Phytochemical analysis and antioxidant activity of *Lycium barbarum* (Goji) cultivated in Greece

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**ABSTRACT**

**Context:** The fruit of *Lycium barbarum* L. (Solanaceae), known as goji berry, has been exploited for a long time in traditional Chinese medicine. In recent decades, it has received much attention as one of the trendiest functional foods with a wide array of pharmacological activities in Western diets.

**Objective:** In this study the phenolic profile and potential antioxidant capacity of *Lycium barbarum* cultivated in Crete (Greece) were investigated.

**Materials and methods:** The berries were defatted with hexane and then extracted with dichloromethane and methanol using a Soxhlet apparatus. Furthermore, the methanol extract was fractionated with ethyl acetate and butanol. All fractions/extracts were tested for their antioxidant activity (DPPH, FRAP, chemiluminescence). Folin–Ciocalteu and LC-DAD-MS analyses were utilized for the identification of the phenolic compounds.

**Results:** The total phenolic content ranged from 14.13 ± 0.40 (water fraction) to 109.72 ± 4.09 (ethyl acetate fraction) mg gallic acid equivalent/g dry extract. Ethyl acetate extract exhibited the highest scavenging activities determined as EC\(_{50}\) (4.73 ± 0.20 mg/mL) and IC\(_{50}\) (0.47 ± 0.001 mg/mL) using DPPH and chemiluminescence assays. Seventeen phenolic compounds, including cinnamoylquinic acids and derivatives, hydrocinnamic acids and flavonoid derivatives, were tentatively identified. To the best of our knowledge, quercetin 3-O-hexose coumaric ester and quercetin 3-O-hexose-O-hexose-O-rhamnose are reported for the first time in goji berry fruits.

**Discussion and conclusion:** The results of this study suggest that consumption of goji berry fruits could serve as a potential source of natural antioxidant compounds and that goji berry phenolic extracts could be exploited for nutritional pharmaceutical purposes.

**Introduction**

Increasing evidence for a relationship between diet and health highlight the importance of plant secondary metabolites and their impact on different physiological functions and health (Verschuren 2002). This knowledge has generated new concepts in nutrition aiming at developing and promoting functional foods, known to be rich with such bioactive compounds (Roberfroid 2002). Goji berry (*Lycium barbarum* L. (Solanaceae)), also known as wolfberry, can be considered one of those foods (Carnés et al. 2013); pharmacological and immunological studies seem to support some of the claims made with regard to their health-promoting properties (Gan et al. 2004; Song et al. 2011).

Since the beginning of the twenty-first century, goji berries became increasingly popular in Europe and North America (Istrati et al. 2013) given its nutritional richness in various vitamins, minerals, antioxidants, and amino acids (Yao et al. 2011; Endes et al. 2015). This might explain the rapid increase in consumption in recent years.

In parallel to all these nutritional benefits, goji fruit might confer many health-protective benefits such as age-related macular degeneration, which can be due to the presence of lutein and zeaxanthin (Bucheli et al. 2011). In addition, it may possess antioxidant and antitumor activities (Gan et al. 2004; Zhang et al. 2010), neuroprotective effects (Lo & Yang 2015), male fertility facilitation (Lau et al. 2012) as well as immunity enhancement (Zhang et al. 2015). However, the complete profile with quality traits, phytochemical composition and antioxidant activity evaluation of these berries is still lacking.

Therefore, the present study aimed at characterizing the phenolic profile, and to evaluate the potential antioxidant activity of goji berries, cultivated on the island of Crete (Greece). The antioxidant activity was evaluated using DPPH scavenging activity, ferric-reducing antioxidant power (FRAP), and luminal-induced chemiluminescence methods. The Folin–Ciocalteu method was used to determine the total phenolic content of each one of the extracts/fractions while the polyphenolic profile was determined using liquid chromatography-diode array-mass spectrometry (LC-DAD-MS). The extracts/fractions examined were prepared using successive solvents of varying polarity and by partitioning the methanol extract with ethyl acetate and butanol.
Materials and methods

Plant material

Fully ripened goji fruits (Lycium barbarum) were picked from an organic farm located in Chania (Crete, Greece) in September 2014. The plant was identified and authenticated by Dr. Theano Samara and a voucher specimen (EK 62) was deposited at the laboratory of ‘Food Quality and Chemistry of Natural Products’ (MAICh, Greece). The berries were left to dry in an oven at 40°C for 48 h. The obtained dry material was ground using an electric blender, weighed and subsequently subjected to further processes.

