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Orange fruit peels from PDO varieties of Ribera (Sicily, Italy): An insight into the chemistry and bioactivity of volatile and non-volatile secondary metabolites extracted using a microwave-assisted method

Gregorio Peron [a,*], Giulia Bernabé [b], Sara Marcheluzzo [c], Gokhan Zengin [d], Kouadio Ibrahime Sinan [d], Jan Hošek [e,f], Jakub Treml [f], Ignis Kaja [e,f], Michela Paccagnella [c], Paola Brun [b], Ignazio Castagliuolo [b], Mirella Zancato [c], Stefano Dall'Acqua [c]

[a] Department of Molecular and Translational Medicine (DMMT), University of Brescia, Viale Europa 11, 25123 Brescia, Italy
[b] Department of Molecular Medicine, University of Padova, Via Gabelli 63, 35121 Padova, Italy
[c] Department of Pharmaceutical and Pharmacological Sciences, University of Padova, Via Marzolo 5, 35131 Padova, Italy
[d] Department of Biology, Science Faculty, Selcuk University, 42130 Konya, Turkey
[e] Veterinary Research Institute, Hudcova 296/70, CZ-621 00 Brno, Czech Republic
[f] Department of Molecular Pharmacy, Faculty of Pharmacy, Masaryk University, Palackého tř. 1946/1, CZ-612 00 Brno, Czech Republic

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ABSTRACT

Peels of Washington Navel (WAS), Navelina (NAV), and Vaniglia Apireno (VAN) orange fruits from Ribera (Sicily, Italy) represent abundant by-products of the Italian agro-food sector. In this work, volatile (VE) and non-volatile (NVE) extracts were obtained from fresh peels using a microwave-assisted protocol, and were characterized for their content in secondary metabolites and bioactivity. VEs exerted antibacterial activity against different pathogens, and were toxic on Caco2 cells. Their main component was limonene (>85 %). NVEs inhibited tyrosinase, amylase, and glucosidase *in vitro*, and exerted significant antioxidant effects on THP-1 XBlue cells. They showed also anti-inflammatory properties, by decreasing NF-κB activity in LPS-stimulated cells. Putative molecular effectors were highlighted by multivariate correlation analysis. Overall, NVEs may represent novel sustainable nutraceutical ingredients with anti-inflammatory and metabolic properties, and can be obtained by using eco-sustainable approaches. Our results will encourage the reuse of by-products of the orange processing chain and will contribute to increase its circularity in the future.

1. Introduction

In the European Union, the cultivation of *Citrus sinensis* (sweet orange fruit) is essentially concentrated in Mediterranean countries such as Italy, Spain, and Greece, and considering the latest USDA reports, the total annual production amounts to about 6.0 Mt. In Italy, cultivation of *C. sinensis* is located mainly in the Southern Regions, and among these, Sicily represents the main area (55,000 ha) (ISTAT, 2023). Thanks to pedoclimatic characteristics of the territory that allow a perfect acclimatization, the production of blonde pulp orange fruits in Sicily finds its specialization in the south-western coast, which is the largest production area of the Navel varieties in Italy (Tudisca et al., 2014). These factors led in 2008 to the establishment of "Orange of Ribera" Protected Designation of Origin (PDO) (Tudisca et al., 2014), which comprises the two varieties named Washington Navel (WAS) and Navelina (NAV). NAV presents some peculiarities that distinguish it from WAS: the former differs substantially for the period in which fruits are harvested (NAV from November, WAS from December), and for a sweeter juice than WAS (Terranova et al., 1983). Vaniglia Apireno (VAN) is another variety that has its origin from Ribera (Figure S1 of the Supplementary Material), although currently it is not registered as a PDO. VAN is characterized by a pale yellow-orange color of the peel, and for a juice low in sugars and acids. Due to their characteristics, a significant amount of Navel and VAN oranges from Ribera undergo processing to obtain

* Corresponding author at: Department of Molecular and Translational Medicine (DMMT), University of Brescia, Viale Europa 11, 25123 Brescia, Italy.
E-mail addresses: gregorio.peron@unibs.it (G. Peron), giulia.bernabe@unipd.it (G. Bernabé), sara.marcheluzzo@studenti.unipd.it (S. Marcheluzzo), gokhanzengin@selcuk.edu.tr (G. Zengin), jan.hosek@vri.cz (J. Hošek), tremlj@pharm.muni.cz (J. Treml), mic.paccagnella@unipd.it (M. Paccagnella), paola.brun.1@unipd.it (P. Brun), ignazio.castagliuolo@unipd.it (I. Castagliuolo), mirella.zancato@unipd.it (M. Zancato), stefano.dallacqua@unipd.it (S. Dall'Acqua).

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juices and other products such as marmalades and jams, thanks also to their lack of seeds.

In general, the industrial processing of *Citrus* fruits generates tons of organic waste residues such as peels, seeds, and pulp every year. The disposal of these by-products represents an environmental issue, and a significant cost for industries. In Sicily, about 1/3 of orange fruits are used to produce orange juice and essential oils (EOs) thus generating 340,000 tons of by-products *per* year, mainly peels (Ciriminna et al., 2024). Although a significant amount of these is reused for different purposes, a part is still destined to landfilling due to disposal costs that are not always affordable, especially for smaller firms and family plants (Cerruto et al., 2016; Ciriminna et al., 2024). This highlights the need to invest in novel strategies for the valorisation and reuse of orange by-products that can benefit also small producers. Up to now, fresh *Citrus* by-products have been largely employed for the recovery of limonene-rich EOs and for the extraction of dietary fibers such as pectin. Due to their high carbohydrate content, they are used also as anaerobic digestion feedstock for the production of biogas (Andrade et al., 2023). Mainly, orange fruit by-products from Sicily are currently destined to these reuses (Ciriminna et al., 2024). However, *Citrus* peels present a valuable content of bioactive phenolic compounds known mainly for their antioxidant properties [e.g., (Singh et al., 2020)]. For this reason, *Citrus* by-products have been recently exploited as sources of nutraceutical ingredients (i.e. non-medicinal products intended to supplement the normal diet and to promote health) (Andrade et al., 2023; Nazir et al., 2022). To note, one ton of orange peels contains 400 g of hesperidin in average, a flavanone used as bioactive ingredient of food supplements that is currently commercialized at €1,560/kg (Ciriminna et al., 2024).

Globally, there is growing awareness of the health effects of increasing life expectancy and poor lifestyle behaviours that characterize urban societies. Consequently, the use of health-promoting products such as nutraceuticals is increasing, and in recent years many botanicals have been proposed in the market with varying claims of health promotion or disease prevention (Chopra et al., 2022). Among these, dietary supplements with hypothetical preventive effects on age-related diseases such as inflammation, metabolic disorders and cardiovascular diseases are gaining popularity (Chen et al., 2022). Nevertheless, these products are often poorly characterized in terms of chemical composition and biological effects, and these factors, combined with their incorrect use and lack of medical supervision, can lead to a lack of beneficial effects and negatively impact the health of consumers (Sut et al., 2016). It appears clear that there is the need of deeper chemical and pharmacological investigations on these products prior to their distribution in the market.

One of the most important sources of natural compounds with potential health promoting effects are fruits, which are part of human diet and afford a significant contribution to a healthy lifestyle. Fruit by-products such as *Citrus* peels are also attractive, since they represent low-value sources of bioactive compounds that could be valorised through sustainable extraction and characterization. During the last years, several authors have attempted to isolate polyphenols and volatile compounds from orange fruit wastes, by using different techniques such as supercritical CO_2, ultrasound-assisted, and microwave-assisted (MAE) extractions (Q. Li et al., 2023). This latter is particularly interesting since it allows to obtain both volatile and non-volatile secondary metabolites from the same material, as already described by the group of Chemat et al. (Boukroufa et al., 2015).

