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Citrus medica essential oil exhibits significant antimicrobial and antiproliferative activity

Gregoria Mitropoulou [a], Eleni Fitsiou [a], Katerina Spyridopoulou [a], Angeliki Tiptiri-Kourpeti [a], Haido Bardouki [b], Manolis Vamvakias [b], Panayiotis Panas [a], Katerina Chlichlia [a], Aglaia Pappa [a], Yiannis Kourkoutas [a, *]

[a] Department of Molecular Biology and Genetics, Democritus University of Thrace, University Campus, Alexandroupolis, GR 68100, Greece
[b] VIORYL S.A., Chemical & Agricultural Industry, Research S.A., Afidnes, GR 19014, Greece

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ABSTRACT

The aim of the study was to investigate the antimicrobial and antiproliferative activity of *Citrus medica* essential oil produced by hydrostillation and evaluate its commercial potential in the food industry. The main constituent of the *C. medica* peels and essential oil identified by SPME GC/MS or GC/MS analysis was limonene (≈88 and 64%, respectively). The antimicrobial properties were assayed and the minimum inhibitory and non-inhibitory concentration values were determined. Both *C. medica* essential oil and limonene were effective against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* Enteritidis, *Salmonella* Typhimurium, *Pseudomonas fragi, Saccharomyces cerevisiae* and *Aspergillus niger*. MIC and MBC values for the oil ranged 845 ± 26–2008 ± 43 mg/l and 4184–8368 mg/l, respectively. *C. medica* essential oil was further assessed in different human cancer cell lines (HepG2, Caco2, MCF-7, and THP-1) and was found to have significant antiproliferative activity, being the most effective against the Caco2 cells. *Citrus medica* oil and limonene were shown to possess cancer-specific activity when tested against the skin melanoma A375 cells and normal skin cells (HaCat). The oil also appeared to inhibit LPS-induced nitric oxide (NO) production in RAW 264.7 cells.

1. Introduction

Nowadays, there has been an increased interest in essential oils from various plant origins as potential antimicrobial, antioxidant, and anti-cancer agents. This trend can be mainly attributed to the rising number and severity of food poisoning outbreaks worldwide along with the recent negative consumer perception against artificial food additives, as the latter have been suspected of residual toxicity and are considered responsible for many carcinogenic and teratogenic attributes.

The use of natural compounds with biological activity has always been an intriguing issue (Nakatsu, Lupo, Chinn, & Kang, 2000; Rios & Recio, 2005). Although spices and herbs have been added to foods, as flavourings, preservatives and healing agents since ancient times, the last decades, their use as food supplements has attracted considerable attention (Burt, 2004). Such interest has been developed primarily from the need of ensuring food quality and safety against pathogenic and spoilage microorganisms and existing reports documenting useful bioactive properties of plant essential oils that can substitute synthetic food additives with inexplicit impact, while at the same time acting as potential preventive and/or therapeutic agents against certain diseases (Akthar & Azan, 2014; Edris, 2007).

Citrus medica (citron) belongs to the *Rutaceae* family and has traditionally been used as sedative, antiemetic, insect repellent, diuretic and antitoxic. In the Greek island of Naxos, citron fruits and leaves are used for the production of traditional alcoholic beverage. Although certain phytochemical and pharmacological properties of *C. medica* have been previously presented (Jayaprakasha & Patil, 2007), this is the first report describing the *in vitro* antimicrobial, antioxidant and antiproliferative properties of *C. medica* essential oil and assessing its commercial potential in the food industry.

* Corresponding author.
E-mail address: ikourkou@mbg.duth.gr (Y. Kourkoutas).

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2. Materials & methods

2.1. Standard compounds

All standard compounds used for identification in GC/MS analysis were kindly provided by Vioryl Chemical and Agricultural Industry, Research S.A.

2.2. Extraction of essential oils by hydrostillation

Citrus medica fruits were supplied directly after their seasonal harvest (at December 2013 and November 2014). All fruits were peeled and chopped into small pieces without further dehydration process involved. A Dean Stark apparatus was used for hydrostillation into which 12.060 kg of *C. medica* peel were placed along with 6 l distilled water. After hydrodistillation (8 h, 90–100 °C), 7 g essential oil were isolated. Subsequently, the essential oil was dried with Na_2SO_4 and collected to sealed vials for further use.

2.3. SPME and GC/MS analysis

C. medica peels were analyzed by SPME GC/MS, while the essential oil was directly injected into the GC/MS.

2.3.1. SPME analysis

For the SPME analysis of *C. medica*, 1.5 g of chopped peels were placed into a 20 ml headspace vial fitted with a Teflon-lined septum sealed with an aluminum crimp seal. The container was then thermostated at room temperature for 45min to reach equilibrium and consequently the SPME syringe needle (bearing a 2 cm DVB/CAR/PDMS 50/30 μm bonded to a flexible fused silica core, Supelco, Sigma-Aldrich, Poole, UK) was introduced. Absorbance of volatiles was performed for 20 min at room temperature. The absorbed volatile analytes were then analyzed by GC/MS, as described below, operated in pulse splitless mode.

