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Research Article

Chemical composition and biological effects of *Citrus aurantium* var. *dulce* essential oil

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Citrus aurantium var. *dulce* (sweet orange) belongs to one of the largest genera of the Rutaceae family. The species of this genus are consumed worldwide fresh or in form of beverages. They include well-known crops lemons, oranges, mandarins, grapefruits, and limes. Essential oils (EOs) obtained from this species have great economic value since they are mainly produced from the peel of the crops, which are considered waste during their industrial processing. Considering, the aim of this study was to evaluate the chemical composition of the essential oil (EO) obtained from the peel of *Citrus aurantium* var. *dulce*, as well as to assess the biological effects by the means of antioxidant and antibacterial activity. Chemical composition analysis performed using GC and GC/MS analysis revealed that this EO is a rich source of limonene presented in this sample in the amount of 93.86% of the total. Obtained results of antioxidant testing indicate better inhibition of $ABTS^{\cdot+}$ (68.32 ±3.54%) compared to the $DPPH^{\cdot}$ (8.60 ±1.52%). Moreover, the results of the antimicrobial assessment using the disc diffusion method displayed almost no inhibition power of this essential oil towards G^- bacteria and yeast strains, while towards G^+ bacterial strains weak inhibition was observed.

Keywords: essential oil, *Citrus*, chemical composition, antioxidant activity, antimicrobial activity

1 Introduction

The emergence of novel therapeutic principles and the need for a healthier lifestyle have again drawn attention to medicinal and aromatic plants. The beneficial properties of plants are known since ancient times. They are used as ornaments, spices, foods, and beverages, as well as in the treatment of many health issues. Such fruitful employment of plants and their products is granted by their production of a wide range of compounds known as secondary metabolites. These compounds are synthesized in plant organs as the result of their interaction with the environment (other plants, soil, pollinators, herbivores, pathogens, etc) (Palazzolo et al., 2013). Essential oils (EOs) represent plant products that have been extensively explored in the pharmaceutical, agronomic, food, sanitary, cosmetic, and perfume industry (Baptista-Silva et al., 2020; Christaki et al., 2021; Palazzolo et al., 2013). These volatile mixtures of diverse compounds are produced in almost all plant organs. Chemically, the main components of EOs are usually terpenes or terpenoids, represented by two or three derivatives in high amounts whose concentration varies in the range of 20–70% (Aljaafari et al., 2021). Current reports regarding the biological features of EOs presented their antimicrobial, antioxidant, anti-inflammatory, antitumor and proapoptotic, antiviral, and enzyme inhibition activity (Aljaafari et al., 2021; Baptista-Silva et al., 2020; Christaki et al., 2021; Popović-Djordjević et al., 2019).

Citrus aurantium var. *dulce* belongs to the *Citrus* genus of the Rutaceae family. This is one of the largest genera consisting of nearly 1,300 species (Bora et al., 2020). Fruits of this genus have great commercial value and include crops like lemons, oranges, mandarins, grapefruits, and limes (Bora et al., 2020; Singh et al., 2021). Their nutritional significance is very important since these fruits are constituents of the human diet, consumed as fresh or used for juice extraction (Singh et al., 2021). Worldwide consumption of *Citrus* fruits is owing to the presence of high levels of bioactive components benefiting human health (Singh et al., 2021), such as phenols, flavonoids, essential oils (EOs), vitamins, and carotenoids. Essential

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oils from *Citrus* species have great industrial value since they are used in food products (for aroma flavor) such as beverages and sweat, as well as in pharmaceutics, cosmetic, and perfumery industries (Bourgou et al., 2012; Bousbia et al., 2009). Besides their wide usage, one of the advantages of *Citrus* species EOs is that they are obtained mainly from the fruit peel, which is considered a waste of citrus processing (Bousbia et al., 2009; de Araújo et al., 2020; Ruiz & Flotats, 2014). This advantage makes them an economic alternative for industrial uses. The chemical profile of peel EOs mainly consists of monoterpenes and sesquiterpenes (hydrocarbons and oxygenated) (Mahato et al., 2019; Ruiz & Flotats, 2014). Moreover, literature data show that the main constituent of these volatile liquids is monoterpene hydrocarbon limonene with a concentration varying between 32% and 98% (de Araújo et al., 2020; González-Mas et al., 2019; Mahato et al., 2019; Ruiz & Flotats, 2014). Such a high occurrence of limonene in citrus peel is of great importance, regarding the numerous proven advantageous effects of limonene on human health (Vieira et al., 2018).