In this work, we hypothesized that fresh peels of WAS, NAV and VAN oranges from the Ribera area (Sicily, Italy) can be used as valuable sources of bioactive compounds, which can be extracted in high yield by using an optimized "green" approach such as the MAE. To achieve this goal, a method for the simultaneous extraction of volatile and non-volatile secondary metabolites was developed and optimized. The bioactivity of volatile extracts (VEs) was assessed by testing their toxic effects on pathogenic bacteria and cancer cells, while non-volatile extracts (NVEs) were studied focusing on their antioxidant and anti-inflammatory effects, together with their inhibitory properties on enzymes of clinical interest such as cholinesterase, glucosidase and tyrosinase.

Since orange fruit peel represents an abundant by-product of the Italian agro-food sector, and of Sicily in particular, we expect that our results will contribute at valorising this waste material and at increasing its economic value.

2. Methods

2.1. Fruit harvesting and sectioning

Fresh orange fruits of WAS, VAN and NAV varieties were harvested in the Province of Agrigento, Sicily Region, Italy. Fruits were produced following the Council Regulation (EEC) No 2092/91 regarding the organic production of agricultural foodstuff, and were harvested at proper ripening stage during October (NAV) and December (WAS and VAN) 2020. Fruits (approximately 20 kg *per* variety, corresponding to 50 fruits) were stored at controlled conditions (T = 7 °C and rh = 75 %) until their delivery to the laboratory.

After washing with fresh tap water, the fruits were cut and squeezed with a manual juicer to extract the juice, which was not considered in this study. Semi-solid residues consisted in pulp, from one side, and peel composed by intact tissues (albedo and flavedo) on the other. Peels were manually sectioned with a cutter to isolate the fresh flavedo, which underwent extraction of VEs and NVEs. The entire procedure was repeated in triplicate.

2.2. Extraction of volatile and non-volatile secondary metabolites from flavedo

The extraction of VEs from flavedo of NAV, VAN and WAS fruits was carried out using a Milestone Ethos X microwave (MW) extractor. MAE is known as an eco-sustainable approach for the extraction of chemical compounds from natural matrices, considering that no organic solvents are used and the time required to achieve high extraction yields is usually low. The extraction parameters (i.e., distillation time and MW power) were optimized with a design of experiment (DoE), where the yield of VEs and the total area of volatile compounds identified by GC–MS were chosen as response variables. To this aim, a response surface methodology (RSM) based on central composite design (CCD) was developed. CCD consisted of a two-level factorial design, a star design in which experimental points are at a distance α from its centre, and the central point. Each factor was varied over 5 levels ($-\alpha$, -1, 0, $+1$, $+\alpha$). The relationship between dependent and independent variables was fitted by a second-order polynomial model. The validation of the quadratic model obtained by RSM was accomplished by analysis of variance (ANOVA). Different combinations of extraction times (15–45 min) and MW power (500–900 W) were tested, and a total of 14 trials were performed in random order. 500 g of flavedo sectioned from commercial oranges were suspended in 1 L of distilled water inside a 2 L glass vessel, that was positioned inside the hoven and connected to a stainless steel condenser and a glass burette, which was used for the collection of the VE. The same procedure allowed also to extract in the suspension water the non-volatile secondary metabolites from the same material. At the end of the distillation, the aqueous extract in the glass vessel was cooled down to RT, filtered with a Buchner apparatus, and lyophilized. The dry powder was finally stored in plastic tubes at −80 °C until further utilization.

2.3. Analytical methods

2.3.1. GC–MS analysis of volatile constituents

The chemical characterization of VEs was performed by gas chromatography coupled to mass spectrometry (GC–MS). Samples were

prepared by mixing 30 μL of VE with 1 mL of n-hexane. Samples were then added of 1 μL of pure nonanol, which was used as internal standard (IS). The GC–MS equipment was an Agilent 7820A coupled to an Agilent 5977B MS. An Agilent DB-5 capillary column (30 m, i.d. 250 mm, 0.25 μm) was used as stationary phase. Helium was used as carrier gas, at a flow rate of 1 mL/min. Sample injection (1 μL) was performed in split mode (1:50). The separation of volatile constituents in GC was achieved using a temperature ramp, as follows: 50 °C for 8 min, then to 250 °C at 4 °C/min; isocratic for 3 min; then to 310 °C at 4 °C/min, and the temperature is maintained for 5 min. Afterwards, the initial conditions are restored. The injector temperature was 210 °C. MS data were acquired in the *m/z* range 45–650 Da. The components were identified by comparing their mass spectra to those of the NIST Library (NIST, 2014), and by evaluating their linear retention indices (LRI) relative to C_6-C_{24} n-alkanes. The percentage composition of VEs was calculated from the GC peak areas using the normalization method without correction factors. The data were finally reported as the mean value of three injections.

2.3.2. Analysis of non-volatile secondary metabolites by chemical assays and HPLC-DAD-MS

Folin-Ciocalteu and $AlCl_3$ assays were used to determine the total phenolic and flavonoid contents, respectively (Zengin & Aktumsek, 2014). For respective assays, results were expressed as gallic acid equivalents (mg GAEs/g dry extract) and rutin equivalents (mg REs/g dry extract).

Non-volatile secondary metabolites of peel extracts were characterized by HPLC-DAD-MS^n. Samples were prepared by suspending 10 mg of lyophilized extracts in 5 mL of methanol using an ultrasound bath. The mixtures were then centrifuged at 9880 relative centrifugal force (rcf) for 10 min, and supernatants were directly injected in the HPLC system. The instrumentation consisted of an Agilent 1260 quaternary pump coupled to an Agilent 1260 diode array detector (DAD) and a Varian MS 500 mass spectrometer (MS) equipped with electrospray (ESI) ion source. A Zorbax SB C18 column (250 × 4.6 mm, 5 μm) was used as stationary phase. A mixture of 0.1 % formic acid in water (A), acetonitrile (B) and methanol (C) was used as mobile phase. The gradient was as follows: 0 min, 90 % A, 7.5 % B, 2.5 % C; 20 min, 80 % B, 20 % C; 22 min, 80 % B, 20 % C; 23 min, 90 % A, 7.5 % B, 2.5 % C. The flow rate was 0.75 mL/min. Injection volume was 10 μL and the column temperature was set at 30 °C. The DAD allowed to acquire chromatographic data in the λ range of 200–640 nm. MS spectra were acquired in both positive and negative ion modes, in the *m/z* range 100–2000. MS parameters were as follows: spray chamber temperature, 50 °C; nebulized pressure, 55 psi; drying gas pressure, 15 psi; needle voltage, ±5 kV; spray shield voltage, ±600 V. The fragmentation pattern of the most intense ion species was obtained using the turbo data depending on scanning (TDDS) function of the instrument. The identification of compounds was obtained based on comparison with the literature and reference standards, when available. For compound quantification, rutin, hesperidin, nomilin, bergapten were used. Standard solutions were prepared in the concentration ranges 1–100 μg/mL and calibration curves were built.

2.4. Antioxidant and enzymatic assays

2.4.1. Antioxidant assays

Antioxidant assays were carried out according to previously reported methodologies (Grochowski et al., 2017). The antioxidant potential was expressed as: mg Trolox equivalents (TE)/g extract in 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging, cupric reducing antioxidant capacity (CUPRAC) and ferric reducing antioxidant power (FRAP) tests, mmol TE/g extract in phosphomolybdenum assay (PDA) and mg ethylenediaminetetraacetic acid equivalents (EDTAE)/g extract in metal chelating assay (MCA).

2.4.2. Inhibition of enzymes of clinical relevance

The enzyme inhibitory assays were carried out according to previously reported methodologies (Grochowski et al., 2017). The acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibition was expressed as mg galantamine equivalents (GALAE)/g extract; tyrosinase inhibition was expressed as mg kojic acid equivalents KAE/g extract; amylase and glucosidase inhibition was expressed as mmol acarbose equivalents (ACAE)/g extract.

