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Edible flowers of *Helichrysum italicum*: Composition, nutritive value, and bioactivities

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ABSTRACT

Helichrysum italicum (*H. italicum*) is a halophyte shrub with bright yellow flowers with a strong curry-like aroma. The essential oils of *H. italicum* have been used in the production of cosmetics and pharmaceuticals, due to their antiallergic and anti-inflammatory properties. In the agri-food sector, *H. italicum* flowers can be used for seasoning and flavoring food, and as natural food preservatives. Here, we report on the composition, bioactive compounds, and nutritive value of *H. italicum* flowers. Flowers were mainly composed of carbohydrates (>80 % dry weight), followed by minerals (6.31 ± 0.95 % dw), protein (5.44 ± 0.35 % dw), and lipids (3.59 % ± 0.53 % dw). High percentages of Fe, Zn, Ca, and K were found in the flower material, along with a high content in antioxidants, polyphenols, and carotenoids, as corroborated by the nuclear magnetic resonance (NMR) data. Flowers were mainly composed of saturated fatty acids (SFAs) (54.50 ± 0.95 % of total FA), followed by polyunsaturated fatty acids (PUFAs) (37.73 ± 1.25 % of total FA) and monounsaturated fatty acids (MUFAs) (7.77 ± 0.34 %), as detected by gas chromatography mass spectrometry (GC–MS). The omega-6 PUFA linoleic acid (22.55 ± 0.76 % of total FA) was the most abundant fatty acid found. Flower extracts showed antimicrobial activity against *Saccharomyces cerevisiae* and *Komagataella phaffii*, as well as against Gram-negative (*Klebsiella pneumoniae*) and Gram-positive (*Staphylococcus aureus*) bacteria. *H. italicum* flower material was nontoxic to human intestinal Caco-2 model cells at concentrations up to 1.0 % w/v.

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1. Introduction

Edible wild plants have been used as food since ancient times and are part of the genetic and gastronomic heritage of different geographical areas (Pinela, Carvalho & Ferreira, 2017). Edible wild plants cannot only be a source of vitamins and trace elements, but also present important therapeutic properties. That is the case of *Helichrysum italicum (H. italicum),* an optional halophilic plant found in southern Europe (Bosnia and Herzegovina, Croatia, Italy, Portugal, and Spain), well known for its intense curry-like aroma (Galbany-Casals, Blanco-Moreno, Garcia-Jacas, Breitwieser, & Smissen, 2011; Viegas, Palmeira-de-Oliveira, Salgueiro, Martinez-de-Oliveira & Palmeira-de-Oliveira, 2014). *H. italicum* has been used to flavor food, such as bakery products and soft drinks. In addition, its therapeutic use, related to analgesic, anti-inflammatory, anti-allergic and antimicrobial properties (Appendino, Ottino, Marquez, Bianchi, Giana, Ballero et al., 2007; Bauer, Koeberle, Dehm, Pollastro, Appendino, Northoff et al., 2011; Djihane, Wafa, Elkhamssa, Maria, & Mihoub, 2017; Rosa, Deiana, Atzeri, Corona, Incani, Melis et al., 2007; Taglialatela-Scafati, Pollastro, Chianese, Minassi, Gibbons, Arunotayanun et al., 2013;), has long been recognized: Theophrastus of Eresos reported in *Historia Plantarum* (c. 350 BCE - c. 287 BCE) the use of "*Heleiochrysos*" mixed with honey for the treatment of burns (Viegas et al., 2014). *H. italicum* is rich in arzanol, a prenylated etherodimeric phloroglucinyl pyrone, with antioxidant (Rosa et al., 2007; Taglialatela-Scafati et al., 2013) and anti-inflammatory properties, given by the inhibition of eicosanoid (leukotriene and prostaglandin) biosynthesis (Appendino et al., 2007; Bauer et al., 2011). However, most of the benefits related to traditional uses of *H. italicum* have not yet been proven scientifically *in vivo*. Therefore, it is paramount to analyze the composition of this plant, to better characterize this species and its nutritional value and beneficial properties.

H. italicum is commonly known as the "curry plant", "perpetual-of-the-sand", "immortelle" and "everlasting" due to its xerophyte behavior and aroma (Galbany-Casals et al., 2011; Viegas et al., 2014). The name *Helichrysum* (from Greek, *helios* = sun; *chrysos* = gold) describes the characteristic flowers of this genus, that flourish from May to September and present a bright yellow color (Viegas et al., 2014). The *Helichrysum* genus contains nearly 500 species distributed globally among different continents (Africa, Asia, Australia, and Europe), with almost 25 species classified as autochthonous of the Mediterranean area (Galbany-Casals et al., 2011; Viegas et al., 2014). In terms of biodiversity, *H. italicum* populates in the form of several subspecies: *H. italicum (Roth) G. Don subsp. italicum; H. italicum* subsp. *microphyllum; H. italicum* subsp. *pseudolitoreum; H. italicum* subsp. *serotinum; H. italicum* subsp. *siculum* and *H. italicum* subsp. *picardii* Franco (Viegas et al., 2014), being the latter endemic to the Atlantic coast of the Iberian Peninsula (Portuguese coast) (Gonçalves, Moreira, Grosso, Andrade, Valentão, & Romano, 2017; Pereira, Barreira, Bijttebier, Pieters, Neves, Rodrigues et al., 2017).

In order to survive and thrive in dry and saline biotopes, *H. italicum* has developed a set of adaptive responses through the production of antioxidants, essential oils and antimicrobial compounds. Some of these components have already been extracted and identified, such as: arzanol (Rosa et al., 2007; Taglialatela-Scafati et al., 2013), quinic acid, pinocembrin (Gonçalves et al., 2017), and essential oils such as terpenes (Conti, Canale, Bertoli, Gozzini, & Pistelli, 2010; Oliva et al., 2020; Pereira et al., 2017), amino-phloroglucinols and acetophenones (D'Abrosca, Buommino, Caputo, Scognamiglio, Chambery, Donnarumma et al., 2016). Nonetheless, other compounds present in the plant, such as minerals and organic acids can also be contributing to the beneficial properties of *H. italicum.* The aerial parts of *H. italicum* can be used in the agri-food sector, as natural food additives or preservatives, due to their antibacterial (e.g., against *Micrococcus luteus*, *Bacillus cereus* and *Pseudomonas aeruginosa),* antifungal (e.g., against *Aspergillus niger* and *Alternaria alternata*) (Djihane et al., 2017) and insecticide properties (Conti et al., 2010). Thus, the objectives of the present study were to evaluate the antioxidant capacity and total phenolic content of *H. italicum* flowers, along with the centesimal composition, content in pigments, minerals, and fatty acid profile. Plant material was also tested against Gram-positive and Gram-negative bacteria, as well as against fungal strains. The Caco-2 cell assay was used to investigate the toxicity imposed by the flower infusions to human model cells (intestinal epithelial cells).

2. Materials and methods

2.1. Plant material and analytical reagents

H. italicum (*H. italicum* subsp. *Picardii* Franco) whole flowers were hand-collected from July to September 2017 at *Praia do Vigão* (Leiria, Portugal), 39°53′28″N 8°57′52″W; 10 m above mean sea level, and taxonomically identified. Once harvested, plant materials were cleansed with ultrapure Milli-Q water, dehydrated at 60 °C, for 12 h (Heraeus D-6450 oven, Hanau, Germany), macerated and homogenized using an herb/coffee grinder (Cookworks Ltd, UK) and stored at −20 °C.

