Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 5738193, 11 pages
http://dx.doi.org/10.1155/2016/5738193

Research Article

The Effect of *Leonurus sibiricus* Plant Extracts on Stimulating Repair and Protective Activity against Oxidative DNA Damage in CHO Cells and Content of Phenolic Compounds

Przemysław Sitarek,[1] Ewa Skała,[1] Halina Wysokińska,[1] Marzena Wielanek,[2] Janusz Szemraj,[3] Monika Toma,[4] and Tomasz Śliwiński[4]

[1]*Department of Biology and Pharmaceutical Botany, Medical University of Łódź, Muszyńskiego 1, 90-151 Łódź, Poland*
[2]*Department of Plant Physiology and Biochemistry, Faculty of Biology and Environmental Protection, University of Łódź, Banacha 12/16, 90-237 Łódź, Poland*
[3]*Department of Medical Biochemistry, Medical University of Łódź, Mazowiecka 6/8, 92-215 Łódź, Poland*
[4]*Department of Molecular Genetics, University of Łódź, Pomorska 141/143, 90-236 Łódź, Poland*

Correspondence should be addressed to Przemysław Sitarek; przemyslaw.sitarek@umed.lodz.pl

Received 22 June 2015; Accepted 10 September 2015

Academic Editor: Ada Popolo

Leonurus sibiricus L. has been used as a traditional and medicinal herb for many years in Asia and Europe. This species is known to have antibacterial, anti-inflammatory, and antioxidant activity and has demonstrated a reduction of intracellular reactive oxygen species. All tested extracts of *L. sibiricus* showed protective and DNA repair stimulating effects in Chinese hamster ovary (CHO) cells exposed to H_2O_2. Preincubation of the CHO cells with 0.5 mg/mL of plant extracts showed increased expression level of antioxidant genes (*SOD2, CAT,* and *GPx*). LC-MS/MS and HPLC analyses revealed the presence of nine phenolic compounds in *L. sibiricus* plant extracts: catechin, verbascoside, two flavonoids (quercetin and rutin), and five phenolic acids (4-hydroxybenzoic acid, chlorogenic acid, caffeic acid, *p*-coumaric acid, and ferulic acid). The roots and aerial parts of *in vitro L. sibiricus* plant extracts, which had the strongest antioxidant properties, may be responsible for stimulating CHO cells to repair oxidatively induced DNA damage, as well as protecting DNA via enhanced activation of the antioxidant genes (*SOD2, CAT,* and *GPx*) regulating intracellular antioxidant capacity. The content of phenolic compounds in *in vitro* raised plants was greater than the levels found in plants propagated from seeds.

1. Introduction

Leonurus sibiricus L., commonly known as "Chinese Motherwort," is a species valued for its medicinal properties, belonging to the family Lamiaceae. The plant naturally grows in Japan, Korea, China, Vietnam, and southern Siberia [1, 2]. The aerial parts of *L. sibiricus* collected during the flowering stage are the raw materials [2] and have been used for the treatment of menstrual irregularities, amenorrhea, malaria, hypertension, and myocardial ischemia [3]. This species is also known to have antibacterial, anti-inflammatory, and antioxidant activity [4–7] and has demonstrated a reduction of intracellular reactive oxygen species [7, 8]. The medicinal properties are attributed mainly to the presence of phenolic acids (e.g., chlorogenic acid, ferulic acid, and caffeic acid), iridoid and phenylpropanoid glycosides (e.g., leonoside A, leonoside E, verbascoside), flavonoids (e.g., wogonin, quercetin, and hyperoside), alkaloids (e.g., leonurine and stachydrine), and labdane diterpenoids (e.g., leosibirone A and leosibirone B) [1, 9]. The aim of this study was to evaluate the antioxidative and DNA repair stimulating abilities of aqueous methanol extracts from the aerial part and roots of *in vitro* raised plants

grown under greenhouse conditions (harvested at flowering stage) against H_2O_2-induced oxidative DNA damage in CHO cells, using comet assay. These properties were compared with those of extracts from seed-derived plants of *L. sibiricus* (*in vivo* plants) which were at the same developmental stages and grown under identical conditions as *in vitro* derived plants. The study also evaluates the expression of three antioxidant genes encoding superoxide dismutase (*SOD2*), catalase (*CAT*), and glutathione peroxidase (*GPx*) in CHO cells using RT-PCR. LC-MS/MS and HPLC methods were used to investigate the production of phenolic compounds (phenolic acids, flavonoids, and phenylethanoid glycoside) in the aerial part and roots of the *L. sibiricus* plants.

2. Materials and Methods

2.1. Establishment of L. sibiricus Plants. *In vitro L. sibiricus* plants were initiated from shoot tips of 5-week-old *in vitro* cultured shoots. Explants of about 1 cm in length were placed on Murashige and Skoog (MS) [10] agar (0.7%) medium supplemented with 0.1 mg/L indole-3-acetic acid (IAA) and 0.5 mg/L 6-benzylaminopurine (BAP) and subcultured every 5 weeks for 6 successive passages. For rooting, 5-week-old shoots longer than 1 cm were excised from shoot cultures and transferred individually into MS agar medium supplemented with 0.5 mg/L IAA. The cultures were maintained under a photoperiod of 16 h light (at 40 μmol m^{-2} s^{-1}) at 26°C ± 2°C in a culture room. After 5 weeks, the rooted shoots were transferred into pots (10 cm diameter) containing a sterilized mixture of soil, sand, and peat (4 : 3 : 3 v/v/v) and kept in a greenhouse for 6 months. *L. sibiricus* plants obtained from seeds from the same source as those used for shoot culture initiation were also grown in greenhouse for 6 months. The botanical identity of plants was confirmed by Skała according to the Flora of China (http://www.efloras.org/). A voucher specimen was deposited at the Department of Biology and Pharmaceutical Botany, Medical University of Łódź, Poland. All plants were harvested at the flowering stage.

2.2. Plant Materials and Extract Preparation. In this study, four different extracts, two from the aerial parts and roots of plants obtained *in vitro* (AP and R *in vitro* plants, resp.) and two from the aerial parts and roots of plant obtained from seeds (AP and R *in vivo* plants, resp.) grown in the greenhouse for 6 months were used. The lyophilized and powdered plant materials (about 10 g dry weight, each) were extracted for 15 minutes with 80% (v/v) aqueous methanol (500 mL) at 35°C using an ultrasonic bath and then twice with 300 mL of the same solvent for 15 min. The extracts were filtered, combined, and evaporated under reduced pressure and then were lyophilized to dryness and kept dry in the dark until further use. The yields (w/w) of extracts were 47.3% and 52.1% for AP and R for *in vitro* plants, respectively, and 46.8% and 49.7% for AP and R for *in vivo* plants, respectively, in terms of initial crude plant material dry weight.

2.3. Cell Culture Assays in Chinese Hamster Ovary Cells. Chinese hamster ovary (CHO) cells were obtained from the American Type Culture Collection (Manassas, VA, USA). CHO cells were grown in Ham F10 medium supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, 100 IU/mL streptomycin, and 2 mM L-glutamine in a 5% CO_2 humidified atmosphere at 37°C. All experiments were performed by seeding 4×10^5 cells per 25 cm^2 flask. Under these conditions, the cell cycle of this line was approximately 12 h.

2.4. MTT Cytotoxicity Assay of L. sibiricus Extracts. The MTT [3-(4,5dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide] cytotoxicity assay was carried out according to Mosmann [11] with some modifications. Approximately 1×10^4 CHO cells were added to each well of a culture microplate. The cells were exposed to AP, R *in vitro* plant and AP, R *in vivo* plant extracts for 24 h. At the end of this period, the cells were incubated with MTT (5 mg/mL) for 4 h. The plates were read in a microplate spectrophotometer (OMEGA) at 550 nm. The IC_{50} values were determined from a dose-response curve based on different extract concentrations (0–6.25 mg/mL). Analyses were performed in triplicate for each concentration.

