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Research Article

Antioxidant and DNA Repair Stimulating Effect of Extracts from Transformed and Normal Roots of *Rhaponticum carthamoides* against Induced Oxidative Stress and DNA Damage in CHO Cells

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Rhaponticum carthamoides has a long tradition of use in Siberian folk medicine. The roots and rhizomes of this species are used in various dietary supplements or nutraceutical preparations to increase energy level or eliminate physical weakness. This is the first report to reveal the protective and DNA repair stimulating abilities of *R. carthamoides* root extracts in Chinese hamster ovary (CHO) cells exposed to an oxidative agent. Both transformed root extract (TR extract) and extract of soil-grown plant roots (NR extract) may be responsible for stimulating CHO cells to repair oxidatively induced DNA damage, but CHO cells stimulated with extract from the transformed roots demonstrated significantly stronger properties than cells treated with the soil-grown plant root extract. These differences in biological activity may be attributed to the differences in the content of phenolic compounds in these root extracts. Preincubation of the CHO cells with TR and NR extracts showed an increase in gene expression and protein levels of catalase (*CAT*) and superoxide dismutase (*SOD2*). *R. carthamoides* may possess antioxidant properties that protect CHO cells against oxidative stress.

1. Introduction

Oxidative stress is mediated by reactive oxygen species (ROS) and is caused by an imbalance between the production of free radicals and the activity of the antioxidant defenses. ROS, free radicals such as superoxide anion radicals ($O_2^{\bullet-}$), and hydroxyl radicals ($OH^{\bullet}$) and non-free radical species such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) may injure cells by causing lipid membrane peroxidation and DNA strand breaks, inducing cellular dysfunction and their consequent apoptosis. The cells must repair these damaged bases, mainly via the base excision repair (BER) pathway [1]. The cellular defense systems against free radicals consist of antioxidant enzymes such as catalase (*CAT*), peroxidase (*POD*), and superoxide dismutase (*SOD2*). These important antioxidant enzymes play a key role in eliminating superoxide and hydrogen peroxide, thereby suppressing potential damage [2, 3].

Oxidative DNA damage is involved in carcinogenesis, mutagenesis, and a variety of associated diseases including coronary diseases, diabetes, cancer, neurodegenerative brain disorders, and ageing [4, 5]. Phenolic compounds widely distributed in plants are known to be effective ROS scavengers. They are natural antioxidants which can prevent oxidative DNA damage and lipid peroxidation. Hence, plants used in the human diet may play an important role in maintaining health and can be used for disease prevention. Of the phenolic compounds, the caffeoylquinic acids (e.g., chlorogenic acid) are free radical and metal ion scavengers and have been shown to modulate the gene expression of antioxidant enzymes [6].

Rhaponticum carthamoides (Willd.) Iljin (Asteraceae) is a rare, endemic, herbaceous species naturally growing in the high-mountain areas of South Siberia, Central Asia, and China [7, 8]. Due to its medical importance, *R. carthamoides* is also cultivated in Central and Eastern Europe. The three- to four-year-old root and rhizome of this plant act as the raw material. This species has been used for a long time in Siberian folk medicine in cases of overstrain and weakness after illness. The extracts from roots and rhizomes of *R. carthamoides* are used in various dietary supplements or nutraceutical preparations to increase energy level and eliminate physical weakness or for recovery after surgery. *R. carthamoides* is also known to have adaptogenic, immunomodulatory, anticarcinogenic, antioxidant, and antimicrobial activities [7]. The antiradical effects of the aerial part of *R. carthamoides* have already been evaluated by the most common radical-scavenging assays based on 2,2-diphenyl-1-picrylhydrazyl (DPPH) [9–12] 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assays [10] and ferric reducing antioxidant power (FRAP) tests [11–13]. The important antioxidant compounds of this plant are polyphenolic compounds such as flavonoids (e.g., quercetin, quercetagetin, luteolin, patuletin, and kaempferol) and phenolic acids (e.g., caffeic, chlorogenic, and ferulic acids and caffeoylquinic acid derivatives) [7, 14].

The aim of the study was to evaluate the repair and protective effects of 80% (v/v) aqueous methanol extract of *R. carthamoides* transformed roots (TR extract) and soil-grown plant roots (NR extract) against H_2O_2-induced oxidative DNA damage in CHO cells, using the alkaline single-cell gel electrophoresis assay (comet assay). The comet assay is a useful, rapid, and sensitive technique for measuring DNA damage and repair in individual mammalian cells. Our previous study [14] showed that the root extracts of *R. carthamoides* produced phenolic compounds mainly caffeoylquinic acid derivatives. The effect of phenolic compounds level on the differences in activity of TR and NR extracts is discussed. The expression of two antioxidant genes encoding superoxide dismutase (*SOD2*) and catalase (*CAT*) and the protein level of both genes in CHO cells was also evaluated.

