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Functional constituents of wild and cultivated Goji (*L. barbarum* L.) leaves: phytochemical characterization, biological profile, and computational studies

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ABSTRACT

Goji (*Lycium barbarum* L.) leaves are emphasized as a functional tea or as dietary supplements. The phenolic compound profile, antioxidant, enzyme inhibitory, antimicrobial, and antimutagenic activities of leaf extracts from two selected cultivars in comparison with wild-growing plants have been evaluated. HPLC-DAD/ESI-ToF-MS analysis revealed the presence of phenolic acids and flavonoids with chlorogenic acid and rutin being the dominant compounds in the cultivated plants, whereas rutin and kaempferol-3-*O*-rutinoside for wild growing ones. In particular, cv. Erma contained the highest amount of chlorogenic acid and showed a strong tyrosinase-inhibitory effect. *Staphylococcus aureus*, *Listeria monocytogenes*, and *Penicillium funiculosum* were the most sensitive strains when exposed to extracts from cultivated plants. Antimutagenic activity was evaluated by Ames' test. The tested extracts provided high protection against mutagenicity induced by 2-anthramine (2-AA) to *Salmonella typhimurium* strains TA 98 and TA 100 (max. inhibition (%) 88% and 74.2%, respectively). Overall, Goji leaves are a rich source of bioactive compounds with functional properties that need further risk/benefit evaluation when used in foods or health-promoting formulations.

Introduction

Functional or bioactive components are biomolecules, mostly comprising phytochemicals that occur in food and possess the ability to modulate one or more metabolic processes or pathways in the human organism resulting in health benefits and promotion of well-being[1,2]. Over the past four decades, several thousands of phytochemicals have been identified in plants. Herbs, herbal extracts, and single phytochemicals are broadly used as foods, drugs, or remedies in traditional medicine. Many herbal products have health-promoting effects and, therefore, are marketed as such[3]. As a consequence, there has been much emphasize in phytochemicals with different biological properties such as antioxidant, antimicrobial, antiviral, antimutagenic, anticancer activity, or their use in pathologies such as Alzheimer's and cardiometabolic disorders, in recent years[4].

Among these phytochemicals, phenolic compounds are widely spread in foods and herbals and have attracted much attention being probably the most investigated molecules of medicinal and nutritional interest[5–7]. Besides, they exhibit various bioactivities such as antimicrobial, antiviral, antiallergic, anti-inflammatory, and protective effects against cell and cutaneous aging[8,9]. Their antioxidant properties as well as their beneficial effects against Alzheimer's disease, diabetes mellitus, and cardiovascular diseases are nowadays highly emphasized[7,10–12]. In recent years, the

possible toxicity of synthetic antioxidants has been reported[13]. Thus, the potential of plant products to serve as natural antioxidants for protecting against various diseases induced by free radicals has been explored. Increased popularity of natural food additives may prompt more food manufacturers to replace synthetic antioxidants with natural ingredients containing natural antioxidative compounds ('clean labeling'). Therefore, research on natural additives has gained momentum as they are perceived as posing no health risk to the consumers[14].

The genus *Lycium* (Solanaceae) comprises approximately 70 species and is disjunctly distributed in temperate to subtropical regions of South America, North America, southern Africa, Eurasia, and Australia[15]. *Lycium barbarum* L. is a defoliated shrubbery that grows in China, Tibet, and other parts of Asia and its fruits are 1–2 cm-long, bright orange-red ellipsoid berries[16]. The original area of *Lycium* spp. is not definitively established, but is likely based in the Mediterranean Basin. Meanwhile, the plant is widely distributed in warm regions of the world, in particular, in the Mediterranean area and Southwest and Central Asia. It is also cultivated in North America and Australia as a hedge plant[17,18]. Goji fruits have been widely used as concentrated extracts, in different beverages, and as ingredients in yogurts for their benefits to anti-aging, kidney, and liver functions[19,20]. Modern pharmacological research indicates effects of Goji fruits on aging, neuroprotection, general well-being, fatigue/endurance, metabolism/energy

expenditure, glucose control in diabetics, antioxidant properties, immunomodulation, antitumor activity, and cytoprotection. *Lycium barbarum* is currently sold world-wide also as a dietary supplement or classified as a nutraceutical food for its long ago and safe use in traditional Chinese medicine[16,18].

However, the leaves of *L. barbarum*, called "tianjingcao" (vitality and vigor of the nature) in traditional Chinese medicine, have been rarely studied, despite their use in alleviating mineral deficiency, combating heat distress, quenching thirst, dispelling wind, and enhancing eyesight[21]. Moreover, *L. barbarum* leaves have also been widely used as tea, medicinal vegetables, and herbal drugs in China, Southeast Asia, and are nowadays highly emphasized in Europe and North America as a functional tea or in dietary supplements. Besides, *L. barbarum* tender leaves have been used to cook soup, or consumed stir-fried, and with boiled eggs in many Asian countries[22].

At the same time, one can notice a competing trend to cultivate medicinal plants or other nutraceuticals away from their native origin and closer to countries where they will be processed and used. Thus, in recent years, there has been a growing trend in introducing Goji cultivation to different pedoclimates (Italy, Romania, Bulgaria, etc.), or even developing new cultivars[18,23–25].

Information about *L. barbarum* leaves' chemical composition or further bioactivities is not comprehensively and quite limited. However, Goji leaves have been described as a sustainable source of antioxidants and antibacterial compounds, recently[22,26].

Considering the under-estimated biological potential of *L. barbarum* leaves, this study aimed at investigating the phenolic profile and antioxidant capacity of leaves from two newly developed Goji cultivars in comparison with spontaneous growing plants. Moreover, a special aim of this study was to assess the cholinesterase, α-amylase, and α-glycosidase inhibitory potential, antimicrobial, and antimutagenic properties of Goji leaves in order to establish a scientific basis for further applications of this plant as a functional food/ingredient. In particular, the evaluation of anti-tyrosinase properties of the extracts could provide novel applications for both food and cosmetic industries. Furthermore, a molecular modeling approach was used to further investigate the enzyme inhibitory effects.

Materials and methods

Plant samples

Leaves of *L. barbarum* from two cultivars and spontaneously growing plants were collected in the summer of 2014 from three origins: (1) cultivar Erma (Lbe) was collected from an ecological culture in North-Vest Romania, Ciuperceni (47°52′14″ N, 23°0′55″ E), Satu-Mare County; (2) cultivar Biglifeberry (Lbb) was collected from an ecological culture in NV Romania, Ploscoș (46°38′33″ N, 23°50′43″ E), Cluj County; (3) wild growing *L. barbarum* leaves (Lbn) were collected in NV Romania, Turda (46°33′52″ N, 23°47′49″ E), Cluj County. Voucher specimens were deposited in the Herbarium of the Department of Pharmaceutical Botany, Faculty of Pharmacy, "Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania. The plant material was dried at room temperature (∼21 °C) in a shaded place for 2 weeks.

Extraction procedure

The ground plant sample (2 g) was extracted with the help of ultrasound with 20 mL of methanol/water (70:30, v/v) at room temperature for 1 h. The extract was filtered through paper filter (MN 615, Macherey-Nagel, Düren, Germany). Following syringe

filtration (0.45 μm nylon membrane), the extracts were analyzed with LC-DAD/ESI-ToF-MS. For evaluating the biological activity, extracts obtained were evaporated under reduced pressure and further kept in a vacuum desiccator to fully remove traces of solvents. For each bioassay where a specific concentration of the extracts is not mentioned, the raw extract was used. In case of antimicrobial and antimutagenic evaluation the concentration of 1 mg/mL was initially considered before subsequent dilutions. For each analysis, three different samples were used and the assays were performed in triplicate.

Separation, identification, and quantification of phenolic compounds

LC-DAD and quantification of phenolic compounds

The phenolic compounds from the different extracts of *L. barbarum* leaves were analyzed using a LC-DAD Smartline series system from Knauer GmbH (Berlin, Germany). The low-pressure gradient was performed with a Smartline manager S5050, pump S1000, autosampler S3950, and diode array detector S2600. The system was controlled with ClarityChrom 3.0 software (Knauer GmbH, Berlin, Germany). The separation was carried out on a Nucleodur® 5 μm C18 HTec (250 mm ×3 mm) column equipped with a C18 security guard (4 mm ×3 mm), both from Macherey-Nagel (Düren, Germany), at a temperature of 21 °C, a flow rate of 0.6 mL/min and UV-detection at 280 nm, 325 nm, 350 nm, and 365 nm. A binary gradient system with eluent (A) 0.1% formic acid in water, eluent (B) 0.1% formic acid in acetonitrile and the following gradient was used for the extracts: 10% B isocratic (0–1 min), 10–40% B (1–30 min), 40% B isocratic (30–33 min), 40–10% B (33–34 min), and 10% B isocratic (34–37 min). For quantitative analysis, a calibration curve ($R^2 \geq 0.99$) for each standard compound available (chlorogenic acid, quercetin-3-*O*-rutinoside, quercetin-3-*O*-glucoside, ferulic acid) was drawn up based on the corresponding UV signals. For a phenolic compound identified for which a commercial standard was not available, the quantification was performed using the calibration curve of another compound from the same phenolic group (e.g. neochlorogenic acid expressed as chlorogenic acid). The results were calculated on a basis of mg per g of dry weight vegetal material.

HPLC-ESI-ToF-MS for the identification of phenolic compounds

The structural characterization of the phenolic compounds in the leaves of *L. barbarum* was carried out on a LC-DAD/ESI-ToF-MS system consisting of an HPLC Agilent 1200 Series with Agilent Mass Hunter B.05.01 software (Agilent, Santa Clara, CA), and an ESI-TOF Agilent 6224 mass spectrometer with software Agilent Mass Hunter B.05.01 (Agilent, Santa Clara, CA), and MestReNova 7 (Mestrelab Research SL, Santiago de Compostela, Spain). The HPLC system consisted of a binary pump, an autosampler, a column compartment, and a diode array detector. The LC-DAD method was similar to the LC-DAD method described above with the exception of a reduced flow rate of 0.5 mL/min. ESI-ToF experiments were recorded in both positive and negative mode and a scan range between 110 and 2000 *m/z*. The capillary voltage was set to 4 kV and the capillary temperature was at 325 °C. N_2 was used as dry gas with a dry flow of 10 L/min and a pressure of 30 psi for the nebulizer.