2.5. DNA Damage and Repair and the Protective Effects of In Vitro and In Vivo Plant Extracts. To assess DNA damage, CHO cells were incubated for 15 min on ice with hydrogen peroxide (H_2O_2) at a final concentration of 75 μM (positive control). To assess the effectiveness of DNA repair in the CHO cell line, both untreated cells and those exposed to H_2O_2 were washed in fresh RPMI 1640 medium (Sigma-Aldrich, Germany/USA), preheated to 37°C, and then various concentrations of AR, R *in vitro* plant and AR, R *in vivo* plant extracts of *L. sibiricus* (0.05, 0.1, or 0.5 mg/mL) were added. PBS was the solvent used in *in vitro* assays as negative control. The samples were centrifuged at 3000 rpm for 10 minutes and washed with PBS. Cells from suspension were taken from suspension and used for testing either immediately or then after 15, 30, 60, and or 120 min. To evaluate the protective effect of the extracts against oxidative DNA damage, CHO cells were preincubated for 15, 30, 60, and 120 min at 37°C, with lyophilized aqueous methanol *L. sibiricus* plant extracts (AP, R *in vitro* plant and AP, R *in vivo* plant extracts) dissolved in PBS and added to the culture medium. The concentrations of plant extracts were 0.05, 0.1, and 0.5 mg/mL. The cells were washed with PBS and treated with H_2O_2 (75 μM) for 15 min on ice. DNA damage was evaluated by comet assay.

2.6. Comet Assay. The alkaline comet assay was performed as described by Cornetta et al. [12] with minor modifications. Exponentially growing CHO cells were treated for 15–120 min with different concentrations (0.05, 0.1, or 0.5 mg/mL) of the AP, R *in vitro* plant or AP, R *in vivo* plant extracts of *L. sibiricus*. The comet assay was carried out under alkaline conditions: CHO cells were suspended in Low Melting Point (LMP) agarose 0.75% in PBS (Ca- and Mg-free) and spread on microscope slides precoated with 0.5% of normal agarose. The slides were then put in a tank filled with lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, and 1% Triton X-100, pH 10) for 1 h at 4°C and incubated in electrophoresis buffer

(0.3 M NaOH and 1 mM EDTA, pH 13) for 30 min to allow unwinding of DNA. Electrophoresis was carried out at 4°C (the temperature of the running buffer did not exceed 12°C) for 25 min at 0.75 V/cm (28 mA). After electrophoresis, the slides were washed in neutralization buffer (0.4 M Tris, pH 7.5), dried, stained with 2 μg/mL DAPI, and covered with a cover slip. To prevent additional DNA damage, all the steps described above were conducted under dimmed light or in the dark. The preparations were observed under 200x magnification. Images of comets for analysis were obtained using a COHU 4910 camera (Cohu, Inc., USA) equipped with a UV-1 filter block comprising an excitation filter (359 nm) and barrier filter (461 nm) connected to a fluorescent microscope (Nikon, Japan). The slides were scored using a Lucia-Comet v. 4.51 PC-based image analysis system (Laboratory Imaging, Czech Republic). Tail DNA was used to quantify the induced DNA damage, which is a measure of the percentage of migrated DNA in the tail [13].

2.7. Expression of SOD2, CAT, and GPx Genes

2.7.1. Treatment of CHO Cells. The experiment was performed by treating CHO cells with 75 μM of H_2O_2 for 15 min on ice. CHO cells were treated with the four *L. sibiricus* plant extracts (AP and R *in vitro* and AP, R *in vivo* plants) at 0.5 mg/mL to a final concentration of <0.1% PBS in the culture medium for 0 and 24 h. The control cells (negative control) were incubated for 0 and 24 hours with the same final amount of 0.1% PBS in the culture medium. After incubation, the cells were harvested and RT-PCR (reverse transcriptase PCR) was used to evaluate the expression of three antioxidant genes: superoxide dismutase, *SOD2*; catalase, *CAT*; and glutathione peroxidase, *GPx*.

2.7.2. RNA Isolation and RT-PCR (Reverse Transcription). An RNA isolation kit (Blirt, Poland) was used to extract total RNA from treated and untreated CHO cells. The purity and concentration of the isolated RNA were determined by reading the absorption at 260 and 280 nm, with a ratio in the range 1.8–2.0 being considered high quality. Next, the same amounts of RNA from each sample were mixed in a 10 μL reaction mixture and single-stranded complementary DNA (cDNA) was synthesized by the Fermentas. Complementary cDNA was synthesized using a First Strand cDNA kit according to the manufacturer's instructions. The total PCR mixture was prepared as 50 μL : 25 mM $MgCl_2$, 10 mM primers, 10 mM dNTP, 10x PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 11 mM $MgCl_2$, and 0.1% gelatin), TaqNova DNA polymerase (Blirt, Poland), and MilliQ water to make up the volume. The obtained cDNA (2 μL) was used for PCR amplification and specific primers belonging to targeted gene regions (*β-actin*, *SOD2*, *CAT*, and *GPx*) were designed to amplify and determine the expression level of targeted genes (Table 1). The PCR thermocycler was programmed to the following conditions: 5 min initial denaturation at 95°C; 40 cycles of denaturation (30 s at 94°C); annealing (30 s at 56°C) and extension (1 min at 72°C); a final extension at 72°C for 10 min. The PCR products were run in 1.5% agarose gel, stained by 2% ethidium bromide, visualized via UVP Gel Doc It Imaging system, and analyzed by densitometry. The mRNA levels were determined by measuring the mean optical density (OD) of the RT-PCR product using Gel-Pro Analyzer 3.0 (Media Cybernetics, USA). The *β-actin* gene was used to evaluate the expression levels of PCR products. The results were normalized with the expression of the *β-actin* gene and in relation to the positive and negative controls (without cDNA) as shown in Figure 5. The experiments were repeated three times for tested cells. The average relative optical density values were subjected to statistical analyses.

Table 1: Primers used for RT-PCR analysis.

Gene	Primer sequence (5′-3′)	(bp)
SOD2	GTGAACAACCTGAACGCCAC CCTACAGGCCCCCAAAACAT	808
CAT	CGGGCCTGGCCGATG GCCATTCATGTGCCGATGTC	853
GPx	CAGTCCACCGTGTATGCCTT GTAAAGAGCGGGTGAGCCTT	344
β-actin	AGATGGTTGCTGCATCTGCT AGGAAAAACTTCATCATATTCCTGC	425

2.8. HPLC and LC-MS/MS Analyses. Chromatographic analysis was carried out using an HPLC system (Dionex, Sunnyvale, USA) equipped with a photodiode-array detector. Separation of the compounds was achieved on an RP column (aQ Hypersil GOLD, 250 × 4.6 mm, 5 μm) linked with a guard column (GOLD aQ Drop-In guards, 10 × 4 mm, 5 μm, Polygen, Poland) at 25°C using a mobile phase composed of water (A) and methanol (B), both with 0.1% formic acid. The linear gradient was started after 2 min of isocratic elution with 5% B and was slowly increased over 30 min to 55% B, followed by 2 min of isocratic elution and increase to 70% within 10 min, and then after an isocratic step with 70% B for 3 min the gradient was returned to initial 5% B within 3 min to reequilibrate the column for the next 2 min. The flow rate was 1 mL/min, and the absorbance was measured at 230, 280 nm and 310, 325, and 375 nm. LC-MS/MS was carried out using API LC/MS/MS system (Applera, USA) with electrospray ionization (ESI) source equipped with Dionex (Germany) HPLC system. Separation was achieved on aQ Hypersil GOLD column (C18, 2.1 mm × 150 mm, 5 μm) at 30°C using a gradient as described above for HPLC and a flow rate of 0.2 mL/min. Detection was performed in the negative ion mode with the following conditions: drying gas (N_2) 11.0 L/min, temperature 350°C, nitrogen nebulizer pressure 40 psi, capillary voltage 4.5 kW, a detector gain of 1600 V, fragmentation voltage 100 V, and full scan range from 100 to 900 m/z. Compounds in *L. sibiricus* extracts were identified by comparing the retention times, UV spectra, and MS spectra of the analyzed samples with those of reference standards. Quantification was based on the calibration curve for standards constructed over the range of 5–200 μg/mL; the linearity of the calibration curve was verified by the correlation coefficient ($r^2 = 0.9994$). The standards of

HPLC-grade purity (≥96%) were purchased from Sigma-Aldrich (Germany/USA).