2. Materials and Methods

2.1. Plant Material and Extract Preparation. The transformed roots of *R. carthamoides* were obtained by the transformation of A4 *Agrobacterium rhizogenes* strain through infection of sterile leaf explants of 4-week-old *in vitro* cultured shoots [14]. The transformed roots were cultured in 300 mL Erlenmeyer flasks containing 80 mL of liquid Woody Plant medium [15] on a rotary shaker at 80 rpm, under a 16/8 h light/dark photoperiod with a light intensity of 40 μmol m^{-2} s^{-1}. The *R. carthamoides* plants were grown in the Medicinal Plant Garden of the Department of Pharmacognosy, Medical University of Łódź (Poland), and were collected in September 2013 after 3 years of cultivation. Botanical identity of plants was confirmed by Skała according to Flora of China (http://www.efloras.org/). The voucher specimen was deposited at the Department of Biology and Pharmaceutical Botany, Medical University of Łódź.

The roots of soil-grown plants and the transformed roots were lyophilized. The dried and powdered materials (10 g) were first extracted with *n*-hexane. After filtration, the *n*-hexane extract was discarded. The defatted sample was sonicated for 15 min 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 combined aqueous methanol extracts were evaporated under reduced pressure and then were lyophilized to dryness and kept dry and in the dark until further use. The yields (w/w) of aqueous methanol extracts were 18.47% for transformed roots (TR extract) and 20.76% for roots of soil-grown plants (NR extract), in terms of initial crude plant material dry weight.

2.2. Phenolic Compounds in TR and NR Extracts. Chemical analysis of the studied extracts of *R. carthamoides* (TR and NR extracts) was performed in our previous work [14]. According to the work, phenolic compounds (caffeoylquinic acid derivatives and flavonoids) were identified by UPLC-PDA-ESI-MS^3 and their contents were determined by HPLC-PDA method.

2.3. Cell Culture of 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's F10 medium (Sigma-Aldrich, USA) supplemented with 10% FCS, 100 IU/mL penicillin, 100 IU/mL streptomycin, and 2 mM L-glutamine in a 5% CO_2 humidified atmosphere at 37°C.

2.4. Cytotoxicity of TR and NR Extracts. The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma-Aldrich, USA) cytotoxicity assay was carried out in accordance with the protocol described by Hansen et al. [16]. Approximately, 1×10^4 CHO cells were added to each well of a culture microplate. The CHO cells were exposed to TR and NR extracts for 24 h. At the end of this period, the cells were incubated with MTT reagent (5 mg/mL in PBS) for 2 h. After incubation, MTT-formazan crystals were dissolved in 20% SDS and 50% DMF at pH 4.7. The plates were read in an ELISA-plate reader (ELX 800, Bio-Tek, USA) at 562 and 630 nm. Cells grown in the absence of plant extracts were used as a control. The values of IC_{50} (the concentration of tested extracts required to reduce the cell survival fraction to 50% of the control) were determined from a dose-response curve by using different concentrations (0–10 mg/mL). The IC_{50} values were calculated from concentration-response curves. Analyses were performed in triplicate for each concentration.

2.5. DNA Damage and Repair and the Protective Effects of NR and TR Extracts. To examine DNA damage, CHO cells were incubated with hydrogen peroxide (H_2O_2) to give a final concentration of 50 μM, for 10 min on ice (positive control). To assess the degree of DNA repair in the untreated CHO cell lines and those exposed to H_2O_2, the cells were washed in fresh RPMI 1640 medium (Sigma-Aldrich, USA) preheated to 37°C. Different concentrations of TR and NR

extracts of *R. carthamoides* (0.05, 0.1, 0.5, and 1.0 mg/mL) were then added. Some cells from suspension were used for testing immediately and then after 15, 30, 60, and 120 min. PBS was the solvent used in *in vitro* assays as negative control. Next, the samples were centrifuged at 1000 ×g for 15 min and washed with PBS.

To evaluate the protective effect of the plant extracts against oxidative DNA damage, CHO cells were preincubated for 24 h at 37°C, with lyophilized aqueous methanol *R. carthamoides* extracts (NR and TR extracts), dissolved in PBS, and added to culture medium. The different concentrations (0.05, 0.1, 0.5, and 1.0 mg/mL) of NR and TR extracts were used. The cells were washed with PBS and treated with H_2O_2 (50 μM) for 10 min on ice. DNA damage was evaluated by comet assay.