Chemicals and reagents

Acetic acid, ethyl acetate, cobalt (II)-chloride hexahydrate, Folin–Ciocalteau reagent, gallic acid, glacial acetic acid (C₂H₄O₂), hydrochloric acid and hydrogen peroxide (H₂O₂, 30%) were purchased from Merck (Germany). Boric acid (H₃BO₃), methanol, butanol, 2,2-diphenyl-1-picrylhydrazyl (DPPH), EDTA (ethylenediamine tetraacetic acid), luminol (3-aminophthalhydrazide), 2,4, 6-tripyridyl-s-triazine (TPTZ), iron (III) chloride hexahydrate (FeCl₃·6H₂O), iron (II) sulfate heptahydrate (FeSO₄·7H₂O), sodium acetate trihydrate (C₂H₃NO₂), sodium carbonate, and sodium hydroxide were purchased from Sigma-Aldrich (Germany).

Extraction and partitioning of extracts

Approximately 20 g of ground fruits were subjected to extraction using a Soxhlet apparatus, with the following solvents in order of increasing polarity: hexane, dichloromethane, and methanol. The methanol extracts were dissolved in 300 mL of distilled water, 10 mL of aqueous sodium carbonate (20% w/v) was added and the mixture vortexed and allowed to stand at room temperature without light for 120 min. The absorbance was read at 750 nm. The total polyphenol content was calculated from a calibration curve (Figure 1) (100–500 μg/mL) and the results were expressed as mg of gallic acid equivalents (GAE)/g dry extract.

Estimation of the total phenolic content by the Folin–Ciocalteu test

The amount of total polyphenols in fruit extracts was measured following the Folin–Ciocalteu procedure (Arnous et al. 2002). In a 1.5 mL Eppendorf tube, 790 μL of distilled water, 10 μL of diluted sample and 50 μL of Folin–Ciocalteu reagent were added and the mixture vortexed. After 1 min, 150 μL of aqueous sodium carbonate (20%) was added and the mixture vortexed and allowed to stand at room temperature without light for 120 min. The absorbance was read at 515 nm. The amount of total phenolics in fruit extracts was measured following the Folin–Ciocalteu procedure (Arnous et al. 2002). In a 1.5 mL Eppendorf tube, 790 μL of distilled water, 10 μL of diluted sample and 50 μL of Folin–Ciocalteu reagent were added and the mixture vortexed. After 1 min, 150 μL of aqueous sodium carbonate (20%) was added and the mixture vortexed and allowed to stand at room temperature without light for 120 min. The absorbance was read at 515 nm. The total polyphenol concentration was calculated from a calibration curve (Figure 1) (100–500 μg/mL) and the results were expressed as mg of gallic acid equivalents (GAE)/g dry extract.

Antioxidant activity determinations

Evaluation of the antioxidant activity using the DPPH* method

The DPPH assay was performed according to the method described by Arnous et al. (2002). Briefly, a methanol DPPH solution (0.1 mM, 975 μL) was added to 25 μL of different concentrations of extracts/fractions. The mixture was shaken vigorously and the decrease in absorbance was measured at 515 nm after 30 min of incubation in the dark with methanol as the blank. The percentage of DPPH inhibition was calculated as follows:

\[
\text{\% DPPH} = \frac{A_{DPPH} - A_S}{A_{DPPH}} \times 100
\]

where \( A_{DPPH} \) is the absorbance of the solution when the sample extract has been added at a particular level and \( A_{DPPH} \) is the absorbance of the DPPH solution. The half maximal effective concentration (EC₅₀) value (mg/mL) is the effective concentration at which the DPPH radical was scavenged by 50% and calculated by interpolation from the data.

The Co (II)/EDTA-induced luminol chemiluminescence method

Co (II)/EDTA-induced luminol chemiluminescence measurements were performed as described earlier (Parejo et al. 2000) with some minor modifications. The chemiluminescence (CL) test consisted of mixing 1 mL of a buffer solution of boric acid (50 mM, pH = 9) containing CoCl₂·6H₂O (2 mg/mL) and EDTA (10 mg/mL) with 100 μL of luminol solution (0.56 mM) in borate buffer (pH = 9). Three different dilutions of each sample were prepared. Thereafter, 25 μL of the diluted sample were added and the mixture was vortexed for 15 s. Then, 25 μL of H₂O₂ aqueous solution (5.4 mM) was added. The instantaneous reduction in the plateau CL intensity, induced by the addition of sample, was recorded as I, and CL intensity, induced in the absence of the sample, was recorded as I₀. The ratio (I₀/I) was plotted vs. the concentration (mg/mL) sample. The equation was established by linear regression and the concentration of sample, which is required to decrease the CL intensity by 50%, was calculated (IC₅₀). The hydroxyl free radical scavenging activity (SA₅₀/FR) was expressed as 1/IC₅₀.

