Research Article

In Vitro Protective Effects of *Lycium barbarum* Berries Cultivated in Umbria (Italy) on Human Hepatocellular Carcinoma Cells

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*Lycium barbarum* is a famous plant in the traditional Chinese medicine. The plant is known to have health-promoting bioactive components. The properties of *Lycium barbarum* berries cultivated in Umbria (Italy) and their effect on human hepatocellular carcinoma cells (HepG2) have been investigated in this work. The obtained results demonstrated that the *Lycium barbarum* berries from Umbria region display high antioxidant properties evaluated by total phenolic content and ORAC method, on hydrophilic and lipophilic fractions. Moreover, on HepG2 cell line *Lycium barbarum* berries extract did not change cell viability analyzed by MTT and Trypan blue exclusion assay and did not induce genotoxic effect analyzed by comet assay. Furthermore, it was demonstrated, for the first time, that the berries extract showed a protective effect on DNA damage, expressed as antigenotoxic activity *in vitro*. Finally, *Lycium Barbarum* berries extract was able to modulate the expression of genes involved in oxidative stress, proliferation, apoptosis, and cancer. In particular, downexpression of genes involved in tumor migration and invasion (CCL5), in increased risk of metastasis and antiapoptotic signal (DUSP1), and in carcinogenesis (GPx-3 and PTGS1), together with overexpression of tumor suppressor gene (MT3), suggested that Umbrian *Lycium barbarum* berries could play a protective role against hepatocellular carcinoma.

1. Introduction

The use of medicinal and edible plants was widely distributed from ancient times to today in Asian countries. During the last twenty years much attention has been paid to plants as novel alternative therapeutic agents and/or as support to the traditional medicine in Europe and North America [1]. The Solanaceae, one of the largest and most important families of flowering plants, includes *Lycium barbarum* species that are recorded in the Chinese Pharmacopoeia [2]. The plant is commonly called Goji [1] and active molecules have been isolated from seeds, fruits, and leaves of *Lycium barbarum*. *In vitro* and *in vivo* studies displayed antihypertensive, antihyperglycemic, antiinflammatory, antimutant, antihyperlipidemia, and anti-Alzheimer activities of *Lycium barbarum* berry (LBB) extract [3].

LBB extract contains high level of health-promoting bioactive components including polysaccharides, flavonoids, and carotenoids [1–4]. Polysaccharides have been considered the major ingredients responsible for the biological activities of LBB extract. Traditional Chinese medicine considered LBBs to have the ability to maintain the function of eyes and strengthen the activity of liver, kidneys, and lungs [3]. In addition, LBB extract has been historically used as anti-inflammation and antiaging agent for thousands of years [5]. In fact, Oh et al. [6] demonstrated that LBB extract has inhibitor effect on proinflammatory mediator production in lipopolysaccharide-stimulated RAW 264.7 cells via blockade on the MAPKs and NF-κB pathways. Additionally, in the last few years, LBBs have been described to modulate the aging by acting on cp53-mediated pathway [7] and on the resistance to the generation of lipid peroxide and other...
substances, which damage cell membrane lipid [8]. LBB extract has been also described for its immune enhancing [9], antioxidant and anticancer [3], and hepatoprotective and neuroprotective [10] properties. Moreover, it has been highlighted that LBB extract has hypoglycemic and hypolipidemic effects by reducing significantly blood glucose levels and serum total cholesterol and triglyceride concentrations [11]. Interestingly it has been reported a protective effect of LBBs against doxorubicin-induced cardiotoxicity through antioxidant-mediated mechanisms. In particular LBBs significantly prevents the loss of myofibrils and improves the heart function of the doxorubicin-treated rats [12]. Finally Wang et al. [13] demonstrated that sulfated LBB polysaccharides significantly inhibit the infectivity of Newcastle disease virus to chicken embryo fibroblast.

The original habitat of *Lycium barbarum* is probably located in the warm regions in Mediterranean area and Southwest and Central Asia [1]. Recently the plant adaptation to different environments has been reported in a study of *Lycium barbarum* cultivation in Tuscany (Italy) [14].