2.3.2. GC/MS analysis

GC/MS analysis of *C. medica* essential oil was carried out in a GC-MS (GC: 6890A, Agilent Technologies, USA; MSD: 5973, Agilent Technologies) using a Factor Four VF 1ms column (25 m, 0.2 mm i.d., 0.33 μm film thickness, Agilent Technologies). 0.1 μl of the essential oil was directly injected and a 1:100 split ratio was applied. The oven temperature was set at 50 °C for 1min, followed by a temperature gradient of 2.5 °C/min to 160 °C for 20min and then 50 °C/min to 250 °C for 15min. Helium was used as carrier gas (flow rate 1 ml/min). Injector and transfer line temperatures were set to 200 °C and 250 °C, respectively. The mass spectrometer was operated in the EI mode (70 eV). Identification was carried out by comparing the retention times and mass spectra of volatiles to Willey/NIST 0.5 and *in-house* created libraries (VIORYL S.A.) and by determining kováts' retention indexes (KI) and comparing them with those reported in the literature.

2.4. Microbial strains and antimicrobial assays

Salmonella enterica subsp. *enterica* ser. Enteritidis FMCC B56 PT4 (kindly provided by Prof. Nychas G.J.E., Agricultural University of Athens, Greece), *Salmonella enterica* subsp. *enterica* ser. Typhimurium DSMZ 554, *Escherichia coli* ATCC 25922, *Listeria monocytogenes* NCTC 10527 serotype 4b, *Staphylocccus epidermidis* FMCC B-202 C5M6 (kindly provided by Dr. Nisiotou A., Athens Wine Institute, ELGO-DIMITRA, Greece) and *Staphylococcus aureus* ATCC 25923 were grown in Brain Heart Infusion (BHI) broth (LABM, UK) at 37 °C for 24 h. Similarly, *Pseudomonas fragi* 211 (also kindly provided by Prof. Nychas G.J.E.) was grown in BHI broth (LABM, UK) at 25 °C for 24 h. *Saccharomyces cerevisiae* uvaferm NEM (Lallemand, Canada) was grown in YPD broth (yeast extract 10 g/l, glucose 20 g/l and peptone 20 g/l) at 28 °C for 3 days. *Aspergillus niger* 19111 (also kindly provided by Prof. Nychas G.J.E.) was grown on Malt extract agar (LABM) for 7 days at 37 °C.

The antimicrobial activity of the tested essential oil was monitored as described previously (Mitropoulou et al., 2015). Briefly, for the antibacterial screening, the disk diffusion assay was performed, using gentamycin (10 μg) (Oxoid Ltd, UK) as positive control and sterile water as negative. After incubation, the inhibition zones were measured in mm.

The same procedure was also followed for screening the activity against yeasts and molds, using *S. cerevisiae* and *A. niger* as model microorganisms. Voriconazole (1 μg) (BioRad Laboratories Inc., USA) was used as positive control and sterile water as negative.

Determination of minimum inhibitory (MIC) and non-inhibitory (NIC) concentrations was carried out as recently described (Mitropoulou et al., 2015), by monitoring changes in optical density of bacterial suspensions in BHI broths containing multiple concentrations (ranging 39–8368 mg/l) of the essential oil or limonene using a microplate reader (Molecular Devices, VERSAmax, USA, Softmaxpro v. 5.0 software). Gentamycin was used as positive control and BHI broths with no inoculum and inoculated BHI broths with no essential oil were used as negative controls. Optical density measurements were carried out every 10 min at 610 nm for 24 h. The calculation of MIC and NIC values was based on the Lambert-Pearson model (LPM) (Chorianopoulos et al., 2006). In brief, the effect on the growth, measured by the optical density method, is manifested by a reduction in the area under the OD/time curve relative to the control well at any specified time. By calculating the area using the trapezoidal rule, the relative amount of growth were obtained using the ratio of the test area to that of the control, termed the fractional area, fa. Data were fitted to the LPM using non-linear least squares regression analysis assuming equal variance.

Minimum bactericidal concentration (MBC) was determined by subculturing 100 μl from each negative well onto BHI agar plates. MBC was defined as the lowest concentration resulting in a negative subculture or giving presence of only one colony after incubation (99.9% of the inoculum is killed). The experiments were carried out at least in four replicates.

2.5. Cell lines and cell cultures

The human hepatocellular carcinoma HepG2, breast adenocarcinoma MCF-7, colon adenocarcinoma Caco2, leukemic monocytic THP-1, malignant melanoma A375 and immortal keratinocyte HaCat cell lines were obtained from the American Type Culture Collection (Rockville, MD). HepG2 and MCF-7 cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) (Biochrom AG, Germany), A375 and HaCat cells in DMEM High Glucose (4.5 g/l), while Caco2 and THP-1 cells were grown and maintained in RPMI-1640 medium, all supplemented with 10% fetal bovine serum (Biochrom AG), penicillin (100 U/ml), and streptomycin (100 mg/ml) (Biochrom AG) and were incubated at 37°C in a humidified atmosphere of 95% O_2 and 5% CO_2. Stock cultures were passaged at 2- to 3-day intervals. Cells were seeded at a density of 5.0×10^3 cells/well in 96-well plates for the sulforhodamine B (SRB) assay. THP-1 cells were seeded at a density of 2.0×10^3 cells/well in round bottom 96-well plates for the XTT assay.

2.6. Antioxidant activity (DPPH assay)

The radical scavenging activity of *C. medica* essential oil or limonene was estimated using the free radical 2,2-diphenyl-1-