All chemicals were of analytical grade or higher. Acetone, ethanol, methanol, diethyl ether, chloroform, gallic acid, [2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)]-diammonium salt (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, cefotaxime, analytical standards Supelco 37 Component Fatty Acid Methyl Esters (FAME) Mix, C17 FAME; Polyunsaturated fatty acid (PUFA) 1 and PUFA 3, D-glucose, peptone, yeast extract, *agar,* fluconazole, Dulbecco's modified Eagle's medium high glucose (DMEM), non-essential amino acids (10 ×) (NEAA), penicillin, streptomycin and trypsin-EDTA solution (10 ×) were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Sodium carbonate was acquired from Scharlau (Scharlab, S. L., Barcelona, Spain) and the *Folin-Ciocalteau* reagent was purchased from Biochem Chemopharma (Nevers, France). Bacterial strains (*Klebsiella pneumoniae* ATCC 27799, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213) were purchased from Microbiologics (St. Cloud, USA). Fungal strains were obtained from the National Collection of Pathogenic Fungi (NCPF, UK). Human colon adenocarcinoma cell line Caco-2 was obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK). Fetal Bovine Serum (FBS) was acquired from Thermo Fisher (Thermo Fisher Scientific Inc., Waltham, USA). Kjeldahl tablets (5 g without Se and Hg) from Merck (Darmstadt, Germany). Isotope-labelled compounds were purchased from Cambridge Isotope Laboratories Inc. (Tewksbury, Massachusetts, USA). Ultrapure Milli-Q (Millipore Sigma, Darmstadt, Germany) Type 1 water (< 0.054 μS/cm) was used in all experiments.

2.2. Centesimal composition

Moisture contents were determined by gravimetry (analytical scale Mettler Toledo AB204-S). Flowers were dried at 100 °C (Heraeus D-6450 oven, Hanau, Germany) for 3.5 h and maintained in a desiccator until obtaining a constant weight. Moisture contents ($\Delta W/W_i \times 100$) were obtained as percentage (%) of the difference ($\Delta W = W_i - W_f$) between initial (W_i) and the final weights (W_f).

Total protein contents of *H. italicum* flowers were determined by the Kjeldahl method (AOAC, 2016). Briefly, dried samples (0.5 g) were digested (Digestor 2006, Foss, Denmark) with 25 mL of sulfuric acid (97 % v/v) and a catalyst tablet, at 400 °C, for 90 min. After cooling, samples were mixed with 70 mL of deionized water and distilled (Kjeltec 2100, Foss, Denmark) under alkaline conditions. Distillates were collected in boric acid (30 mL at 4.0 % v/v) and the resulting solutions were titrated with standard chloride acid 0.1 mol/dm^3, using methyl red and bromocresol green as pH indicators. Control assays were prepared similarly but without sample addition. Protein contents expressed as percentage of dry weight (% dw) were calculated based on nitrogen (^{14}N) content, using a conversion factor of 6.25, according to equation (1), where V_S and V_C, stand for volumes (mL) of HCl used in samples and control titrations, respectively; [HCl], for HCl concentration (mol/dm^3); and *m*,

for sample weight (mg):

$$Protein(\%dw) = \frac{(V_S - V_B) \times [HCl] \times 14 \times 6.25}{m} \times 100 \quad (1)$$

The lipid content of *H. italicum* flowers was determined by the Folch method (AOAC, 2016) with modifications. Dried samples were mixed with the Folch extraction solvent ($CHCl_3$:CH_3OH 2:1) under vortex stirring for 5 min. After addition of 0.8 % (w/v) NaCl, mixtures were vortexed for 2 min and centrifuged (5 min at 4000 × *g*) for phase separation. The lower phase was carefully collected and filtered through an anhydrous sodium sulphate column. Then, 99.5 % (v/v) chloroform (5 mL) was added to the remaining mixtures and the extraction procedure was repeated. Finally, the solvent was removed in a rotary evaporator (Heidolph 2, LAB1ST, Shanghai China) and the lipid extract was dried at 40 °C until constant weight (~2 h). Total fat content was expressed as % dw. The ash content (% dw) was obtained from dried samples of flowers weighed in a porcelain crucible (3 h, 200 °C) and incinerated in a muffle (Nabertherm, Liliemthal/Bermen, Germany), at 500 °C, for 12 h. Total carbohydrates (% dw) were obtained by subtracting the sum of crude protein, fat, and ash and as a value from the total dry matter, according to the AOAC (2016).

2.3. Quantification of pigments

Chlorophylls and carotenoids of *H. italicum* flowers were extracted with pure acetone (≥ 99.5 % v/v) from macerated and homogenized dried samples (0.2 g) by vortex stirring for 5 min. After centrifugation (10 min at 10000 × *g*) supernatants were filtered through cotton and collected in a volumetric flask. Then, the volume of the extracts was adjusted to 10 mL with acetone ≥ 99.5 % (v/v) and absorbance at 470, 644.8 and 661.6 nm was read (Thermo Scientific, Evolution 201 Spectrophotometer). Concentration of chlorophylls and carotenoids (μg/mL) in the acetonic extracts were calculated using equations (2), (3) and (4) and the amount of pigments (μg/g dw) by equation (5), where A_n, is absorbance at the corresponding wavelength (n); C_a; C_b and $C_{(x+c)}$ stand for the concentration (μg/mL) of chlorophyll *a*, chlorophyll *b* and total carotenoids (xanthophylls and carotenes) in the extracts, respectively; *V*, is the total volume of extract (mL); and *m*, weight of dried sample (g):

$$c_a\ (\mu g/mL) = 11.24 \times A_{661.6} - 2.04 \times A_{644.8} \quad (2)$$

$$c_b\ (\text{Â}\mu g/mL) = 20.13\text{â}\ddot{A}_{644.8} - 4.19\text{â}\overline{\ddot{A}_{661.6}} \quad (3)$$

$$c_{(x+c)}\ (\mu g/mL) = (1000 \times A_{470} - 1.90c_a - 63.14c_b)/214 \quad (4)$$

$$Pigment(\mu g/g) = \frac{C \times V}{m} \quad (5)$$

2.4. X-ray fluorescence (μ-EDXRF) analysis

Mineral contents of *H. italicum* flowers were determined by micro-Energy Dispersive X-ray Fluorescence (μ-EDXRF) (Bruker M4 TornadoTM, Bruker, Germany) equipped with a Rh-anode X-ray tube, powered by a low-power HV generator. Dehydrated samples were previously macerated with a mortar and pestle and transformed into pellets of 2 cm in diameter and 1 mm in thickness. For analysis, pellets were glued onto a mylar sheet in a plastic frame and placed directly onto the X-ray beam. A minimum of three pellets was prepared for each sample and submitted to analysis. High intensity radiation covered areas of 25 μm in diameter. A XFlash® SDD detector with energy resolutions above 145 eV was used for detection. Spectral acquisition times were of 1,000 s. Elemental maps (pixel spacing of 15 μm, with a measuring time of 6 ms/pixel) were acquired to obtain average spectra, representative of the whole pellets. Quantification of the spectra was performed by making use of the built-in ESPRIT software and the recovery rate was checked against a set of standards: orchard leaves (NBS 1571), poplar leaves (GBW 07604) and bush branches (GBW 07603). Detection limits were of 0.8, 1.0, 55, 35, 4.0 and 15 μg/g, for Rb, Sr, K, Ca, Mn, and Fe, respectively, and of 2.0 μg/g for Cu, Ni and Zn. Results were expressed as μg/g dw. Since μ-EDXRF experiments required the preparation of dried pellets of plant material, values per gram of apparent "fresh" weight were estimated using equation (6), where M_x represents the mineral to be quantified and "W" the moisture (%) of plant material:

$$M_x(\mu g/gfw) = M_x(\mu g/gdw) \times \left(\frac{100 - W}{100}\right) \quad (6)$$