In this paper we report for the first time that LBBs, cultivated in Umbria (Italy), have very good antioxidant properties, evaluated by two different methods. Moreover, LBBs are able to protect *in vitro* HepG2 cells from genotoxicity induced by 1,2,4-benzenetriol (BT) and stimulate MT3 tumor suppressor gene, suggesting that LBBs could play a specific role in maintaining cell health.

## 2. Materials and Methods

### 2.1. Materials.

LBBs cultivated in Umbria were provided by Impresa Agricola of Gianluca Bazzica, Foligno (Italy); commercial LBBs were bought in pharmacy, Perugia (Italy). Human Caucasian hepatocyte carcinoma HepG2 cells were purchased from Istituto Zooprofilattico Sperimentale della Lombardia e dell’Emilia Romagna “Bruno Ubertini” (Brescia, Italy). Eagle’s Minimum Essential Medium (MEM), L-glutamine, trypsin, and ethylenediaminetetraacetic acid disodium and tetrasodium salt (EDTA) were from Microtech Srl (Pozzuoli, NA, Italy). Fetal Bovine Serum (FBS) and penicillin-streptomycin were from Microtech Srl (Brescia, Italy); Impresa Agricola of Gianluca Bazzica, Foligno (Italy); commercial LBBs were bought in pharmacy, Perugia (Italy). Mycology, trypsin, and ethylenediaminetetraacetic acid disodium, and sodium hydroxide were purchased from Sigma-Aldrich Srl (Milan, Italy). Dimethyl sulfoxide (DMSO), ethanol, hydrochloric acid, sodium chloride, and sodium hydroxide were purchased from Carlo Erba Reagents Srl (Milan, Italy). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), Trypan blue solution 0.4%, acridine orange, 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI), ethidium bromide, low- and normal-melting-point agarose (LMPA and NMPA), 1,2,4-benzenetriol (BT), staurosporine, tris(hydroxymethyl)aminomethane (Tris), Triton X100, valinomycin, Folin-Ciocalteu, 2,2'-azobis (2-methylpropionamide) dihydrochloride (AAPH) and 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2-tetrazolium bromide (MTT) were obtained from Sigma-Aldrich Srl (St. Louis, MO, USA).

### 2.2. Preparation of LBB Extract.

Berries from *Lycium barbarum* were collected from 3-year-old trees growing in Foligno, Umbria Region, Italy (42°55′26.9″ north, 12°39′29.2″ east, and altitude 230 m). In this area, the climate is warm and temperate according to Cfa class in Köppen and Geiger classification [15]. The average annual temperature and mean annual rainfall are 14.3 °C and 706 mm, respectively. The global solar radiation (on the ground) is 5235 MJ/m² (dates from ENEA, http://clisun.casaccia.enea.it/).

Commercial LBBs were used as controls. Umbrian and commercial LBBs (1 g) were homogenized in physiological solution (10 mL) with Ultra Turrax 725 Basic homogenizer (Ika Labortechnik, Staufen, Germany) at room temperature for 1 min followed by centrifugation at 3150 × g for 30 min and the supernatant was used for all experiments.

### 2.3. Total Phenolic Content (TPC).

The total phenolic content (TPC) of commercial and Umbrian LBB extract was determined using the Folin-Ciocalteu colorimetric method described by Rashidinejad et al. [16] with modifications [17]. Gallic acid stock solution (5 mg/mL) and working standard concentrations of 0, 10, 25, 50, 100, 250, and 500 μg/mL were prepared in deionized water. The Folin-Ciocalteu procedure consisted of transferring 20 μL standard or sample into 4.5 mL borosilicate tube, followed by addition of water (1.58 mL) and Folin-Ciocalteu reagent (100 μL). After mixing the samples, 300 μL of 20% Na₂CO₃ was added and the samples mixtures were kept for 30 min at 40 °C. The total phenols were determined at 765 nm. Total phenol values are expressed in terms of gallic acid equivalent (GAE), which is a common reference compound.