Total phenolic content (TPC)

The TPC was determined using the Folin–Ciocâlteu method as reported previously[27,28]. For a high throughput of samples, a

Synergy HT Multi-Detection Microplate Reader with 96-well plates (BioTek Instruments, Inc., Winooski, VT) was used. A mixture solution consisting of 25 μL sample extract, 125 μL of Folin–Ciocâlteu reagent (10-fold diluted from the stock solution), and 100 μL of sodium carbonate (Na_2CO_3) solution (7.5% w/v) was homogenized and incubated at room temperature in the dark for 2 h. The absorbance was measured at 760 nm against a solvent blank. Gallic acid was used as a standard, and the content of TPC was expressed as gallic acid equivalents (GAE) in mg/g dry weight (dw) of vegetal material.

Total flavonoid content (TFC)

The TFC of the extracts was determined according to methods described previously[28,29]. An 100 μL aliquot of 2% $AlCl_3$ ethanolic solution was added to 100 μL of the extracts and mixed. After incubating for 1 h at room temperature, the absorbance at 420 nm was measured against a solvent blank. The TFC was expressed as quercetin equivalents (QE) in mg/g dry weight (dw) of vegetal material.

Trolox equivalent antioxidant capacity (TEAC) assay

The TEAC of the different *Lycium* extracts against the stable synthetic $ABTS^{\bullet+}$ radical cation was assessed using the method described by Barakat and Rohn[29] and Rohn et al.[30] with some modifications. A trolox calibration curve was plotted as a function of the percentage of ABTS radical cation scavenging activity. The final results were expressed as milligrams of trolox equivalents (TE) per gram of vegetal material (mg TE/g dw).

Electron paramagnetic resonance (EPR) spectroscopy

To compare with the results of the photometric measurements, the radical scavenging activity of different *L. barbarum* leaves extracts was additionally measured with EPR spectrometry using the stable synthetic radical Fremy's salt as reported previously[29,31]. Primarily, the Lbb and Lbe extracts were diluted 100-fold and the Lbn 50-fold (v/v), respectively. A mixture of 25 μL of the dilution and a 25 μL of a 1 mM Fremy's salt solution [potassium nitrosodisulfonate, $K_2NO(SO_3)_2$] in phosphate buffer (pH 7.4) were filled into a 50 μL capillary. After a reaction time of 30 min, the EPR spectrum of Fremy's salt radical was recorded and intensity was obtained by integration of the signal. The antioxidant activity of the extracts, expressed as milligrams of Fremy's salt equivalents per gram dry matter (mg FSE/g dw), was calculated in comparison to a control reaction with the solvent. Spectra were recorded at 21 °C on a Miniscope MS 200 X-band spectrometer (Magnettech GmbH, Berlin, Germany) with the microwave power set to 10 dB and using modulation amplitude of 1500 mG.

Enzyme inhibitory activity

Cholinesterase (ChE) inhibition

ChE inhibitory activity was measured using Ellman's method, as reported previously[32,33]. Sample solution aliquot (50 μL, 2 mg/mL) was mixed with 125 μL 5,5-dithio-bis(2-nitrobenzoic) acid (DTNB) and acetylcholinesterase (AChE from Electric eel, Type-VI-S, EC 3.1.1.7, Sigma, Saint Louis, MO), or butyrylcholinesterase (BChE from horse serum, EC 3.1.1.8, Sigma, Saint Louis, MO) solution (25 μL) in Tris-HCl buffer (pH 8.0) in a 96-well microplate and incubated for 15 min at 25 °C. The reaction was then initiated with the addition of acetylthiocholine iodide (ATCI) or butyrylthiocholine chloride (BTCl) (25 μL). Similarly, a blank was prepared by adding sample solution to all reaction reagents without enzyme(s) (AChE or BChE) solution. The sample and blank absorbances were recorded at 405 nm after 10 min incubation at 25 °C. The absorbance of the blank was subtracted from that of the sample and the cholinesterase inhibitory activity was expressed as milligrams of galanthamine equivalents (mg GALAE/g extract).

α-Amylase inhibition

α-Amylase inhibitory activity was performed using Caraway–Somogyi iodine/potassium iodide (IKI) method[32,34]. Sample solution (25 μL, 2 mg/mL) was mixed with α-amylase solution (porcine pancreas, EC 3.2.1.1, Sigma, Saint Louis, MO) (50 μL) in phosphate buffer (pH 6.9 with 6 mM sodium chloride) in a 96-well microplate and incubated for 10 min at 37 °C. After pre-incubation, the reaction was initiated with the addition of starch solution (50 μL, 0.05%). Similarly, a blank was prepared by adding sample solution to all reaction reagents without enzyme solution. The reaction mixture was incubated 10 min at 37 °C, then stopped by adding HCl (25 μL, 1 M). This is followed by the addition of the iodine–potassium iodide solution (100 μL). The sample and blank absorbances were recorded at 630 nm. The absorbance of the blank was subtracted from that of the sample and the α-amylase inhibitory activity was expressed as millimoles of acarbose equivalents (mmol ACE/g extract).

α-Glucosidase inhibition

α-Glucosidase inhibitory activity was performed as previously described by Zengin et al.[32] and Zengin[35]. Sample solution (50 μL) was mixed with glutathione (50 μL, 2 mg/mL), 50 μL α-glucosidase solution (from *Saccharomyces cerevisiae*, EC 3.2.1.20, Sigma, Darmstadt, Germany) in phosphate buffer (pH 6.8), and 50 μL of 10 mM PNPG (4-N-trophenyl-α-D-glucopyranoside) (Sigma-Aldrich, Darmstadt, Germany) solution in a 96-well microplate and incubated for 15 min at 37 °C. Similarly, a blank was prepared by adding sample solution to all reaction reagents without enzyme solution. The reaction was stopped with the addition of sodium carbonate (50 μL, 0.2 M). The sample and blank absorbances were recorded at 400 nm. The absorbance of the blank was subtracted from that of the sample and the α-glucosidase inhibitory activity was expressed as millimoles of acarbose equivalents (mmol ACE/g extract).

Tyrosinase inhibition

Tyrosinase inhibitory activity was measured using the modified dopachrome method with L-DOPA as substrate, as previously reported[32] with slight modifications. A sample solution (25 μL, 2 mg/mL) was mixed with tyrosinase solution (40 μL) and phosphate buffer (100 μL, pH 6.8) in a 96-well microplate and incubated for 15 min at 25 °C. The reaction was then initiated with the addition of L-DOPA (40 μL). Similarly, a blank was prepared by adding sample solution to all reaction reagents without enzyme (tyrosinase) solution. The sample and blank absorbances were recorded at 492 nm after incubating 10 min at 25 °C. The absorbance of the blank was subtracted from that of the sample and the tyrosinase inhibitory activity was expressed as equivalents of kojic acid (mg KAE/g extract).

Molecular modeling studies

Molecular modeling studies were carried out in order to confirm the role of chlorogenic acid in the inhibition of the tested
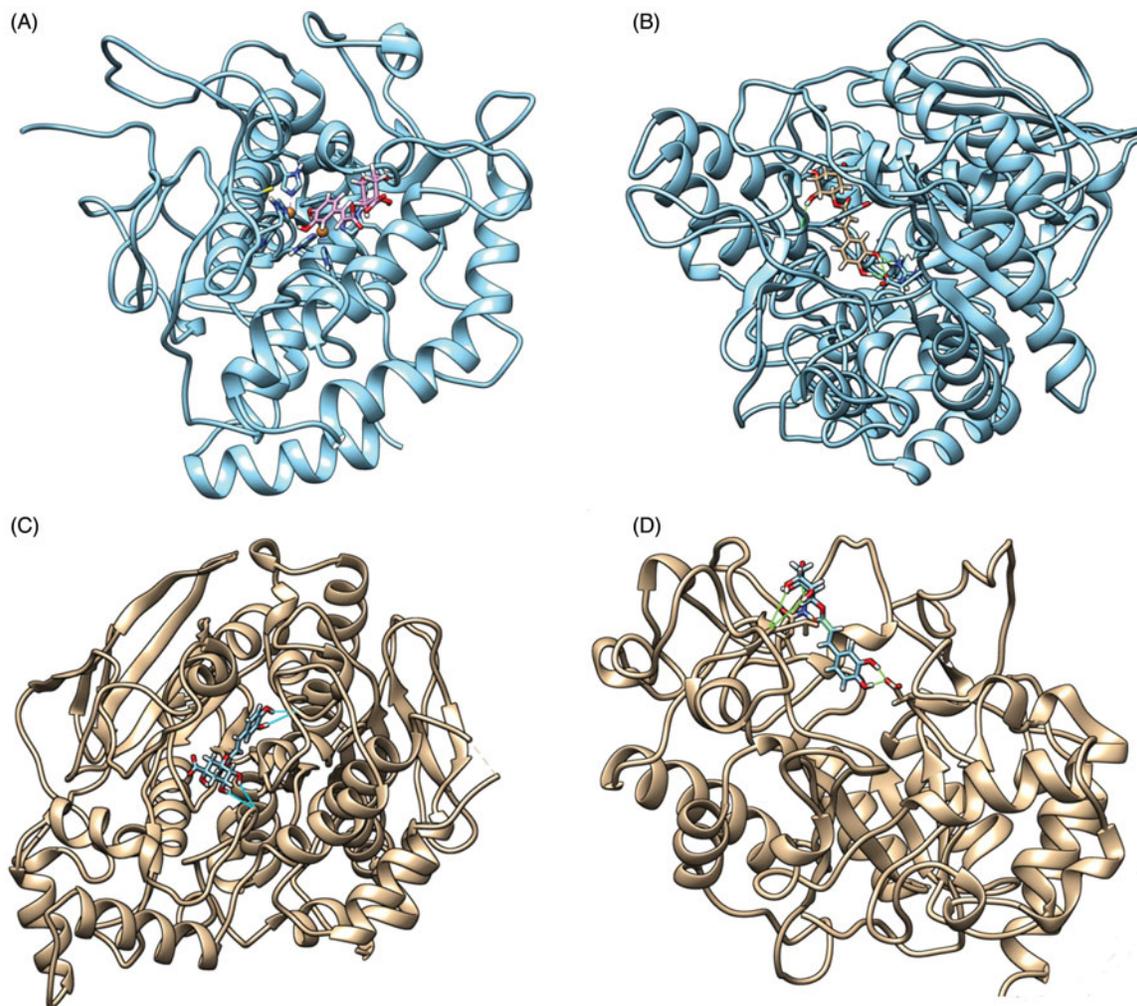
Figure 1. Best docking pose found between chlorogenic acid and tyrosinase (A), α-glucosidase (B), butyrylcholinesterase (C) and a-amylase (D).