2.9. Statistical Methods. The values in this study were expressed as mean ± SE (standard error) from three experiments; that is, the results of three experiments were pooled and the statistical parameters were calculated. The Mann-Whitney U test was used to determine differences between samples with a nonnormal distribution (Kolmogorov-Smirnov test), while the differences between samples with a normal distribution were evaluated using Student's t-test. The results were analyzed using STATISTICA 6.0 software (Statsoft, USA). Statistical comparisons of relative optical densities (OD) were performed using a one-factor analysis of variance (ANOVA) and Dunnett's Multiple Comparison Test ($p < 0.001$).

3. Results

3.1. In Vitro Plant Regeneration. L. sibiricus plants were propagated *in vitro* using shoot tips. Axillary shoots were multiplied on MS medium supplemented with 0.5 mg/L BAP and 0.1 mg/L IAA. The type and concentration of growth regulators were selected on the base of preliminary studies (data not shown). After 5 weeks, from one explant an average of 4.5 shoots, about 3 cm in length were formed. The shoots were rooted on MS medium containing 0.5 mg/L IAA. In total, 97% of the shoots formed an average of 3.2 roots (about 2 cm in length) within 5 weeks. The plantlets were transferred into soil and grown in pots in the greenhouse for 6 months and flowered. The survival rate of the plantlets after 6 months after transfer into *ex vitro* conditions was about 90%.

3.2. Effect of Plant Extracts on CHO Cells Viability. The readings of the cytotoxicity assay determined spectrophotometrically showed that, after 24 h of treatment, AP and R *in vitro* plant extracts (range 0–6.25 mg/mL) exerted a cytotoxic effect on CHO cells in both dose-dependent manners (Figure 1). The cell viability was notably decreased at concentrations of 0.47 mg/mL for the R and 1.00 mg/mL for the AR *in vitro* plant extracts. IC_{50} values were 1.7 mg/mL and 2.8 mg/mL for the R and AP *in vitro* plant extracts, respectively (Figure 1). Therefore, concentrations at 0.05, 0.1, and 0.5 mg/mL were chosen for further study by comet assay. CHO cells viability after treatment of R and AP *in vivo* plant extracts was similar to that obtained for R and AP *in vitro* plant extracts (data not shown).

3.3. Effect of L. sibiricus Extracts on DNA Damage Repair and Protective Ability against DNA Strand Breaks Induced by H_2O_2. The antioxidant influence of *L. sibiricus* R, AP *in vitro* plant and R, AP *in vivo* plant extracts was investigated in CHO cells. The percentage of DNA in the comet tail corresponds to the level of DNA strand breaks after H_2O_2 treatment. Before treatment with plant extracts, the cells were exposed to H_2O_2 at a concentration of 75 μM for 15 minutes at 4°C. Figures 2(a)–2(c) show the % DNA in tail of the CHO cells incubated with R, AP *in vitro* plant and R, AP *in vivo*

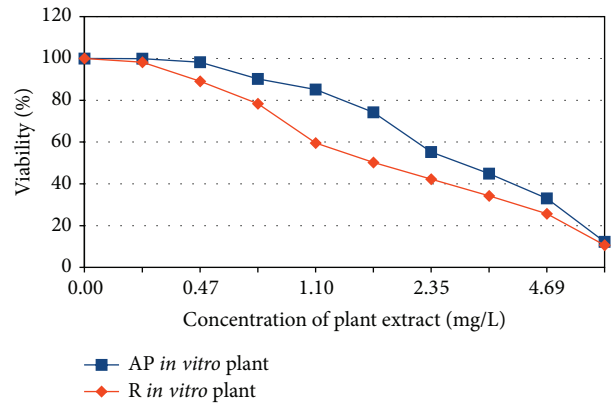

FIGURE 1: Cytotoxicity/viability of CHO cells treated with AP or R *in vitro* plant extracts (0–6.25 mg/mL) for 24 h. AP and R *in vitro* plant extracts—80% aqueous methanol extracts of aerial parts and roots of micropropagated plants of *L. sibiricus*.

plant extracts (0.05–0.5 mg/mL) immediately at time 0, as well as after 15, 30, 60, and 120 min incubation. CHO cells treated with H_2O_2 showed incomplete repair within 2 h and about 5% of the DNA was present in the tail. After incubation, the cells pretreated with H_2O_2 and plant extract exhibited more efficient DNA repair than those treated with hydrogen peroxide alone (Figure 2). The level of DNA damage induced by H_2O_2 was decreased and was proportional to increasing concentrations of plant extracts. The relative decrease of DNA damage observed during repair incubation was significantly higher after treatment with the R *in vitro* plant extract. At the optimal concentration (0.5 mg/mL), the levels of DNA damage were significantly increased to 50% after 15 and 30 min of DNA repair incubation time (Figure 2). After 60 min of the repair incubation, the percentage of DNA in the tail was about 10%. At the same time, the level of DNA damage in CHO cells treated with only H_2O_2 was about 25% (Figure 2(c)). Pretreatment with R, AP *in vitro* plant and AP, R *in vivo* plant extracts resulted in a protective effect against oxidation in CHO cells which increased proportionally with increasing extract concentrations (Figures 3(a)–3(c)). The R and AP *in vitro* plant extracts had a significantly stronger protective effect than R and AP *in vivo* plant extracts (Figure 3(c)). The strongest effect was observed for R *in vitro* plant extracts. After 15 and 30 min of DNA repair incubation time, the levels of DNA damage were significantly increased to 20–30% (Figure 3). After 60 min of the repair incubation, the percentage of DNA in tail was about 5–10%. CHO cells treated with H_2O_2 and pretreated with extracts showed complete repair within 2 h.