### 2.4. Antioxidant Assay by Oxygen Radical Absorbance Capacity (ORAC).

The antioxidant capacity of Umbrian LBB extract was determined using the ORAC method [18]. The hydrophilic and lipophilic fractions were extracted according to Prior et al. [19]. A duplicate extraction was performed for each sample and used to evaluate the lipophilic (L-ORACFL) and hydrophilic ORACFL (H-ORACFL) values [19]. Evaluations of the lipophilic and hydrophilic ORACFL in the LBBs samples were performed separately, and the total antioxidant capacity (TAC) was calculated by adding the L-ORACFL and H-ORACFL values [20]. The ORACFL assays were carried out on a FLUOstar OPTIMA microplate fluorescence reader (BMG LABTECH, Offenburg, Germany) at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The procedure was based on the method of Zulueta et al. [21] with slight modifications. Briefly, 2,2'-azobis (2-methylpropionamide) dihydrochloride (AAPH) was used as a peroxyl radical generator, Trolox was used as a reference antioxidant standard, and fluorescein was used as a fluorescent probe. The data are expressed as micromoles of Trolox equivalents (TE) per gram of sample (μmol TE/g).

### 2.5. Cell Culture and Treatments.

HepG2 cells were grown in monolayer cultures in 25 cm² tissue flasks, with MEM supplemented with 10% heat-inactivated FBS, 1 mmol/L of sodium pyruvate, 2 mM of L-glutamine, and antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin). The cells were maintained in a cell incubator at 37 °C in a humidified
atmosphere containing 5% CO₂. When the cells reached 80–90% of confluence, the routine culture medium was aspirated and the HepG2 cells were washed with PBS IX. The cells were then harvested by 0.05% trypsin in 0.02% Na₂EDTA for 5 min at 37°C and suspended in 1:3 supplemented growth medium to be maintained in the exponential growth phase.

2.6. Cell Viability. Cell viability was tested by MTT and Trypan blue exclusion assay.

2.6.1. MTT Assay. Cellular viability was assessed by the reduction of MTT to formazan [22]. HepG2 cells were seeded onto 96-well plate at a density of 1 × 10⁴ cells/well with MEM complete medium. After 24 h in each well culture medium was replaced with fresh complete medium containing different concentrations (400, 600, 800, 1000, 1200, 1400, 1600, 1800, 2000, 2200, 2400, 2600, and 2800 μg/mL) of Umbrian LBB extract and incubated for additional 24 h. Then, MTT reagent was dissolved in PBS 1x and added to the culture at 0.5 mg/mL final concentration. After 3 h incubation at 37°C, the supernatant was carefully removed and formazan salt crystals were dissolved in 200 μL DMSO added to each well. The absorbance (OD) values were measured spectrophotometrically at 540 nm using an automatic microplate reader (Eliza MAT 2000, DRG Instruments, GmbH). Each experiment was performed two times in quadruplicate. Cell viability was expressed as a percentage relative to that of the control cells set at 100%.

2.6.2. Trypan Blue Exclusion Assay. Trypan blue was performed according to Srivastava et al. [23] with modifications. Cytotoxicity using the Trypan blue exclusion assay was measured using a Countess™ (Invitrogen Srl, Milan, Italy) automated cell counter. Briefly, 50 μL of HepG2 cell suspensions was mixed with equal volumes of 0.4% Trypan blue and loaded onto a Countess cell counting chamber slide. The instrument is equipped with a camera that acquires images from cell samples on the chamber slide, and the image analysis software automatically analyzes acquired cell images and measures cell count and viability.