2.5. Bioactivity assays on volatile extracts in vitro

2.5.1. Antibacterial activity on pathogens of clinical interest

The antimicrobial activity of VEs was determined on both gram + and gram- bacteria by the microplate dilution method, following a previously reported protocol (Faggian et al., 2021). Methicillin-resistant *Staphylococcus aureus* (MRSA), *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*, *Aeromonas caviae*, and *Salmonella enterica* were cultured in Luria Bertani broth (LB). Bacteria cultures were growth at 37 °C for 16 h with shaking. After incubation, bacteria were collected by centrifugation and resuspended at a final concentration of 1×10^6 CFU/mL. Diluted bacteria were added to 96-well plates, where VEs were added at a final concentration ranging from 10 % to 0.01 %. After 16 h incubation at 37 °C, the optical density at 620 nm (as measure of total bacterial cells) was determined with Varioskan LUX multimode multiplate reader to evaluate the effect of VEs on bacterial growth. Bacteria incubated only with growth medium were used as control and their growth was arbitrarily set at 100 %. The effect of VEs on bacterial growth was calculated by comparison with not treated control using the following formula:

$$\%Bacterial\ growth = (OD_{620}sample/OD_{620}control) * 100$$

2.5.2. Cytotoxicity on Caco-2 cells

The procedure has been already described in another article (Bernabè et al., 2021). Briefly, human epithelial colorectal adenocarcinoma cells (Caco-2, ATCC# R HTB-37TM) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20 % foetal bovine serum (FBS) and 1 % penicillin/streptomycin. Cells were seeded in 96-well plates with the proper culture media. When they reached the confluence, cells were treated with VEs at concentrations ranging from 10 to 0.01 %. The culture medium was removed after 24 h at 37 °C and replaced with fresh complete medium. To measure cell viability, a MTT [3-(4,5-dimethylthiazol-2-yL)-2,5-diphenyltetrazolium bromide] solution (5 mg/mL) was added to each well and maintained at 37 °C for additional 4 h. Formazan crystals were then solubilized in 100 μL of SDS 10 % w/v HCl 0.01 N. The absorbance was recorded 16 h later at 590 nm using a Varioskan LUX multimode multiplate reader. Vitality of cells incubated with DMEM plus ethanol was set as 100 %.

2.6. Bioactivity assays on non-volatile extracts in vitro

2.6.1. Cell culture, maintenance, and viability determination

Human monocytic leukaemia cell line THP1-Blue™ NF-κB (Invivogen, San Diego, CA, USA) was routinely cultivated in RPMI 1640 medium with stabile glutamine, 10 % foetal bovine serum (FBS) and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin; Merck, St. Louis, MO, USA). Cells were passaged twice a week and their viability was regularly controlled by Trypan Blue staining.

The effect of test extracts on cell viability was evaluated using Cell Counting Kit 8 (CCK8; Abcam, Cambridge, UK) according to manufacturer's manual, as we described previously (Kos et al., 2021). Briefly, cells were seeded in the concentration of 5×10^4 cells/well in 96-well plate, resuspended in a serum-free cultivation medium. The test material dissolved in DMSO (Merck) were added after 2 h recovery span. The maximum concentration of DMSO in the assays never exceeded 0.1 % (v/v). Cell viability was measured 24 h later and the IC_{50} values (the

concentration of test nanoparticles, which caused 50 % decrease of metabolic active cells in the comparison with untreated cells) were calculated according to four parameters logistic (4PL) analysis, excluding outstanding values (ROUT algorithms, Q = 5 %) in Prism 7.01 software (GraphPad Software, Inc., San Diego, CA, USA).

2.6.2. Determination of anti-NF-κB activity

The effect of the tested extracts on the activity of pro-inflammatory transcription factor NF-κB was determined on lipopolysaccharide (LPS)-challenged THP1-Blue™ NF-κB cells as we described previously (Leláková et al., 2020). Briefly, the cells were pre-treated by the tested material dissolved in DMSO at concentration of 10 μM for 1 h. Then, LPS from *Escherichia coli* 0111:B4 (Merck) dissolved in serum-free RPMI 1640 medium (1 μg/mL) was added. After 24 h incubation, the activity of NF-κB was determined as the amount of secreted embryonic alkaline phosphatase using Quanti-Blue™ medium (Invivogen). The relative NF-κB activity was determined as a ratio between tested group and untreated LPS-challenged (DMSO) group.

2.6.3. Cellular antioxidant activity (CAA) assay

The cellular antioxidant activity of the test extracts was measured in THP-1 XBlue cells using a method reported previously (Malaník et al., 2020).

2.7. Statistical analysis

All experiments were conducted in triplicate, and expression of the results was as mean ± SD (standard deviation). One-way variance analysis (ANOVA) was used to detect the differences among examined data, followed by Tukey's honest significant difference post hoc test. Differences with a p-value < 0.05 were considered significant. Statistical analyses were performed using the SPSS version 14.0 program. Construction of the graphs was performed by GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA).

To investigate the differences in chemical composition of VEs and NVEs, a multivariate approach was used. GC–MS and HPLC-MS data were transformed in numerical matrices that were exported as.csv files. These were then analysed using the MetaboAnalyst v. 5.0 platform (https://www.metaboanalyst.ca/). Prior to analysis, data were sum-normalized, log transformed and Pareto scaled. The dataset was first submitted to ANOVA with the Fisher's Least Significant Difference (LSD) post-hoc test. P-values adjusted for false discovery rate (FDR) were evaluated and FDR-adjusted $p < 0.05$ was considered as statistically significant. The same data were then explored by using an unsupervised analysis in order to identify putative molecular markers of each orange fruit variety, and results were visualized using heatmaps.

Correlations between chemical composition of NVEs and their bioactivity were assessed performing a Pearson's rank correlation test using the MetaboAnalyst platform. For every correlation, the Pearson's coefficient (r) was calculated. $-1 < r < 0$ indicated a negative correlation between the variables studied, while $0 < r < 1$ was associated to positive correlations. A correlation heatmap was built to facilitate data visualization and interpretation.

3. Results and discussion

3.1. Optimization of microwave-assisted distillation

MAE is a type of hydro-distillation that uses as heating system a MW oven. The advantages of such method can be summarised in the higher energy efficiency of extraction compared to traditional methods, and in the opportunity of controlling extraction conditions, that allow to develop tailor-made extractions. MW allows to obtain VEs from natural matrices by using lower amounts of water compared to traditional hydro-distillation, and are usually less time-consuming (Golmakani & Rezaei, 2008). Furthermore, the same procedure allows to extract non-volatile metabolites from the same natural materials, simultaneously to the distillation. In this work, with the aim to ensure an efficient extraction of VEs, the MAE method was optimized by using the response surface methodology. Extraction time, MW power and biomass/water ratio in the extraction vessel are reported as the most critical parameters for VE yield (Bustamante et al., 2016). In our study, a preliminary evaluation of the biomass/water ratio was performed independently from the study of the other two parameters, and biomass/water ratios of 1:1, 1:2, 1:3 and 1:4 *w/v* were evaluated. Results showed that the 1:2 ratio was the most suitable, not only for the highest yield (data not reported), but also for the feasibility of VE recovery, considering that higher volumes of water in the vessel lead to the condensation of greater volumes of water in the collector tube.

As shown in Fig. 1, the estimated optimum values for extraction time and MW power were 25 min and 900 W, respectively. Using these parameters, the highest extraction yield was obtained, i.e. 0.65 % of fresh material. Such results were expected, considering the MAE methods already reported in literature for *Citrus* peels [e.g., (Bustamante et al., 2016)]. The adequacy of the model was evaluated by using the coefficient of determination (R^2), and assessing the regression fitness. The two-factor interaction model (2FI) showed statistical valid fitness ($p < 0.0001$ for the regression F test) and non-significant lack of fit ($p = 0.70$), and adjusted R^2 was 0.92.

The effect of time and MW power on the extraction of volatile compounds was also evaluated comparing total number of chromatographic peaks and total peak area obtained after GC–MS analysis of the obtained VEs. The results showed a non-significant effect on peak numbers, while the effect on total area was significant ($p = 0.0015$). The highest total AUC was obtained from the VE obtained using a MW power of 900 W for 25 min (Fig. 1). The parameters that were optimized in this study for optimal VE extraction from orange fruit peels are in line with those already reported by other authors (Auta et al., 2018).