3.4. Changes in Expression Level of Antioxidant Genes (SOD2, CAT, and GPx). The mRNA levels of three antioxidant genes, *SOD2*, *CAT*, and *GPx*, were also determined in CHO cells incubated with 0.5 mg/mL of *L. sibiricus* R, AP *in vitro* plant and R, AP *in vivo* plant extracts after 0 and 24 hours. Before treatment with the plant extracts, the cells were exposed to H_2O_2 at 75 μM for 15 min. The gene expression level of the *SOD2*, *CAT*, and *GPx* in CHO cells exposed to the R, AP *in*

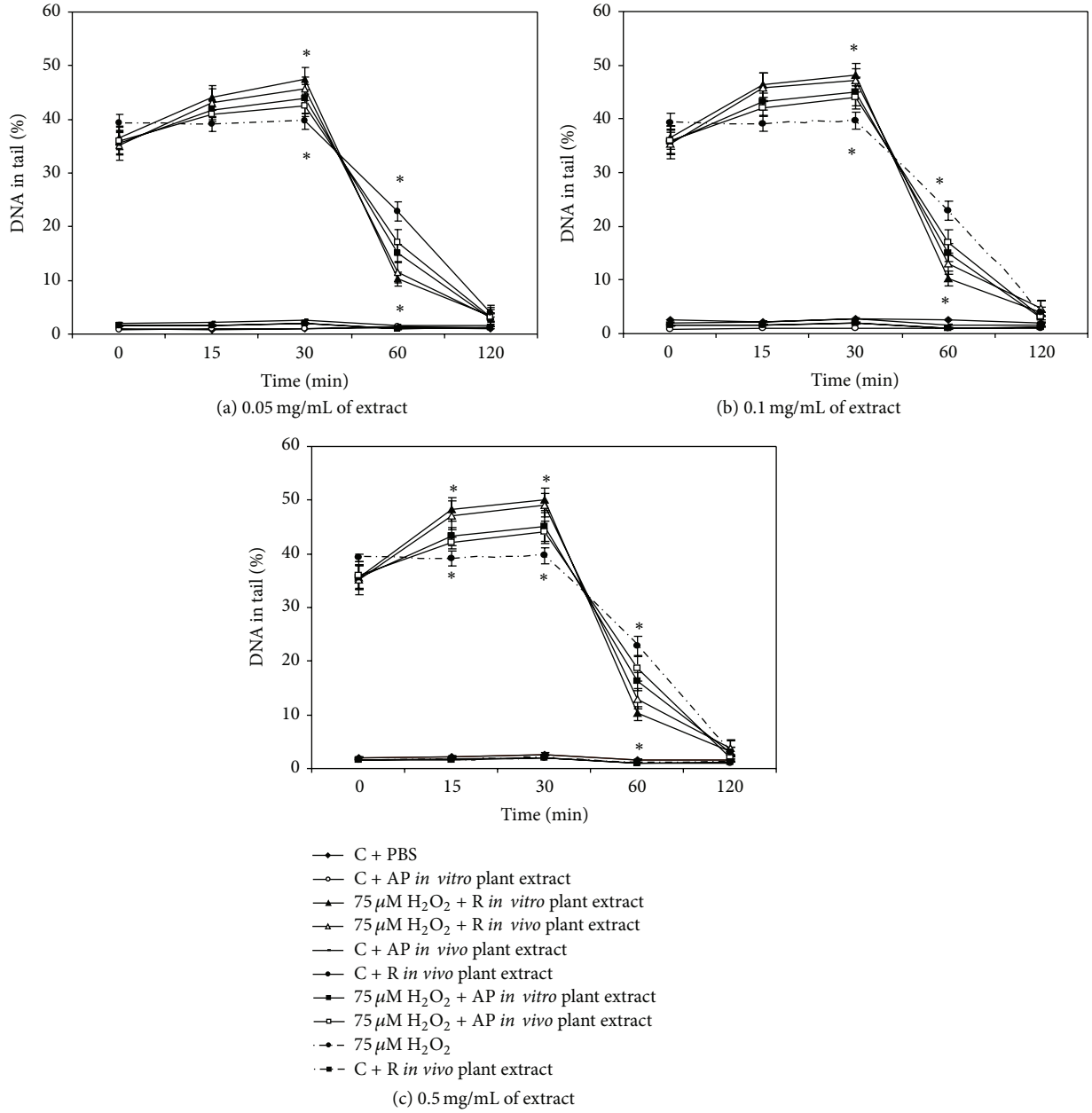

Figure 2: DNA damage and repair in CHO cells with 0.05 mg/mL (a), 0.1 mg/mL (b), and 0.5 mg/mL (c) of *L. sibiricus* AP, R *in vitro* plant and AP, R *in vivo* plant extracts. DNA damage (strand breakage) was induced or not by H_2O_2 at a concentration of 75 μM at 4°C on ice and measured as a percentage of the tail DNA in the alkaline comet assay. $^*P < 0.001$ by comparing AP *in vitro* plant with AP *in vivo* plant extracts and R *in vitro* plant with R *in vivo* plant extracts at the appropriate time of incubation. The number of cells scored for each individual was 100. Data is expressed as means ± SE of at least three independent experiments.

vitro plant or R, AP *in vivo* plant extracts immediately after incubation (0 hours) did not differ significantly compared with that observed in control cells. Figure 4 shows that all of the tested extracts were able to increase the expression level of *SOD2*, *CAT*, and *GPx* genes about 2–5-fold after a 24-hour incubation period.

3.5. Identification and Quantification of Phenolic Compounds in L. sibiricus Extracts. The phenolic compounds present in all plant extracts were identified by comparison of retention times, online UV absorption spectra, and mass data with those of the of authentic standard compounds (Table 2). The structural elucidation of phenolic compounds found in *L. sibiricus* extracts was achieved by negative ionization LC-ESI-MS/MS. MS data showed the quantities of molecular ions [M-H]- and their fragmentation patterns to be consistent with these obtained for nine phenolic standards (Table 2). The main phenolic metabolites in *L. sibiricus* extracts were identified as the hydroxycinnamic acid derivatives, chlorogenic acid (**3**) and caffeic acid (**4**), as well as the flavonol quercetin (**8**), in extracts from the aerial parts. Quercetin