enzymes, exemplarily. Docking experiments with the ligand chlorogenic acid have been performed with butyrylcholinesterase (pdb code: 4BDS)[36], α-amylase (pdb code: 1VAH)[37], α-glucosidase (pdb code: 3AXI)[38], and tyrosinase (pdb code: 2Y9X)[39]. The receptors have been prepared by using the Maestro 11.0 (free academic licence), protonated at neutral pH and all the crystals errors were corrected by hand. The docking experiments have been carried out using Gold suite 6 software[40], using the scoring function chemscore, which is designed to work in the presence of metallo-enzymes as in the case of tyrosinase. The docking grid has been automatically calculated by Gold, centered on the crystallographic ligand, the best docking pose found for each enzyme-chlorogenic acid complex is depicted in Figure 1.

Molecular dynamics of the chlorogenic acid-tyrosinase complex

In order to gain further information about the time-dependent behavior of the chlorogenic acid–tyrosinase complex found in the docking experiments, a molecular dynamics experiment was carried out using the software Amber 14[41]. The partial charges of the ligand (chlorogenic acid) were determined by using the AM1-BCC method of Antechamber suite. The complex tyrosinase–chlorogenic acid was prepared by leap suite, hydrated in a cubic water box of 20 Å, and the net charge neutralized by adding Na^+ ions. Then, the complex was minimized for 3000 steps of steepest descent followed by conjugate gradient until convergence of 0.05 kcal/Å mols. In a second stage, the complex was subjected to molecular dynamic

simulations. Then, molecular dynamics trajectory was run. The simulation was analyzed for 10 ns with recording one frame every 0.1 ns.

Antimicrobial activity

Bacteria and culture conditions

For this bioassay, six bacterial strains were used: four Gram-positive bacteria: *Staphylococcus aureus* (ATCC 49444), *Bacillus cereus* (ATCC 11778), *Listeria monocytogenes* (ATCC 19114), and *Enterococcus faecalis* (ATCC 29212), and three Gram-negative bacteria: *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 14028), and *Escherichia coli* (ATCC 25922), as well as two anaerobic strains: *Fusobacterium nucleatum* (ATCC 25586) and *Peptostreptococcus anaerobius* (ATCC 27337). All the tested microorganisms were obtained from Food Biotechnology Laboratory, Life Sciences Institute, University of Agricultural Sciences and Veterinary Medicine Cluj Napoca, Romania. The aerobic strains were cultured on Muller–Hinton agar and cultures were stored at 4 °C and subcultured once a month. Anaerobic bacteria were cultured overnight at 37 °C on thioglycollate broth with resazurin media at 37 °C.

Microdilution method

The modified microdilution technique was used to evaluate the antimicrobial activity of the samples as reported previously by

Mocan et al.[42,43]. Briefly, fresh overnight cell suspensions were adjusted with sterile saline solution to a concentration of approximately 2×10^5 CFU/mL in a final volume of 100 µL per well. The inoculum was stored at 4 °C for further use. Dilutions of the inoculums were cultured on solid Muller–Hinton (MH) agar for bacteria to verify the absence of contamination and to check the validity of the inoculums. Determinations of minimum inhibitory concentrations (MICs) were performed by a serial dilution technique using 96-well plates. Different dilutions of the extracts were carried out with wells containing 100 µL of MH broth and afterwards, 10 µL of inoculum was added to all the wells. The microplates were incubated for 24–48 h at 37 °C. The MIC of the samples was detected following the addition of 20 µL (0.2 mg/mL) of resazurin solution to each well, and the plates were incubated 2 h at 37 °C. A change from blue to pink indicates reduction of resazurin and, therefore, bacterial growth. The MIC was defined as the lowest drug concentration that prevented this color change. The minimum bactericidal concentrations (MBCs) were determined by serial subcultivation of a 2 µL into microtitre plates containing 100 µL of broth per well and further incubation for 48 h at 37 °C. The lowest concentration with no visible growth was defined as MBC, indicating 99.5% killing of the original inoculum. Streptomycin (Sigma P 7794, Santa Clara, CA) (0.05–3 mg/mL) was used as positive control for bacterial growth. Water was used as negative control.

Antifungal activity

For this bioassay, following fungi were used: *Aspergillus flavus* (ATCC 9643), *Aspergillus niger* (ATCC 6275), *Candida albicans* (ATCC 10231), *Candida parapsilosis* (ATCC 22019), and *Penicillium funiculosum* (ATCC 56755), all bought from the same source as mentioned above. Cultures were maintained on malt agar at 4 °C and subcultured every month. Spore suspension (1.0×10^5) was obtained by washing agar plates with sterile solution containing (0.85% saline, 0.1% Tween 80 (v/v)), then added to each well to a final volume of 100 µL. Inocula were screened for contamination by culturing on a solid medium. The minimum inhibitory (MIC) and minimum fungicidal (MFC) concentrations assays were performed using the microdilution method by preparing a serial of dilutions in 96-well plates. The extracts were diluted in 0.85% saline (10 mg/mL), then added to microplates containing Broth Malt medium with inoculum and incubated for 72 h at 28 °C on a rotary shaker. The lowest concentrations without visible growth (at the binocular microscope) were defined as minimal inhibitory concentrations (MICs). The fungicidal concentrations (MFCs) were determined by serial sub-cultivation of 2 µL of tested extracts dissolved in medium and inoculated for 72 h, into microtiter plates containing 100 µL of broth per well and further incubation 72 h at 28 °C. The lowest concentration with no visible growth was defined as MFC indicating 99.5% killing of the original inoculum. The fungicide fluconazole (Sigma F 8929, Santa Clara, CA) was used as positive control (1–3500 µg/mL). All the experiments were performed in duplicate and repeated thrice. Water was used as a negative control.

Antimutagenicity

Microbial mutagenicity was assayed by the standard Ames test (standard plate incorporation assay) according to Maron and Ames[44] and Ong et al.[45]. To avoid an erroneous result caused by antimicrobial effect of tested samples towards test strains, a preliminary toxic dose range experiment was performed to determine an appropriate dose range for the Ames test. Samples were prepared with 0.1 mL of 10 h-cultured test strain (approx. 10^8 cells/mL), 0.1 mL of the test extracts (5 mg/plate), 0.1 mL of phosphate buffer (0.2 M, pH 7.4) and 0.5 mL of S9 mix or phosphate buffer. The serial dilutions were immediately made with phosphate buffer, and then 1 mL of the aliquot was mixed with 12 mL of nutrient agar. After incubation at 37 °C for 48 h, the number of colonies was counted. A toxicity effect was confirmed when the standard plate count of the tested compound was lower than that of the control (with no compound added).

A mixture containing 0.1 mL of the extracts (5 mg/plate), 0.5 mL of S9 mix or phosphate buffer, 0.2 mL of 0.5 mM histidine-biotin and 0.1 mL 10 h-cultured test strain (approximately 10^8 cells/mL) was added to a tube containing 2 mL of top agar. The tube was then gently vortexed and poured onto the MA plate. The extracts were tested with and without S9 mix, and triplicate plates are poured for each dose of extracts. Reference mutagens, 2-AA (2-anthramine) (5 µg/plate or 2 µg/plate) were prepared by dissolving in DMSO and served as positive control chemicals. After incubation at 37 °C for 48 h, the number of revertants was counted. An extract was considered as a mutagen when there was a two-fold increase in the number of revertants, comparing with the number of spontaneous revertants (negative control). The antimutagenic effect of the tested sample was assessed using the Ames test, except for the addition of reference mutagens, 2-AA in each tested plate. The antimutagenicity of the reference mutagens in the absence of the extract was defined as 0% inhibition, and the antimutagenicity was calculated according to the formula given by Ong et al.[46]: % Inhibition $= [1 - T/M] \times 100$, where T is the number of revertants per plate in the presence of mutagen and the extract and M is the number of revertants per plate in the positive control (without extract). The tests were performed in duplicate with three sub-samples each, and the data was presented as mean ± standard deviation (SD).

Statistical analysis

The standard deviation was calculated and the averaged values along with the standard deviations (SD) are documented in the respective tables or figures. One-way analysis of variance (ANOVA) was used to detect any significant differences among different means as well as interactions between the variables used in biochemical analyses.