2.7. Comet Assay. Cells, for genotoxic and antigenotoxic assays, were analyzed by comet assay [24]. For genotoxicity testing, HepG2 cells were seeded onto 6-well plate at a density of 1 × 10⁵ cells/well with MEM complete medium. After 48 h, in each well culture medium was replaced with fresh complete MEM containing different concentrations (200, 600, 1000, 1400, and 1800 μg/mL) of Umbrian LBB extract and incubated for 4 h. Negative (MEM) and positive 100 mM of l,2,4-benzenetriol (BT) controls were included in each experimental set [25]. Each experimental set was repeated at least 3 times. For antigenotoxicity testing, HepG2 cells were cultured for 15 days in the presence of 1800 μg/mL of LBB extract, added to the medium as a nutritional supplement. After the treatment, LBBs extract was removed from the medium to avoid scavenger effects. The HepG2 cells were divided into two groups, the first one (negative control) grown only in complete medium (MEM) and the second one grown with MEM added with LBB extract. Both groups were used to perform comet assay and incubated 4 h with only MEM or with 100 mM of BT. Each experimental set was repeated at least 3 times [26].

For both experiments cells were collected by centrifugation at 70 g for 8 min at 4°C and then processed in the comet assay under alkaline conditions (lysis at pH 10, unwinding and electrophoresis at pH > 13). The comet assay was carried out basically following the original procedure [24], with minor modifications [27] using the double-spot system. Briefly, cell pellets were gently resuspended in 0.7% LMPA in PBS maintained at 37°C. Then, the cell suspensions were rapidly layered onto agarized microscope slides. After the gels were allowed to solidify, the slides were immersed in cold, freshly prepared cellular lysis solution (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris-HCl; pH 10; and 1% Triton X100 added just before use) overnight at 4°C. After the membranes lysis, the slides were placed in a horizontal electrophoresis box (HU20, Scie-Plas, Cambridge, UK) filled with a freshly prepared electrophoresis solution (10 mM Na₃EDTA, 300 mM NaOH; pH > 13). After 20 min of preelectrophoresis to allow DNA unwinding and expression of alkali-labile damage, electrophoresis runs were performed in an ice bath for 20 min by applying an electric field of 1 V/cm and adjusting the current to 300 mA (Power Supply PS250, Hybaid, Chesterfield, MO, USA). The microwells were then neutralized with 0.4 M Tris-HCl buffer (pH 7.5). For scoring, the slides were stained with 50 μL of EtBr (20 μg/mL). The comets in each microgel were analyzed (blind), at 200x magnification, with an epifluorescent microscope (BX41, Olympus Co., Tokyo, Japan) under a 100 W high-pressure mercury lamp (HSII-1030-L, Ushio Inc., Tokyo, Japan), using appropriate optical filters (excitation filter 510–550 nm and emission filter 590 nm). The microscope, equipped with a high sensitivity black and white CCD camera (PE2020, Pulinx Europe Ltd., Basingstoke, UK), was connected to a computerized analysis system (“comet assay III,” Perceptive Instruments, Suffolk, UK). The tail intensity, that is, percent of fluorescence migrated in the comet tail, which is considered to be the most useful parameter system [28], was used to evaluate DNA damage. A total of 100 randomly selected comets (50 cells/replicate spot) were evaluated for each experimental point. For each independent test, the median tail intensity of 50 cells/spot was assessed and the average of 2 replicated spots was calculated as a summary statistic [29].

2.8. Acidine Orange and DAPI Staining. In order to determine cell viability, the same samples, which contained cells in suspension, used for comet assay were mixed with a solution of acidine orange (30 μg/mL) and DAPI (100 μg/mL). Acidine orange is necessary to stain the entire population of cells, while DAPI is used to stain nonviable cells. Briefly, for each sample 5 μL of mixture of dyes was added to 95 μL of cell suspension. Then the samples were immediately loaded into the NC-Slides A8 and read with the NucleoCounter NC-3000 analysis system (ChemoMetec A/S, Denmark). The system recognizes and counts all cells (green fluorescence) and the nonviable cells (blue fluorescence), subtracting the
latter value to the first automatically and then returning the data related to the viability of each sample.