2.6. Oxidative DNA Damage. The alkaline version (pH > 13) of the comet assay recognizes both double- and single-strand breaks along with alkali-labile sites; it does not detect oxidative DNA damage. Thus, the level of this kind of DNA damage was assessed by the use of 2 DNA glycosylases: 8-oxoguanine DNA glycosylase (hOGG1) or Nth (endonuclease III) (New England Biolabs, Ipswich, MA, USA) according to the procedure described earlier [17]. Both enzymes are bifunctional glycosylases and they possess AP-lyase activity. Therefore, after the recognition and removal of the damage bases, they introduce single-strand breaks, which can be detected by the alkaline version of the comet assay. hOGG1 recognizes and removes 8-oxoadenine when paired with cytosine, 7,8-dihydro-8-oxoguanine (8-oxoguanine) when paired with cytosine, methy-fapy-guanine, and foramidopyrimidine (fapy)-guanine [18, 19]. Nth recognizes and removes urea, thymine glycol, 5,6-dihydroxythymine, uracil glycol, 5-hydroxy-5-methylhydanton, 6-hydroxy-5,6-dihydrothymine, and methyltartronylurea [20–22]. To examine the oxidative DNA damage, the CHO cells were incubated with 50 μM H_2O_2, for 10 min on ice (positive control). Then, the cells were treated with the plant extracts (1.0 mg/mL) to a final concentration of 0.1% PBS in the culture medium for 24 h. The control cells (negative control) were incubated with the same final amount of 0.1% PBS in the culture medium. The CHO cells were suspended in 1% low gelling temperature agarose type XI (Sigma-Aldrich, Germany/USA) in PBS and spread on microscope slides precoated with 0.5% low electroendosmosis agarose type I (Sigma-Aldrich, Germany/USA) in distilled water. The slides were submerged in a lysis solution (100 mM EDTA, 2.5 mM NaCl, 10 mM TRIS, and 1% Triton X-100, pH 10) and stored at 4°C overnight. Then, the enzyme-treated samples were washed with enzyme buffer (0.5 mM EDTA, 40 mM HEPES, 0.1 M KCl, and 0.2 mg/mL bovine serum albumin, pH 8). 100 μL of the buffer containing 1 U of either hOGG1 or Nth enzymes, or as a control, the buffer alone, was placed onto gels. Those samples were covered with cover glasses and incubated at 37°C for 60 min. Then, coverslips were removed and the slides were submerged in electrophoresis buffer (1 mM EDTA and 300 mM NaOH, pH > 13) and left for 20 min for unwinding of DNA. DNA of undigested samples was unwound directly after the lysis step. Then, electrophoresis was conducted at an electric field strength of 0.73 V/cm (300 mA) for 20 min.

2.7. Comet Assay. The alkaline comet assay was performed as described by Singh et al. [22] with minor modifications [23]. Exponentially growing CHO cells were treated for 15–120 min with different concentrations (0.05, 0.1, 0.5, or 1.0 mg/mL) of the TR or NR extract of *R. carthamoides*. The comet assay was carried out under alkaline conditions: CHO cells were suspended in Low Melting Point (LMP) agarose (Blirt, Poland) 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 an 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 for 25 min at 0.75 V/cm (28 mA): the temperature of the running buffer did not exceed 12°C. 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 coverslip. 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). Measurements were made for 100 cells per slide and the percentage of DNA in the comet tail was used as a quantitative measure of DNA damage.

2.8. Gene Expression of SOD2 and CAT

2.8.1. Cell Treatment. To test the effect of *R. carthamoides* TR and NR extracts on the level of genes activity, CHO cells were treated with the plant extracts (1.0 mg/mL) to a final concentration of 0.1% PBS in the culture medium for 24 h. The control cells (negative control) were incubated for 24 h with the same final amount of 0.1% PBS in the culture medium. Before treatment with plant extracts, the cells were exposed to H_2O_2 at 50 μM for 10 min. The cells were also incubated only with TR and NR extracts or were treated only with 50 μM H_2O_2. After incubation, the cells were harvested and RT-PCR method was used to evaluate the expression of two antioxidant genes: superoxide dismutase (*SOD2*) and catalase (*CAT*).

2.8.2. RNA Isolation and RT-PCR (Reverse Transcription). Total RNA was extracted from CHO cells treated and nontreated with plant extracts using an RNA isolation kit (Blirt, Poland). 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. The same amounts of RNA from each sample were mixed in a 20 μL reaction mixture and single-stranded

complementary DNA (cDNA) was synthesized by the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas Life Sciences, USA) according to the manufacturer's instructions. The total PCR mixture was prepared as 25 μL: 2.5 mM $MgCl_2$, 2 mM primers, 0.2 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 (3 μL) was used for PCR amplification and specific primers against target gene regions (*β-actin, SOD2*, and *CAT*) were designed to amplify and determine the expression level of targeted genes. The sequences of primers and PCR amplification were performed according to the procedure described by Sitarek et al. [24]. The PCR products were run on 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) values of the RT-PCR product using Gel-Pro® Analyzer 3.0 (Media Cybernetics, USA). The expression of each target gene was standardized with internal control gene (*β-actin*) and represented as a ratio. The results were normalized with the expression of the *β-actin* housekeeping gene and in relation to the positive and negative controls (without cDNA) as shown in Figure 6. The experiments were repeated three times for CHO cells. The average relative optical density values were subjected to statistical analyses.