3.2. Chemical and pharmacological analyses of VEs

3.2.1. Chemical characterization

The chemical profile of VEs from the peels of VAN, NAV and WAS varieties was elucidated by using GC–MS. Results are reported in Table S1 and Fig. S2-3 of the Supplementary Material. More than 98 % of the VEs composition was elucidated, and for all the orange varieties limonene was the major compound, as expected, contributing for the 85–87 %. Significant inter-variety differences were observed especially for sesquiterpenes, that were more abundant in WAS and NAV (Table S1). Specifically, the compounds α-copaene, β-cubebene, germacrene D, valencene, and γ-cadinene were those contributing significantly to these differences. Total monoterpene content was also differing, being significantly higher in WAS and VAN. α-Thujene, myrcene, α-terpinene, and γ-terpinene were the compounds underlying these differences. Moreover, α-thujene (associated to wood, green, and herb odours; https://flavornet.org/flavornet.html) was detected only in VAN, hence it can be considered as a putative marker of this variety. Similarly, the amounts of valencene (associated to green and oil odours; https://flavornet.org/flavornet.html) were at least six times higher in WAS and NAV compared to VAN (Figures S2 and S3), hence it can be regarded as a putative volatile marker of these Navel varieties. Nevertheless, further studies will be required to validate our results.

Comparing our data with previously published results, it can be noticed that, except for the high contents in limonene, there are differences in the less abundant volatile constituents of the three orange varieties [NAV: (Ferrer et al., 2022); WAS: (Njoroge et al., 2005; Sawamura et al., 2005); VAN: no data]. First of all, this may be due to the different extraction procedures used. Furthermore, the intra-species variability in the composition of EOs and VEs has been widely described in literature, and it has been associated to several aspects correlated to environmental and climatic conditions, harvesting periods, and extraction methods (Barra, 2009).

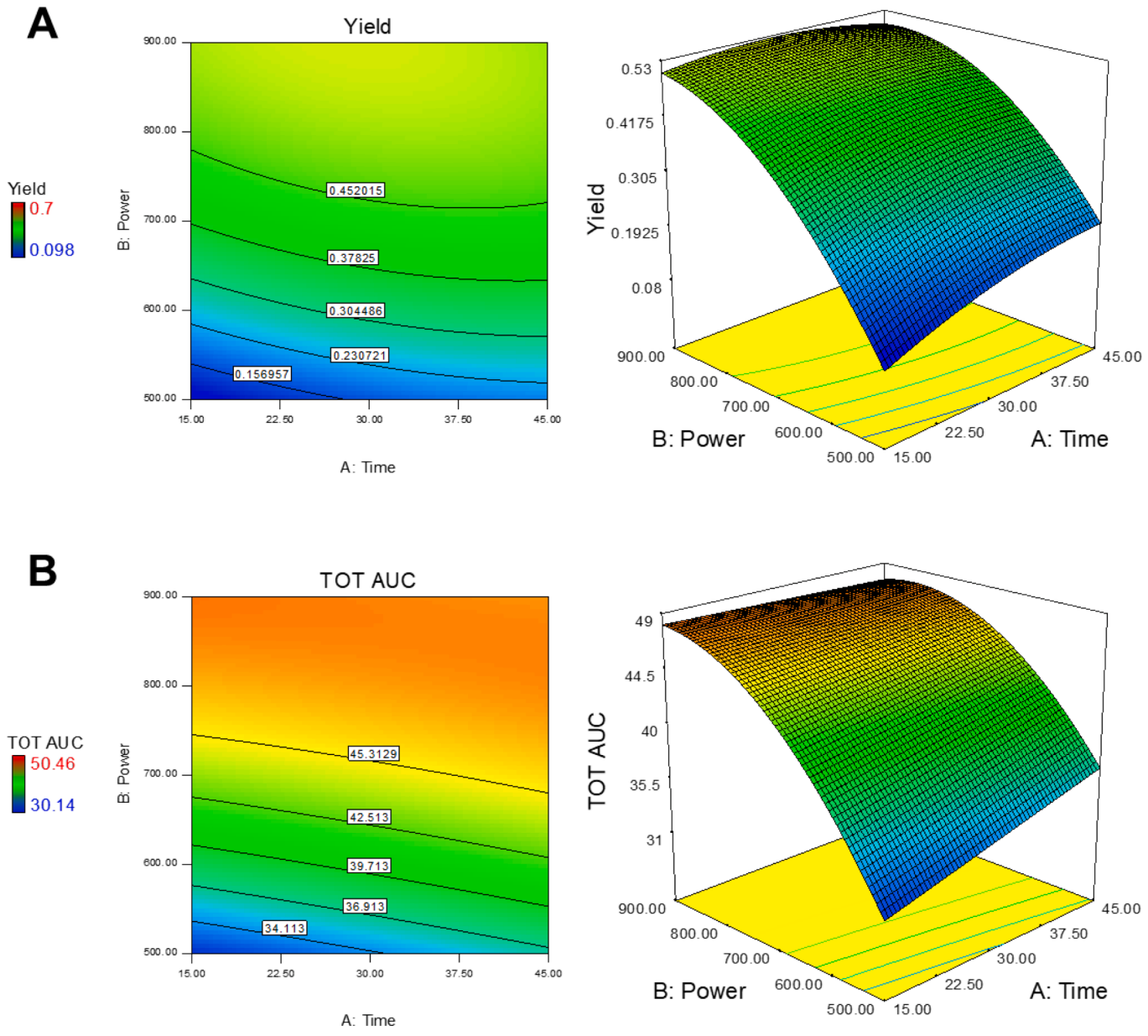

Fig. 1. Response surface plots from the two-factor interaction model showing the effect of time (min) and microwave power (W) on the extraction yield of volatile compounds from fresh material (panel A), and total chromatographic area (AUC; panel B). Total AUC was calculated by the sum of the peak areas normalized for the AUC of the peak corresponding to the internal standard.

3.2.2. *Antioxidant activity of VEs*

Three different assays were performed, namely DPPH, ABTS, and carotene bleaching assays. The β-carotene bleaching technique is more suitable for non-polar antioxidants. In fact, it employs an emulsified system, so the activity depends on the substrate polarity. Non-polar antioxidants can exhibit stronger antioxidant properties in emulsions because they concentrate at the lipid phase, while polar antioxidants remain in the aqueous phase and are thus less effective in the lipid protecting (Nickavar & Esbati, 2012). On the other hand, the antioxidant potential of compounds in DPPH and ABTS assays is not dependent on their polarity (Nickavar & Esbati, 2012), hence a broader variety of chemical compounds can be evaluated. Our results showed no antioxidant potential of the VEs from NAV, WAS and VAN fruits in the DPPH and ABTS assays. According to previously published data, this result was expected. In fact, the VEs from orange fruit peels are rich in monoterpenes that cannot neutralize the radical species by donating a hydrogen (Magalhães et al., 2020). Regarding the carotene bleaching assay, VEs from the three orange varieties showed a mild and comparable antioxidant potential, with IC_{50} = 23.10 ± 1.32 %, 27.22 ± 2.00 %, and 20.11 ± 1.74 % for NAV, WAS, and VAN, respectively. Literature data regarding carotene bleaching assay on VEs and EOs from *Citrus* species are heterogeneous, and they highlight that their chemical composition is determinant. Magalhães and coll. reported recently no antioxidant potential of EO from the peels of *C. sinensis* Osbeck in this assay (Magalhães et al., 2020). The EO was mainly composed of monoterpenes such as limonene (95.12 %), α-pinene (0.35 %), sabinene (0.54 %), and myrcene (1.07 %). Conversely, Ben Miri et al. evaluated the antioxidant activity of EO from *C. sinensis* var. Valencia and obtained positive results by this method, showing higher antioxidant potential (55.56 % inhibition of oxidation) compared to those observed for VEs from WAS, NAV and VAN (Ben Miri et al., 2018). However, also in this case the volatile composition of the EO significantly differed from our volatile extracts, with lower amounts of monoterpenes hydrocarbons (86.3 % vs. 93 %, respectively) and higher abundancy of oxygenated

monoterpenes (9.7 % vs. 2.5 %). As stated above, these differences may be due also to the different extraction procedures used. A mild antioxidant activity was also reported by Toscano-Garibay and coll. for the EO from Mexican *C. sinensis*, although data regarding its chemical composition was limited to the high amount of R-(+)-limonene (96 %) (Toscano-Garibay et al., 2017).