2.5. Fatty acid profile

Analysis of the fatty acid profile of *H. italicum* flowers was carried out as described previously (Brito, Bertotti, Primitivo, Neves, Pires, Cruz et al., 2021), with some modifications. Briefly, fatty acid methyl esters (FAMEs) were obtained by direct transmethylation of dried *H. italicum* flowers with 2.0 % v/v H_2SO_4 in methanol, at 80 °C, and collected in hexane phase containing C17 FAME as internal standard. The mixture of FAMEs was analyzed by Gas chromatography (GC), in a Finnigan Ultra Trace gas chromatograph (GC), equipped with a Thermo TR-FAME capillary column (length 60 m, 0.25 mm internal diameter, 0.25 μm membrane thickness), AS 3000 auto sampler (Thermo Electron Corporation) and a flame ionization detector (FID). Temperatures of detector and injector (splitless mode) were set at 280 °C and 250 °C, respectively. Oven temperature program: 100 °C for 0.1 min, increased at 10 °C/min to 150 °C, maintained for 1 min; second increase at 5 °C/min to 200 °C (maintained for 15 min); and a final increase at 2 °C/min until 235 °C (maintained for 5 min). Helium was used as the carrying gas at 1.2 mL/min. Synthetic air and hydrogen were used for the detector flame, flowing at 350 mL/min and 35 mL/min, respectively. Xcalibur software (Thermo Fisher Scientific Inc., Waltham, USA) was used for acquisition and analysis. Fatty acids (FAs) were identified by comparison of retention times with those of reference samples (Supelco 37, PUFA 1 and PUFA 3 standards). Results were expressed as percentage of total area (% Total FA). Amounts of fatty acid (μg/mg dw) were calculated by the internal standard method according to expression (7), where FA_x, stands for the fatty acid to be quantified; [IS] for the concentration of IS (internal standard) (μg/mL); V_S, for the volume of hexane used for the extraction of FAME from samples (mL); m_S, for sample weight (mg); *RRf* for the theoretical relative response factor related with the IS (C17 FAME) (Tvrzická, Vecka, Staňková, & Žák, 2002) and *K*, for the FAME/FA mass ratio:

$$FA_x(\mu g/mg) = \frac{Area(FAME_x) \times [IS] \times V_s \times RRf}{Area(IS) \times m_s \times K} \quad (7)$$

All results corresponded to the mean ± standard deviation (sd) of four independent samples. The ratio of hypocholesterolemic and hypercholesterolemic FAs (H/H); atherogenicity index (AI) and thrombogenicity index (TI) were calculated from percentage (%) data, as described in Brito et al., (2021), via equations (8), (9) and (10):

$$H/H = \frac{(C18:1n9 + C18:2n6 + C20:4n6 + C18:3n3 + C20:5n3 + C22:5n3 + C22:6n3)}{(C14:0 + C16:0)} \quad (8)$$

$$AI = \frac{(C12:0 + 4 \times (C14:0) + C16:0)}{(\sum MUFA + \sum n3 + \sum n6)} \quad (9)$$

$$TI = \frac{(C14:0 + C16:0 + C18:0)}{\left(0.5 \times \sum MUFA + 3 \times \sum n3 + 0.5 \times \sum n6 + \frac{\sum n3}{\sum n6}\right)} \quad (10)$$

FAME identification was confirmed twice on an Agilent 6890 N GC (Agilent Technologies, California, USA), equipped with a flame ionization detector (FID) and a 7683B series injector, and on an Agilent 7820A GC with a 7693A autoinjector and an 5977E quadrupole mass spectrometer. Both systems contained 25 m long Agilent J&W Ultra 2 capillary columns. Each FAME peak was identified by the PLFAD1 method. The inlet of the GC–MS was set at 250 °C, the MS source at 230 °C and the MS quad at 150 °C, with the mass transfer line set at 280 °C.

2.6. Total phenolic content

Flower extracts of *H. italicum* in water (infusion, 1:200 ratio) and in 70% (v/v) acetone (1:10 ratio), were tested for phenolic compounds by the Folin-Ciocalteau assay, as described by Brito et al. (2021). *H. italicum* aqueous extracts (infusions) were prepared in Milli-Q water (15 min), at 90 °C. Extracts in 70 % (v/v) acetone were prepared by stirring (2 h) in a magnetic stirrer, 9000 rpm, at 20 °C. Samples were filtered through qualitative paper filters (~22 μm) and centrifuged for 20 min, at 4000 × *g* (Heraeus Labofuge 200, Hanau, Germany). Small aliquots of plant extracts and standard solutions of gallic acid were mixed with the Folin-Ciocalteau reagent. Sodium carbonate (70.0 g/L) and Milli-Q water were added to the mixture and let to incubate (60 min), at 20 °C. Absorbance was read at 750 nm (Varian Cary 50 UV–Visible spectrophotometer). Total phenolic content was expressed as milligrams of gallic acid equivalents per gram (mg GAE/g), by making use of a calibration curve.

2.7. Antioxidant activity

The antioxidant capacity of *H. italicum* flowers was evaluated by two assays, based on the reduction of the ABTS●+ and DPPH● radicals, as described by Brito et al., (2021). The ABTS radical (ABTS●+) was prepared by reaction of 7.0 mM ABTS with 2.45 mM potassium persulfate. The mixture was held in the dark (16 h), at room temperature, filtered through a sterile filter (0.22 μm) (Whatman, Ohio, USA) and diluted in water, to an absorbance of 0.700 ± 0.020, at 734 nm. Standards and flower extracts in water (infusions) and in 70 % (v/v) acetone (prepared as in the previous section for the Folin-Ciocalteau assay) were added to the ABTS solution. After a 6-minute reaction period, absorbance was read at 734 nm. Regarding the DPPH assay, stock solutions of 0.099 mM DPPH in 70 % (v/v) acetone, were prepared to obtain an absorbance of 0.500 ± 0.040, at 515 nm. Standards and flower extracts in water (infusions) and in 70 % (v/v) acetone were added to the DPPH solution and let to rest in the dark (30 min), at room temperature. Absorbance was measured at 515 nm. Vitamin C was used as standard for the calibration curve in both (ABTS and DPPH) procedures. Control samples were prepared with ultrapure Milli-Q water and 70 % (v/v) acetone. The results report on antioxidant activity expressed in milligrams of Vitamin C (Vitamin C Equivalent Antioxidant Capacity, VCEAC) per gram of sample (mg VCEAC)/ g), by making use of the calibration curve.

2.8. NMR analysis

H. italicum flowers were freeze-dried in a Kinetics Ez-Dry freeze dryer (Kinetics, Dresden, Germany). NMR solutions were prepared as described by Brito et al. (2021), by dissolving freeze-dried samples in deuterated 70 % (v/v) acetone-d_6 and centrifuging for 20 min, at 4000 × *g* (Heraeus Labofuge 200, Hanau, Germany). NMR spectra were obtained on a Bruker Avance III 400 spectrometer (Bruker, Massachusetts, USA) operating at a ^{1}H frequency of 400.133 MHz, at 25 °C. One-dimensional (1D) ^{1}H NMR data acquisition used *zgpr* pulse sequences with 32 k complex points, 64 scans, a spectral width of 4401.41 Hz and a recycle delay of 2 s. Two-dimensional (2D) [^{1}H–^{1}H]-COSY experiments were collected using gradient pulses for selection and presaturation during relaxation, for solvent signal suppression. Data was acquired with 2 k complex points in t2 and 512 increments (16 scans each), a spectral width of 4401.41 Hz (11 ppm) in both dimensions, and a relaxation delay of 1.4 s. Spectra were apodised with a SINE window function and zero filled to 1 k, in both dimensions. Final data was processed and analyzed with Topspin v4.0 (Bruker) and MestReNova 9.1 (Mestrelab) software. Chemical shifts were reported in ppm, relative to methyl group –CH_3 ^{1}H ($\delta = 2.05$ ppm) resonances of acetone-d_6, used as external reference.

2.9. Antibacterial capacity

A two-fold broth microdilution method was used to determine the bacterial activity of extracts of *H. italicum* flowers in water (infusion) and in 70 % (v/v) acetone against *Escherichia coli (E. coli)*, *Klebsiella pneumoniae (K. pneumoniae)* and *Staphylococcus aureus (S. aureus)*. Extracts in acetone were vacuum dried, while aqueous extracts were freeze-dried. Dried samples were dissolved in the cell culture medium. Tests were performed in 96-wells microtiter plates, with concentrations of flower extracts ranging from 2.63 μg/mL to 1,346 μg/mL for flower material extracted with water and from 3.06 μg/mL to 1,566.15 μg/mL for flower material extracted in 70 % (v/v) acetone. Microorganisms were grown overnight in Mueller Hinton Agar (MHA), at 37 °C, and two to three colonies were used to inoculate Muller-Hinton broth (MHB) until reaching the 0.5 McFarland Standard (DEN-1B, Grant Bio, UK) under incubation, at 37 °C, 250 rpm. Bacterial suspensions were inoculated in the microtiter plate wells to a final concentration of 5 × 10^5 cfu/mL in each well. Microtiter plates were incubated at 37 °C (24 h) and the microbial growth was determined by measuring optical density at 585 nm. Results were expressed as the minimal inhibitory concentration (MIC) of the extracts and as the inhibitory concentration to 50 % of the cell growth (IC_{50}). Sterility controls (culture medium without bacterial suspension), positive controls (medium with bacterial suspension) and quality controls (medium with the antibiotic cefotaxime inoculated with bacterial suspension) were performed.