2.9. PCR-Array Analysis. HepG2 cells cultured in the absence or presence of Umbrian LBB extract were used for total RNA extraction performed by using RENaQueous®-4PCR kit (Ambion Inc., Austin, Texas) as previously reported [30]. Samples were treated with RNase-free DNase to prevent amplification of genomic DNA. Samples were dissolved in RNase-free water and total RNA was quantified by measuring the absorbance at 260 nm ($A_{260}$). The purity of RNA was evaluated by using the $A_{260}/A_{280}$ ratio. $A_{260}/A_{230}$ ratio also was used as indicator of chemical contaminants in nucleic acids. The extracted RNA was immediately frozen and maintained at −80°C. Before cDNA synthesis, the integrity of RNA was confirmed by denaturing electrophoresis in TAE 1.2% agarose gel [31]. cDNA was synthesized using 1 μg total RNA for all samples by High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) under the following conditions: 50°C for 2 min, 95°C for 10 min, 95°C for 15 sec, and 60°C for 1 min for 40 cycles. RTqPCR was performed using Master Mix TaqMan® Gene Expression and 7300 RT-PCR instrument (Applied Biosystems), targeting genes in TaqMan Array 96-Well Plate P/N: 4414250.

2.10. Statistical Analysis. Data were reported as the mean ± SD of experiments conducted in triplicate. The significance of treatment was analyzed using the Student t-test ($p$ value was <0.001).

3. Results and Discussion

*Lycium barbarum* cultivated in Umbria grows up to 2 meters similarly to Chinese one [1]. The plant produces a bright orange-red, oval berry 1.5 cm long and possesses a sweet taste. In the traditional East Asian medicine the LBB extract is known to have beneficial effects for the health, thanks to their antioxidant properties [32]. Thus, we first evaluated the total phenolic content (TPC) of LBBs cultivated in Umbria in order to make a comparison with commercial LBBs produced in Asia. TPC value is 1278.247 ± 29.60 mg GAE/100 g dry weight (DW), using 80% ethanol for the extraction (Figure 1). It has been reported that dehydrated LBBs had the TPC value of 351 ± 7.25 mg GAE/100 g, performing the extraction with 80% methanol [33]. The influence of the solvent, used for the extraction on the TPC value, has been previously investigated and it was found a reduction of 1.3-fold or 2.2-fold using methanol instead of ethanol [34, 35]. In any case, also taking into account the variability due to different method of extraction, the Umbrian LBBs have a TPC higher than commercial one. To confirm these result 80% ethanol extract of commercial LBBs was prepared. The results show a TPC value of 712.01 ± 29.12 mg GAE/100 g, similar to that reported in the literature [23], confirming the highest TPC of Umbrian LBBs. It is difficult to establish exactly the reason of this difference, but we can hypothesize that the climate, the season, and therefore the hours of sunshine could contribute positively to the result of TPC.

The Umbrian LBB extract exhibits antioxidant activity value of 22507.03 ± 1402.02 μmol TE/100 g DW with ORAC method (Figure 1), whereas the value of commercial LBB extract is 26502 ± 3807 μmol TE/100 g DW. Thus, the values obtained for LBBs cultivated in Umbria and commercial LBBs, in terms of antioxidant activity, were similar despite the different TPC. It is possible to conclude that both TPC and antioxidant activity are very high in LBBs cultivated in Umbria ground. These results do not mean that TPC and ORAC are directly correlated because TPC evaluates only the polyphenol antioxidant properties, whereas ORAC indicates the total antioxidant properties.