3.2.3. Antibacterial and anti-proliferative activities of VEs

The potential of VEs from NAV, VAN, and WAS peels to inhibit bacterial growth was tested on six species of clinical relevance, namely the gram + MRSA and *E. faecalis*, and the gram- *A. caviae, S. enterica, K. pneumoniae,* and *P. aeruginosa*. Overall, results reported in Fig. 2 show comparable antimicrobial activity of the three extracts tested. The most significant differences were observed on *S. enterica*, where VEs from VAN and WAS were active from a concentration of 0.05 %, while activity for NAV was observed only for VE at the highest concentration tested (10 %). Conversely, this latter VE was the most active against *A. caviae* (from 0.1 %). All the VEs failed to interfere with *P. aeruginosa* growth. The antibacterial activity of volatile extracts and EOs from *C. sinensis* peel against different bacterial species has been previously reported in literature, although except for the Washington Navel variety (Settanni et al., 2012), to the best of our knowledge there are no available data for the others considered in our study. Hence, our data are the first to show the antibacterial properties of volatile extracts from fresh NAV and VAN.

To note, the three VEs exhibited relevant cytotoxic effects in Caco-2 cells at concentrations higher than 0.1 %, i.e. those that showed antibacterial activities against most of the species tested (Fig. 3). At 0.5 %, VEs from NAV and VAN reduced cell viability by almost 20 %, while a more marked effect was observed for WAS (30 %). This latter revealed to be the most toxic, although at the highest concentrations (5 and 10 %) all the VEs where characterized by high toxicity (cell viability was reduced to 20–5 %). Although the high amount of articles proposing potential therapeutic or food applications of *C. sinensis* peel EOs and volatile extracts, the cytotoxicity of effective doses in cellular or animal models has been scarcely reported, and only recently it has been shown in Caco-2 cells. In a study by Lanzerstorfer and coll., the authors showed that *Citrus* EO (species not specified) is toxic to Caco-2 cells at concentrations higher than 0.1 %, after which their viability is rapidly reduced

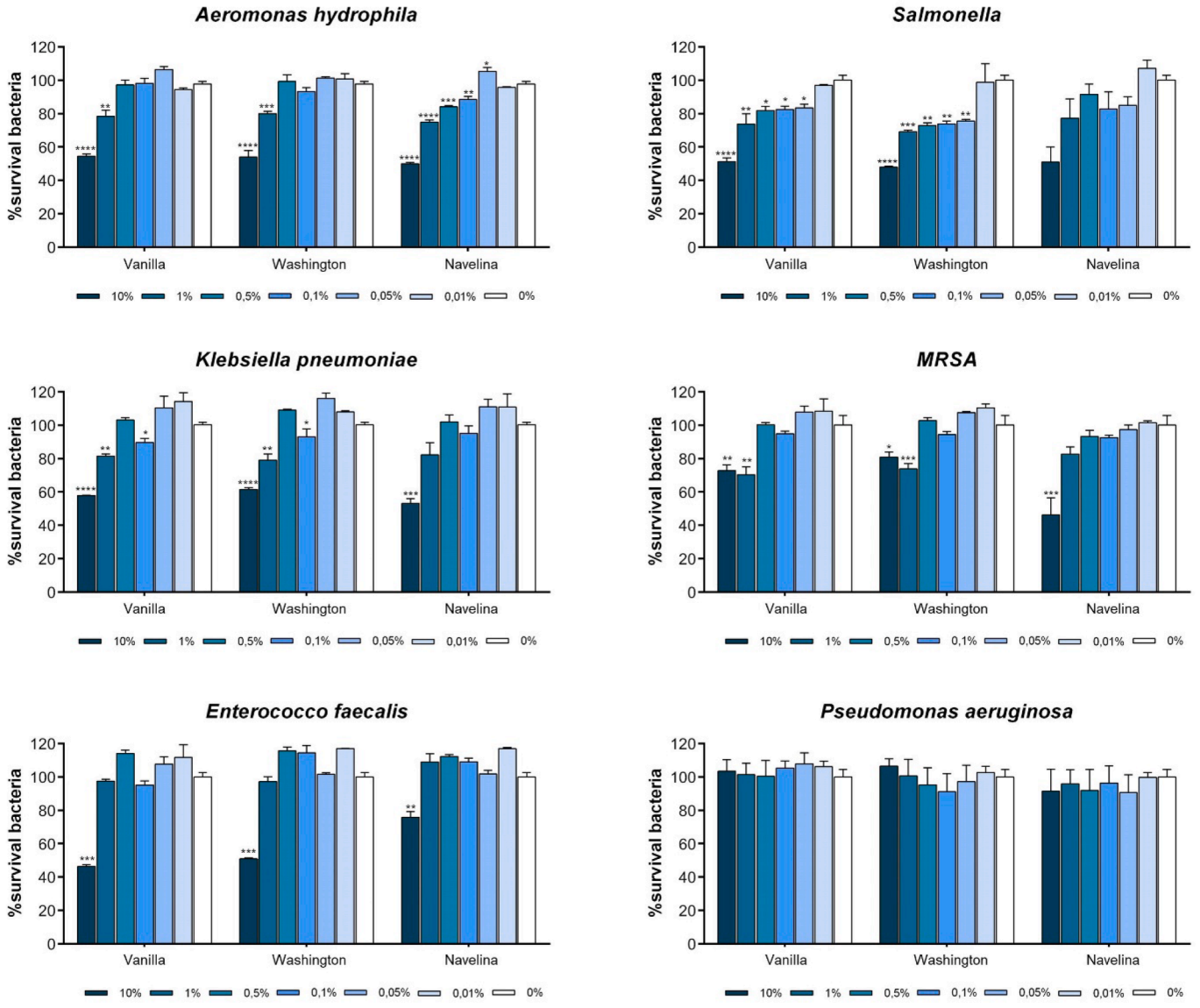

Fig. 2. Antibacterial activity of orange peel volatile extracts tested against different species of clinical relevance. Bacteria (10^6 CFU/ml) were seeded in 96-wells plate and VEs were added at the specified concentration. Plates were incubated at 37 °C for 24 h and then OD was measured at 620 nm using a Varioskan LUX multimode multiplate reader as a measure of bacterial growth. White bars indicate control, while the colour intensity of other bars indicate the increasing VE concentration (%). *P < 0.05, **P < 0.01, ***P < 0.001 vs control.