2.10. Antifungal capacity

Several *H. italicum* flower extracts were prepared with different solvents (ethanol, methanol, acetone, diethyl ether, chloroform, and dimethyl sulfoxide). Solvents were added to macerated and homogenized whole plant material in a 1:10 (w/v) plant:solvent ratio, for 60 min on a roller at 40–45 rpm. Negative controls were performed with the extraction solvent only, and quality controls were performed with the antifungal fluconazole, at 1.0 mg/mL. Strains were cultured from NCPF stocks. Stock cultures of *Komagataella phaffii* (*K. phaffii),* wild type *Saccharomyces cerevisiae (S. cerevisiae)* BY4141 and *S. cerevisiae* AD1-9 were cultured in standard complete media with 2 % D-glucose, 0.67 % yeast nitrogen base without amino acids, complete supplement mixture of leucine drop out, L-leucine 80 mg, adenine sulphate 80 mg, pH 6.5. YPD agar and broth (for stock and strain maintenance). The MIC assay was performed in a 96-well microtitre plate with a gradient created by 1:1 serial dilution from 11.2 % to 0.011 % (w/v) with the last set of wells acting as controls. Cell dilutions used in the assay were of 1 in 1000. Data was collected at 24 h and at 48 h, 28 °C, by resuspending any cells settled at the bottom of the wells at 1000 rpm, for 60 s, using an Ascent Microplate Reader (Thermo Fisher Scientific Inc., Waltham, USA), by absorbance at 595 nm. IC_{50}s were calculated after normalization by the absorbance in the positive control.

2.11. Cell viability assays

The toxicity of *H. Italicum* flowers was assessed by evaluating the viability of human intestinal Caco-2 cells, after exposure to plant material. Cells were maintained in T75 cm^2 flasks in Dulbecco's modified Eagle's medium (DMEM) medium supplemented with 10 % (v/v) heat-inactivated Fetal Bovine Serum (FBS), 1 % (v/v) non-essential amino acids (NEAA) and 1% penicillin–streptomycin, in a humidified atmosphere of 5 % CO_2, at 37 °C. Cells were subcultured at 80–90 % confluence, twice a week (at a subculture ratio 1:8), using a 2.5 % (w/v) trypsin-EDTA solution (10 ×) for cell detachment. Caco-2 cells were seeded in 96-well plates at a density of $1x10^4$ cells/well, maintained in culture for 4 days, with medium renewals every two days. Caco-2 cells were incubated for 24 h with increasing concentrations of freeze-dried *H. italicum* flower material added to DMEM (0.3 to 10.0 % w/v). After exposure to the plant material, cells were washed with PBS and incubated for 4 h with 0.5 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide), at 37 °C. The MTT solution was discarded, and the formazan precipitates dissolved in DMSO. Absorbance was measured at 570 nm (BioTek Synergy HT microplate reader, Biotek Instruments, VT, USA). Cell incubation with DMEM alone was used as negative control (100 % cell viability).

2.12. Statistical analysis

All experiments were performed at least in triplicate, and results reported as mean ± standard deviation (sd). Data were analyzed by one-way Analysis of variance (ANOVA). Effects were compared using a Student's *t* test, and deemed statistically significant at $p < 0.05$. Correlations were based on Pearson's coefficients. Calculations were performed with the *R* software (R Foundation for Statistical Computing, Vienna, Austria).

3. Results and discussion

3.1. Centesimal composition and mineral content

H. italicum whole flowers showed a higher moisture content (39.14 ± 1.34 %) than other natural food coloring and flavoring spices, such as commercially available turmeric (*Curcuma Longa*) powder (10.34 %) (Behera, Sutar, & Aditya, 2017). *H. italicum* flowers were also characterized in terms of total protein, fat, ash, and carbohydrate contents, expressed as percentage of dry weight (% dw) (Table 1). Flowers were mainly composed of carbohydrates (84.66 ± 0.66 % dw), followed by ash (6.31 ± 0.67 % dw), protein (5.44 ± 0.35 % dw) and lipids (3.59 ± 0.53 % dw). To the best of our knowledge there are no published studies indicating the centesimal composition of *H. italicum sp*. However, its centesimal composition is close to the amounts of protein (7.17 ± 0.22 % dw), ash (6.76 ± 0.29 % dw) and lipids (2.60 ± 0.10 % dw) found in other *Helichrysum* species, namely, *Helichrysum petiolare*, from South Africa (Aladejana, Bradley, & Afolayan, 2021). Also, similarly, to other edible wildflowers, such as dog rose (*Rosa canina L.)* (carbohydrates: 86.12 ± 0.80 % dw, ash: 4.29 ± 0.10 % dw, protein: 7.58 ± 0.84 % dw; lipids: 2.01 ± 0.04 % dw) and marigold (*Calendula officinalis L.)* (carbohydrates: 81.32 ± 0.75 % dw, ash: 6.93 ± 0.14 % dw, protein: 6.43 ± 0.68 % dw; lipids: 5.33 ± 0.45 % dw) (Pires, Dias, Barros, & Ferreira, 2017), *H. italicum* flowers show, besides water, a high content in carbohydrates and a low content in protein and lipids, accompanied by a variable content and/or composition in minerals.

Table 1
Pigments and centesimal composition of *H. italicum* subsp. *Picardii* Franco flowers.

Moisture (%)		39.14	±	1.34
Centesimal Composition (% dw)	Proteins	5.44	±	0.35
	Lipids	3.59	±	0.53
	Ash	6.31	±	0.67
	Carbohydrates	84.66	±	0.66
Pigments (μg/g dw)	Chlorophyll *a*	30.9	±	6.8
	Chlorophyll *b*	26.1	±	9.6
	Total carotenoids	67.9	±	4.9

Values represent the mean ± sd of triplicates of three independent samples.

Table 2
Mineral composition of *H. italicum* subsp. *picardii* Franco flowers (HiP) and *H. italicum (Roth) G. Don fil* (HiD) aerial parts.

Mineral	HiP (μg/g dry weight)	HiP (μg/g app. fresh weight)[a)]	HiD (μg/g fresh weight)[b)]
Calcium (Ca)	32,000 ± 1,500	19,475 ± 913	4,500 – 10,100
Phosphorus (P)	215.0 ± 8.2	130.8 ± 5.0	1,100 – 4,100
Potassium (K)	31,000 ± 1,400	18,867 ± 852	5,200 – 28,300
Sulfur (S)	391.0 ± 16	238.0 ± 9.7	*n.d.* [c)]
Copper (Cu)	92.0 ± 4.3	56.0 ± 2.6	3.8 – 28
Iron (Fe)	586.0 ± 18	356.6 ± 11	88.5 – 950
Manganese (Mn)	216.0 ± 5.7	131.5 ± 3.5	74.0 – 751
Nickel (Ni)	23.6 ± 0.9	14.36 ± 0.55	*n.d.*[c)]
Rubidium (Rb)	17.4 ± 0.6	10.59 ± 0.37	*n.d.* [c)]
Strontium (Sr)	109.0 ± 4.8	66.3 ± 2.9	*n.d.* [c)]
Zinc (Zn)	310.0 ± 10	188.7 ± 8.8	27.8 – 240

Values in mean ± sd of triplicates of three independent samples.
[a)] Apparent fresh weight considering the moisture content found in the plant material (flowers) as estimated by equation (6) (in Materials and methods),
[b)] Range of values determined by Bianchini et al., 2009;
[c)] *n.d.*, not determined by Bianchini et al., 2009.