FRAP assay

The FRAP assay was carried out according to Benzie and Strain (1999) with slight modifications. The FRAP reagent was prepared from acetate buffer (pH 3.6), 10 mmol TPTZ (2, 4, 6-tripyridyl-s-triazine) solution in 40 mmol HCl and 20 mmol FeCl₃·6H₂O (ferric chloride solution) in proportions of 10:1:1 (v/v), respectively. The FRAP reagent was prepared fresh daily and was warmed to 37°C in a water bath prior to use. One-hundred microliters of sample was added to 3 mL of FRAP reagent. The reaction mixture was incubated for 4 min at room temperature. The absorbance of the reaction mixture was measured at 593 nm. The standard curve was obtained using FeSO₄·7H₂O (concentration...
range: 100–1000 μmol·L⁻¹). The results were expressed in millimoles of Fe²⁺ equivalents per g of dry extract.

**Statistics**

All the measurements were means of triplicate measurements (n = 3), including standard deviation (± SD). Data were performed by Microsoft Excel 2010 (Redmond, WA).

**Survey of the phenolic profile by LC-DAD-MS (ESI+)**

Methanol extract fractions with the highest antioxidant capacity (ethyl acetate and butanol fractions) were further screened by LC-DAD/MS (ESI+). The LC-DAD-MS system used was a Finnigan MAT Spectra System P4000 pump, in conjunction with a Finnigan Spectra System UV 6000LP diode array detector and a Finnigan AQA mass analyzer (San Jose, CA). The separation was performed on a Superspher, 100 RP-18, 4 μm end-capped (125 × 2.0 mm) (phenomenex) column with a flow rate was 0.3 mL/min and kept at 40°C.

The mobile phase was a linear gradient system of 2.0% acetic acid in water (v/v) (solvent A) and methanol (solvent B). The elution was achieved with the following stepwise gradient: 2 min, 100% A; 10 min, 75% A; 25 min, 75% A; 50 min, 0% A; 60 min, 0% A; 65 min, 100% A. Mass spectra were obtained at the positive ion mode, with acquisition set at 20 and 80 eV, capillary voltage 4.00 kV, source voltage 25 V, detector voltage 650 V and probe temperature 350°C.

**Results and discussion**

**Phenolic content**

The total phenolic content values of goji berry fruit extracts ranged from 14.13 to 109.72 mg GAE/g dry extract (Figure 2). Results were classified according to the solvents that were used for extraction. Ethyl acetate seems to be the solvent that best concentrates the phenolic substances of intermediate polarity. This is in line with previous findings (Termentzi et al. 2006).

**Antioxidant activity assays**

Since the antioxidant capacity of food is determined by a mixture of different antioxidants with different mechanisms of action, among which are included synergistic interactions, it is necessary to combine more than one method in order to determine in vitro the antioxidant capacity of food stuffs. Therefore, it is necessary to perform more than one type of antioxidant capacity measurement to take into account the various mechanisms of antioxidant action (Frankel & Meyer 2000; Pérez-Jiménez et al. 2008). In this study, the antioxidant activity of the phenolic extracts/fractions isolated from L. barbarum fruits were analyzed using DPPH⁺, luminol-induced chemiluminescence and FRAP assays. Table 1 shows the results of the antioxidant activity measured by the three assays.

According to the DPPH assay in which values are expressed as EC₅₀, the ethyl acetate fraction exhibited the highest scavenging activity, followed by the dichloromethane extract, butanol fraction and water fraction, which had the lowest value. This result confirms that most antioxidants were partitioned into the methanol extract. The hydroxyl radical scavenging efficiency, as evidenced from the IC₅₀ values obtained with the luminol chemiluminescence test, was found to vary from 0.47 to 7.70 mg dry extract/mL. The SA₅₀ showed the same pattern and the ethyl acetate fraction had the higher values, while water had the lowest.

As displayed in Table 1, the reduction potential of the iron by the extracts increased in the following order: water fraction < butanol fraction < dichloromethane extract < ethyl acetate fraction. The ability to reduce the FeIII-TPTZ complex can be attributed to the donation of electrons/hydrogen, the mechanism of action that is related to the antioxidant activity of phenolic compounds, which indicates the presence of reducing agents. The ethyl acetate expressed a higher capacity to reduce Fe³⁺ to Fe²⁺, with a value of 1.62 mmol Fe²⁺/g extract, which may be related to the increased concentration of phenolics in this fraction.