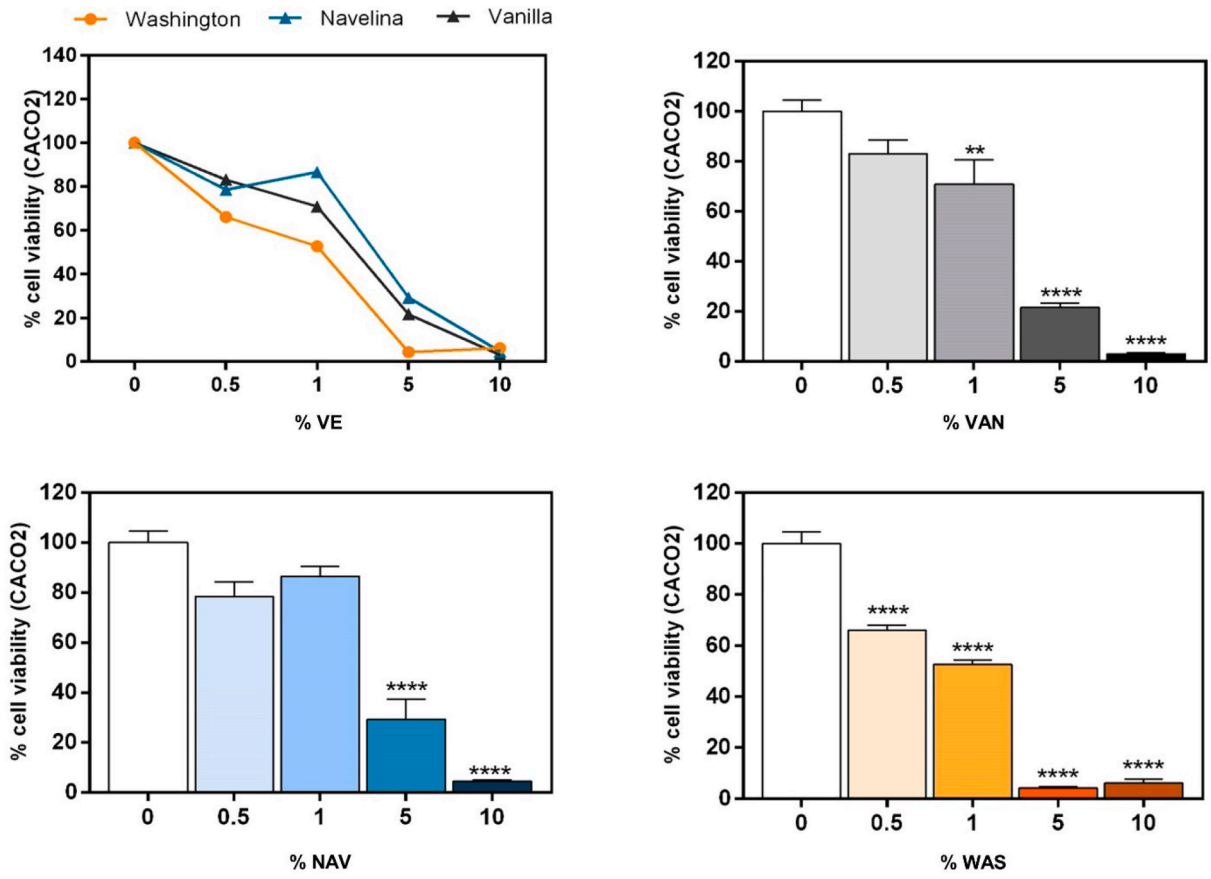

Fig. 3. Cytotoxicity of volatile extracts (VE) from Washington Navel (WAS), Navelina (NAV), and Vaniglia Apireno (VAN) peels in Caco-2 cell model. Confluent Caco-2 monolayers were expose to VEs (0 to 10 %). MTT solution was added to each well, and after 4 h 100 μL of SDS 10 % w/v HCl 0.01 N were added to solubilize formazan crystals. The absorbance was recorded using a Varioskan LUX multimode multiplate reader. **P < 0.01, ****P < 0.0001 vs control.

(to 80 % when treated with 0.25 % dose, and 20 % with a 0.5 % dose, similarly to what was observed in our study) (Lanzerstorfer et al., 2021). The authors concluded that detailed toxicological assessments are highly recommended for this EO and for single intended application.

Considering its toxicity on different cell models, other authors have proposed the EOs from *C. sinensis* peels as anti-proliferative agents (Singh et al., 2021). The high concentration of limonene has been considered as responsible of the toxic effects. This abundant monoterpene acts through different pathways: it can induce cell apoptosis by increasing the expression of Bax and activating the caspase pathway; it can inhibit tumour growth by increasing the expression of p53 protein and decreasing the activity of Ras/Raf/MEK/ERK and PI3K/Akt pathways; finally, it can interfere with tumour vascularization, by decreasing the expression of the VEGF (de Araújo-Filho et al., 2021).

In light of our results, we conclude that the use of NAV, VAN and WAS peel VEs as antibacterial agents *in vivo* seems not feasible, due to the significant toxicity observed at the effective doses. However, alternative applications as antibacterial could be tested in the future, such as in detergents and sanitizing products. Furthermore, the same VEs can be investigated in future to assess their potential as natural anti-proliferative agents.

3.3. Chemical and pharmacological analyses of NVEs

3.3.1. Chemical characterization

The MAE protocol, which was optimized for the extraction of VEs from fresh orange peels, allowed to recover also non-volatile secondary metabolites from the same material, in a single and fast extraction step. The optimal conditions used to maximise VE distillation (900 W for 25 min) allowed to obtain a NVE with a mean yield of 8.2 % on fresh weight. The residual water in the loading vessel was lyophilized and the obtained powder was used for chemical characterization and biological assessment. Results from TPC and TFC determinations indicated that the contents of both phenols and flavonoids in NAV were almost double compared to WAS and VAN. Namely, TPC in NAV was 33.09 ± 0.53 mg GAE/g DW, compared to 18.77 ± 0.27 and 17.82 ± 0.13 mg GAE/g DW of WAS and VAN, respectively. Regarding TFC, the amount was 9.39 ± 0.09 mg RE/g DW in NAV, compared to 5.55 ± 0.04 and 5.9 ± 0.05 mg RE/g DW in WAS and VAN, respectively.

Phytochemical screening was performed by HPLC-DAD-MS^n, and data were acquired in both negative and positive ion modes. The two ionization modes were used to cover the broadest molecular range as possible, considering that instrumental sensitivity for (poly)phenols such as flavonoids and flavanes is usually higher in negative mode, while positive mode is more suitable for methoxylated flavonoids and (furo) coumarins. Table S2 of the Supplementary Material summarizes the results, while exemplificative chromatograms are reported in Figure S4. Significant differences among the three orange varieties in matter of non-volatile secondary metabolites were observed (Figure S5 of the Supplementary material). The most abundant compounds identified were flavones, with an average amount around 4 mg/g of fresh peels, followed by flavanes (1.4 mg/g), and limonoids (0.4 mg/g). Low amounts of coumarins and furocoumarins were also detected, i.e. 0.1 mg/g. The content of furocumarins in products for human use derived from *Citrus* species is usually carefully evaluated, since they have controversial effects on humans. In fact, they are potential photosensitizers, and they act as inhibitors of the intestinal cytochrome P450-3A4, hence causing interference with the metabolism of several drugs (Arigò

et al., 2021). However, *C. sinensis* has been reported to be almost devoid of these compounds (Bruni et al., 2019), as shown in a recent publication on *C. sinensis* from Colombia, where furanocoumarins amount in peels was comparable with those quantified in NAV, WAS, and VAN varieties (0.093 mg/g) (Ramírez-Pelayo et al., 2019).

Data were analysed by multivariate statistics. The resulting heatmap shows two clusters, one formed by the Navel varieties and one by VAN (Figure S5). The two Navel varieties form two distinct sub-clusters, indicating that also the composition of their peels in non-volatile secondary metabolites differs. These differences are driven by the amounts of the identified metabolites in the three orange varieties. Among the most significant ($p < 0.01$) are eriocitrin/neoeriocitrin, 5,7,3′,4′-tetramethoxyflavone and obacunoic acid, which are characteristic of peels from VAN. Other few compounds are more abundant in the peels from the two Navel varieties, namely the flavone diosmetin-6–8-di-C-glucoside, the limonoid limonin and the megastigmane roseoside. However, several significant metabolites are characteristic of the NAV variety, and among these are mainly flavones (Table S2, and Figures S6 and S7).

3.3.2. Antioxidant activity of NVEs

Results show a higher antioxidant capacity of NAV in all the assays performed (Table 1). The reason of this result resides in the different chemical composition of orange fruit peels, as indicated by the correlation heatmap in Fig. 4. TPC and TFC, higher in NAV, are positively correlated to antiradical, metal complexing and reducing activities. Among specific constituents, several flavones such as luteolin, luteolin-7-O-neohesperidoside, quercetin-dihexoside and apigenin-6–8-di-C-glucoside are positively correlated with the antioxidant activities. Among the other constituents, all the identified limonoids except obacunoic acid are associated to the antioxidant effects.

The correlation between phenolic content of orange fruit peel extracts and their antioxidant activity has been already reported in literature, considering for instance the varieties Washington Navel (Lagha-Benamrouche & Madani, 2013), Osbeck cv. Gannanzao (Long et al., 2021), and Newhall (Guo et al., 2020). However, to the best of our knowledge, our data represent the first regarding NAV and VAN from Sicily, Italy. Furthermore, literature data deal with extracts obtained with organic solvents, instead of more eco-sustainable approaches such as MAE.

