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Assessment of the antioxidant and antimutagenic activity of extracts from goji berry of Greek cultivation

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ABSTRACT

The aim of this study was to assess the antioxidant and antimutagenic activities of ultrasound assisted aqueous extracts from dry goji berry fruits cultivated in Greece. The extracts' free radical scavenging activity was assessed by the DPPH· and ABTS·$^+$ assays. The results from both assays demonstrated that the extracts exhibited strong radical scavenging activity with IC_{50} values ranging from 1.29 to 3.00 mg/ml for DPPH· and from 0.39 to 1.10 mg/mL for ABTS·$^+$ assay. The investigated extracts also inhibited free radical-induced DNA damage induced by peroxyl (ROO·) radicals with IC_{50} ranging from 0.69 to 6.90 mg/mL. The antioxidant activity of the goji berry extract exhibited the highest potency in the above assays was also examined in muscle cells. In particular, muscle C2C12 cells were treated with the selected extract at non cytotoxic concentrations for 24 h and four oxidative stress markers were measured: total reactive oxygen species (ROS), glutathione (GSH), lipid peroxidation and protein carbonyl levels. The results showed that the extract at 25 and 100 μg/mL increased GSH levels up to 189.5% and decreased lipid peroxidation and protein carbonyls by 21.8 and 29.1% respectively. The present study was the first on the antioxidant effects of ultrasound assisted aqueous extracts from goji berry fruits in muscle cells.

1. Introduction

The production of reactive oxygen species (ROS) occurs physiologically in living organisms. ROS are useful molecules at low concentrations, since they regulate growth, differentiation, proliferation, and apoptosis. However, when there is an excess production of ROS, a pathological condition called oxidative stress, then several diseases (e.g. cardiovascular, cancer and neurodegenerative) may be caused [1–3].

One of the tissues that are especially susceptible to oxidative stress is skeletal muscle [4]. In skeletal, muscle, overproduction of ROS may occur even under physiological processes such as exercise [5]. During exercise there is a high rate of O_2 consumption in skeletal muscles, that may cause incomplete O_2 reduction and electron leakage from the electron transfer chain, leading to the generation of ROS and oxidative stress [5]. In turn, oxidative stress results in cell damage and muscle fatigue [6]. Thus, antioxidant supplementation has been suggested for counteracting oxidative stress-induced damage to skeletal muscles [7].

Moreover, it is well documented that several traditional herb and plant extracts have antioxidant properties and are potential candidates for the prevention and treatment of ROS-induced diseases [8–10]. One of those herbs is goji berry (*Lycium barbarum*), whose extracts have been shown to protect from damage caused by ROS [11]. For example, goji berry's extracts have been reported to prevent oxidative stress-induced disorders (e.g. neurological) and pathological conditions (e.g. aging) [12].

Both *in vitro* and *in vivo* studies have provided evidence for these antioxidant effects of goji berry's extracts. For instance, the antioxidant capacity of *L. barbarum* polysaccharides (LBPs) has been demonstrated by *in vitro* methods including superoxide, DPPH· and ABTS·$^+$ radical scavenging activity and reducing power [13]. In addition, other studies with rats and mice have reported that LBPs protected from DNA damage and inhibited lipid peroxidation and damage in hepatic and renal tissues caused by chronic hyperglycemia-induced oxidative stress in a high-fat diet [14,15]. Moreover, LBPs protected against oxidative damage in skeletal muscle, caused by exhaustive exercise [16]. Apart from

Abbreviations: DPPH·, 2,2-diphenyl-1-picrylhydrazyl; ABTS·$^+$, 2,2′-Azino-bis-(3-ethyl-benzthiazoline-sulphonic acid; ROS, reactive oxygen species; GSH, glutathione; TBARS, thiobarbituric acid-reactive substances

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polysaccharides, other antioxidant compounds found in goji berry are carotenoids (e.g. zeaxanthin) and polyphenols. It is believed that the antioxidant properties of the polysaccharides are likely attributed to low molecular weight phenolic substances that bind to them during the extraction process [17].

Most studies on goji berry's extracts have used plants from cultivations in China, which is the main supplier of this fruit. In the present study, we used extracts from *L. barbarum and L. chinensis* dry fruits from Greek cultivations and investigated their antioxidant and antimutagenic activity using molecular and cell culture methods. It is known that the climatic and soil conditions may affect the chemical composition of a plant, and so the bioactivity of its different parts [18,19]. The extracts were produced by an ultrasound assisted extraction process using water as solvent, optimized for giving extracts with maximum antioxidant activity as we have described previously [20].

2. Materials and methods

2.1. Chemicals

Folin Ciocalteu, sodium carbonate, gallic acid, ethanol, 2,2-diphenyl-1-picrylhydrazyl (DPPH), methanol, 2,2′-Azino-bis-(3-ethylbenzthiazoline-sulphonic acid) (ABTS), horseradish peroxidase enzyme (HRP), hydrogen peroxide (H_2O_2), 2,7-dichlorofluorescein diacetate (DCF-DA) and mercury orange, were purchased from Sigma Aldrich (St Louis, MO, USA). Dulbescco's modified Eagle medium (DMED), fetal bovine serum (FBS) and phosphate buffered saline (PBS) of analytical grade were purchased from Gibco (UK). Cell proliferation kit II (XTT) was purchased from Roche Diagnostics (Mannheim, Germany).