**Qualitative analysis of phenolics and flavonoid compounds by HPLC-DAD-MS (ESI+)**

Seventeen different phenolic compounds were traced in our samples and their identification was achieved by studying their retention times, UV–Vis absorption spectrum in correlation to proposed MS fragmentation mechanisms and comparison to the scientific literature (Fang et al. 2002; Sakakibara et al. 2003). Typical HPLC–DAD–MS chromatograms are presented in Figures 3 and 4.

It has been observed, upon the comparison of LC-DAD-MS (ESI+) chromatogram data of methanol extract fractions (ethyl acetate and butanol fractions), that different compounds were present in these fractions (Tables 2 and 3). However, besides this difference in their content, some compounds were seen in both the fractions.
Figure 3. HPLC chromatogram of the ethyl acetate fraction of *L. barbarum* at 278 nm and 340 nm, respectively.

Figure 4. HPLC chromatogram of the butanol fraction of *L. barbarum* at 278 nm and 340 nm, respectively.
The compound eluted at Rt 3.43 min was suggested to be a derivative of a dihydroisoferulic acid (no.1). It showed [M + H]⁺ at m/z 197, [M + Na]⁺ at m/z 219 and a fragment ion at m/z 153, which corresponds to the loss of the CO₂ group from the carboxylic acid function (Sánchez-Rabanela et al. 2004). The peak at 9.40 min was detected to be coumaric acid derivative (no. 12) as its absorption maxima is 292 nm, which is typical for coumaric acid (Sakakibara et al. 2003). At Rt 12.35 min a coumaroyl-, caffeoyl-caffic acid (no.4) was detected. It yielded a pseudomolecular ion [M + H]⁺ at m/z 489, a sodium adduct ion [M + Na]⁺ at m/z 511 and a fragment ion at m/z 194 [caffeic acid + CH₄]⁺ and m/z 164, which corresponds to the molecular weight of coumaric acid.

The ethyl acetate fraction contains tentatively identified coumaroyl-, isofuroyl-caffic acid/coumaroyl-, feruloyl-caffic acid (no.5) at Rt 14.65 min with [M + H]⁺ 485. Fragments at m/z 339 are due to the loss of the coumaroyl unit, m/z 177 [ferulic/iso ferulic acid – OH]⁺, m/z 147 [coumaric acid – OH]⁺ and m/z 165 [caffeic acid – OH]⁺. Esters of hydrocinnamic and dihydroxybenzoic acids with quinic acids were dominant in our fractions (Figure 5). In most cases the quinic acid moiety does not give a fragment ion in ESI + analysis (Fang et al. 2002).

Ethyl acetate and butanol fractions have in their chromatograms three peaks at Rt 9.90, 11.82 and 12.61 min that correspond to a coumaroyl-caffic acid (no. 2) and a coumaroyl-caffic acid derivative (no. 16 and no. 17). The UV spectra are similar to that of the aforementioned p-coumaric acid in the literature (max 310 nm) (Clifford et al. 2006). MS showed a protonated molecular ion at m/z 339 compatible with a p-coumaroylquinic acid, with fragment ions at m/z 163 originating from the coumaric acid moiety, m/z 192 which was attributed to quinic acid, m/z 267 [M – (CH=CH – COOH)]⁺, and m/z 311 [M – CO + H]⁺. Derivatives of p-coumaric acid have been reported to be present in Lycium genus (Inbaraj et al. 2010; Abdennacer et al. 2015).

The butanol fraction contained a coumaroyl-, dimethylcaffeoquinic acid (no. 13) at Rt 10.02 min, where [M + H]⁺ is 529. The fragmentation pattern of the proposed compound showed a fragment ion at m/z 511 that results from the loss of water. Coumaric acid gave a typical fragment at m/z 147, while the fragment ion at m/z 163 is [dimethylcaffic acid – COOH]⁺. Loss of the methoxy group from the dimethylcaffic unit (m/z 177) is observed as well.

The peak at Rt 10.92 min, revealed in the butanol fraction, and at Rt 11.50 min, revealed in the ethyl acetate fraction, shared the same UV spectra (λmax 246, 316 nm, shoulder at 292 nm), although the MS spectra showed a pseudomolecular ion [M + H]⁺ at m/z 355 corresponding to chlorogenic acid (no. 3 and 15). The fragmentation pattern of this assumed compound showed the fragment ions with the strongest and most distinguishable peaks at m/z 163 and 377, corresponding to the caffeoyl moiety and [M+Na]⁺, respectively.