Results and discussion

TPC and TFC

In this study, Goji leaves have been characterized. The total phenolic and flavonoid contents of the three *Lycium* leaves extracts are presented in Figure 2. The two cultivars Lbb and Lbe provided similar values in terms of TPC (11.14 mg GAE/g dw and 11.98 mg GAE/g dw, respectively), while wild-growing Lbn had a significant lower amount (2.49 mg GAE/g dw). A similar trend was observed for the TFC (Figure 2). These results are in agreement with a study by Dong et al.[47] who found that total flavonoids in the leaves of cultivated *L. barbarum* were significantly higher than those in wild *L. barbarum*, suggesting that cultivated Goji leaves varieties can be promising raw materials for being used in functional foods. Higher yields of TPC and TFC contents were reported by Abdennacer et al.[48] in leaves of wild-growing Tunisian *Lycium intricatum* Boiss. while a similar trend was also described by Simirgiotis et al.[49] concerning South American berries and leaves of *Luma* spp. In this case, the observed differences for total bioactive components in
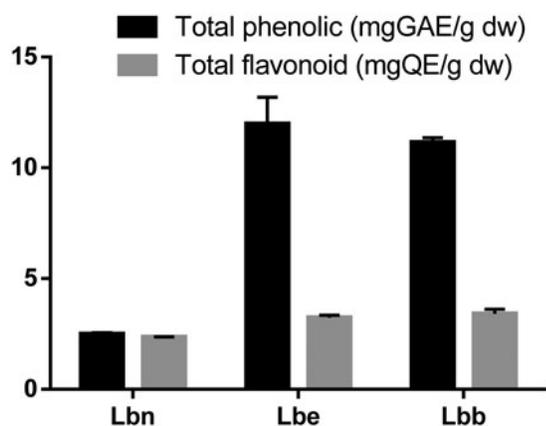
Figure 2. Total phenolic and flavonoid contents of the three extracts from *Lycium barbarum* leaves (mean ± SD).

these extracts may be explained with agro-climatic conditions including rainfall, altitude, or soil composition as well as genetic differences. Similar trends were reported by several researchers[50,51].

Characterization of phenolic compounds

The characterization of the single phenolic compounds in the *Lycium* leaves extracts was performed by HPLC-DAD-ESI-ToF-MS analysis. Retention times, λ_{max}, mass spectrometric characteristics, identification, and their distribution in the samples are presented in Tables 1 and 2. Exemplarily, the HPLC phenolic profile of Lbb recorded at 325 nm is illustrated in Figure 3. UV and mass spectra showed that the phenolic composition of the leaves of *L. barbarum* is mainly characterized by the presence of several hydroxycinnamic acids and flavonoid glycosides.

Hydroxycinnamic acids

From the hydroxycinnamic acids identified, compounds correspond to caffeoylquinic (compounds **3** and **8–11** in Table 1) or ferulic acid derivatives (compound **13** in Table 1). Compound **8** was identified as 5-*O*-caffeoylquinic acid (5-CQA, chlorogenic acid) by comparison with an authentic standard. Chlorogenic acid have been previously reported in fruits of *L. barbarum*, *L. chinense*, and *L. rhutenicum* as well as in *L. intricatum* fruits, where it was described as being the main compound[18,48,52,53]. Besides, it was also described as dominant in the leaves of wild *L. barbarum*, *L. intricatum,* or cultivated *L. chinense*[26,48,54]. Contents of chlorogenic acid in Chinese wild-growing *L. barbarum* leaves range between 2.09 mg/g dw and 4.28 mg/g dw, as reported by Dong et al.[54], whereas the value from the present study of wild-growing *L. barbarum* leaves is comparatively lower (510.68 µg/g dw). However, with regard to the two cultivars Lbe and Lbb, chlorogenic acid was found as the dominant compound with yields approximately 48 times higher than in the wild-growing plant (24 887 µg/g dw for Lbe and 17 811 µg/g dw for Lbb, respectively). This fact may suggest that besides culture conditions, geography, ecophysiology, harvesting time, and storage, genotype most significantly affects the composition of bioactive compounds. Compound **3** (*m/z* 353 [M–H]$^-$) was, based on its MS fragmentation pattern, identified as 3-*O*-caffeoylquinic acid (neochlorogenic acid) yielding a peak at *m/z* 191 with an abundance of 45.6% and two further ions, one at *m/z* 179 with an abundance of 23.4% and the other at *m/z* 135 with the abundance of 15.7%. The same compound presented also a dimeric adduct ([2M–H]$^-$ at *m/z* 707 with an abundance of 16.4%). This fragmentation behavior is considered characteristic for neochlorogenic acid as previously reported by Clifford et al.[55,56])

Compound **10** ($\lambda_{max}=326$; 296sh) was easily distinguished among the other caffeoylquinic acids because of its signal at *m/z* 173 [quinic acid–H–OH]$^-$ (15.8%), accompanied by another fragment ion at *m/z* 179 (13.7%), which allowed identifying it as 4-*O*-caffeoylquinic acid (cryptochlorogenic acid) according to the fragmentation pattern described by Clifford et al.[55,56]. Compound **11** showed similar fragmentation pattern and fragment abundances as those obtained for 5-*O*-caffeoylquinic acid (chlorogenic acid), indicating the presence of an isomer. However, according to Barros et al.[57], and based on its fragmentation pattern and small amounts, it was tentatively assigned as *cis*-5-*O*-caffeoylquinic acid. Results of the present study are in line with previous findings of Abdennacer et al.[48] who reported the presence of chlorogenic acid and its isomers in the leaves of Tunisian *Lycium intricatum*. Compound **9** had a pseudomolecular ion at *m/z* 499 that led to further fragments at *m/z* 353 ([caffeoylquinic acid–H]$^-$), and 191 and 179, corresponding to deprotonated quinic acid and deprotonated caffeic acid, respectively, which allowed identifying it as a further 3-*O*-caffeoylquinic acid derivative[58]. Compound **13** ($\lambda_{max}=325$; 296sh and [M–H]$^-$ at *m/z* 367) was identified as 5-*O*-feruloylquinic acid according to Clifford et al.[55], and based on its MS fragmentation, presenting a fragment at *m/z* 191 (34%) resulting from a loss of a feruloyl moiety, and two additional fragments at *m/z* 173 and *m/z* 735, the latter indicating a dimeric adduct of the feruloylquinic acid molecule. These data are consistent with the feruloylquinic acid isomers found previously in leaves of *Lycium intricatum* by Abdennacer et al.[48].

Flavonoid glycosides

In the present study, 11 quercetin and kaempferol glycosides have been tentatively identified in the two orchard cultivars and the wild-growing *Lycium* leaves. A total of 10 flavonol glycosides were detected in naturally growing *Lycium* leaves. For cultivars Lbb and Lbe, the flavonoid profile was consisted of only five or two dominant compounds, respectively. Detailed mass spectra with fragmentation patterns, depending on the compound, maximum wavelength of their UV–Vis spectra, retention times, and quantitative data are presented in Tables 1 and 2. Generally, all mass spectra were characterized by their deprotonated molecular ions [M–H]$^-$ or by their protonated molecular ions [M + H]$^+$. However, due to the fact that in these experiments, the compounds presented higher ionization yields in the positive mode, the data obtained in this mode were further used for discussions related to compounds assignment, as previously reported by Dong et al.[47]. The assignment of different sugar substitutions at the flavonoid aglycons was carried out in accordance with previous studies[48,59,60]. Those studies demonstrated that the first fragmentation of the quasi-molecular ion [M–H]$^-$/[M + H]$^+$ is due to the breakdown at the 7-*O* position leading to a peak ion [M + H-146]$^+$ for pentosides, [M + H-162]$^+$ for monohexosides or [M + H-324]$^+$ for dihexosides, and the remaining sugars at the flavonoid molecule are to be linked to the hydroxyl group at the 3-*O* position of the flavonol skeleton. Compound **14** was identified as rutin (quercetin-3-*O*-rutinoside) by comparing with an authentic standard. Rutin was detected in all samples. The highest amount was found in cultivar Lbe (9339 µg/g dw). These results are in agreement with Dong et al.[47,54] who reported that levels of rutin in leaves of cultivated Chinese *Lycium barbarum* are much higher than those in wild-growing plants and are influenced by ecophysiological factors and geographical origin. Compound **1** ([M + H]$^+$ at *m/z* 789) and compound **2** ([M + H]$^+$ at *m/z* 773) provided similar fragmentation behavior, each yielding a fragment at *m/z* 465 (67.9%), and 449 (93.2%), respectively, characterized by

Table 1. Phenolic compounds in *Lycium barbarum* leaves, retention times (R_t), wavelengths of maximum absorption (λ_{max}), mass spectral data and identification.