All these findings prompted us to investigate the chemical composition of *Citrus aurantium* var. *dulce* essential oil and to evaluate its antioxidant potential by means of the neutralization of $DPPH^{\cdot}$ radical and $ABTS^{\cdot+}$ radical cation. Moreover, diverse bioactivity profiles of EOs encouraged us to assess the antimicrobial activity of this EO towards G^+ and G^- bacterial strains, and yeasts.

2 Material and methods

2.1 Essential oil

EO prepared from *Citrus aurantium* var. *dulce* was purchased from Hanus, s. r. o. (Nitra, Slovakia) and was extracted by steam distillation of peal. The sample was stored in the dark at 4 °C throughout the analyses.

2.2 Gas chromatography-mass spectrometry and gas chromatography analyses

To determine the chemical composition of essential oil obtained from *Citrus aurantium* var. *dulce*, the Agilent Technologies (Palo Alto, Santa Clara, CA, USA) 6890 N gas chromatograph operated by an interfaced HP Enhanced ChemStation software (Agilent Technologies) and equipped with a quadrupole mass spectrometer 5975 B (Agilent Technologies, Santa Clara, CA, USA) was employed. Volatiles were separated using the HP-5MS capillary column (30 m × 0.25 mm × 0.25 µm), while helium 5.0 was used as a carrier gas, with a flow rate set at 1mL.min^{-1}. Prior to analysis, the essential oil sample was diluted in hexane (10% solution). The injection volume of the tested sample was 1 µL. The total run time for analysis was 52 min and the temperature program was 50 °C to 70 °C (rate of increase 4 °C.min^{-1}), held for 2 min at 70 °C, 70 °C to 120 °C (rate of increase 5 °C.min^{-1}) held 1 min at 120 °C, and 120 °C to 290 °C (rate of increase 5 °C.min^{-1}). The temperatures of the split/splitless injector, the MS source, and the MS quadruple were set at 280 °C, 230 °C, and 150 °C respectively. The solvent delay time was 3.2 min, while the mass scan range was 35–550 amu at 70 eV, and the split ratio was 40.8 : 1.

Identification of volatile compounds present in the essential oil sample was determined by the means of their retention indices (RI) in comparison with the reference spectra reported in the literature and the ones stored in the MS library (Wiley7Nist) (Adams, 2007; van den Dool & Dec. Kratz, 1963). Using GC-FID with the same HP-5MS capillary column performed was semi quantification of the components, considering amounts higher than 0.1%.

2.3 Assessment of antioxidant activity

The antioxidant potential of *Citrus aurantium* var. *dulce* was determined using two assays: 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2-azinobis-3-ethylbenzothiazoline- 6 acid ($ABTS^{\cdot+}$).

The DPPH assay was performed with some modifications as described previously (Galovičová et al., 2022). The solution of $DPPH^{\cdot}$ (Sigma Aldrich, Schnelldorf, Germany) in the concentration of 0.025 g.L^{-1} in methanol (Uvasol® for spectroscopy, Merck, Darmstadt, Germany) was diluted to the absorbance of 0.8 at 515 nm prior to analysis. In a 96-well microtiter plate added was 190 µL of $DPPH^{\cdot}$ solution and 10 µL of essential oil. The reaction mixture was incubated in the dark at room temperature for 30 min with shaking at 1,000 rpm after which the absorbance was measured at 515 nm using a microplate reader.

For the ABTS assay, $ABTS^{\cdot+}$ radical cation was generated according to the already described procedure (Proestos et al., 2013). The prepared radical cation was diluted prior to the analysis up to an absorbance value of 0.7 at 744 nm. The 190 µl of this solution was mixed with 10 µl of EO (in a 96-well microtiter plate) for 30 min with continuous shaking at 1,000 rpm at room temperature in the dark. A decrease in absorbance at 744 nm was registered using a microplate reader and the results are presented as a percentage of $ABTS^{\cdot+}$ inhibition.