In addition, a detailed analysis of the mineral composition of *H. italicum* flowers was conducted by μ-EDXRF. Contents (μg) in calcium (Ca), copper (Cu), iron (Fe), manganese (Mn), nickel (Ni), phosphorus (P), potassium (K), strontium (Sr), sulfur (S), rubidium (Rb), and zinc (Zn), were obtained per gram of dw (Table 2). *H. italicum* flowers showed higher contents of Fe (586 ± 18.1 μg/g dw), Zn (310 ± 10.2 μg/g dw), Ca (32,000 ± 1,500 μg/g dw), and K (31,000 ± 1,400 μg/g dw), than other natural food coloring and flavoring spices, such as oven-dried turmeric (*Curcuma longa*) (Fe: 230.0 μg/g dw; Zn: 70.0 ± 0.0 μg/g dw; Ca: 9,060 ± 0.00 μg/g dw; K: 6,430 ± 20 μg/g dw) (Braga, Vieira, & de Oliveira, 2018). Likewise, the mineral contents found in *H. italicum* subsp. *picardii* Franco (from the Atlantic coast of the Iberian Peninsula) studied herein are within the range of values described for *H. italicum (Roth) G. Don fil* from Corsica Island, France (Bianchini, Santoni, Paolini, Bernardini, & Mouillot, 2009) (Table 2). Even if μ-EDXRF experiments require the preparation of dried pellets of plant material, an apparent "fresh" weight can be estimated by considering the moisture content (Table 2). *H. italicum* subsp. *picardii* Franco flowers showed amounts of K, Fe, Mn, and Zn close to the values registered for fresh aerial parts of *Helichrysum italicum (Roth) G. Don fil* harvested at 48 different locations of Corsica Island (Table 2). Exceptions were only found for Ca, Cu and P. While higher values (~50% higher) were detected here for Ca and Cu; lower values were found herein for P (~90% lower). These dissimilarities can be attributed to the different edaphoclimatic conditions (temperature, soil, water composition) of the two regions. Additionally, some sulfur was also detected in the *H. italicum* flowers studied herein (391.0 ± 15.9 μg/g dw), which was present in higher amounts than in Braeburn apples (144.3 μg/g dw) (Doleman, Grisar, Van Liedekerke, Saha, Roe, Tapp et al., 2017) but in much lower amounts than in tea flowers (*Camellia sinensis (L.) O. Kuntze,* 2,648.2 μg/g dw) (Jia et al., 2016) or in alliaceous and cruciferous vegetables (Doleman et al., 2017).

It was also possible to detect other trace elements, such as Ni (23.6 ± 0.9 μg/g dw), Rb (17.4 ± 0.6 μg/g dw), and Sr (109.0 ± 4.8 μg/g dw) (Table 2). Contents in Ni, Rb, and Sr are rather high but do not seem to compromise health safety regarding the use of the flower of *H. italicum* as an aromatic herb. Nickel is ubiquitously present in the soil and used in plant metabolism. It is found in foodstuffs at 0.20 to 16 μg/g and in

vegetables (in Europe) at 0.001 to 10 μg/g of fresh weight (Squadrone, Crescio, Brizio, Dutto, Bocca, Stella, et al., 2020). Ni concentrations must be permanently monitored in plants for human consumption and should not exceed 300 μg/day (WHO, 1991). Regarding Rb, coffee (40 μg/g dw), asparagus (60 μg/g dw), and black tea (100 μg/g dw) are examples of Rb-rich food and *H. italicum* flowers show Rb contents similar to the amounts present in fresh carrots (20 μg/g dw) (Anke & Angelow, 1995). In what regards Sr, the content found in *H. italicum* flowers is close to the amount present in cinnamon (*Cinnamomum verum*, 81.3 ± 2.1 μg/g dw) and in other medicinal plants of the Mediterranean basin (e.g.: liquorice, *Glycyrrhiza glabra,* 110 ± 4 μg/g dw) (Khuder, Sawan, Karjou, & Razouk, 2009), but lower than the amount found in peppermint (*Mentha piperita*, 349 ± 16 μg/g dw), basil (*Ocimum basilicum*, 374 ± 15 μg/g dw), or horsetail (*Equisetum arvense*, 669 ± 63 μg/g dw) (Tokalıoğlu, 2012).

3.2. Photosynthetic pigments and antioxidant capacity

Plants are particularly rich in photosynthetic pigments, with green tissues richer in chlorophylls, and non-green tissues richer in carotenoids. Table 1 shows the amounts of carotenoids and chlorophylls present in *H. italicum* flowers. Chlorophyll *a* (30.9 ± 6.8 μg/g dw) and chlorophyll *b* (26.1 ± 9.6 μg/g dw) are present in similar and smaller amounts, whereas higher amounts of carotenoids were detected (67.9 ± 4.9 μg/g dw) in *H. italicum* flowers, like those found in other edible flowers, such as centaurea (*Centaurea cyanus L.*, 58.0 ± 10.0 μg/g dw) (Fernandes et al., 2020). Thus, besides anthocyanins and betalains, the presence of carotenoids in *H. italicum* flowers is consistent with the bright yellow color showed in the petals. High contents in xanthophylls and in α-, β- and ζ-carotenes have been associated with the expression of yellow phenotypes (de Carvalho & Caramujo, 2017; Tanaka, Sasaki, & Ohmiya, 2008), and identified among other edible yellow flowers, like *Cosmos sulphureus cav.* (cosmos yellow) and *Primula* × *polyantha* (primula) (Chensom, Okumura, & Mishima, 2019). Carotenoids act as photoprotectors, color attractants, hormone precursors and antioxidants (de Carvalho & Caramujo, 2017; Tanaka et al., 2008). Hence, they also contribute for the antioxidant capacity exhibited by the plant.

The antioxidant capacity showed by *H. italicum* flowers was evaluated by two radical scavenging methods (ABTS and DPPH assays). Since the chemical composition of the extracts greatly depends on the extraction method and solvent used (Gonçalves et al., 2017), two solvents were applied: water (infusion) and 70 % (v/v) acetone. The antioxidant capacity of the flower extracts is shown in Table 3, expressed in milligrams of Vitamin C Equivalent Antioxidant Capacity per gram of flower material (mg VCEAC/g). Similar values of antioxidant capacity were found by the two methods, as previously reported for different plant parts of *H. italicum* subsp. *Picardii* Franco (Pereira et al., 2017). Nonetheless, the DPPH assay detected slightly higher VCEAC values in the extracts in acetone (52.7 ± 9.2 mg VCEAC/g) than in the infusions (39.5 ± 1.1 mg VCEAC/g), showing a negative (-0.75) Pearson's correlation between the two methods. Thus, acetone was more efficient in extracting lipophilic compounds and exhibited higher antioxidant capacity by the DPPH method (since the DPPH radical is less soluble in aqueous solutions and thus more appropriate to detect more lipophilic compounds). On the other hand, aqueous extraction by infusion led to higher antioxidant capacities by the ABTS assay (51.9 ± 4.6 mg VCEAC/g). Thus, while the ABTS assay can be detecting higher amounts of phenolic acids and of other water-soluble antioxidants, the DPPH assay can be detecting higher amounts of lipophilic compounds, such as carotenoids.

Table 3
Phenolic content and antioxidant capacity of *H. italicum* subsp. *picardii* Franco.

H. italicum flower extract	Total phenolic content		Antioxidant capacity (mg VCEAC/g)	
	(mg GAE/ 200 mL)	(mg GAE/ g)	ABTS	DPPH
Infusion	79.7 ± 1.4	39.9 ± 0.6[a]	51.9 ± 4.6[a]	39.5 ± 1.1[a]
Acetone 70 % v/v	*n.a.*[#]	41.8 ± 2.9[a]	37.8 ± 9.8[a]	52.7 ± 9.2[a]

[#] *n.a.*, not applicable. Values in mean ± sd of triplicates of three independent samples. GAE, Gallic Acid Equivalents. VCEAC, Vitamin C Equivalent Antioxidant Capacity. Different letters mean significant differences ($p < 0.05$).