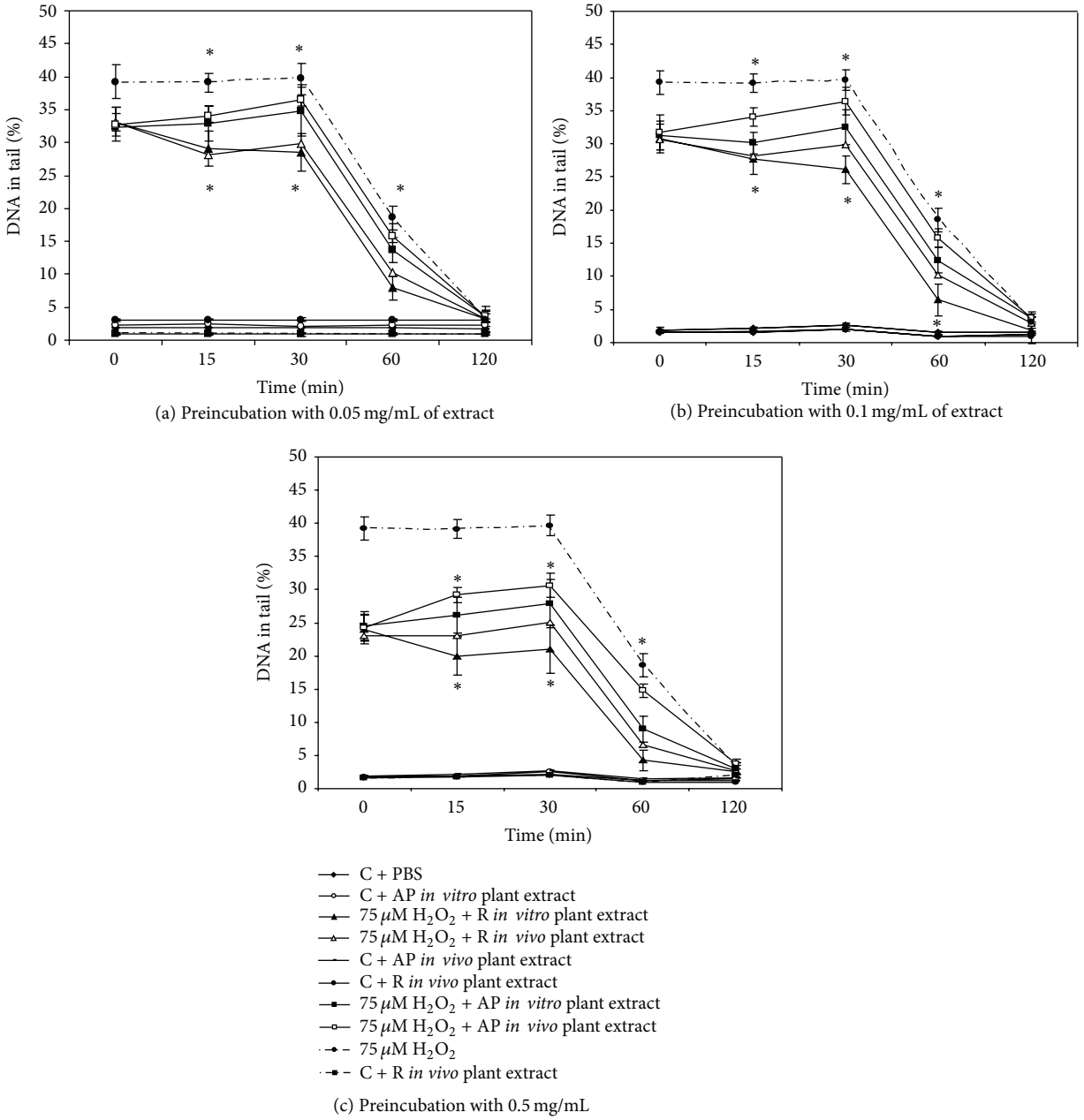

FIGURE 3: The protective effect on CHO cells treatment 0.05 mg/mL (a), 0.1 mg/mL (b), and 0.5 mg/mL (c) of *L. sibiricus* AP, R *in vitro* plant and AP, R *in vivo* plant extracts after 24 h preincubation. DNA damage (strand breakage) was induced or not by H_2O_2 at a concentration of 75 μM at 4°C and measured as a percentage in the tail DNA in the alkaline comet assay. $^*P < 0.001$ by comparing AP *in vitro* plant with AP *in vivo* plant extract and R *in vitro* plant with R *in vivo* plant extracts at the appropriate time of incubation. The number of cells scored for each individual was 100. Data is expressed as means ± SE of at least three independent experiments.

was also found in glycoside form, as rutin (**7**). Verbascoside (**9**) was another glycosylated compound identified in the extracts. Additionally, other minor compounds were detected in plant extracts: 4-hydroxybenzoic acid (**1**), (+)-catechin (**2**), *p*-coumaric acid (**5**), and ferulic acid (**6**). A typical chromatogram of the methanolic extract of the AP *in vivo* plants is shown in Figure 5. The contents of five phenolic acids (4-hydroxybenzoic acid, chlorogenic acid, caffeic acid, *p*-coumaric acid, and ferulic acid), two flavonoids (quercetin and rutin), verbascoside, and catechin in aqueous methanolic AP and R *in vitro* plant and AP and R *in vivo* plant extracts of *L. sibiricus* were determined by HPLC method. The results demonstrated that the aerial parts of the *in vitro* and *in vivo* plants contained significantly different levels of phenolic acids, flavonoids, verbascoside, and catechin than the roots (Table 3). In the extracts from the aerial parts, the flavonoid fraction was dominant (2.8–9.7 mg/g DW) with quercetin as the main constituent. In the roots of both the *in vitro* and *in*

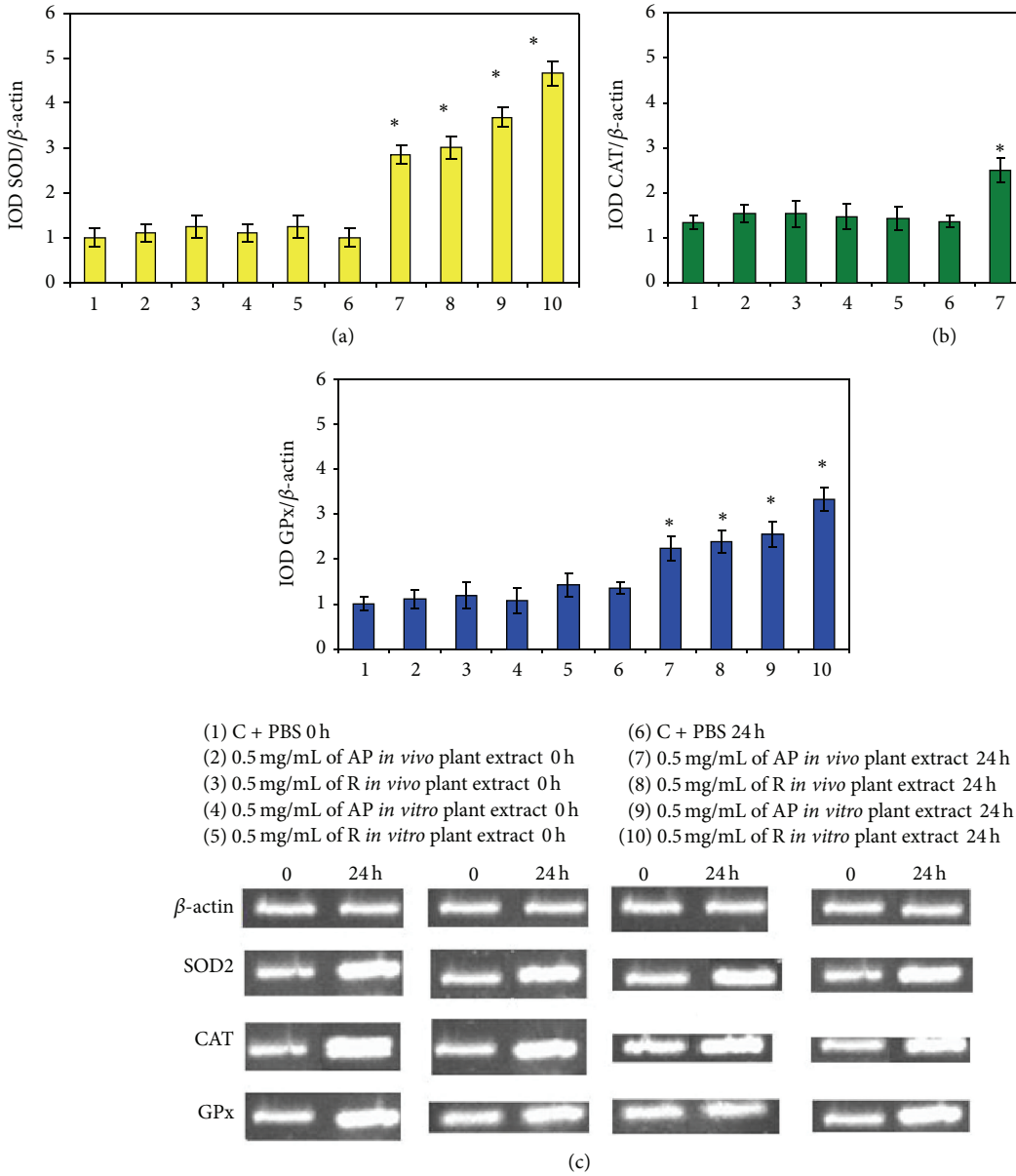

Figure 4: The *SOD2* (a), *CAT* (b), *GPx* (c), and *β-actin* mRNA levels in the CHO cell line, incubated in the presence of 0.5 mg/mL AP, R *in vitro* plant and AP, R *in vivo* plant extracts of *L. sibiricus* for 24 h. *β-actin* was used as an internal control for integrity and equal amount of cDNA used in each PCR. Representative gels from three independent experiments are shown. Before treatment with plant extracts, the cells were exposed to H_2O_2 at 75 μM for 15 min. The bar graph shows a semiquantitative comparison of the *SOD2*, *CAT*,and *GPx* to *β-actin* optical density ratio. $^*P < 0.001$ as compared to AP, R *in vitro* plant and AP, R *in vivo* plant extracts with control the appropriate time of incubation.

vivo plants, the phenolic acids (7.6–12 mg/g DW) were found to be the most prominent fraction, with chlorogenic acid and caffeic acid as the major compounds (4.4 and 6.5 mg/g DW and 3.2 and 5.5 mg/g DW, resp.) (Table 3). Also, verbascoside was accumulated in higher amounts in the root extracts (0.26–0.37 mg/g DW) than the extracts from aerial parts (0.18–0.19 mg/g DW). Significant quantitative differences in the content of phenolic compounds between *in vitro* and *in vivo* plant extracts were also observed. As shown in Table 3, the content of quercetin in the AP *in vitro* plants extract (9.7 mg/g DW) was about 3.5 times greater than in the AP *in vivo* plants extract (2.8 mg/g DW). Similarly, the phenolic acid content in the R *in vitro* plants extract (12.1 mg/g DW) was found to be 1.5 times higher than that of the R *in vivo* plants extract (7.6 mg/g DW).