Recently, LBBs have been described to have apoptotic and antiproliferative effects on cancer cells in vitro and in vivo [3]. Based on these results, we investigated the cytotoxicity, genotoxicity, and antigenotoxicity of Umbrian LBB extract in HepG2, human hepatocellular carcinoma cells. This cell line has been chosen for its high degree of morphological and functional differentiation in vitro and also because it is a suitable model to study drug and plant metabolites targeting in vitro [36–38]. MTT assay has been used to test cell viability at different concentration of LBB extract (400, 600, 800, 1000, 1200, 1400, 1600, 1800, 2000, 2200, 2400, 2600, and 2800 μg/mL) after 24 h of culture (Figure 2). With low concentrations (400–800 μg/mL) and with high concentrations (1800–2800 μg/mL) of LBB extract, the cell viability did not change in comparison with control cells (CTRLs). At 1000, 1200, 1400, and 1600 μg/mL concentrations the cell viability was reduced by 12%, 11%, 14%, and 18%, respectively, indicating that highest inhibitory effect of LBBs was at 1600 μg/mL concentration. As shown in Figure 4 the highest concentrations (from 1800 to 2800 μg/mL) have a very important standard deviation. Given the nature of cell type, that is, an immortalized cell line, the variability is normal and the standard deviation conforms to the experimental system. Anyway the cell viability is more than 80% in overall concentration used. The behavior of the LBBs assays by Trypan blue exclusion test appears similar to that obtained by MTT assay (Figure 2). The vitality percentage with Trypan blue assay evaluated at critical concentration (from 800 to
The subsequent objective was to test the potential genotoxic effect of LBB extract on cell viability. HepG2 cells are treated for 24 h with different concentrations of LBBs (from 400 μg/mL to 2800 μg/mL). Cell viability is measured by MTT assay (A) and by Trypan blue exclusion assay (B). The values are reported as % viability of the control sample set at 100%. Data are expressed as mean ± SD of four independent experiments (∗ p < 0.001).

2000 μg/mL) is lower than the vitality percentage obtained with MTT assay according to previous observations [39].

The increase of cell viability at concentration up to 1600 μg/mL could be explained by the nonlinear dose-response of plants and other natural products [40]. Gan et al. [41] demonstrated that 10 mg/kg dose of Chinese LBBs was more effective than 5 and 20 mg/kg doses in the reduction of sarcoma weight and in improving the immune system in the mice.

Thus, we demonstrated that Umbrian LBBs weakly influence HepG2 cell viability in a dose depending manner but without any cytotoxic effect at all concentration considered. The subsequent objective was to test the potential genotoxic effect on HepG2 cell line at different concentrations of LBB extract (200, 600, 1000, 1400, and 1800 μg/mL) after 4 h of treatment. The reason for selecting this exposure time (4 h) is to avoid the initiation of DNA repair events that would lead to the formation of numerous free radicals inside the cells, able to cause oxidative damage to DNA [25].

For real-time PCR, mRNA levels were normalized using GAPDH as internal control. The results show that few specific genes are modulated by LBBs. As shown in Figure 6, where the gene expression is referred to that of untreated cells, CCL5, DUSP1, GPX3, and PTGS1 genes are downexpressed by 0.44 ± 0.08%, 0.43 ± 0.05%, 0.52 ± 0.12%, 0.33 ± 0.08%-fold, respectively, and MT3 gene is overexpressed by about 4.0 ± 1.89%-fold. CCL5 is an 8kDa protein classified as a chemotactic cytokine or chemokine that exerted protumoral effects on human hepatoma cells through its G protein-coupled receptor, CCRI, and is involved in HepG2, Hep3B, and Huh7 human hepatoma cell migration, invasion, or spreading induced by the chemokine [43]. Dual-specificity phosphatases I (DUSP1) belong to a protein family responsible for dephosphorylating threonine/serine and tyrosine residues on their substrates; it is associated with different kinds of cancers and with an increased risk of metastasis and shorter overall survival [44]. In HepG2, DUSP1 prevents the apoptotic effect which is mycotoxin-induced [45]. GPx-3 is a
Figure 3: Effect of LBB extract in HepG2 cells. (a) Genotoxic effect, determined by comet assay, after 4 h of treatment with different concentrations of LBBs (200, 600, 1000, 1400, and 1800 μg/mL). MEM is used as negative control, and 1,2,4-benzenetriol (BT), known to induce oxidative damage to DNA, is used as positive control. Results are expressed as mean ± SD of three independent experiments (* p < 0.05); (b) acridine orange and DAPI staining for negative control (MEM); (c) same staining for positive control (100 mM of 1,2,4-benzenetriol, BT); (d) same staining for LBB extract (1800 μg/mL). The arrows indicate the death cells stained in blue with DAPI.