The UV and MS characteristics show feruloylquinic acid ester (no. 6) (Fang et al. 2002) in the ethyl acetate fraction at Rt 16.55 min. [M + H]⁺ is 369 and [M + Na]⁺ 391. Fragments at m/z 195 correspond to the ferulic acid molecular ion, m/z 177 [ferulic acid – OH]⁺ and m/z 149 [ferulic acid – COOH]⁺. The mass spectral analysis of the compound with Rt =19.17 min (ethyl acetate fraction) associated with the UV–vis spectrum (λmax 296) identified a protonated molecular ion at m/z 517 that was attributed to isofuroyl-, dihydrocoumaroyl-quinic acid (no. 7). The fragmentation pattern suggested that the fragment ion at m/z 369 corresponded to the loss of the dihydrocoumaroyl moiety. The ion at m/z 195 comes from the isofuric molecular ion and m/z 166 corresponds to the molecular weight of the dihydrocoumamic acid.

In the same fraction, the compound eluting at Rt 32.41 min was tentatively assigned the structure of isofuroyl-, benzoyl-, protocatechyquinic acid (no. 9). It yielded a pseudomolecular ion [M + H]⁺ at m/z 609, a sodium adduct ion [M+Na]⁺ at
m/z 649 and fragment ions at m/z 627 [M + H₂O + H]⁺, m/z 473 [609 - protocatechyl moiety]⁺, m/z 368 [609 - protocatechyl moiety - benzoyl moiety]⁺, m/z 314 [368 - 54]⁺ (54 fragments from quinic acid), m/z 177 [isoferulic acid - OH]⁺ and m/z 149 [isoferulic - COOH]⁺.

The ethyl acetate fraction also contained dihydroisofluroyl-, coumaroyl-, protocatechyl-quinic acid (no. 10) eluted at Rt 38.14 min and was characterized by a molecular ion at m/z 653 and [M + Na]⁺ at m/z 675. The fragmentation pattern showed fragment ions at m/z 625 [M - CO + H]⁺, m/z 197 [dihydroisorfeleric acid + H]⁺ and m/z 165 [coumaric acid + H]⁺. The UV spectrum and the mass spectrum for the peak eluting at Rt 39.43 min suggested an isomer (no. 11) of the above-mentioned compound (no.16) with the acids in different positions.

Figure 5. Proposed chemical structure of some compounds identified by the LC-DAD/MS of L. barbarum fractions.
The compound in the butanol fraction eluted at Rt 13.18 min was identified as a quercetin 3-O-hexose-O-hexose-O-rhamnose (no. 18) based on its pseudomolecular ion [M + H]^+ at m/z 773 with three noticeable fragments at m/z 627 = [M + H] – rhamnol hexose moiety^+ , m/z 465 [(M + H) – rhamnol hexose moiety – hexosyl moiety]^+ and m/z 303 for the aglycon.

Rutin and quercetin 3-O-hexose coumaric ester present the same mass and the same first parent ion (m/z = 611) but were distinguished by comparing the UV absorption, the Rt and the MS fragmentation. The presence of the coumaroyl moiety bonded to the hexose retained the molecule longer in the reverse phase HPLC column compared to rutin, so we conclude that the molecule eluted at Rt 27.48 and 26.81 min in both the ethyl acetate and butanol fractions is quercetin 3-O-hexose coumaric ester (no. 8 and 19). It exhibited an intense molecular ion [M + H]^+ at m/z 611 and [M + Na]^+ at m/z 633. The ion at m/z 465 comes from the loss of the coumaroyl unit and the ion at m/z 303 corresponds to the aglycon.

**Conclusion**

LC-DAD-MS (ESI+) profiling led to the identification of esters of hydroxycinnamic and dihydroxybenzoic acids with quinic acids as the main antioxidant components. Among the flavonoids identified in this study, the presence of quercetin 3-O-hexose coumaric ester and quercetin 3-O-hexose-O-hexose-O-rhamnose, is reported for the first time in *Lycium barbarum*. Phenolic acids and their derivatives were among the compounds detected in this study. Coumaric, isofloric, and caffeic acids, and their derivatives, were found to be the dominant ones. The presence of these compounds in *L. barbarum* may explain some of the health benefits observed in its traditional applications. Further investigations are needed to isolate and confirm the structural identity of the whole range of compounds detected in this study. It is worth noting that several compounds remained unidentified. Concerning the antioxidant activity, the ethyl acetate fraction was shown to have the best antioxidant activity, which can be related to the total content of phenolic compounds and flavonoids in this fraction. To conclude, these results suggest that consumption of goji berry fruits could serve as a good source of natural antioxidant compounds and that goji berry phenolic extracts could potentially be exploited for nutritional pharmaceutical purposes.

**Disclosure statement**

The authors report no conflicts of interest. The authors are alone responsible for the content and writing of the paper.

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