2.2. Goji berry fruits

Goji berry fruits from *L. barbarum and L. chinensis* were collected from a 3 year old experimental plantation, located in the region of Thessaly, and they were subsequently sun-dried. The moisture content of all samples was measured after fruits were drying to constant weight at 105 °C following the AOAC 935.29 official method. The samples were then stored under refrigeration (0–4 °C) for further analysis.

2.3. Extraction process

To optimize the extraction process the extraction conditions were varied as previously described [20]. In particular, dried whole fruits of goji berry were frozen by liquid nitrogen and were pulverized in mortar. From each sample, 2.5 g were extracted with distilled water (using 50, 75, 93, 99 or 100 mL of water,) in a 250 mL glass beaker. The samples were preheated to the selected experimental temperature (45, 55, 57 or 65 °C), and then they were introduced to the ultrasound extraction device (Hielscher UP400 S). The ultrasound power was set at 138.0, 220.0, 253.0, 360.0, 366.7 or 368.0 W/cm^2, while the extraction time was set at 23, 30, 33.4, 35 or 40 min, according to the chosen experimental conditions. The obtained extracts were centrifuged at 12,000 rpm for 15 min and the supernatant was collected and kept in a freezer (−20 °C) until further use.

2.4. Determination of the total carbohydrate content of the extracts

Phenol/sulfuric method was used for the determination of total carbohydrates and was carried out according to Dubois et al. [21]. Briefly, 1 mL of each extract was mixed with 0.5 mL of 4% phenol and 2.5 mL of 95% sulfuric acid, and after 10 min incubation the optical density was measured at 490 nm. The total carbohydrate content was calculated on the basis of a calibration curve of D-glucose (concentration range: 0.01–0.1 mg/L; $R^2 = 0.991$) and were expressed as g of carbohydrates/L of extract. The assay was repeated three times.

2.5. Determination of the total polyphenolic content (TPC) of the extracts

The method of Singleton et al. [22] modified by Waterhouse [23] was used to determine the TPC. Briefly, 20 μL of each extract was mixed with 1.58 mL water, and then with 100 μL of Folin- Ciocalteu reagent (0.2 N). Subsequently, 300 μL of Na_2CO_3 solution (200 g/L) was added and after 120 min incubation in dark, the optical density was measured at 765 nm. TPC was calculated on the basis of a calibration curve of gallic acid (concentration range: 50–500 mg/L, $R^2 = 0.991$) and expressed as gallic acid equivalents (GAE)/mg of dried extract. The assay was repeated three times.

2.6. DPPH· radical scavenging activity assay

The DPPH· radical scavenging activity of extracts was assessed as described previously by Spanou et al., [24]. Briefly, 1 mL of freshly made methanolic solution of DPPH· radical (100 μM) was mixed with the tested goji berry extracts dissolved in distilled water at different concentrations and after 20 min incubation in dark, the optical density was measured at 517 nm. The percentage of radical scavenging capacity (RSC) of the tested extracts was calculated according to the following equation:

$$\%\ \text{DPPH}\cdot\ \text{radical scavenging activity} = [(\text{Abs}_{control} - \text{Abs}_{sample})/\text{Abs}_{control}] \times 100$$

where Abs $_{control}$ and Abs $_{sample}$ are the absorbance values of the control and the tested sample respectively. Moreover, in order to compare the radical scavenging efficiency of the extracts, the IC_{50} value showing the concentration caused 50% scavenging of DPPH· radical was estimated. All measurements were carried out in triplicate and at least in two independent experiments.

2.7. ABTS$\cdot^+$ radical scavenging activity assay

ABTS$\cdot^+$ radical scavenging activity was measured as described by Kerasioti et al., [25]. The RSC percentage of the ABTS$\cdot^+$ radical scavenging activity and the IC_{50} values were determined as described above for the DPPH· method. All measurements were carried out in triplicate and at least in two independent experiments.

2.8. Peroxyl radical-induced DNA plasmid strand cleavage

The assay was performed as described previously by Priftis et al., [26]. The preventive activity of the tested extracts against peroxyl radical-induced DNA strand breakage was based on the inhibition of the conversion of supercoiled form to the open-circular. The analysis was performed using an AlphaImager EC photodocumentation system and the amounts of supercoiled and open-circular forms were analyzed with the Alpha View software (AlphaInnotech, CA, USA).

The percentage inhibition was calculated using the following formula:

$$\%\ \text{inhibition} = [(S - So)/(Scontrol - So)] \times 100$$

where *Scontrol* is the percentage of supercoiled DNA of the negative control sample (plasmid DNA alone), *So* is the percentage of supercoiled plasmid DNA of the positive control sample (without tested extracts but in the presence of the radical initiating factor), and *S* is the percentage of supercoiled plasmid DNA of the sample with the tested extracts and the radical initiating factor. Each experiment was carried out three times.

2.9. Total solid assay

Total solids of extract no. 5 were measured according to the method of Symons and Morey [27]. The extract was well-mixed and evaporated

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