No.	Phenolic compound	R_t (min)	λ_{max} (nm)	M_W (g/mol)	$[M–H]^-$	$[M + H]^+$	MS fragments (m/z)
1′	Quercetin-3-O-Sophtr-7-O-Soph	4.27	Coeluting with 1	1112	1111	1113	789 (100) $[M_7-324]^+$; 627 (14.16) $[M_7-324; M_3-162]^+$; 465 (67.93) $[M_7-324; M_3-324]^+$; 303 (21.21) $[M_7-324; M_3-486]^+$
1	Quercetin-3-O-Glu-7-O-Soph	4.27	256; 266sh; 352	788	787	789	627 (14.16) $[M_3-162]^+$; 465 (67.93) $[M_7-324]^+$; 303 (21.21) $[M_7-324; M_3-162]^+$; 1577 (2.60) $[2M + H]^+$
2	Kaempferol-3-O-Glu-7-O-Soph	5.21	267; 286sh; 341	772	771	773	611 (21.79) $[M_3-162]^+$; 449 (93.21) $[M_7-324]^+$; 287 (40.79) $[M_7-324; M_3-162]^+$; 1567 (1.24) $[2M + Na]^+$
3	3-O-Caffeoylquinic acid (neochlorogenic acid)	6.26	296sh; 324	354	353	377 $[M + Na]^+$	191 (45.59) $[M-H-caffeoyl]^-$; 179 (23.39) $[M-H-quinic]^-$; 173 (3.64); 161 (2.94); 135 (15.70); 707 (16.37) $[2M-H]^-$
4	Unknown compound	6.86	256; 287; 315sh; 340	324	323	347 $[M + Na]^+$	–
5	Quercetin-3-O-Rut-7-O-Glu	7.1	255; 266sh; 354	772	771	773	611 (4.54) $[M_7-162]+$; 465 (6.63) $[M_3-308]+$; 303 (3.30) $[M_7-162; M_3-308]+$ 1567 (1.25) $[2M + Na]+$
6	Quercetin-3-O-Soph-7-O-Rha	7.45	255; 266sh; 354	772	771	773	627 (4.53) $[M_7-146]^+$; 465 (4.29) $[M_7-146; M_3-162]^+$; 303 (2.10) $[M_7-146; M_3-324]^+$ 1567 (1.03) $[2M + Na]^+$
7	Kaempferol-3-O-Rut-7-O-Glu	8.13	265; 340	756	755	757	611 (10.14) $[M_7-162]^+$; 449 (8.16%) $[M_3-308]^+$; 287 (5.39) $[M_7-162; M_3-308]^+$
8[*]	5-O-Caffeoylquinic acid (chlorogenic acid)	8.46	244; 296sh; 320	354	353	355	191 (100) $[M-H-caffeoyl]^-$; 161 (1.04); 707 (95.59) $[2M-H]^-$
9	3-O-Caffeoylquinic acid derivative	8.96	265; 296sh; 326	500	499		353 (100) $[caffeoylquinic acid-H]^-$; 191 (41.67); 179 (15.19); 173 (15.96)
10	4-O-Caffeoylquinic acid (cryptochlorogenic acid)	9.26	244; 296sh; 326	354	353	377 $[M + Na]+$	191 (11.58) $[M-H-caffeoyl]^-$; 179 (13.67) $[M-H-quinic]^-$; 173 (15.75) 161 (1.00); 707 (13.90) $[2M-H]^-$
11	5-O-Caffeoylquinic acid isomer	10.38	296sh; 318	354	353		191 (100) $[M-H-caffeoyl]-$; 179 (0.91) $[M-H-quinic]^-$; 161 (2.98); 707 (3.90) $[2M-H]^-$
12	Quercetin-3,7-O-diGlu	11.88	256; 266sh; 352	626	625	627	465 (17.90) $[M_7-162]^-$; 303 (100) $[M_7-162; M_3-162]^-$; 1275 (1.96) $[2M + Na]^+$
13	5-O-Ferruloylquinic acid	12.48	296sh; 325	368	367	369	191 (34.06) $[M-H-ferruloyl]^-$; 173 (3.34); 735 (3.79) $[2M-H]^-$
14[*]	Quercetin-3-O-Rut	14.24	256; 266sh; 354	610	609	611	303 (37.34) $[M_3-308]^+$; 1243 (2.80) $[2M + Na]^+$
15	Quercetin-3-O-Glu-7-O-Rha	14.65	255; 296; 341	610	609	611	465 (14.21) $[M_7-146]^+$; 303 (44.25) $[M_7-146; M_3-162]^+$
16	Kaempferol-3-O-Rhu	15.5	265; 296sh; 344	594	593	595	287 (70.66) $[M_3-308]^+$
17	Kaempferol-3-O-Glu-7-O-Rha	16.2	265; 296sh; 344	594	593	595	449 (19.58) $[M_7-146]^+$; 287 $[M_7-146; M_3-162]^+$

Compounds with * were identified with references accurately, and the others were tentatively assigned based on literature data. Compounds were organized in order of their retention times (R_t). Numbers for some of the compounds also refer to chromatogram from Figure 3. M_7 indicates a sugar loss from 7-O position; M_3 indicates a sugar loss from 3-O position.

Table 2. Phenolic compounds (μg/g dw) and their distribution in *Lycium barbarum* leaves (mean ± SD).

| No.	Phenolic compound	Lbb	Lbe	Lbn
1	Quercetin-3-O-Glc-7-O-Soph	2406.38 ± 65	nd	222.58 ± 24
2	Kaempferol-3-O-Glc-7-O-Soph	186.46 ± 12	nd	146.5 ± 31
3	3-O-Caffeoylquinic acid (neochlorogenic acid)	804.4 ± 22	346.52 ± 41	nd
4	Unknown compound	nd	nd	nd
5	Quercetin-3-O-Rut-7-O-Glu	nd	nd	757.54 ± 45
6	Quercetin-3-O-Soph-7-O-Rha	5216.34 ± 98	nd	908.8 ± 84
7	Kaempferol-3-O-Rut-7-O-Glu	tr	nd	478.53 ± 35
8	5-O-Caffeoylquinic acid (chlorogenic acid)	17811.31 ± 102	24887.11 ± 205	510.68 ± 54
9	3-O-Caffeoylquinic acid derivative	248.1 ± 12	tr	nd
10	4-O-Caffeoylquinic acid (cryptochlorogenic acid)	1565.15 ± 32	1145.21 ± 120	nd
11	5-O-Caffeoylquinic acid isomer	343.19 ± 14	262.56 ± 51	nd
12	Quercetin-3,7-O-diGlu	810.82 ± 23	nd	171.75 ± 25
13	5-O-Ferruloylquinic acid	857.77 ± 32	917.22 ± 16	242.33 ± 12
14	Quercetin-3-O-Rut	1430.60 ± 41	9339.17 ± 112	1847.79 ± 101
15	Quercetin-3-O-Glu-7-O-Rha	nd	nd	867.16 ± 54
16	Kaempferol-3-O-Rhu	nd	nd	1063.34 ± 87
17	Kaempferol-3-O-Glu-7-O-Rha	nd	307.77 ± 31	447.98 ± 12

nd: not determined; tr: traces.
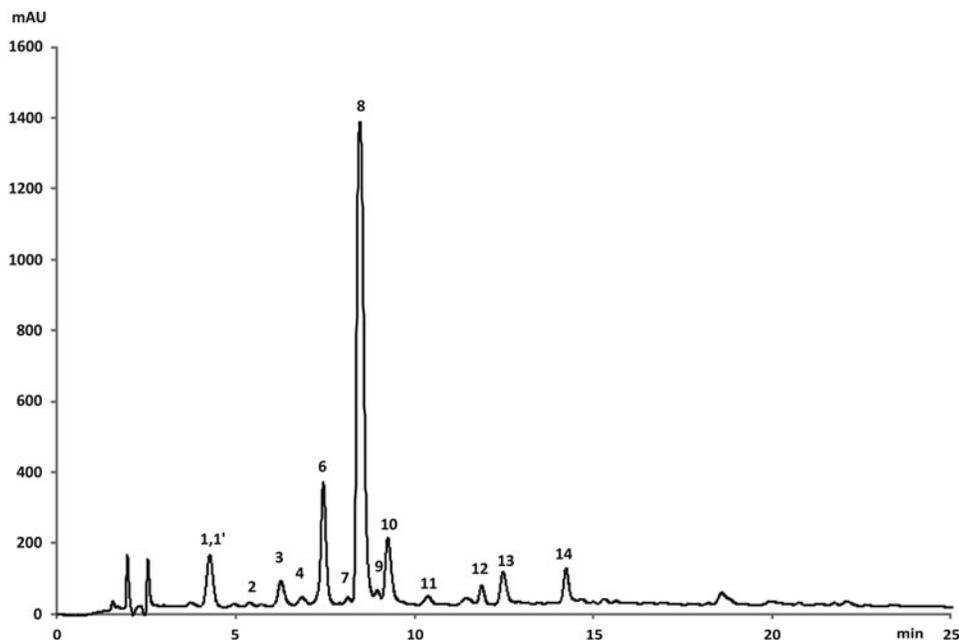
Figure 3. LC-DAD chromatogram (at 325 nm) of the phenolic compounds from *L. barbarum* leaves (cultivar Lbb- "Bigligeberry"). For compounds numbers refer to Table 1.

the loss of a dihexoside from the 7-*O* position. Additional fragments were observed at *m/z* 303 ([aglycone + H]$^+$) and *m/z* 287 ([aglycone + H]$^+$), respectively, indicating a supplementary loss of a glucose moiety (−162 amu) from the 3-*O* position. Thus, compound **1** was identified as quercetin-3-*O*-glucoside-7-*O*-sophoroside and compound **2** as kaempferol-3-*O*-glucoside-7-*O*-sophoroside, respectively. Compound **1'** ($\lambda_{max}=352$; 266sh and [M + H]$^+$ at *m/z* 1113) co-eluted with compound **1** and was assigned based on its UV–vis maxima and mass spectra as quercetin-3-*O*-sophorotrioside-7-*O*-sophoroside. Compounds **5** ([M + H]$^+$ at *m/z* 773) and **7** ([M + H]$^+$ at *m/z* 757), identified as quercetin-3-*O*-rutinoside-7-*O*-glucoside and kaempferol-3-*O*-rutinoside-7-*O*-glucoside provided significant similarities in their fragmentation behavior. Thus, both produced a fragment at *m/z* 611 indicating the loss of 162 amu from the 7-*O* position, corresponding to a glucose moiety. Moreover, additional fragments consistent with the loss of a rutinoside moiety (308 amu) were observed at 465 *m/z* and 449 *m/z*, respectively. In fact, these two compounds were previously reported by Yao et al.[53] in leaves of European wild growing *L. halimifolium*. The fragmentation behavior of compound **6** was consistent with quercetin-3-*O*-sophoroside-7-*O*-rhamnoside, by yielding a fragment at *m/z* 627 resulting from a loss of a rhamnose moiety (−146 amu) from the 7-*O* position and two additional fragments at *m/z* 465 (M-146; M-162) and *m/z* 303 (M-146; M-324), suggesting in this case a gradual oligosaccharide breakdown from the 3-*O* position. Sophorosides of quercetin and kaempferol were previously reported by Terauchi et al.[61] in the leaves of *L. chinense*. Compounds **12** ([M + H]$^+$ at *m/z* 627) and **15** ([M + H]$^+$ at *m/z* 611) were tentatively identified as quercetin-3,7-*O*-diglucoside and quercetin-3-*O*-glucoside-7-*O*-rhamnoside based on their UV–vis maxima and MS behavior. Both presented a fragment at *m/z* 465 indicating the loss of a glucose (−162 amu) and rhamnose (−146 amu) moiety, respectively, from the 7-*O* position, and the protonated aglycone at *m/z* 303 via an additional loss of a glucose (−162 amu) from the 3-*O* position. Finally, compounds **16** and **17** presented UV–Vis absorption maxima and MS characteristics coherent with kaempferol-3-*O*-rutinoside (nicotiflorin) and kaempferol-3-*O*-
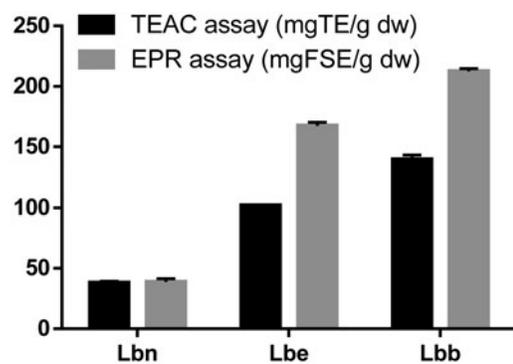

Figure 4. Total antioxidant capacity of *Lycium barbarum* leaves measured with TEAC and EPR spectroscopy. Results were expressed as mg TE/g dw, and as mg FSE/g dw. The error bars are the result of a triple determination.

glucoside-7-*O*-rhamnoside. Thus, both compounds showed a fragment at *m/z* 287 indicating the protonated aglycone via the loss of the sugar substituents. Additionally, a fragment at *m/z* 449 was observed for compound **17** suggesting the loss of a rhamnose moiety (−146 amu) from the 7-*O* position. Nicotiflorin has been reported to be present in wild growing *L. halimifolium* leaves as well in aerial parts of *L. arabicum*[53,62]. Finally, compound − ([M–H]$^-$ at *m/z* 323) presented no characteristic fragments in its MS spectra, and thus remained unidentified.