Table 1
Antioxidant activity and enzyme inhibitory properties of orange peel extracts from NAV, WAS, and VAN. Results are reported as mean ± S.D. of three independent measurements. n.a.: not active; TE: Trolox equivalents; ETDAE: ethylenediaminetetraacetic acid equivalents; GALAE: galantamine equivalents; ACAE; acarbose equivalents; KAE: kojic acid equivalents.

	Assay	NAV	WAS	VAN
Antioxidant potential	DPPH (mg TE/g)	13.32 ± 0.59	6.81 ± 0.71	6.40 ± 0.03
	ABTS (mg TE/g)	48.63 ± 0.76	23.11 ± 0.79	20.49 ± 0.66
	CUPRAC (mg TE/g)	45.44 ± 0.60	30.84 ± 0.16	30.23 ± 0.09
	FRAP (mg TE/g)	39.06 ± 0.34	25.54 ± 0.30	24.14 ± 0.31
	MCA (mg EDTAE/g)	21.74 ± 0.16	8.76 ± 0.12	10.75 ± 0.12
	PBD (mg TE/g)	1.63 ± 0.05	0.89 ± 0.03	0.82 ± 0.00
Enzyme inhibition	AChE (mg GALAE/g)	n.a.	n.a.	n.a.
	BChE (mg GALAE/g)	n.a.	n.a.	n.a.
	Tyrosinase (mg KAE/g)	15.91 ± 1.17	11.1 ± 0.94	16.83 ± 1.90
	Amylase (mg ACAE/g)	0.04 ± 0.00	0.04 ± 0.00	0.03 ± 0.00
	Glucosidase (mg ACAE/g)	0.63 ± 0.05	1.05 ± 0.10	0.62 ± 0.05

The same extracts were also evaluated for their antioxidant activity in cells. Since the CAA assay is based on generation of peroxyl radicals in a cell culture, its results are considered to be more relevant for *in vivo* conditions compared to traditional chemical-based assays (Treml et al., 2021). In THP-1 XBlue cells, all the tested extracts (10 μg/mL) showed an activity of about 60 % compared to the positive control (quercetin at 5 μM; CAA = 85.7 ± 3.5). Significant differences among the tested extracts from different orange varieties were not observed, although VAN showed the highest activity (CAA = 53.5 ± 6.1). Results are graphically shown in Fig. 5A.

3.3.3. Inhibitory activity against enzymes of therapeutic interest

Results, reported in Table 1, show different inhibitory properties of the three peel extracts against specific target enzymes. Regarding AChE and BChE, no activity was detected. However, a different scenario was observed for tyrosinase, amylase and glucosidase. NAV and VAN showed a significantly higher inhibitory potential against tyrosinase compared to WAS, which in turn was more active than the positive control kojic acid. Conversely, glucosidase was significantly inhibited only by WAS (1.05 mmol ACAE/g of dried peel extract). Finally, the three extracts showed a low activity against amylase, comprised between 0.03 and 0.04 mmol ACAE/g of peel extract. The anti-tyrosinase property of orange peel extracts has been already documented, although for different varieties than those studied here. Guo et al. recently reported an IC_{50} value of 108.24 μg/mL for the ethyl acetate fraction of a 95 % ethanol extract of Newhall navel peels (compared to 5.21 μg/mL of kojic acid) (Guo et al., 2020). Authors indicated sinensetin, 4′,5,6,7-tetramethoxyflavone, nobiletin, 3,3′,4′,5,6,7-hexamethoxyflavone, and narirutin as secondary metabolites associated to this bioactivity (Guo et al., 2020). Nobiletin was reported as anti-tyrosinase also by Sasaki and Yoshizaki (IC_{50} = 18.6 μg/mL), in a work where a *Citrus* peel extract (species not reported) was tested. The activity of nobiletin was comparable of that of kojic acid (Sasaki & Yoshizaki, 2002).

Biological data were correlated to the chemical composition of peel extracts in order to undercover possible molecular effectors. Results are reported in the heatmap in Fig. 4. These plots show that the methoxylated flavones homoeriodictyol and 4′,5,6,7-tetramethoxyflavone were positively correlated to anti-tyrosinase activity, as already observed by other authors (Dej-adisai et al., 2022; Guo et al., 2020). This result strengthens the indication that these compounds, secondary metabolites typical of orange fruit peels, are among the most important tyrosinase inhibitors present in this natural matrix. However, other polymethoxylated flavones showed negative correlations with anti-tyrosinase activity, although not significant. In light of these results, future research focused on structure–activity correlation are needed, especially to explain the influence of methoxyl- groups distribution in the flavone scaffold on the enzyme inhibitory effects. Concerning anti-amylase and anti-glucosidase activities, 5,6,7,3′,4′-pentamethoxyflavanone and roseoside were positively correlated. These compounds, being more abundant in peels from WAS oranges, revealed to be the main responsible of its bioactivity. The role of roseoside as an inhibitor of glucosidase has been already reported in previous studies (Fawzi Mahomoodally et al., 2020; Zhang et al., 2021), hence our data further support the potential usefulness of this compound and its natural sources in the prevention and management of diabetes and other metabolic disorders.

3.4. Evaluation of anti-NF-κB activity

The transcription factor NF-κB is one of the key regulator of inflammatory response and it drives the expression of more than 150 genes (Perkins & Gilmore, 2006). Because it acts mainly in a pro-inflammatory manner, it is a favourite target of anti-inflammatory therapy.

All the tested orange extracts were able to decrease NF-κB activity in LPS-stimulated cells by 10 % (Fig. 5B). According to our best knowledge, this is the first observation of direct inhibition activity of NF-κB by

