of the triplicate analyses.

Analysis of Eos

EOs were dissolved in ethyl ether and analyzed by GC-FID and GC–MS with the use of both Shimadzu QP5000 Plus and GCMS2010 Plus (Shimadzu Corporation, Kyoto, Japan) systems. Temperature of the column in GC-FID was programmed to rise from 60 to 240°C at 3°C/min and was held at 240°C for 5 min; the carrier gas was H_2 at a flow rate of 1.0 mL/min. The equipment was set to operate in the injection mode; the injection volume was 0.1 µL (split ratio of 1:10), while injector and detector temperatures were 240 and 280°C, respectively. Relative concentrations of components were obtained by normalizing peak areas (%). Relative areas consisted of the average of triplicate GC-FID analyses. GC-MS conditions and identification of EOs have been previously reported (Fernandes et al., 2020). Identification of volatile components of EOs from *Citrus* spp. was based on their retention indices on an Rtx-5MS (30 m X 0.25 mm; 0.250 µm) capillary column under the same operating conditions used for GC relative to a homologous series of *n*-alkanes (C_8-C_{20}). Structures were computer-matched with Wiley 7, NIST 08 and FFNSC 1.2. Their fragmentation patterns were compared with literature data (Adams 2007).

Antibacterial activity

The following microorganism strains of foodborne pathogens and food spoilage bacteria were provided by the American Type Culture Collection (ATCC, Rockville, MD, USA): *Yersinia enterocolitica* (ATCC 9610), *Staphylococcus aureus* (ATCC 9144), *Clostridium botulinum* (ATCC 19397), *Bacillus cereus* (ATCC 10987) and *Listeria monocytogenes* (ATCC 15313).

MIC values were determined by the broth microdilution method, as recommended by the literature (Fernandes et al.,

2021), with modifications. Assays were performed in Mueller Hinton broth. Inoculums were adjusted to 75% transmittance at 660 nm, which corresponds to the 0.5 MacFarland standard (equivalent to 1.5×10^8 CFU/mL). Samples of EOs from *Citrus* were prepared in absolute ethanol and the initial concentration was 8 mg/mL. Ethanol provides proper solubility of EOs from *Citrus* in the aqueous culture medium used in the MIC assay. Dilutions were performed on 96-well microplates to obtain serial dilutions of EOs from *Citrus* and reach final concentrations from 0.39 to 400 µg/mL (eleven serial dilutions). Microplates were incubated at 35 ± 2 °C for 24 h. A solution of resazurin (0.02%) was used for determining microbial growth, which was visually indicated by changes in color (from blue to pink). The lowest concentration at which color did not vary was considered the MIC value. Positive control was penicillin (Sigma-Aldrich, St. Louis, MO, USA), at concentrations ranging from 1.25 to 5.9 µg/mL. The antimicrobial assay was performed in triplicate. The solvent (absolute ethanol), antimicrobial standards and culture media were used as controls.

Anti-Malassezia furfur activity

In order to evaluate anti-*Malassezia furfur* activity, the methodology described by Demitto et al. (2012) – the broth microdilution test, with modifications – was used. Standard strains of *Malassezia furfur* (ATCC 14521) provided by the American Type Culture Collection were used. The microdilution test was carried out in agreement with the M27-A3 issued by the CLSI (2008). The positive control was fluconazole (Pfizer). The culture medium was RPMI-1640 (Gibco), buffered with morpholino propanesulfonic (Sigma) pH 7.0 and supplemented with 2% glucose, as recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST 2002). Yeast suspension was adjusted by a spectrophotometer (530 nm) to reach initial concentration from 0.5 to 2.5×10^3 cells/mL. The test was conducted on 96-well microplates; incubation was carried out at 35ºC for 48 h, with constant agitation. Readings were performed by a microplate reader (E & K Scientific, Santa Clara, CA®) at 490 nm and minimum inhibitory concentration (MIC) was defined as the lowest concentration of the antifungal fluconazole and EOs from *Citrus*, which were capable of inhibiting 50% of the fungus.

Anti-inflammatory activity

The chemotaxis model was used for evaluating anti-inflammatory activity. It was investigated by the Boyden chamber assay (Boyden, 1962), as described by Castilhos et al. (2007). This technique enabled the anti-inflammatory potential of EOs from *Citrus* (100 µg/mL) to be evaluated by means of its inhibitory influence on neutrophil motility in suspension (upper compartment) towards the chemotactic agent fMLP 10^{-8} M (lower compartment), determined by the space used by neutrophils through the polycarbonate filter placed between the chamber compartments. An optical microscope (40x magnification) was used for reading the filters. Its focus was initially adjusted to the upper plane of the filter and then slowly deepened to focus on two cells. Reading was carried out in five fields of every filter and the migrating capacity of neutrophils was evaluated in terms of distance, as micrometers, measured between the upper plane of the filter and the final plane of observation (Zigmond & Hirsch, 1973). Results showed the mean of distances found by the readings. Results were expressed as mean+/-standard deviation, analyzed by the Student's t-test and compared with the mean of distances determined for non-treated control cells (Snedecor & Cochran, 1974). The difference was considered statistically significant when $p \leq 0.05$.

Evaluation of acetylcholinesterase inhibition

The assay was carried out by the Ellman spectrophotometric method, modified by Lopes et al. (2020). The enzyme was acetylcholinesterase (AChE) from *Electrophorus electricus* VI (Sigma®). On the microplate, 25 µL iodized acetylcholine 15 mM, 125 µL 5,5'-dithiobis(2-nitrobenzoic-acid) (DTNB), 50 µL buffer Tris-HCl 50 mM (pH 8.0) with 0.1 % Bovine Serum Albumin (BSA) and EOs from *Citrus* at final concentrations in the test solution ranging from 31.25 to 1000 μg/mL (20 μL) were added to 2 μL phosphate buffer pH 8 and pre-incubated in ice bath at 4°C for 30 min. Duplicate tubes were also treated this way with 20 mL physostigmine (0.1 mM) to allow interference of the test compounds in the assay under evaluation and to control any acetylcholine hydrolysis which is not due to enzyme activity. Absorbance was measured 5 times at 405 nm by the microplate reader (Biotek, Elx800), at 15-s intervals. After the readings, 25 µL acetylcholinesterase 0,22 $U.mL^{-1}$ was added to the wells. Absorbances were then measured 9 times at 405 nm, at 15-s intervals. Increases in absorbance due to spontaneous hydrolysis of the substrate were corrected by subtraction of the reaction rate before enzyme addition. Inhibition percentage was calculated by comparing rates of samples and the ones of the control. All assays were carried out in triplicate. Enzyme activity and inhibition percentage of AChE were calculated by the Microsoft® Excel program.

Conclusions

EOs from *Citrus limonia, C. latifolia, C. sinensis* and *C. deliciosa* exhibited chemical compositions which were very close to findings that had already been described by the literature. Limonene was the major constituent of oils extracted from the four *Citrus* species. All EOs exhibited either good or moderate antibacterial activity against foodborne pathogens and food spoilage bacteria. They also showed efficient anti-*Malassezia furfur* and antiacetylcholinesterase activities. In short, all EOs were found to have anti-inflammatory activity, which was shown by the Boyden chamber assay as neutrophil motility in suspension. Other *in vivo* studies should be carried out to confirm the efficacy of EOs under investigation and to determine their mechanisms of action.

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