All in all, the present results suggest great differences between cultivated and wild growing *L. barbarum* leaves with regard to amount and composition of hydrocycinnamic acids and flavonol glycosides. As a peculiarity, on one side, a total number of 10 flavonoids were tentatively assigned in wild-growing *L. barbarum* in comparison with five in cultivar Lbb and only two in cultivar Lbe. On the other side, it was observed that cultivated plants contain significantly higher amounts of bioactive compounds than the wild growing ones. Nonetheless, concerning hydroxycinnamic acid derivatives, cultivated Goji leaves were richer, although the proportions of the different hydroxycinnamic acid derivatives varied, significantly.
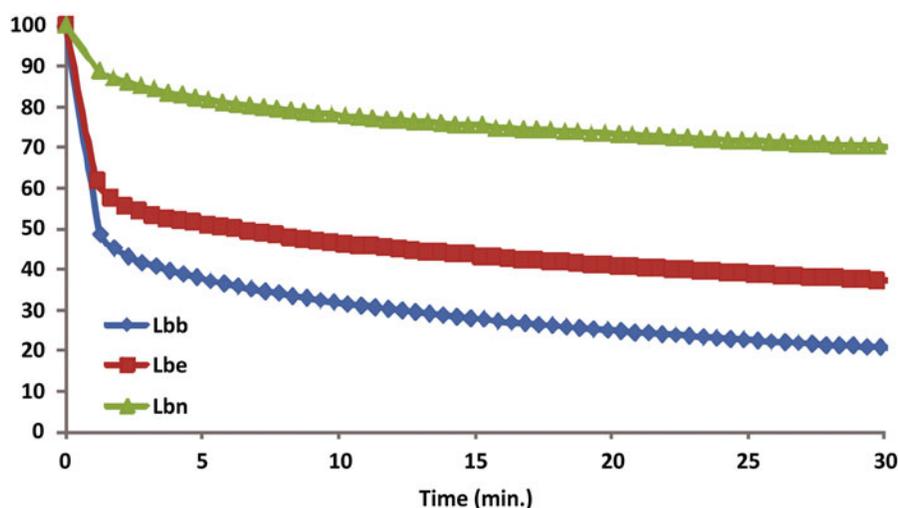
Figure 5. Degradation kinetics of the free radical Fremy's salt by *Lycium* leaves.

Antioxidant activity assays

The antioxidant activities of the extracts were measured using two assays in the present study: Trolox equivalent antioxidant capacity (TEAC) and EPR (electron paramagnetic resonance) spectroscopy.

Trolox equivalents antioxidant capacity (TEAC) assay

The TEAC assay is based on electron transfer reactions to evaluate radical scavenging activity of hydrophilic and lipophilic compounds or complex samples[9]. The antioxidant activity against the stable synthetic ABTS radical cation of the different *Lycium* leaves extracts is summarized in Figure 4. Significant differences have been found in the antioxidant activities of the leaves from wild-growing plants and the two cultivars. The highest antioxidant activity was found for cultivar Lbb (140 mg TE/g dw), and the lowest in the sample coming from wild growing plants (38 mg TE/g dw). While the differences between the samples can be correlated with the amounts of the phenolic components, the highest antioxidant activity of cultivar Lbb can be more specifically related to the presence of flavonoid glycosides. Generally, the ability of flavonoids to be effective antioxidants depends on: the presence of hydrogen-/electron-donating substituents able to reduce free radicals, and the ability of the flavonoid to delocalize the unpaired electron leading to the formation of a stable phenoxyl radical. Moreover, it is generally accepted that the excellent antioxidant properties of flavonoids are due to the presence of catechol groups in the B-ring[14,63].

Previous data concerning the antioxidant activity of *Lycium* spp. were obtained by Abdennacer et al.[48] on Tunisian *L. intricatum* when using the DPPH, the TEAC, and the FRAP assay. These authors indicated that leaves of the investigated species possess a higher antioxidant activity than the fruits. Additionally, Donno et al.[18] investigated the antioxidant capacity of Goji berries cultivars grown in Italy. Nevertheless, it is not possible to compare the present results with those from the literature, because of the different assays, ways of expression, and/or type of sample (preparation).

Electron paramagnetic resonance spectroscopy (EPR)

To support the results from the TEAC and the Folin-Ciocâlteu assays, all *Lycium* extracts were additionally analyzed using EPR with Fremy's salt as a stable radical. EPR is a very common method to assess the antioxidant activity of different samples. The main advantage of this assay is the matrix-independent measurement of the reaction between potential antioxidants and radicals in an electro-magnetic field instead of the absorbance of light[29,64,65]. The degraded amount of Fremy's salt after 30 min incubation time was 212 mg FSE/g dw for Lbb, 167 mg FSE/g dw for Lbe cultivar and just 39 mg FSE/g dw for the leaves coming from wild growing plant (Figure 4). Additionally, the Lbb extract led to an 80% degradation of the Fremy's salt, Lbe extract to 63%, while Lbn extract only 30%, as seen in Figure 5. As already shown for the TEAC and the TPC, EPR results revealed a similar trend with regard to the antioxidant activity of the *Lycium* leaves extracts. The EPR is a common, well-characterized, established methodology for detecting radicals and their kinetics. Unfortunately, it is not so frequently used in combination with other assays to detect the overall antioxidant activity. Therefore, in this case, a comparison with the results of other researchers with regard to *Lycium* samples is lacking.

Inhibitory activities against selected carbohydrate hydrolases, cholinesterases, and tyrosinase

Enzyme inhibitory assays have become a very prominent tool to assess the potential health benefits of herbals, dietary supplements, and nutraceuticals for the development of functional foods[66,67]. Moreover, most usual assays involve key enzymes relevant in metabolic disorders such as diabetes mellitus (α-amylase, α-glucosidase)[68,69], hypertension (angiotensin-converting enzyme), and Alzheimer's disease (cholinesterases)[70,71].

α-Amylase and α-glucosidase

The inhibition of α-amylase and α-glucosidase delays the digestion and absorption of carbohydrates and subsequently suppress postprandial hyperglycemia[72]. The α-amylase and α-glucosidase inhibitory activities of *Lycium* leaves extracts are listed in Table 3. The three extracts provided higher inhibitory activity on α-glucosidase than on α-amylase. In fact, the highest inhibitory potential on both enzymes was exhibited by Lbb with values of 5.38 (for α-glucosidase) and 0.26 (for α-amylase) mmol ACAE/g extract, leaves extracts from wild growing plants provided similar results for α-amylase and two times lower for α-glucosidase (2.25 mmol ACAE/g extract). As a matter of fact, the inhibitory activity of the Lbb and Lbe extracts on α-glucosidase might be related to their high contents in phenolic compounds, which has been demonstrated to possess ability to bind to active protein pocket of the

Table 3. The enzyme inhibitory effects of *Lycium* leaves.

Samples	AChE (mg GALAE/g extract)	BChE (mg GALAE/g extract)	Amylase (mmol ACAE/g extract)	Glucosidase (mmol ACAE/g extract)	Tyrosinase (mg KAE/g extract)
Lbb	na	0.39 ± 0.1	0.26 ± 0.01	5.38 ± 0.09	12.84 ± 0.66
Lbe	na	0.66 ± 0.08	0.26 ± 0.01	4.92 ± 0.05	16.81 ± 0.58
Lbn	na	1.02 ± 0.17	0.24 ± 0.01	2.25 ± 0.07	0.72 ± 0.01

Three parallel experiments $\pm$ SD. GALAE: galanthamine equivalents; ACAE: acarbose equivalents; KAE: kojic acid equivalents; na: not active.

protein[73]. Previous reports indicated that phenolic compounds have lower α-amylase inhibitory activity and a stronger inhibition activity against yeast α-glucosidase[66]. Moreover, chlorogenic acid and rutin, the two dominant compounds in Lbb and Lbe, were previously described as potent α-glucosidase inhibitors by Wang et al.[72] and Zhang et al.[74].

Cholinesterases

To date, cholinesterase inhibitors are still the best available option for the treatment of patients suffering from Alzheimer's disease, although currently used cholinesterase inhibitors, such as tacrine (9-amino-1,2,3,4-tetrahydroacridine), produce certain side effects such as gastrointestinal disturbances and cell toxicity[75]. Enzyme inhibitors providing antioxidant properties are promising in the case of neurodegenerative diseases, due to their protective role against radical species[76]. However, the extracts used in the present study did no show any activity against acetylcholinesterase, while Lbb and Lbe had a weak and Lbn a moderate inhibitory effect on butyrylcholinesterase. In fact, when comparing the results from the present study with other researchers' results it seems that not always edible plants rich in phenolic compounds possess potent inhibitory effects on cholinesterase[76,77].