Figure 4: Antigenotoxic effect of LBB extract (1800 μg/mL for 15 days) and the cotreatment for 4 h with BT (1,2,4-benzenetriol) that induced DNA damage in HepG2 cells. MEM is used as negative control, and 1,2,4-benzenetriol (BT), known to induce oxidative damage to DNA, is used as positive control. Each result is expressed as the mean ± SD of three independent experiments (* p < 0.001).

Selenoprotein belonging to the glutathione peroxidase family upregulated in HepG2 cells, indicating its role in the development of liver carcinogenesis [46]. Prostaglandin H synthase 1 (PTGS1) is implicated in colorectal carcinogenesis [47] and it is considered a good target for cancer therapy [48].

Metallothionein 3 (MT3) is considered a putative tumor suppressor gene [49]. Decreased expression of MT3 has been found in gastric cancer, esophageal adenocarcinoma, and squamous cell cancer [50, 51]. Therefore, downexpression of genes involved in tumor migration and invasion (CCL5), in increased risk of metastasis and antiapoptotic signal (DUSP1), and in carcinogenesis (GPx-3 and PTGS1) together with overexpression of tumor suppressor gene (MT3) suggests that Umbrian LBBs play an anticancer role. However, potential cancer-suppressive effects of LBBs should be further evaluated in in vivo and in vitro experiments. If you consider the high antioxidant activity of LBBs, it is possible to suppose that their potential anticancer role in vitro could be due to the high content of polyphenols. In fact, Chen et al. described a correlation between flavonoids and antiproliferative activities of Rhamnus davurica [52] and Xia et al. demonstrated a potential antihepatocellular carcinoma agent of flavonoids, using HepG2 cell line [38]. Among flavonoids, gallic acid is known to be an anticancer agent since it reduces cell survival, proliferation, and invasion in PC3 cells by downregulating IL-6 with consequent reduction of pSTAT3, pERK1/2, and pAKT signaling proteins [53]. At the moment the phytochemical composition of LBBs from Umbria is unknown. For further experiments it will be useful to clarify this point.
4. Conclusion

In conclusion the *Lycium barbarum* plant, originally cultivated in East Asia, has Umbrian environment adaptability. This could be due to the ability of the plants to learn from experience and to memorize previous experiences in order to optimize the acclimation to environmental stresses. This behavior is considered a form of intelligence of the plants [41]. The overall results show, for the first time, that the LBBs cultivated in Umbria have not only high antioxidant properties, but also a significant antigenotoxic effect. Finally LBBs appear to regulate the expression of genes involved in tumor progression and metastasis. However, prospective cancer-suppressive effects of LBBs should be further evaluated in *in vivo* and *in vitro* experiments.

**Abbreviations**

AAPH: 2,20-Azobis(2-methylpropionamide) dihydrochloride  
BT: 1,2,4-Benzodinitriol  
cDNA: Complementary DNA  
DAPI: 4',6-Diamidine-2'-phenylindole dihydrochloride  
DW: Dry weight  
FBS: Fetal Bovine Serum  
HepG2: Human Caucasian hepatocyte carcinoma  
LMPA: Low-melting-point agarose  
LBBs: *Lycium barbarum* berries  
MEM: Eagle's Minimum Essential Medium  
MTT: 3-[4,5-Dimethyl-2-thiazolyl]-2,5-diphenyl-2-tetrazolium bromide  
NMPA: Normal-melting-point agarose  
ORAC: Antioxidant assay by oxygen radical absorbance capacity  
PBS: Phosphate-buffered saline  
RTqPCR: Real-time polymerase chain reaction  
TE: Trolox equivalents  
TPC: Total phenolic content  
Trolox: 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid  
Tris: Tris(hydroxymethyl)-aminomethane.
Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper and the mentioned received funding in Acknowledgments.

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