For both methods, Trolox (Sigma Aldrich, Schnelldorf, Germany) was used as the standard reference substance (1–5 mg.L^{-1}), and methanol was used as blank. From the Trolox calibration curve results were obtained for TEAC and IC_{50} values.

The percentage of inhibition of DPPH$^{\cdot}$ and ABTS$^{\cdot+}$ was calculated according to the following formula:

$$(A_0 - A_A)/A_0 \times 100 \quad (1)$$

where: A_0 – the absorbance of DPPH or ABTS$^{\cdot+}$; A_A – the absorbance of the sample

All measurements were performed in triplicate and the results were presented as mean values ± standard deviation (SD) of three independent measurements.

2.4 Assessment of antimicrobial activity using disc diffusion method

The antimicrobial activity of *C. aurantium* var. *dulce* was determined by the disc diffusion method using bacteria cultivated on Tryptone Soya Agar (TSA, Oxoid, Basingstoke, method UK) at 37 °C for 24 h, while yeasts were cultivated on the Sabouraud dextrose agar (Oxoid, Basingstoke, UK) at 25 °C for the same period. For this purpose we have used the following microorganisms Gram-negative (G^-) bacteria *Yersinia enterocolitica* CCM 5671, Gram-positive (G^+) bacteria *Enterococcus faecalis* CCM 4224, and yeasts *Candida krusei* CCM 8271, *Candida albicans* CCM 8186, *Candida tropicalis* CCM 8223, and *Candida glabrata* CCM 8270 obtained from the Czech collection of microorganisms. For inoculation on Mueller Hinton agar (MHA, Oxoid, Basingstoke, UK) 100 µL of prepared inoculum was used (optical density of 0.5 McFarland standard). Blank discs with a diameter of 6 mm were saturated using 5 µL of tested EO and placed on prepared agar. Incubation of bacteria lasted 24 h at 37 °C, while yeasts were incubated at 25 °C. The inhibitory activity was detected in the following criteria: zone inhibition diameter above 5 mm is considered weak, above 10 mm moderate, and above 15 mm very strong. Each test was performed in triplicate.

3 Results and discussion

3.1 Chemical composition of C. aurantium var. dulce essential oil

Results obtained using GC and GC/MS analysis are presented in Table 1, while Table 2 shows the percentage of the abundance of different classes of compounds identified in this EO sample. Corresponding chromatograms obtained from the analysis are presented in Fig. 1 and Fig. 2. As can be seen from Table 1, 11 compounds were identified in this sample, which represent 99.9% of the total. Monoterpene hydrocarbon limonene was the main component of this EO identified in the amount of 93.86%. Alongside limonene, the chemical composition of *C. aurantium* var. *dulce* EO was characterized by relatively high amounts of monoterpene hydrocarbons β-myrcene (3.1%) and α-pinene (1.0%), while other components were identified in amounts below 1.0%. Considering the results shown in Table 2 it is evident that monoterpene hydrocarbons found in the amount of 99.2% of the total represent the main class of compounds in this sample.