Tyrosinase

Tyrosinases are copper-containing polyphenol-oxidases that play an essential role in melanogenesis. They convert L-tyrosine to L-DOPA and oxidize L-DOPA to form dopachrome, which induces the production of melanin pigments[78]. The present study found that all tested extracts inhibited the oxidation of L-DOPA catalyzed by mushroom tyrosinase. Lbe extract was the most active (16.81 mg KAE/g), followed by Lbb extract (12.84 mg KAE/g) and Lbn, which showed a very low activity ($<$ 1 mg KAE/g). In fact, a positive correlation between total phenolic content and tyrosinase inhibition activity was shown in several studies[76,79]. Moreover, chlorogenic acid, the dominant compound in Lbe and Lbb, was previously reported by Karioti et al.[80] as a potent tyrosine inhibitor[81].

However, an actual literature survey illustrated that enzyme inhibitory activities of *L. barbarum* leaves were not previously been reported.

Molecular modeling studies and dynamics

In the nutraceutical field, computational techniques have been successfully used for the prediction of ligand-target binding affinity and to better understand the molecular basis of the biological responses. *In silico* studies also provide additional insight into the possible mechanism of action and binding mode of active compounds against metabolic key enzymes[82].

The analysis of the interactions of butyrylcholinestarase revealed that chlorogenic acid penetrates the enzymatic pocket with the catechol function. This pose is stabilized by four hydrogen bonds and several other hydrophobic interactions. A similar path has been found for the interaction of chlorogenic acid with amylase and glucosidase, revealing an interesting ability to interact both with the catechol portion and with the glycoside moiety (Figure 6). As already reported herein, a strong correlation between the inhibition of tyrosinase and the concentration of chlorogenic acid in the plant extracts has been found. In fact, the binding pose of chlorogenic acid in the enzymatic pocket of tyrosinase appears to be very effective, and the catechol ring is able to bind to at least to one copper atom with a coordination bond and interacts with several other amino acids present in the binding pocket by forming hydrogen bonds and aromatic $\pi-\pi$ stacks (Figure 7B and C).

For further investigating the stability of this pose, the binding mode of chlorogenic acid to tyrosinase was further evaluated by a molecular dynamics study. A molecular docking simulation (10 ns) was carried using the Amber biomolecular simulation programs.
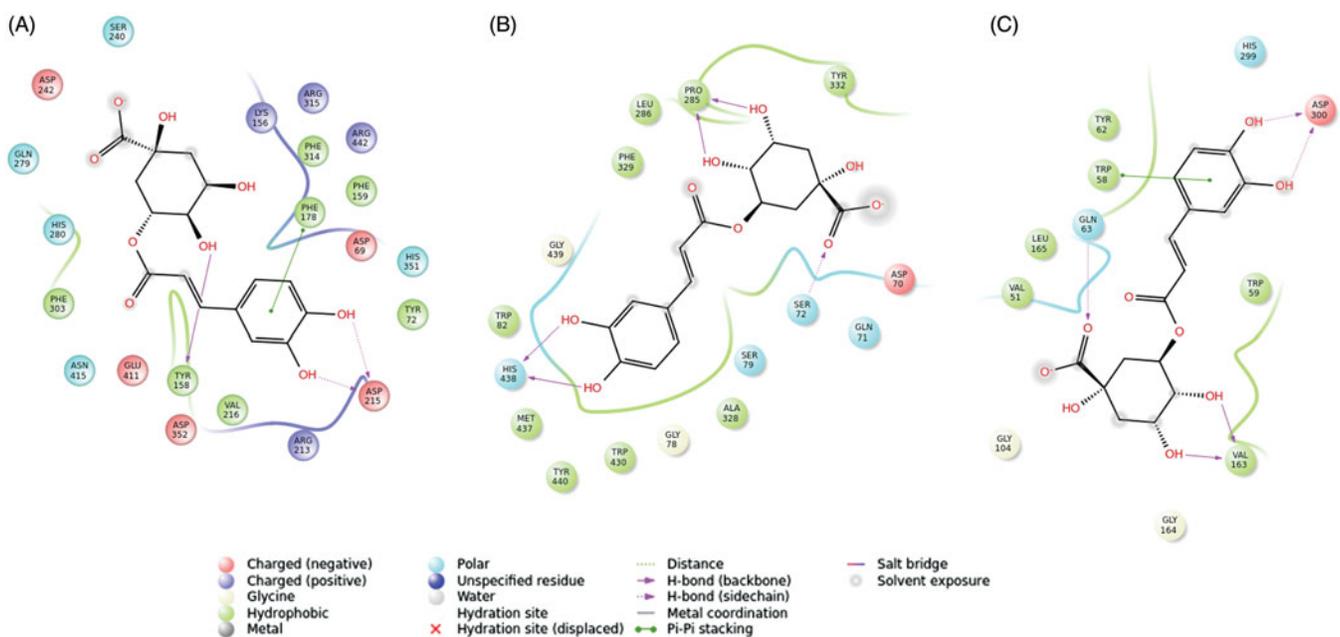

Figure 6. Binding interactions of the best pose of chlorogenic acid in complex with: α-glucosidase (A), butyrylcholinesterase (B), and α-amylase (C) (cut-off 4 Å).
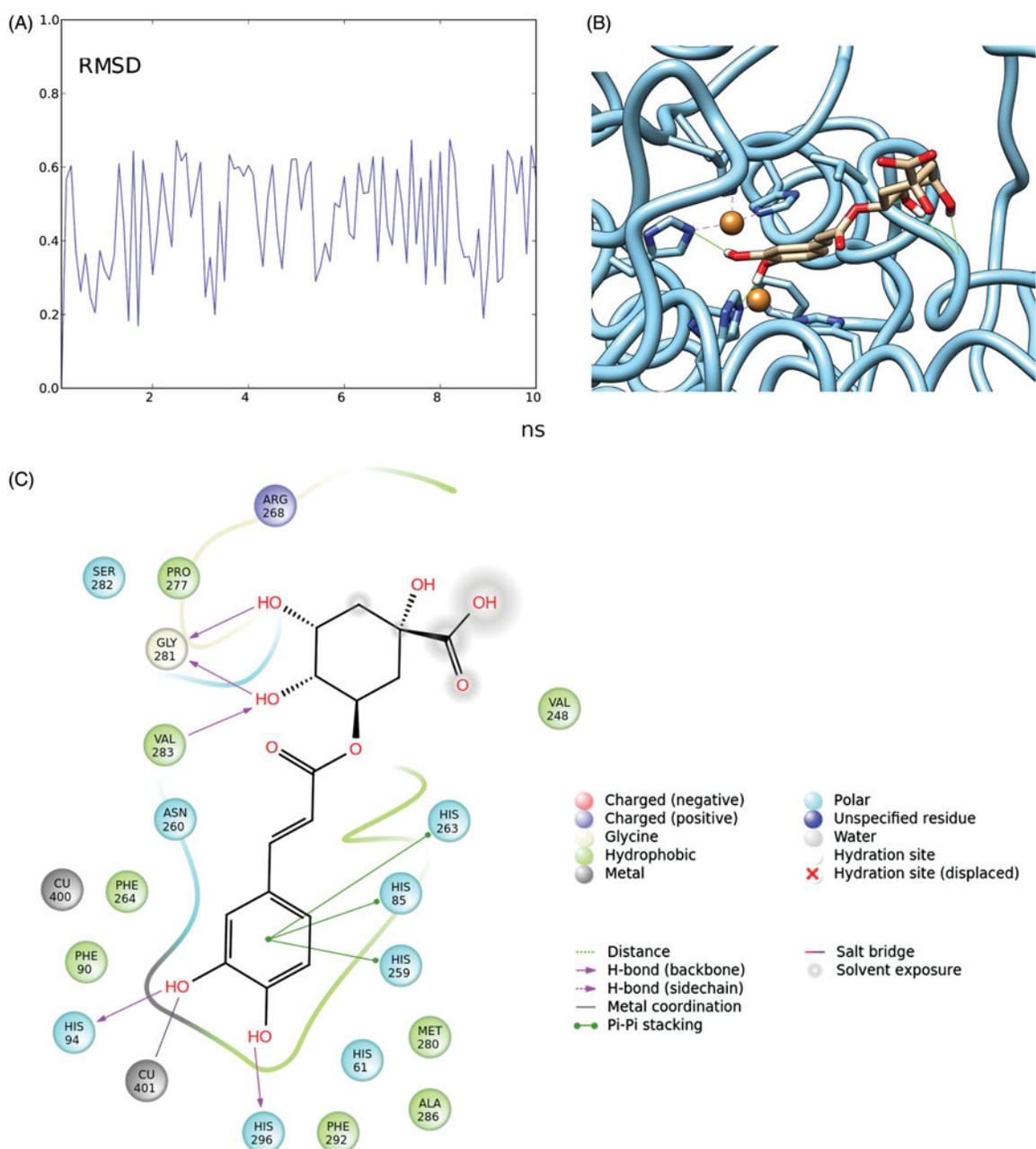
Figure 7. RMSD (in Angstrom) time-dependent plot of chlorogenic acid fluctuation docked to tyrosinase (A); close-up of the complex chlorogenic acid-tyrosinase (hydrogen bonds are reported as green lines) (B); details of the interactions formed by chlorogenic acid with the residues present in the binding pocket (cut-off = 4 Å from the ligand) (C).

The ligand partial charges were determined by Antechamber suite (AM1-BCC method). The system ligand-complex was prepared by tleap suite. General Amber force field (GAFF) parameters were assigned to the ligands and ff14SB to the proteins. The complexes were hydrated in a cubic water box of 20 Å, and the net charge of the system was neutralized by adding Na$^+$ or Cl$^-$ ions to the complex. Then the complex was minimized for 10 000 steps of steepest descent followed by conjugate gradient. Then the molecular dynamic trajectory was run by using the CUDA version of pmemd (pmemd.cuda) implemented in Amber 14, and the simulations were analyzed for 10 ns, by recording one frame every 0.1 ns[83]. The MD trajectories were analyzed by using UCSF Chimera[84], the *root mean square deviation* (RMSD) fluctuation of chlorogenic acid (expressed in Angstroms) was plotted versus time (expressed in ns) (Figure 7A). The RMSD found during

the simulation was in the range of ±0.4 Å, and the fluctuation was homogeneous in the time, thereby confirming that the pose found by the docking experiments between chlorogenic acid and tyrosinanse is stable, also the coordination bond of the Cu atom is maintained for the time of the simulation. Thus, one can hypothesize that chlorogenic acid is capable to enter in the enzymatic pocket of tyrosinase and chelate a Cu atom with the cathecol moiety of the molecule, while the other parts of the molecule contribute to stabilize the binding interactions.