3.3. Phenolic content

Quantification of total phenolic content (TPC) was performed by the Folin-Ciocalteu method in two solvents: aqueous (infusions) and 70 % (v/v) acetone. Results are expressed in Table 3. *H. italicum* flowers presented a similar TPC in the acetonic extracts (41.8 ± 2.9 mg GAE/g), when compared to infusions (39.5 ± 1.1 mg GAE/g). Additionally, the TPC is within the range of previously reported data for *H. italicum* subsp. *picardii* Franco flowers (Pereira et al., 2017). The TPC per "cup-of-tea" (mg/200 mL) of infusion, considering 1 g of flower material (69.9 ± 3.9 mg GAE/200 mL) found by Pereira et al. (2017), when applying the same methodology, is close to the one determined here (79.7 ± 0.03 mg GAE/200 mL). In addition, the values found here are also higher than the ones obtained with rooibos tisane (*Aspalathus linearis*) infusions (in mg/cup-of-tea) (43.1 ± 3.39 mg GAE/200 mL) (Pereira et al., 2017). Moreover, even if different extracting media influence the type and extent of phenolics obtained, the TPC extracted with 70 % (v/v) acetone, is close to that reported for other spices, such as turmeric (*Curcuma longa)* (66.8 ± 1.0 mgGAE/g) and ginger (*Zingiber officinale)* (41.0 ± 0.7 mgGAE/g) (Tinello & Lante, 2019). Likewise, the TPC obtained agrees with the antioxidant capacity shown by the flowers. Positive (+0.70) and negative (-0.76) correlations were found between TPC and antioxidant capacity detected by the DPPH and the ABTS assays, respectively, when comparing the extracts in the two solvents (Table 3). Acetonic extracts showed slightly higher amounts of TPC and of DPPH antioxidant capacity, when compared to infusions, reinforcing the presence of more lipophilic phenols, such as curcuminoids, also explaining the high antioxidant power and bright yellow color of the flowers.

In addition, proton NMR experiments were also conducted to identify major groups of phenolic compounds present in the *H. italicum* flowers. The aromatic region (δ 6.0 to 8.0 ppm) of the 1D ^{1}H NMR spectrum of the plant material in 70 % (v/v) deuterated acetone-d_6 is shown in Figure S1 (supp. material). Different classes of compounds were identified, such as phenolic acids, curcuminoids and carotenoids. Phenolic acids typically present doublet (d) signals assigned to ortho- and *meta*-protons of the hydroxybenzoic moiety (at chemical shift (δ), multiplicity, vicinal coupling constants $^{3}J_{HH}$: 7.67 (d, $J = 9.0$ Hz), 7.11 (d, $J = 2.0$ Hz), 6.98 (dd, $J = 7.9$ Hz, 2.0 Hz), 6.89 (d, $J = 9.0$ Hz) and 6.81 ppm (d, $J = 7.9$ Hz)) and to protons of the acrylic group, in the case of hydroxycinnamic acids (7.54 (d, $J = 16.8$ Hz) and 6.26 (d, $J = 16.8$ Hz)) (Brito et al., 2021), as seen in Figure S1.A and Table S2. In turn, curcuminoids, *i.e.*, curcumin and curcumin derivatives are diferuloylmethanes. Thus, beyond the NMR signals characteristic of hydroxybenzoic moieties described above (from 6.8 to 7.2 ppm), an additional intense signal detected at 4.26 ppm (singlet) was attributed to the methoxy group (OMe); whereas signals at 7.68 ppm (singlet) and 7.54 (d, $J = 15.8$ Hz), and 6.34 ppm (d, $J = 15.8$ Hz)), were attributed to vinyl ketone (keto-enol/β-diketone tautomers) groups (Prasad, Praveen, Mahapatra, Mogurampelly, & Chaudhari, 2021). Moreover, signals observed at 6.45 ppm (d, $J = 17.0$ Hz) and 6.79 ppm (d, $J = 17.0$ Hz) with large coupling constants (Putzbach, Krucker, Albert, Grusak, Tang, & Dolnikowski, 2005), as confirmed by the cross peaks observed in the 2D ^{1}H–^{1}H COSY spectrum (Figures S1.B, S2 and Table S1), were attributed to the isoprene/terpene units of carotenoids, also attesting for the presence of this class of compounds in the flower extracts.

Table 4
Fatty acid profile of *H. italicum* subsp. *picardii* Franco flowers.

Fatty acid	% total FA			µg FA/mg dw		
SFA						
C10:0	0.91	±	0.11	0.07	±	0.01
C12:0	3.47	±	0.21	0.27	±	0.02
C13:0	0.15	±	0.02	0.01	±	0.00
C14:0	10.47	±	0.40	0.78	±	0.05
C15:0	0.28	±	0.19	0.03	±	0.02
C16:0	16.45	±	0.20	1.19	±	0.08
C16:0 2OH	3.29	±	0.19	0.24	±	0.01
C17:0	0.67	±	0.03	0.05	±	0.00
C18:0	2.84	±	0.17	0.21	±	0.02
C20:0	2.79	±	0.11	0.20	±	0.02
C21:0	6.81	±	0.04	0.48	±	0.04
C22:0	4.01	±	0.17	0.28	±	0.03
C23:0	0.67	±	0.05	0.05	±	0.00
C24:0	1.68	±	0.14	0.12	±	0.02
MUFA						
C15:1n-6	0.10	±	0.07	0.01	±	0.01
C16:1n-7	1.22	±	0.11	0.09	±	0.01
C18:1n-9	4.49	±	0.15	0.32	±	0.03
C18:1n-7	0.76	±	0.03	0.06	±	0.01
C20:1n-9	0.15	±	0.10	0.01	±	0.01
C20:1n-8 or n-11	0.26	±	0.05	0.02	±	0.01
C24:1n-7 and n-9	0.79	±	0.07	0.06	±	0.01
PUFA						
C16:3n-6	1.56	±	0.14	0.11	±	0.02
C18:2n-6,9	22.55	±	0.76	1.60	±	0.17
C18.3n-3,6,9	12.40	±	0.59	0.89	±	0.07
C20:2n-6	0.33	±	0.10	0.02	±	0.01
C22:5n-3	0.90	±	0.10	0.06	±	0.01
Totals						
ΣSFA	54.50	±	0.95	4.06	±	0.28
ΣMUFA	7.77	±	0.34	0.57	±	0.05
ΣPUFA	37.73	±	1.25	2.73	±	0.26
Σn-3	13.29	±	0.68	0.96	±	0.08
Σn-6	24.44	±	0.85	1.78	±	0.19
Ratios						
H/H	1.50 ± 0.06					
AI	1.36 ± 0.07					
TI	0.53 ± 0.02					
n-6/n-3	1.84 ± 0.09					
PUFA/SFA	0.69 ± 0.03					

Values in mean ± sd of triplicates of three independent samples. FA, fatty acid. SFA, saturated fatty acid. MUFA, monounsaturated fatty acid. PUFA, polyunsaturated fatty acid. dw, dry weight.

3.4. Fatty acid content

The fatty acid (FA) composition of *H. italicum* flowers was mainly comprised by saturated fatty acids (SFA) corresponding to 54.50 ± 0.95 % of total FA, followed by polyunsaturated (PUFA) and monounsaturated (MUFA) fatty acids, representing 37.73 ± 1.25 % and 7.77 ± 0.34 % of total FA, respectively, (Table 4).

Linoleic acid (C18:2n-6), was the most abundant FA detected in *H. italicum* flowers attaining values of 1.60 ± 0.17 µg/mg dw (22.55 ± 0.76 % of total FA). Alpha-linolenic acid (C18:3n-3) was also present in a high content (0.89 ± 0.07 µg/mg dw), whereas other PUFAs, namely docosapentaenoic (C22:5n-3), hexadecatrienoic (C16:3n-6) and eicosadienoic (C20:2n-6) acids were found in minor amounts.