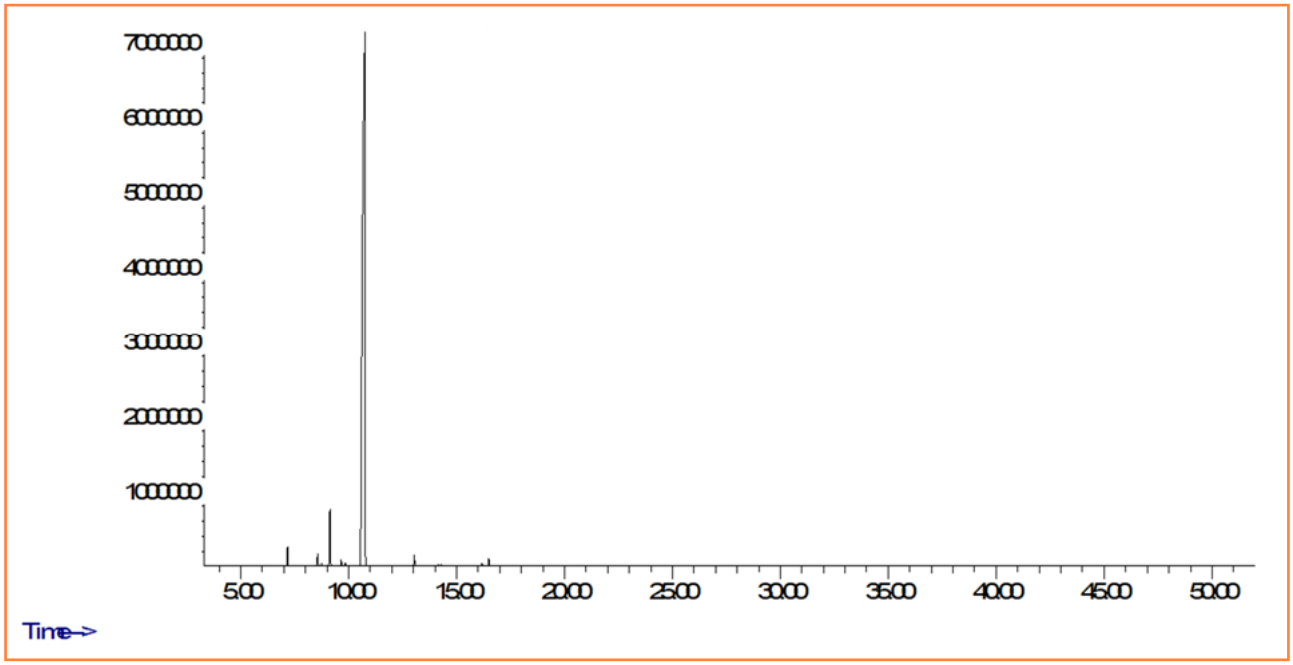


Figure 1 GC/MS chromatogram of *C. aurantium* var. *dulce* EO for calculation of Kovats retention indices

Table 1 Chemical composition of essential oil from *C. aurantium* var. *dulce*, with corresponding literature and calculated retention indices (RI)

No	Compound*	(%)	RI (lit.)	RI (calc.)**
1	α-pinene	1.0	939	938
2	sabinene	0.7	975	977
3	β-pinene	tr***	979	981
4	β-myrcene	3.1	990	991
5	octanal	0.3	998	1,003
6	α-phellandrene	tr	1,002	1,005
7	δ-3-carene	tr	1,011	1,011
8	limonene	93.9	1,029	1,038
9	α-terpinolene	0.5	1,088	1,100
10	*cis*-limonene oxid	tr	1,136	1,136
11	decanal	0.4	1,201	1,205
Total (%)	99.9			

* identified compounds; ** values of retention indices on HP-5MS column; *** tr – compounds identified in amounts less than 0.1%

It is well known that the chemical composition of the EOs depends on many factors. Some of them are harvesting time, part of plant organ used for EO extraction, extraction method, geographical, and environmental factors. However, all previously published papers agree that these volatile extracts obtained from the peel of *Citrus* species are characterized as a source of limonene (Bora et al., 2020; Geraci et al., 2017; González-Mas et al., 2019; Mahato et al., 2019; Palazzolo et al., 2013; Ruiz & Flotats, 2014). This monoterpene with empirical formula $C_{10}H_{16}$ is considered responsible for these EOs' biological effects. However, a synergistic effect of compounds presented in minor amounts could not be neglected.