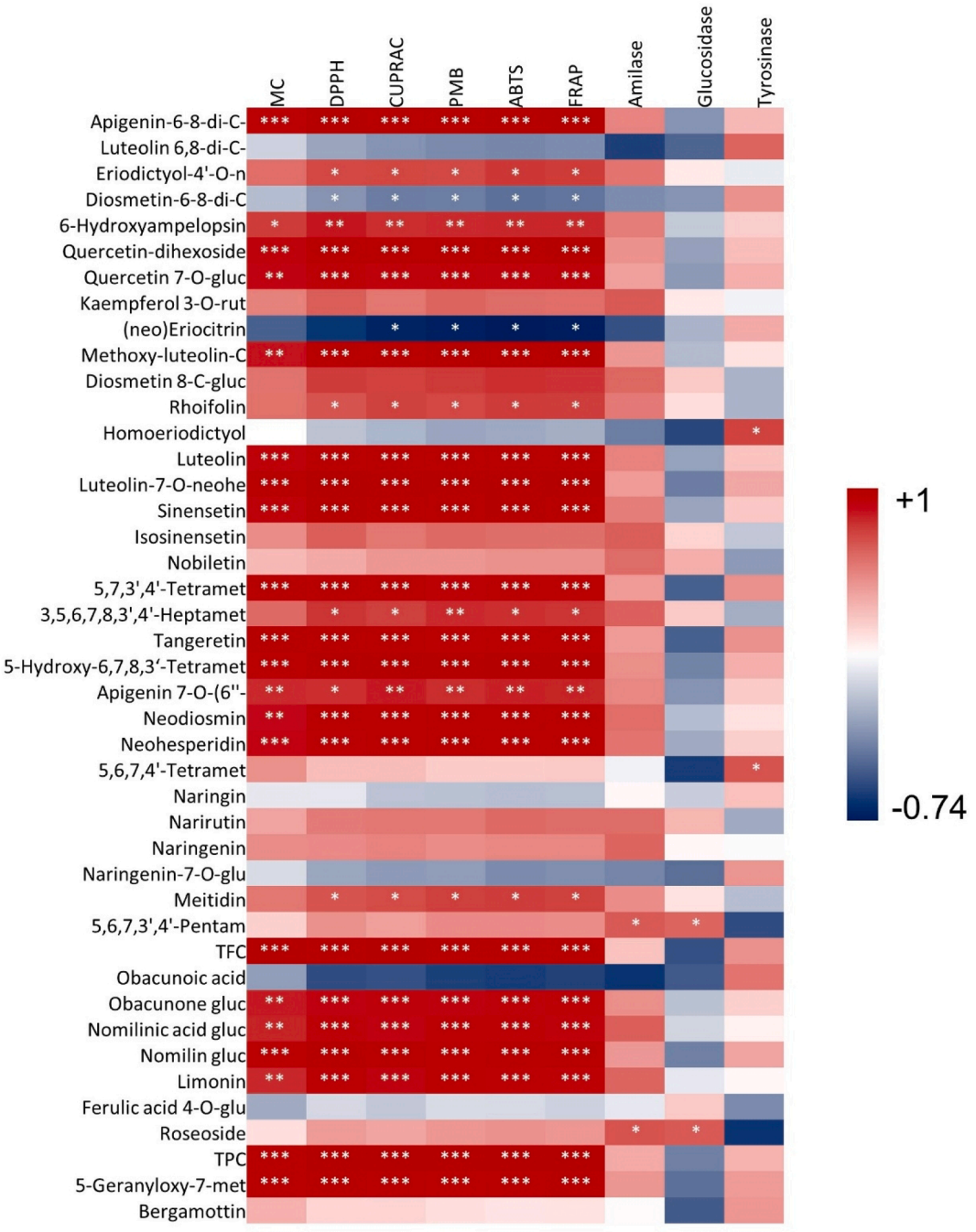

Fig. 4. Heatmap showing the correlations between chemical constituents of the non-volatile extracts of orange peels and their bioactivities. Positive correlation is indicated by red colour, while negative correlation by blue colour. Correlation coefficients are comprised between + 1 (strong positive correlation) and −0.74 (strong negative correlation). *: $P < 0.05$; **: $p < 0.01$. ***: $p < 0.001$.

orange peel extracts. Previous studies described only the reduction of NF-κB mRNA expression in the liver tissues of mice with oxidative damage (Li et al., 2021), and inhibition of its nuclear translocation in LPS-stimulated RAW264.7 cells (Etoh et al., 2013). All these results indicate that the interaction of orange peel extracts with the NF-κB intracellular signalling pathway could be one of their possible anti-inflammatory mechanisms of action.

4. Conclusion

To sum up, in this work we demonstrated that a fast and sustainable approach such as the MAE can be efficiently used to extract bioactive volatile and non-volatile secondary metabolites from fresh orange fruit peels in a single step, using water as solvent. In future studies, the MAE method can be further optimized by considering also the yield of the NVE as a variable. Our results show that both VEs and aqueous NVEs from the peels of WAS, NAV and VAN varieties are rich in secondary metabolites and can be used for different purposes. In virtue of their cytotoxicity and the high concentration of limonene, VEs can find a use as natural anti-proliferative agents. Furthermore, considering the toxic effect on different bacterial species of clinical relevance, they can be used as active ingredients of sanitizing products and disinfectants, for example. On the other hand, NVEs exerted multiple bioactivities, comprising antioxidant, anti-NF-κB, and enzyme-inhibitory properties,

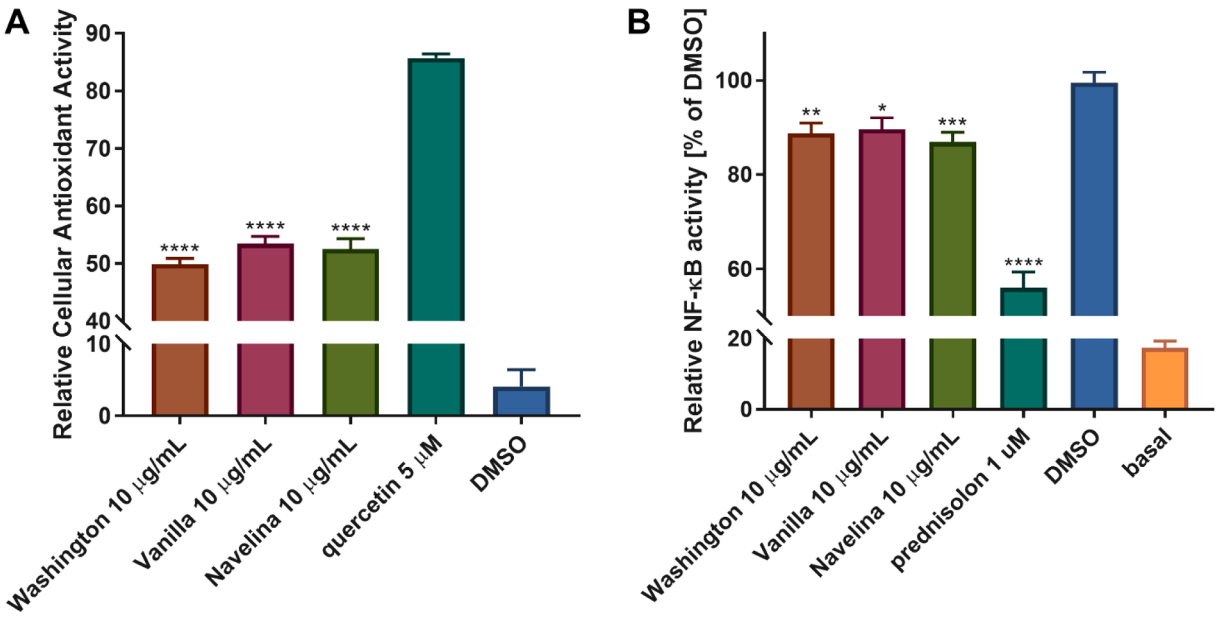

Fig. 5. Results from cellular assays. Panel A: evaluation of relative cellular antioxidant activity of orange peel extracts (10 μg/mL) and the positive control quercetin (5 μM). Panel B: the effect of orange peel extracts on the relative activity of NF-κB in LPS-stimulated cells. Cells were pre-treated by extracts at the concentration of 10 μg/mL, prednisolone (1 μM) or vehicle (DMSO) only for 1 h and then they were challenged to LPS for 24 h (except of *basal* group, which remained without LPS stimulation). *: statistical significant difference compared to DMSO ($p < 0.05$); **: $p < 0.01$; ***: $p < 0.001$); ****: $p < 0.0001$. Data are presented as mean ± SD.

hence they may be assessed as candidate ingredients for the development of novel nutraceuticals. The anti-tyrosinase effects demonstrated to be particularly relevant, and significantly correlated to several polymethoxylated flavonoids. It is interesting to note that compounds presenting the methoxyl groups in different positions of their structures showed opposite correlations with this activity, suggesting different mechanisms of interaction with the same enzyme. This aspect can represent a matter of study for further works.

Overall, the results here presented may be of interest for other two aspects: 1) elucidation of chemical composition and biological properties of peels of WAS, NAV, and VAN orange varieties typical of Sicily, which until now have been scarcely investigated; 2) valorisation and reuse of waste materials derived from their industrial processing, hence contributing positively to the sustainability of the entire orange production and processing chain.

CRediT authorship contribution statement

Gregorio Peron: Writing – original draft, Visualization, Software, Methodology, Formal analysis, Data curation, Conceptualization. **Giulia Bernabé:** Writing – original draft, Methodology, Investigation. **Sara Marcheluzzo:** Investigation. **Gokhan Zengin:** Writing – review & editing, Investigation, Data curation. **Kouadio Ibrahime Sinan:** Investigation. **Jan Hošek:** Writing – review & editing, Methodology, Investigation, Data curation. **Jakub Treml:** Methodology, Investigation. **Ignis Kaja:** Investigation. **Michela Paccagnella:** Methodology, Investigation. **Paola Brun:** Methodology, Investigation. **Ignazio Castagliuolo:** Supervision, Resources, Methodology, Data curation. **Mirella Zancato:** Writing – review & editing, Supervision, Resources, Conceptualization. **Stefano Dall'Acqua:** Writing – review & editing, Supervision, Resources, Methodology.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2024.106147.

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