The molecular modeling and dynamic experiments are in full agreement with the experimental data, in which chorogenic acid is a strong inhibitor of tyrosinase, as effectively binding to the enzymatic pocket of tyrosinase based on several hydrogen bonds and a direct coordination to the central Cu atom of the enzyme.

Table 4. Antimicrobial activity of the three *Lycium* leaves extracts.

Microorganism	Lbn		Lbe		Lbb		Streptomycin/ Fluconazole (μg/mL)	
	MIC	MBC/MFC	MIC	MBC/MFC	MIC	MBC/MFC	MIC	MBC/MFC
Listeria monocytogenes	0.0039	0.0078	0.0019	0.0038	0.0039	0.0078	0.015	0.03
Escherichia coli	0.062	0.124	0.031	0.062	0.0039	0.0078	0.12	0.24
Salmonella typhimurium	0.031	0.062	0.062	0.124	0.062	0.124	0.06	0.12
Pseudomonas aeruginosa	0.031	0.062	0.031	0.062	0.015	0.031	0.06	0.12
Staphylococcus aureus	0.031	0.062	0.0039	0.0078	0.0019	0.0038	0.03	0.06
Bacillus cerreus	0.062	0.124	0.031	0.062	0.0078	0.0156	0.015	0.03
Enterococcus faecalis	0.124	0.248	0.062	0.124	0.062	0.124	0.012	0.024
Fusobacterium nucleatum	0.124	0.248	0.124	0.248	0.062	0.124	0.12	0.24
Peptostreptococcus anaerobius	0.124	0.248	0.062	0.124	0.062	0.124	0.12	0.24
Aspergillus flavus	0.062	0.124	0.062	0.124	0.124	0.248	0.15	0.3
Aspergillus niger	0.062	0.124	0.062	0.124	0.124	0.248	0.15	0.3
Candida albicans	0.031	0.062	0.062	0.124	0.124	0.248	0.10	0.2
Candida parapsilosis	0.015	0.031	0.062	0.124	0.124	0.248	0.10	0.2
Penicillium funiculosum	0.031	0.062	0.031	0.062	0.124	0.248	0.15	0.3

MIC, minimal inhibitory concentration; MBC/MFC, minimal bactericidal/fungicidal concentration (mg/mL).

Table 5. Antimutagenic properties of *Lycium* leaves extracts on *Salmonella typhimurium* TA 98 and TA 100.

	Number of colonies (CFU/plate)					Inhibition (%)	
	TA 98		TA100				
Sample	S9−	S9+	S9−	S9+	Treatment group (mg/plate)	TA 98	TA100
Control*	49 ± 2‡	32 ± 1‡	110 ± 4‡	124 ± 4‡	Control*	0	0
Lbn	46 ± 3‡	34 ± 1‡	109 ± 2‡	132 ± 2‡	Mutagen + Lbn	88 ± 3‡	73 ± 0‡
Lbe	44 ± 1‡	32 ± 2‡	110 ± 1‡	120 ± 1‡	Mutagen + Lbe	83.2 ± 2‡	72 ± 1‡
Lbb	50 ± 3‡	36 ± 1‡	113 ± 1‡	135 ± 3‡	Mutagen + Lbb	87 ± 1‡	74.2 ± 2‡
2-AA (0.005)†	–	763 ± 23	–	–			
2-AA (0.002)†	–	–	–	885 ± 19			

*Negative control: without extract; treated with DMSO; spontaneous revertants/plate.
†Positive control: 2-anthramine (2-AA).
‡Significantly different from the control (negative) group at $p < 0.001$.
Inhibition (%) = [1 − (His + revertants in sample of test − number of His revertant colonies/His + revertants in control of test − number of His revertant colonies)].

Antibacterial and antifungal activity

Testing the antibacterial and antifungal activities of plants extracts offers a scientific basis for their applications in food industry as natural preservatives[85,86]. The antimicrobial and the antifungal activity of the *Lycium* extracts were tested against a panel of nine bacteria (seven aerobic and two anaerobic strains) and five fungi, selected on the basis of their relevance for public health. Up to date, very little is reported concerning the bacterial inhibitory properties of leaves from the genus *Lycium*[26,87,88], while information referring to its antifungal properties is not available. *Listeria monocytogenes* and *Staphylococcus aureus* were the most sensitive strains towards Lbe and Lbb extracts with similar values of MIC (0.0019 mg/mL) and MBC (0.0038 mg/mL), respectively (Table 4). These results are in line with previous findings of Terauchi et al.[87] who reported that both Gram-positive and Gram-negative bacteria were sensitive towards tea brewed from leaves of *Lycium chinense*. The antibacterial activity of chlorogenic acid, the main phenolic compound in Lbb and Lbe, has already been demonstrated in various studies. According to Lou et al.[89], chlorogenic acid induced lethal effect on both Gram-positive and Gram-negative bacteria by provoking irreversible permeability changes in the cell membrane, causing the cells to lose the ability to maintain membrane potential. As a result, the antimicrobial activity of *Lycium* leaves could be attributed at least in part to chlorogenic acid. Nevertheless, other constituents should be taken into consideration for the total activity, e.g. the high amounts of rutin or further flavonoid glycosides[90].

However, the most resistant strains were *Enterococcus faecalis* and *F. nucleatum* with MICs = 0.12 mg/mL and MBCs = 0.25 mg/mL, as shown in Table 4. Concerning the antifungal activity, *Candida parapsilopsis* provided the highest sensitivity to the Lbn extract with MIC = 0.02 mg/mL and MFC = 0.03 mg/mL. Moreover, *Candida albicans* and *Penicillium funiculosum* were similarly susceptible to the inhibitory (MICs = 0.031 mg/mL) and fungicidal effects (MFCs = 0.062 mg/mL) of Lbn extract. Nonetheless, the most resistant fungal strains were *Aspergillus flavus* and *Aspergillus niger* towards Lbb extract with MICs = 0.12 mg/mL and MFCs = 0.25 mg/mL, respectively.

Antimutagenicity testing

Antimutagens from natural dietary sources that can counteract the promutagenic and carcinogenic effects of mutagens present increased significance[91]. Moreover, current research suggests that the use of antimutagens in daily diets can be an effective method for preventing cancer and genetic diseases[92]. The Ames test is a generally accepted short-term bacterial assay for identifying substances that can produce genetic damage that leads to mutations. The test uses *Salmonella* strains with preexisting mutations that leave the bacteria unable to synthesize the required amino acid, histidine, and, therefore, unable to grow and form colonies in its absence. New mutations at the site of these pre-existing mutations, or nearby in the genes, can restore the gene's function and allow the cells to synthesize histidine. These newly mutated cells can grow in the absence of histidine and form colonies[45].

In this study, the mutagenicity of *Lycium* leaves extracts against *S. typhimurium* TA 98 and TA 100, both with and without metabolic activation was tested by comparing the numbers of induced revertants and spontaneously revertants. According to Table 5,

TA98 and TA100 strains did not increase in the number of revertant colonies compared with the negative control when the bacteria was treated with *Lycium* extracts at 5000 μg/plate concentration both with and without metabolic activation enzymes (S9), indicating the absence of any mutagenic activity.

As reported in Table 5, tested extracts displayed protective effects against 2-AA induced mutagenicity. According to Negi et al.[93], a value smaller than 25% inhibition of the mutagen activity indicates a weak or non-antimutagenic effect, a moderate effect when the value is between 25% and 40% and strong antimutagenic potential when the value is greater than 40%. In the present study, all extracts showed strong inhibitory effects against the direct-acting mutagen 2-AA in both test-strains with values ranging between 83.2% for Lbe and 88% for Lbn (strain TA 98); 72% for Lbe and 74.2% for Lbb (strain TA100), respectively. As the extracts plus bacterial strains treated plate did not produce any change in the background growth of bacteria with respect to the plate used to detect the spontaneous revertant, the antimutagenic activity of extract cannot be correlated to its toxicity to bacterial strains.

The antimutagenic activity of the extracts is again related to their constituents, in particular to the major phenolic compounds chlorogenic acid and flavonoid glycosides. In fact, chlorogenic acid and flavonoids have been already suggested as potent antigenotoxic agents[94,95]. Therefore, taking into consideration the concentration of these compounds in the extracts, it can be suggested that they could be an essential part of the antimutagenic activity of *Lycium* leaves.

Conclusions

In the present study, the bioactive composition, antioxidant, enzyme-inhibitory, antimicrobial, and antimutagenic properties of two selected Goji cultivars in comparison with leaves from wild-growing plants were evaluated. The present study showed significant variations in the chemical composition between leaves of cultivated and wild-growing Goji plants. The results showed that leaves from cultivated plants contain higher amounts of chlorogenic acids and flavonoid glycosides than wild-growing plants, but the latter had a more diversified phenolic profile. In particular, cultivar Erma, containing the highest amount of chlorogenic acid, showed a strong tyrosinase-inhibitory effect. Furthermore, the interaction between chlorogenic acid and this enzyme was evaluated by molecular docking studies. The antimicrobial status of the studied extracts presented potent activity against the Gram-positive bacteria *L. monocytogenes* and *S. aureus* for cultivated leaves extracts, and a moderate inhibition for anaerobic strains. Nevertheless, wild-growing leaves presented potent activity against *C. parapsilosis*, and cv. Erma on *P. funiculosum*. The exhibited antimutagenic activity of the extracts might be ascribed to their constituents, in particular to the major phenolic compounds chlorogenic acid and flavonoid glycosides. To the best of our knowledge, this is the first comparison describing thoroughly the phytochemical composition and further health-promoting effects of leaves from selected *L. barbarum* cultivars and wild-growing plants. The results indicate that cultivated *L. barbarum* leaves have the potential to be marketed as ingredients for functional beverages and to be used as a valuable source of bioactive compounds in further foods or health-promoting formulations.

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