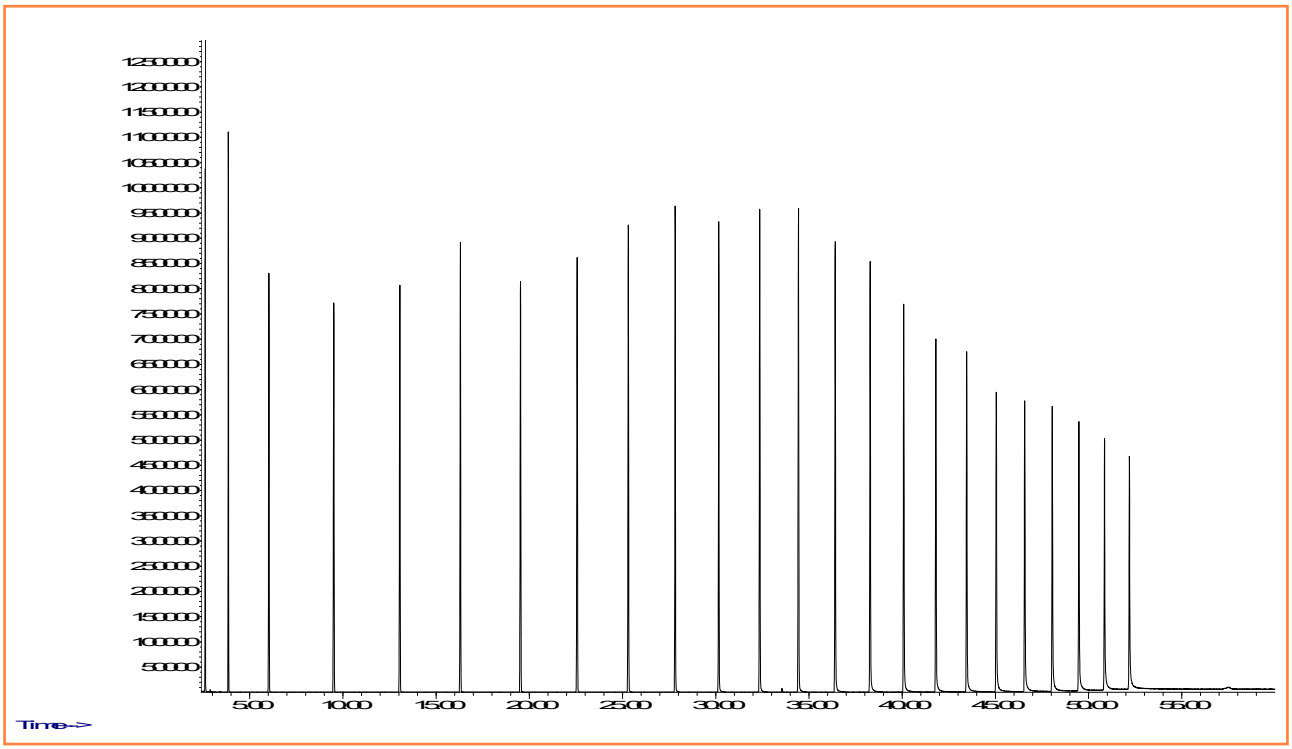


Figure 2 GC/MS chromatogram of series of n-alkanes (C7–C35) for calculation of Kovats retention indices for *C. aurantium* var. *dulce* EO

Table 2 Percentage composition of each class of identified compounds

Class of compounds	(%)
Monoterpenes	99.2
Monoterpene hydrocarbons	99.2
Oxygenated monoterpenes	tr
Monoterpene epoxide	tr
Non-terpenic	0.7
Aldehydes	0.7
Total	99.9

3.2 Antioxidant activity

The antioxidant potential of the *C. aurantium* var. *dulce* essential oil was assessed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2-azinobis-3-ethylbenzothiazoline-6 acid (ABTS) assays. Here, the reducing potency of the EO is measured, i.e., the ability to donate hydrogen to the radical species. Generally, in the presence of an antioxidant, the neutralization of the $DPPH^{\cdot}$ is observed with the change of the solution colour from purple to yellow, whereas the reduction of the $ABTS^{\cdot+}$ (bluish-green) occurs with the decolorization of the sample. The obtained results expressed as % of inhibition are presented in Table 3. Here, in the case of $DPPH^{\cdot}$, the lower activity of EO was determined in comparison to the standard reference compound Trolox, since the pure EO could not reach the 50% inhibition of $DPPH^{\cdot}$ (8.60 ±1.52%). On the other hand, the investigated EO expressed higher inhibition activity against ABTS radical cation than Trolox with TEAC 3.83 ±0.03 $mg.L^{-1}$, which is equivalent to 68.32 ±3.54% of inhibition. Previous findings show that the ABTS assay is superior to the DPPH assay in the examination of plant foods containing hydrophilic, lipophilic, and high-pigmented antioxidant compounds (Floegel et al., 2011). This can explain the differences in the antioxidant activity results obtained by using two different assays in this study. Moreover, as limonene is considered not to play an essential role in the antioxidant mechanism of EOs it can explain the low antioxidant capabilities of the tested EO (Bendahaa et al., 2016).