4. Discussion

L. sibiricus, commonly known as Chinese Motherwort, has important antibacterial, anti-inflammatory, anticarcinogenic, and antioxidant properties [4–7]. Our study had three general aims. Firstly, we investigated DNA repair stimulating and

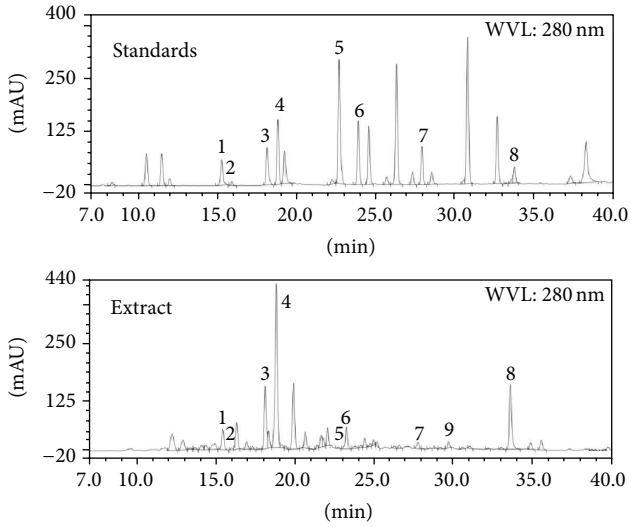

Figure 5: HPLC chromatograms of mixture of standard compounds and extract from AP *in vivo* plant of *L. sibiricus*. Description of numbered compounds in Table 2.

protective effects of aqueous methanol *L. sibiricus* extracts on H_2O_2-induced DNA damage in CHO cells. Secondly, we evaluated the expression level of free antioxidant genes encoding *SOD2*, *CAT*, and *GPx* after preincubation with H_2O_2 of CHO cells with extracts of this plant. Thirdly, we determined the phenolic acids, flavonoids, and verbascoside content in aqueous methanol extracts from the aerial parts and roots of *in vitro* plants grown under greenhouse conditions and compared them with those from the aerial part and roots of seed-derived plants by HPLC and LC-MS/MS methods.

All of tested extracts (AP, R *in vitro* plants and AP, R *in vivo* plants) protected CHO cells from H_2O_2-induced oxidative DNA damage and were able to stimulate DNA repair in these cells, but stronger effects were observed for extracts from the roots and aerial parts of *in vitro* plants than those from *in vivo* plants. This could be associated with two times higher contents of phenolic compounds (calculated as the sum of phenolic acids and flavonoids) in *in vitro* plant extracts (16 mg/g DW in the aerial part and 12.6 mg/g DW in the roots) than in *in vivo* plant extracts (7.6 mg/g DW in the aerial part and 7.7 mg/g DW in the roots). Additionally, the roots extracts were found to have stronger activity than the aerial part extracts. These differences may be attributed to quantitative variations of individual compounds which were characterized by high antioxidant activity such as chlorogenic acid and caffeic acid. For example, the roots of *in vitro* derived plants produced 6.53 mg/g DW of chlorogenic acid, while the concentration of the compound in the overground parts of the plants was 4.9 mg/g DW. The same tendency was observed for caffeic acid, its level being 4.5 times higher in the R extract than the AP extract from the *in vitro* plant. Although phenolic compounds had previously been detected in the aerial parts and roots of *L. sibiricus* [14], the present study is the first to confirm their presence in the overground and underground parts of micropropagated plants. The polyphenolic compounds are known to be strong antioxidants which act by providing hydrogen atoms from their phenolic hydroxyl groups to scavenge hydroxyl radicals generated from hydrogen peroxide [15, 16] and thus protect DNA from damage induced by H_2O_2. On the other hand, the mechanism of interactions between these compounds, and any other possible synergistic effects, may enhance the protective effects. Several *in vitro* and *in vivo* studies have used comet assay to evaluate the protective activities of phenolic acids such as ferulic acid, chlorogenic acid, and caffeic acid against oxidative DNA damage [17–19], but this is the first study to report the ability of *L. sibiricus* extracts to protect against DNA damage and stimulate its repair in CHO cells exposed to an agent inducing oxidative stress. The antioxidant properties of the *L. sibiricus* aerial part extracts have also been confirmed by Chua and Aminah [20], who report that ethanol and aqueous extracts of *L. sibiricus* have strong antioxidant activities in the autooxidation of the linoleic acid lipid system (LP) and demonstrate xanthine oxidase superoxide scavenging activity (XOD) and 1,2-diphenyl-2-picrylhydrazyl radical scavenging activity (DPPH).

H_2O_2 is known to induce oxidative stress and consequent cell damage via the modulation of gene expression (e.g., *SOD2*, *CAT*, and *GPx*), and this is believed to contribute to the development of inflammation, carcinogenesis, and other conditions [21–23]. The present study uses RT-PCR to analyze changes in genes expression after treatment with extracts from the roots and aerial parts of *L. sibiricus* plants grown *in vitro* and *in vivo*. 24 h incubation of CHO cells with 0.5 mg/mL extracts resulted in a significant increase in mRNA levels of all tested genes as compared with the β-actin gene, but OD (optical density) was significantly higher for both *in vitro* plant extracts than both *in vivo* plant extracts. It suggested probably that phenolic compounds contained in *L. sibiricus* extract may inhibit ROS production and upregulation of *SOD*, *CAT*, and *GPx* genes expression mediated by H_2O_2. Marimoutou et al. [24] report that the protective action of polyphenolic compounds was associated with an increase in *SOD* gene expression in 3T3L1 preadipocyte cells. Similarly, Yang et al. [25] revealed that grape seed extract induced a prominent increase in the expression levels and activity of *CAT* and *SOD* in H_2O_2-pretreated PMC cells. The expression of a number of genes involved in antioxidant defense has been found to be influenced by treatment with oxidizing agents. For example, Kobayashi et al. [26] report increased expression of glutamate-cysteine ligase (GCL) along with decreased expression of *GPx* after treatment with H_2O_2 of 3T3-L1 cells. The authors speculate that these changes may be associated with increased intracellular concentrations of reduced glutathione (GSH) under oxidative stress.

5. Conclusion

In conclusion, this work demonstrates that all the tested extracts of *L. sibiricus* protect CHO cells from H_2O_2-induced oxidative DNA damage. The protective property of these extracts could be caused by the presence of phenolic acids

Table 2: Phenolic compounds identified in *L. sibiricus* extracts using HPLC-ESI-MS/MS method.