Palmitic (C16:0) and myristic (C14:0) acids were the major SFAs detected in *H. italicum* flowers, reaching values of 1.19 ± 0.08 µg/mg dw (16.45 ± 0.20 %) and 0.78 ± 0.05 µg/mg dw (10.47 ± 0.40 %), respectively. These SFAs were also the most abundant in other edible flowers, such as anchusa plants (e.g. *Anchusa azurea*), sulla (*Hedysarum coronarium)*, and rosemary (*Rosmarinus officinalis)* (Loizzo, Pugliese, Bonesi, Tenuta, Menichini, Xiao et al., 2016). The odd henicosanoic acid (C21:0) was also found in the flowers in significant amounts, attaining values of 0.48 ± 0.04 µg/mg (6.81 ± 0.04 %). Other SFAs, namely behenic (C22:0), lauric (C12:0), stearic (C18:0), arachidic (C20:0) lignoceric (C24:0) acids and the hydroxi fatty acid C16:0 2OH were present in low amounts. In turn, oleic acid (C18:1n-9) was the most abundant MUFA of *H. italicum* flowers corresponding to 0.32 ± 0.03 µg/mg dw (4.49 ± 0.15 %). Other MUFAs, namely palmitoleic (C16:1n-7), tetracosenoic (C24:1n-9 and n-7), vaccenic (C18:1n7), eicosenoic (C20:1n-9 and n-11), and pentadecenoic (C15:1n-6) acids were detected in low amounts.

To the best of our knowledge, this is the first study describing the fatty acid content of *H. italicum* flowers. Also, reports regarding FA contents in other *Helichrysum* species are scarce. Nevertheless, when comparing fatty acid contents from different *Helichrysum* species, *H. italicum* flowers have shown to be a source of nutritionally valuable FAs. Umaz (2020) studied the FA content of *H. arenarum* collected from two locations. This species, when compared with *H. italicum,* had lower values of PUFAs and higher values of MUFAs and SFAs, independently of location. Thus, *H. italicum* flowers showed higher PUFA/SFA (0.69 ± 0.03) and H/H ratios (1.50 ± 0.06), and lower atherogenicity (AI) (1.36 ± 0.07) and thrombogenicity (TI) (0.53 ± 0.02) indexes, when compared with *H. arenarum, H. chionophilum* and *H. plicatum* flowers (Umaz, 2020). Even if palmitic acid (C16:0) was the most abundant SFA in all species, *H. italicum* showed a higher percentage of PUFAs and a lower percentage of SFAs. Nonetheless, although within the same range of values, the MUFA content of *H. italicum* flowers (7.77 ± 0.34 %) was higher than in *H. chinophilum* (2.17 ± 0.18 %), but lower than in *H. plicatum* (11.50 ± 0.19 %) (Acet et al., 2020). Still in what concerns MUFAs, C18:1n-9 was the most abundant FA in *H. italicum* (4.49 ± 0.15 %), while in the other *Helichrysum* species, this FA was not detected or was present in much lower percentage. Again, while C18:2n-6 (22.55 ± 0.76 %) and C18.3n-3 (12.40 ± 0.59 %) were the most abundant PUFAs in *H. italicum*, the *Helichrysum* species *H. chionophilum*, *H. plicatum* and *H. arenarium* showed lower amounts of C18:2n-6, and the presence of C18:3n-3 was not detected (Umaz, 2020; (Acet et al., 2020)). Exceptions were only observed for *H. plicatum* that presented roughly twice as much the percentage of C18:1n-9 (8.25 ± 0.05 %) when compared with *H. italicum* (4.49 ± 0.15 %). Additionally, in what concerns other edible flowers, *H. italicum* presented higher percentages of PUFAs and MUFAs than common mallow (*Malva sylvestris)*, or rosemary (*Rosmarinus officinalis)* (Loizzo et al., 2016).

3.5. Antibacterial activity

The antibacterial activity of the *H. italicum* flower extracts was tested against *Klebsiella pneumoniae (K. pneumoniae)*, *Escherichia coli (E. coli)* and *Staphylococcus aureus* (*S. aureus*). MICs and IC_{50}s are shown in Table S2. Aqueous extracts showed antibacterial activity against *S. aureus* solely; while extracts in acetone showed antibacterial activity against *K. pneumoniae* and *S. aureus*. The two-fold broth microdilution method showed that aqueous extracts had *S. aureus* antimicrobial activity at an IC_{50} of 842 µg/mL, with a MIC higher than 1,600 µg/mL; whereas acetonic extracts proved to have greater inhibitory capacity (48.0 µg/mL IC_{50}, 1,578 µg/mL MIC) against the same microorganism (Table S2). Moreover, even if aqueous flower extracts were not effective in controlling the growth of *K. pneumoniae*; acetonic flower extracts were able to inhibit 50 % of *K. pneumoniae* growth at a concentration of 1,447 µg/mL (MIC > 1,600 µg/mL) (Table S2). Conversely, *E. coli* was the most resistant microorganism to both extracts, with no inhibitory effects observed at the concentrations tested. This might be due to the activation of one of the many efflux pumps, known to be present in *E. coli* strains. These efflux pumps are responsible for the elimination out of the cell of certain metabolites, antimicrobials, and toxic substances (Chetri, Bhowmik, Paul, Pandey, Chanda, Chakravarty, Bora et al., 2019). Other possibility for the resistance presented by *E. coli* might be a reduced

permeability of the cell to compounds present in the extracts (Delcour, 2009).

Previous studies showed that *H. italicum* exhibited antimicrobial properties against *Staphylococci, Streptococci* (D'Abrosca et al., 2016; Djihane et al, 2017; Nostro, Cannatelli, Musolino, Procopio, & Alonzo, 2002) *Bacilli, Enterococci, Micrococcus luteus, Pseudomonas aeruginosa* and *Proteus mirabilis* (Djihane et al, 2017). Extracts of the aerial parts of *H. italicum (Roth) G. Don* in diethyl ether had inhibitory effects against various *S. aureus* strains, through reduction of bacterial growth and via the inhibition of enterotoxin production (Nostro et al., 2002). Moreover, essential oils extracted from the aerial parts of *H. italicum (Roth) G. Don* exhibited antimicrobial activity against Gram-positive and Gram-negative bacteria (Djihane et al, 2017). The authors reported MICs for *H. italicum* essential oils, diluted in 10 % DMSO, ranging from 0.79 μg/mL against *Enterococcus cereus* to 50.6 μg/mL against *Staphylococcus aureus*. In addition, no effective inhibition against *E. coli* and *K. pneumoniae* was reported. Thus, the antibacterial activity observed herein against *K. pneumoniae* may be associated to the presence of higher amounts of lipophilic compounds with antibacterial properties, specifically present in the flower material, such as curcuminoids, when compared to the compounds globally present in the aerial parts (mixture of flowers and leaves), tested by the previous authors (Djihane et al, 2017).