3.3 Antimicrobial activity

The results of the antimicrobial activity of *C. aurantium* var. *dulce* essential oil are summarized in Table 4.

Table 4 Disk diffusion method in the *C. aurantium* var. *dulce*, EO against G^+, G^- bacteria, and yeasts

Microorganism	Zone inhibition (mm ±SD)
Yersinia enterocolitica	0.67 ±0.58
Enterococcus faecalis	2.67 ±0.58
Candida albicans	0.33 ±0.58
Candida glabrata	0.33 ±0.58
Candida krusei	0.33 ±0.58
Candida tropicalis	0.00 ±0.00

Obtained results show very weak inhibition of all tested microorganisms. Considering G^- bacterial *Y. enterocolitica* and all yeast strains, this essential oil showed almost no inhibitor activity, with the inhibition zones in a range from 0.00 ±0.00 to 0.67 ±0.58 mm. Moreover, very weak inhibition was also noted for the G^+ bacteria *Enterococcus faecalis* with a zone of inhibition of 2.67 ±0.58 mm. Previous results of the antibacterial activity of this EO mainly differ. In one study performed by Fisher & Phillips (2006), sweet orange oil showed good activity towards *L. monocytogenes* and weak activity against *C. jejuni*, *E. coli*, *S. aureus*, and *B. cereus*, while results obtained by Friedman et al. (2002) showed that this EO exhibited high activity against *C. jejuni*, low against two *L. monocytogenes* strains, and was merely active against *E. coli* and *S. aureus*. Moreover, some previous studies show that G^+ bacteria strains are more susceptible to the treatment of *Citrus* essential oils compared to G^- bacteria (Akarca & Sevik, 2021; Geraci et al., 2017). Considering the yeast strains reported studies also show low inhibition power of this EO on *C. tropicalis* strain (Akarca & Sevik, 2021).

Even though the mechanisms of action of EOs are not well understood yet, obtained results can be attributed to the low antimicrobial activity of its main constituent, limonene (Thielmann & Muranyi, 2019). Studies made until now, regarding the antimicrobial activity of limonene, show that high concentrations of this monoterpene hydrocarbon are necessary to achieve a significant antimicrobial effect (Thielmann & Muranyi, 2019). Although some studies show that the presence of limonene can change the lipid profile of some bacteria, it is still not possible to fully designate its antimicrobial mode of action (Ruiz & Flotats, 2014).

Table 3 *In vitro* antioxidant activity of *C. aurantium* var. *dulce* essential oils

	% of inhibition	TEAC ($mg.L^{-1}$)	Trolox (IC_{50}) ($mg.L^{-1}$)
DPPH	8.60 ±1.52	1.12 ±0.11	4.39 ±0.13
ABTS	68.32 ±3.54	3.83 ±0.03	2.96 ±0.01

4 Conclusion

This study presents the results of the chemical composition and biological effects of sweet orange (*C. aurantium* var. *dulce*). Results obtained from GC and GC/MS analysis show the presence of 11 compounds, with a very high abundance of limonene (93.86%), followed by notable amounts of monoterpene hydrocarbons β-myrcene (3.1%) and α-pinene (1.0%). In antioxidant investigations, the EO expressed lower antioxidant activity towards $DPPH^{\cdot}$ than the reference compound Trolox (8.60 ±1.52%), whereas against $ABTS^{\cdot+}$ the activity of EO was higher than the reference compound (68.32 ±3.54%). The results obtained from the disc diffusion method showed no or very weak inhibition of microorganisms in the treatment with *C. aurantium* var. *dulce* essential oil which can be attributed to the low antimicrobial effects of limonene.

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