Peak number	Phenolic compounds	max (nm)	RT (min)	Molecular (precursor) ion $[M-H]^-$ (m/z)	Main fragment ions (m/z)
1	4-Hydroxybenzoic acid	211, 253	15.26	137	93 $[M-CO_2-H]^-$
2	(+)-Catechin (flavan-3-ol)	230, 278	15.90	289	245 $[M-CH_2-CH-OH-H]^-$
3	Chlorogenic acid	217, 241 294, 231	18.13	353	191 $[QA-H]^-$; 179 $[CA-H]^-$ 173 $[QA-H_2O-H]^-$; 135 $[CA-CO_2-H]^-$
4	Caffeic acid	215, 241 291, 319	18.81	179	135 $[M-CO_2-H]^-$; 107 $[M-CO_2-CO-H]^-$ 89 $[M-CO_2-CO-H_2O-H]^-$
5	*p*-Coumaric acid	207, 226 291, 307	22.67	163	119 $[M-CO_2-H]^-$
6	Ferulic acid	214, 239 291, 325	23.88	193	179 $[M-CH_2-H]^-$; 149 $[M-CO_2-H]^-$ 135 $[M-CO_2-CH_2-H]^-$
7	Rutin	255, 265 294, 352	27.89	609	301 [M-rutinose-H]$^-$
8	Quercetin	253, 268 297, 368	33.58	301	271 $(M-CH_2O-H]^-$; 179 $[M-B_{ring}-H]^-$ 151 $[M-B_{ring}-CO-H]^-$
9	Verbascoside	237, 296, 330	29.63	623	669 $[M+HCOO]^-$; 461 $[M-H-CA]^-$ 161 $[CA-H_2O-H]^-$

CA: caffeic acid; QA: quinic acid.

Table 3: Contents of phenolic compounds in extracts from aerial part and roots of micropropagated and seed-derived plants of *L. sibiricus*.

Number	Phenolic compounds	AP *in vitro* mg/g DW	R *in vitro* mg/g DW	AP *in vivo* mg/g DW	R *in vivo* mg/g DW
1	4-Hydroxybenzoic acid	$0{,}090 \pm 0{,}004^c$	$0{,}041 \pm 0{,}0017^b$	$0{,}16 \pm 0{,}0073^d$	$0{,}004 \pm 0{,}0001^a$
2	(+)-Catechin	$0{,}071 \pm 0{,}0033^b$	$0{,}1 \pm 0{,}004^c$	$0{,}184 \pm 0{,}0083^d$	$0{,}054 \pm 0{,}0024^a$
3	Chlorogenic acid	$4{,}912 \pm 0{,}246^b$	$6{,}532 \pm 0{,}306^c$	$3{,}125 \pm 0{,}054^a$	$4{,}380 \pm 0{,}187^b$
4	Caffeic acid	$1{,}235 \pm 0{,}060^a$	$5{,}480 \pm 0{,}244^c$	$1{,}361 \pm 0{,}066^a$	$3{,}222 \pm 0{,}127^b$
5	*p*-Coumaric acid	—	$0{,}011 \pm 0{,}0004^a$	$0{,}019 \pm 0{,}0006^a$	—
6	Ferulic acid	$0{,}034 \pm 0{,}0152^b$	$0{,}028 \pm 0{,}0011^b$	$0{,}027 \pm 0{,}0010^b$	$0{,}020 \pm 0{,}0006^a$
7	Rutin	$0{,}018 \pm 0{,}0008^a$	$0{,}502 \pm 0{,}022^b$	$0{,}014 \pm 0{,}0005^a$	$0{,}172 \pm 0{,}0051^c$
8	Quercetin	$9{,}712 \pm 0{,}372^b$	—	$2{,}848 \pm 0{,}108^a$	—
9	Verbascoside	$\mathbf{0{,}188 \pm 0{,}008}^a$	$\mathbf{0{,}373 \pm 0{,}013}^c$	$\mathbf{0{,}180 \pm 0{,}007}^a$	$\mathbf{0{,}255 \pm 0{,}002}^b$
10	Sum of phenolic acids	$\mathbf{6{,}273 \pm 0{,}301}^b$	$\mathbf{12{,}093 \pm 0{,}568}^c$	$\mathbf{4{,}694 \pm 0{,}192}^a$	$\mathbf{7{,}628 \pm 0{,}299}^b$
11	Sum of flavonoids	$\mathbf{9{,}730 \pm 0{,}370}^d$	$\mathbf{0{,}502 \pm 0{,}022}^b$	$\mathbf{2{,}862 \pm 0{,}107}^c$	$\mathbf{0{,}172 \pm 0{,}0051}^a$

AP and R *in vitro* plant extracts: 80% aqueous methanol extracts of aerial parts and roots of micropropagated plants.
AP and R *in vivo* plant extracts: 80% aqueous methanol extracts of aerial parts and roots of seed-derived plants.
Different superscript letters within the rows indicate significant differences in the mean values at $p < 0.05$ (one-way ANOVA by the Mann-Whitney U test).

and flavonoids. The antioxidant and DNA repair stimulating mechanism of the R *in vitro* plant extract is presented in Figure 6. The potential mechanism of activity shows that the R *in vitro* plant extract, which had the strongest antioxidant properties, may be responsible for stimulating CHO cells to repair oxidative DNA damage, or protecting DNA from oxidative lesions, by elevating the activation of antioxidant genes (*SOD2*, *CAT*, and *GPx*) regulating intracellular antioxidant capacity. Our results also support the hypothesis that natural compounds like polyphenols may actively accumulate in discrete cell structures and display a pleiotropic action in addition to their antioxidant effects on tested cells. However, further *in vitro* studies are necessary to better clarify its antioxidative mechanism of action.

Abbreviations

CHO:	Chinese hamster ovary cells
DW:	Dry weight
AP and R *in vitro* plant extracts:	80% aqueous methanol extracts of aerial part and roots of micropropagated plants
AP and R *in vivo* plant extracts:	80% aqueous methanol extracts of aerial part and roots of seed-derived plants
HPLC:	High-performance liquid chromatography
RT-PCR:	Reverse transcriptase polymerase chain reaction.

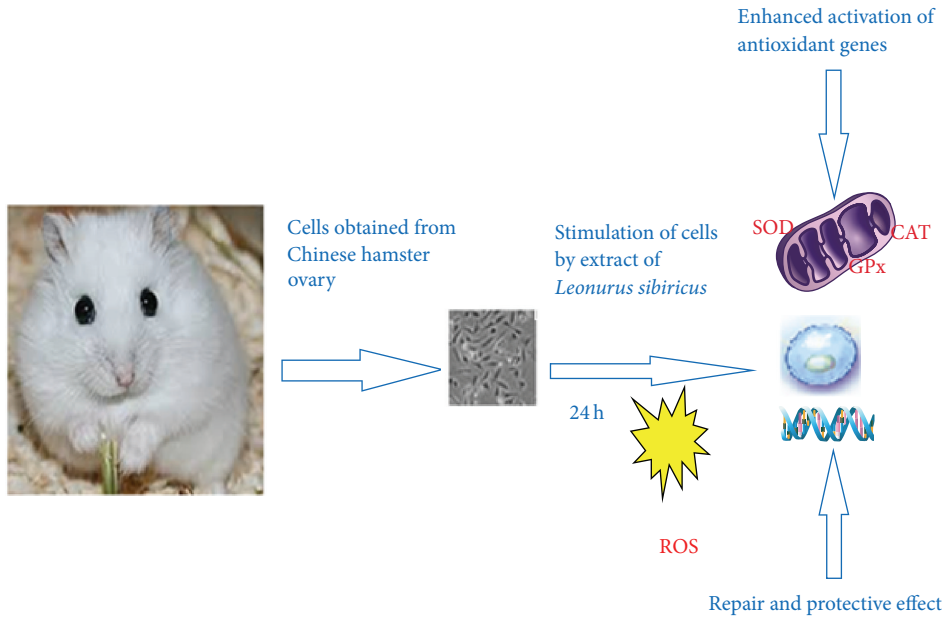

Figure 6: Schematic illustration of the possible mechanism of the DNA repair stimulating and protective effect of R *in vitro* plant extract from *Leonurus sibiricus* against oxidative DNA damage caused by H_2O_2 in CHO cells.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

This work was financially supported by Medical University of Łódź (Grant no. 503/3-012-01/503-36-001).