3.6. Antifungal activity

As described in the preceding section, *H. italicum* flower extracts in acetone have shown antibacterial activity against both Gram-positive and Gram-negative bacteria, in agreement with previous studies performed with extracts in ethanol, diethyl ether and DMSO (Djihane et al., 2017; Nostro et al., 2002; Taglialatela-Scafati, et al., 2012). In what respects antifungal activity, previous authors have also shown that DMSO extracts of the essential oils of the aerial parts of *H. italicum* presented inhibitory effects against certain fungal species, such as *Candida albicans*, *Saccharomyces cerevisiae*, *Fusarium solani* var. *coeruleum*, *Aspergillus niger*, *Alternaria alternata* and *Ascochyta rabiei* (Djihane et al., 2017). Nevertheless, although DMSO is frequently used as solvent in microbiological assays, it is also known to be considerably toxic to certain microbial species, aside from presenting a high boiling point of 189 °C, that can compromise the isolation of several bioactive compounds (with antioxidant and antimicrobial properties). Therefore, to further examine the antimicrobial capacity of *H. italicum* whole flowers, we have assayed samples extracted in different solvents (acetone, chloroform, diethyl ether, ethanol, methanol, and water) against different fungal species. Figure S3 (supp. material) presents the effect of the plant material on the growth of wildtype *Saccharomyces cerevisiae* (*S. cerevisiae)* BY4741, *S. cerevisiae* AD1-9 and *Komagataella phaffii* (*K. phaffii)*, after a 48-hour incubation period. *S. cerevisiae* AD1-9 is a drug hypersensitive strain in which the genes for 9 plasma membrane–bound PDR transporters (drug efflux pumps) were knocked out (Millson, Prodromou, & Piper, 2010). This strain allows compounds to accumulate intracellularly and to better characterize the antifungal potential of the extracts. The results showed that *H. italicum* extracts in acetone, ethanol, and methanol were able to inhibit the growth of the three fungal strains tested; while extracts in chloroform, diethyl ether and water had no inhibitory effect (Figure S3). Table S3 presents the MICs and IC_{50}s values obtained. Extracts in methanol, ethanol and acetone presented lower MICs for all strains, being *K. phaffii* (aka *Pichia pastoris*) the most sensitive one (MICs of 175 μg/mL, for the three extracts) and wild type *S. cerevisiae* BY4741 the most resistant (MICs of 1,400 μg/mL for extracts in ethanol and methanol, and of 5,600 μg/mL for extracts in acetone). In addition, wildtype *S. cerevisiae* (BY4141) showed IC_{50} values of 1,100 μg/mL and 1,200 μg/mL for ethanol and methanol respectively, when compared to the mutated *S. cerevisiae* AD1-9 strain (180 μg/mL and 210 μg/mL), which leads to roughly a sixfold difference in sensitivity. Interestingly, an even greater difference in sensitivity between the two strains (about 25-fold) was detected for the extracts in acetone. In table S3, it is also worth noting that *K. phaffii* had IC_{50} values of 670 μg/mL, 770 μg/mL and 1,300 μg/mL for extracts performed in ethanol, methanol, and acetone, respectively. Thus, this *K. phaffii* sensitivity to *H. italicum* highlights the potential of using this herb as a food preservative. Methanol, ethanol, and acetone are powerful extractants and are well known for their ability to extract different types of antioxidants and essential oils, since they dissolve both hydrophilic and lipophilic components. In addition, given that acetone can efficiently extract polyphenols and flavonols, the TPC, antioxidant capacity and content on curcuminoids and carotenoids found herein correlate well with the antimicrobial properties presented by the plant. Indeed, even higher antifungal activities were obtained against *S. cerevisiae* and other fungi, by previous authors (Djihane et al., 2017), when using the essential oils solely, extracted in DMSO from the aerial parts of the plant (MIC of 6.325 μg/mL), reinforcing the potential of *H. italicum* as a natural antifungal agent.

3.7. Effect in Caco-2 cells

Caco-2 cells were used to examine possible toxic effects caused by *H. italicum* flower material. Fig. 1 shows the percentage of cell viability according to the MTT assay. As shown in Fig. 1, concentrations under 1.0 % w/v of whole flower material showed high cell viabilities, close to 100 %. The total herb and spice consumption is on average of 0.4 g/person/meal, which corresponds to concentrations in the range of 0.5 to 1.0 % w/v of plant material (Carlsen, Blomhoff, & Andersen, 2011). Nonetheless, only very low amounts (in the μg-ng range) would be available for intestinal absorption and be in the range of safe concentrations for ingestion. Thus, at low concentrations, *H. italicum* flowers (<1.0 % w/v) have shown to be nontoxic to Caco-2 model cells.

Previous *in vitro* and *in vivo* studies performed with commercial extracts of *H. italicum* showed that the plant was able to induce digestive enzyme inhibition and to reduce glucose uptake (Garza, Etxeberria, Palacios-Ortega, Haslberger, Aumueller, Milagro et al., 2014). The authors reported inhibitory activities for α-glucosidase and α-amylase, along with reduction of SGLT1-mediated methylglucoside uptake (56 % inhibition) in Caco-2 cells. Additionally, *in vivo* studies demonstrated that *H. italicum* extracts improved postprandial glycemic control by decreasing blood glucose levels, after an oral maltose tolerance test; reduced postprandial glucose levels after the oral starch tolerance test, as well as improved hyperinsulinemia (in 31 %) in overweight and

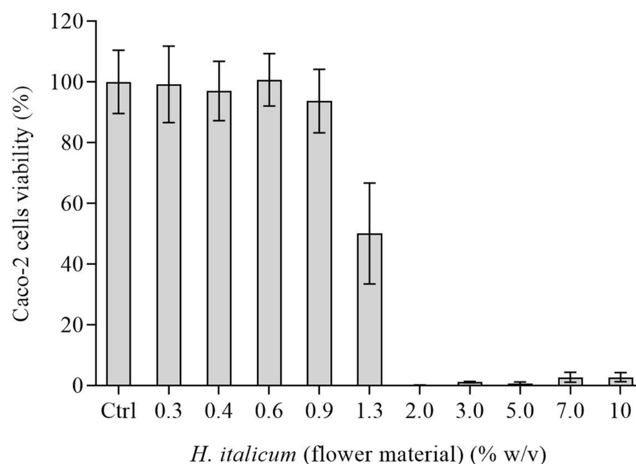

Fig. 1. MTT viability assay of Caco-2 cells upon exposure to different concentrations (in % w/v) of *Helichrysum italicum* subsp. *picardii* Franco flower material in DMEM culture medium, for 24 h. Control (Ctrl) refers to DMEM medium solely (100 % viability). Results are expressed as cell viability and presented as mean ± standard deviation of triplicates of three independent samples.

diabetic db/db mice (Garza, et al., 2014). Other authors have shown that at non-cytotoxic concentrations, the pyrone–phloroglucinol heterodimer arzanol, extracted form *H. italicum* showed anti-inflammatory properties and protective effects against lipid oxidation. Arzanol was able to inhibit the transcription nuclear factor-κB and the biosynthesis of pro-inflammatory lipid mediators like PGE(2) both *in vitro* (alveolar cells) and *in vivo* (Wistar Han rats) (Bauer et al., 2011), and to decrease the formation of oxidative products (hydroperoxides and 7-ketocholesterol) from the degradation of unsaturated FAs and cholesterol, in monkey kidney cells and Caco-2 cells (Rosa et al., 2007). Moreover, Appendino et al. (2007) also showed that crude acetonic extracts of the aerial parts (flowers and leaves) of *H. italicum* ssp. *microphyllum*, as well as extracted/purified arzanol, were able to inhibit TNFR-induced HIV-1-LTR transactivation in human T-lymphocytes, also exhibiting relevant antiviral properties, that substantiate the potential health beneficial effects of this plant.

4. Conclusion

Herbs and spices can act as natural food additives and preservatives, replacing other regulatory-approved synthetic chemicals, which are less beneficial to human/animal health. At low concentrations, *H. italicum* flowers are a good source of minerals, omega-6, and omega-3 PUFAs, polyphenols, carotenoids, curcuminoids and other bioactive compounds with relevant antibacterial, antifungal and antioxidant properties. Thus, the rational use of *H. italicum* flowers can bring color, aroma, and flavor to culinary dishes, while contributing to expand the shelf-life of food products.

CRediT authorship contribution statement

Maria João Primitivo: Investigation, Writing – original draft, Writing – review & editing. **Marta Neves:** Investigation, Formal analysis, Writing – review & editing. **Cristiana L. Pires:** Investigation, Writing – review & editing. **Pedro F. Cruz:** Investigation, Writing – review & editing. **Catarina Brito:** Investigation, Writing – review & editing. **Ana C. Rodrigues:** Investigation, Writing – review & editing. **Carla C.C.R. de Carvalho:** Investigation, Formal analysis, Writing – review & editing. **Megan M. Mortimer:** Investigation, Formal analysis, Writing – review & editing. **Maria João Moreno:** Validation, Supervision, Resources, Writing – review & editing. **Rui M.M. Brito:** Validation, Resources, Writing – review & editing. **Edward J. Taylor:** Validation, Supervision, Writing – review & editing. **Stefan H. Millson:** Validation, Supervision, Resources, Writing – review & editing. **Fernando Reboredo:** Validation, Supervision, Resources, Writing – review & editing. **Maria Jorge Campos:** Validation, Supervision, Resources, Writing – review & editing. **Daniela C. VAZ:** Validation, Supervision, Conceptualization, Writing – review & editing. **Vânia S. Ribeiro:** Validation, Supervision, Conceptualization, Writing – review & editing.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2022.111399.

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