References

[1] H. Wu, F. R. Fronczek, D. Ferreira, C. L. Burandt Jr., and J. K. Zjawiony, "Labdane diterpenoids from *Leonurus sibiricus*," *Journal of Natural Products*, vol. 74, no. 4, pp. 831–836, 2011.

[2] Y. Narukawa, A. Niimura, H. Noguchi, H. Tamura, and F. Kiuchi, "New diterpenoids with estrogen sulfotransferase inhibitory activity from *Leonurus sibiricus* L.," *Journal of Natural Medicines*, vol. 68, no. 1, pp. 125–131, 2014.

[3] S. Schmidt, M. Jakab, S. Jav et al., "Extracts from *Leonurus sibiricus* L. increase insulin secretion and proliferation of rat INS-1E insulinoma cells," *Journal of Ethnopharmacology*, vol. 150, no. 1, pp. 85–94, 2013.

[4] M. A. Islam, F. Ahmed, A. K. Das, and S. C. Bachar, "Analgesic and anti-inflammatory activity of *Leonurus sibiricus*," *Fitoterapia*, vol. 76, no. 3-4, pp. 359–362, 2005.

[5] F. Ahmed, M. A. Islam, and M. M. Rahman, "Antibacterial activity of *Leonurus sibiricus* aerial parts," *Fitoterapia*, vol. 77, no. 4, pp. 316–317, 2006.

[6] H.-Y. Shin, S.-H. Kim, S.-M. Kang et al., "Anti-inflammatory activity of Motherwort (*Leonurus sibiricus* L.)," *Immunopharmacology and Immunotoxicology*, vol. 31, no. 2, pp. 209–213, 2009.

[7] M.-J. Lee, H.-S. Lee, S.-D. Park, H.-I. Moon, and W.-H. Park, "*Leonurus sibiricus* herb extract suppresses oxidative stress and ameliorates hypercholesterolemia in C57BL/6 mice and TNF-α induced expression of adhesion molecules and lectin-like oxidized LDL receptor-1 in human umbilical vein endothelial cells," *Bioscience, Biotechnology and Biochemistry*, vol. 74, no. 2, pp. 279–284, 2010.

[8] U. Singh and I. Jialal, "Oxidative stress and atherosclerosis," *Pathophysiology*, vol. 13, no. 3, pp. 129–142, 2006.

[9] X. Shang, H. Pan, X. Wang, H. He, and M. Li, "*Leonurus japonicus* Houtt.: ethnopharmacology, phytochemistry and pharmacology of an important traditional Chinese medicine," *Journal of Ethnopharmacology*, vol. 152, no. 1, pp. 14–32, 2014.

[10] T. Murashige and F. Skoog, "A revised medium for rapid growth and bio assays with tobacco tissue cultures," *Physiologia Plantarum*, vol. 15, no. 3, pp. 473–497, 1962.

[11] T. Mosmann, "Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays," *Journal of Immunological Methods*, vol. 65, no. 1-2, pp. 55–63, 1983.

[12] T. Cornetta, L. Padua, A. Testa et al., "Molecular biomonitoring of a population of nurses handling antineoplastic drugs," *Mutation Research: Fundamental and Molecular Mechanisms of Mutagenesis*, vol. 638, no. 1-2, pp. 75–82, 2008.

[13] A. R. Collins, "The comet assay for DNA damage and repair: principles, applications, and limitations," *Applied Biochemistry and Biotechnology*, vol. 26, no. 3, pp. 249–261, 2004.

[14] P. Sheng-Ming, H. Dingb, W. L. Changa, and H. Lina, "Phenols from the aerial parts of *Leonurus sibiricus*," *The Chinese Pharmacutical Journal*, vol. 58, pp. 35–40, 2006.

[15] K. D. Croft, "The chemistry and biological effects of flavonoids and phenolic acids," *Annals of the New York Academy of Sciences*, vol. 854, pp. 435–442, 1998.

[16] M. P. Kähkönen, A. I. Hopia, H. J. Vuorela et al., "Antioxidant activity of plant extracts containing phenolic compounds," *Journal of Agricultural and Food Chemistry*, vol. 47, no. 10, pp. 3954–3962, 1999.

[17] Y.-T. Szeto and I. F. F. Benzie, "Effects of dietary antioxidants on human DNA *ex vivo*," *Free Radical Research*, vol. 36, no. 1, pp. 113–118, 2002.

[18] F. Guglielmi, C. Luceri, L. Giovannelli, P. Dolara, and M. Lodovici, "Effect of 4-coumaric and 3,4-dihydroxybenzoic acid on oxidative DNA damage in rat colonic mucosa," *British Journal of Nutrition*, vol. 89, no. 5, pp. 581–587, 2003.

[19] S. Schaefer, M. Baum, G. Eisenbrand, H. Dietrich, F. Will, and C. Janzowski, "Polyphenolic apple juice extracts and their major constituents reduce oxidative damage in human colon cell lines," *Molecular Nutrition and Food Research*, vol. 50, no. 1, pp. 24–33, 2006.

[20] H. P. Chua and A. Aminah, "Determination of antioxidant activities of dried kacangma (*Leonurus sibiricus*) extract in three bioassay systems," *Journal of Tropical Agriculture and Food Science*, vol. 41, no. 2, pp. 221–229, 2013.

[21] M. S. Cooke, M. D. Evans, M. Dizdaroglu, and J. Lunec, "Oxidative DNA damage: mechanisms, mutation, and disease," *The FASEB Journal*, vol. 17, no. 10, pp. 1195–1214, 2003.

[22] K. Vandenbroucke, S. Robbens, K. Vandepoele, D. Inzé, Y. Van De Peer, and F. Van Breusegem, "Hydrogen peroxide-induced gene expression across kingdoms: a comparative analysis," *Molecular Biology and Evolution*, vol. 25, no. 3, pp. 507–516, 2008.

[23] D. Closa and E. Folch-Puy, "Oxygen free radicals and the systemic inflammatory response," *International Union of Biochemistry and Molecular Biology Life*, vol. 56, no. 4, pp. 185–191, 2004.

[24] M. Marimoutou, F. Le Sage, J. Smadja, C. Lefebvre d'Hellencourt, M. Gonthier, and C. Robert-da Silva, "Antioxidant polyphenol-rich extracts from the medicinal plants *Antirhea borbonica*, *Doratoxylon apetalum* and *Gouania mauritiana* protect 3T3-L1 preadipocytes against H_2O_2, TNFα and LPS inflammatory mediators by regulating the expression of superoxide dismutase and NF-κB genes," *Journal of Inflammation*, vol. 12, article 10, 2015.

[25] T. Yang, X. Li, W. Zhu et al., "Alteration of antioxidant enzymes and associated genes induced by grape seed extracts in the primary muscle cells of goats *in vitro*," *PLoS ONE*, vol. 9, no. 9, Article ID A1499, 2014.

[26] H. Kobayashi, M. Matsuda, A. Fukuhara, R. Komuro, and I. Shimomura, "Dysregulated glutathione metabolism links to impaired insulin action in adipocytes," *American Journal of Physiology: Endocrinology and Metabolism*, vol. 296, no. 6, pp. E1326–E1334, 2009.