2.8.3. Western Blot Analysis. CHO cells were treated with the plant extracts (1.0 mg/mL) to a final concentration of 0.1% PBS in the culture medium for 24 h. The control cells (negative control) were incubated for 24 h with the same final amount of 0.1% PBS in the culture medium. Before treatment with plant extracts, the cells were exposed to H_2O_2 at 50 μM for 10 min. The cells were also incubated only with TR and NR extracts and were treated only with 50 μM of H_2O_2. After incubation, the cells were harvested and washed with cold PBS. The cells were lysed with RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS) containing protease and phosphatase inhibitor cocktails (Roche, Germany) and centrifuged at 16000 ×g for 10 min at 4°C. The collected supernatants were used for Western blotting. The protein concentration in each sample was measured by using Bio-Rad Protein Assay Reagent (Bio-Rad Laboratories) according to the manufacturer's instructions. Western blots were performed by standard protocols. In brief, total protein was resolved on SDS 10% polyacrylamide gel and transferred to nitrocellulose membranes. The membranes were blocked with 5% BSA in TBS containing 0.1% Tween-20 (TBST) for 1 h at room temperature and then incubated sequentially with primary antibodies at 4°C overnight. The levels of CAT, SOD2, and β-actin proteins were analyzed with the following antibodies: SOD2 (Abcam, UK), CAT (LSBio, USA), and β-actin (Abcam, UK), followed by detection with ECL plus Western-blotting detection reagents (GE Healthcare, Piscataway, NJ). The membranes were scanned on a Bio-Rad Versa Doc Imaging System (model 4000), and the intensity of the protein bands was analyzed by using the Bio-Rad Quantity One software (version 4.5.1).

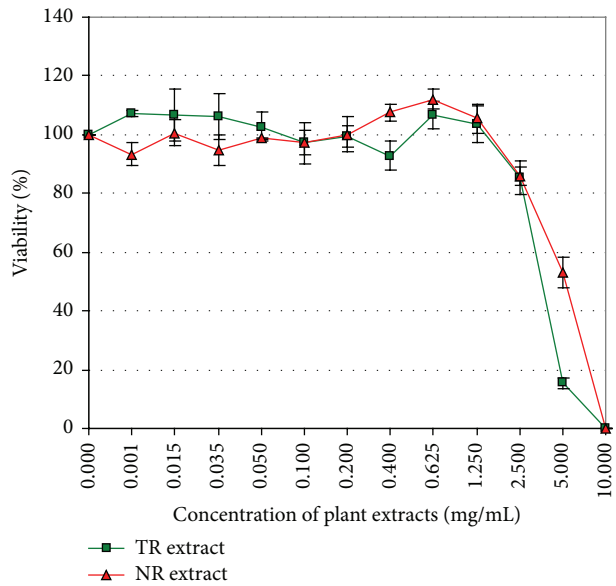

Figure 1: The viability of CHO cells treated with TR or NR extracts (0–10 mg/mL) for 24 h. The viability was determined by MTT assay. The values are the mean ± SE of three independent experiments.

2.9. Statistical Analysis. The values in this study were expressed as mean ± SE (standard error) from three experiments. The Mann-Whitney *U* test was used to determine differences between samples with a nonnormal distribution (Shapiro-Wilk test), while the differences between samples with a normal distribution were evaluated using Student's *t*-test. The results were analyzed using STATISTICA 12 software (StatSoft, Poland). 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 and Discussion

3.1. Effect of Plant Extracts on CHO Cell Viability. MTT assay was used to determine the cytotoxic effect of *R. carthamoides* transformed roots extract (TR extract) and roots of soil-grown plant extract (NR extract) (range: 0–10 mg/mL) after 24 h incubation with the Chinese hamster ovary cells (CHO cells) (Figure 1). Cell viability notably decreased when the TR and NR extracts were applied at 2.5 mg/mL. The IC_{50} values were 3.76 mg/mL and 5.29 mg/mL for the TR and NR extracts, respectively. Hence, 0.05, 0.1, 0.5, and 1.0 mg/mL concentrations were chosen for the comet assay.

3.2. Effect of R. carthamoides Extracts on DNA Damage Repair and Protective Ability against DNA Damage Induced by H_2O_2. This is the first study to investigate the ability of aqueous methanol extracts of *R. carthamoides* transformed roots and normal roots of soil-grown plants to stimulate DNA repair and protect against the effects of H_2O_2-induced DNA damage in CHO cells. Our results show that NR and TR extracts protect CHO cells from H_2O_2-induced oxidative DNA damage and improve the ability of CHO cells to repair

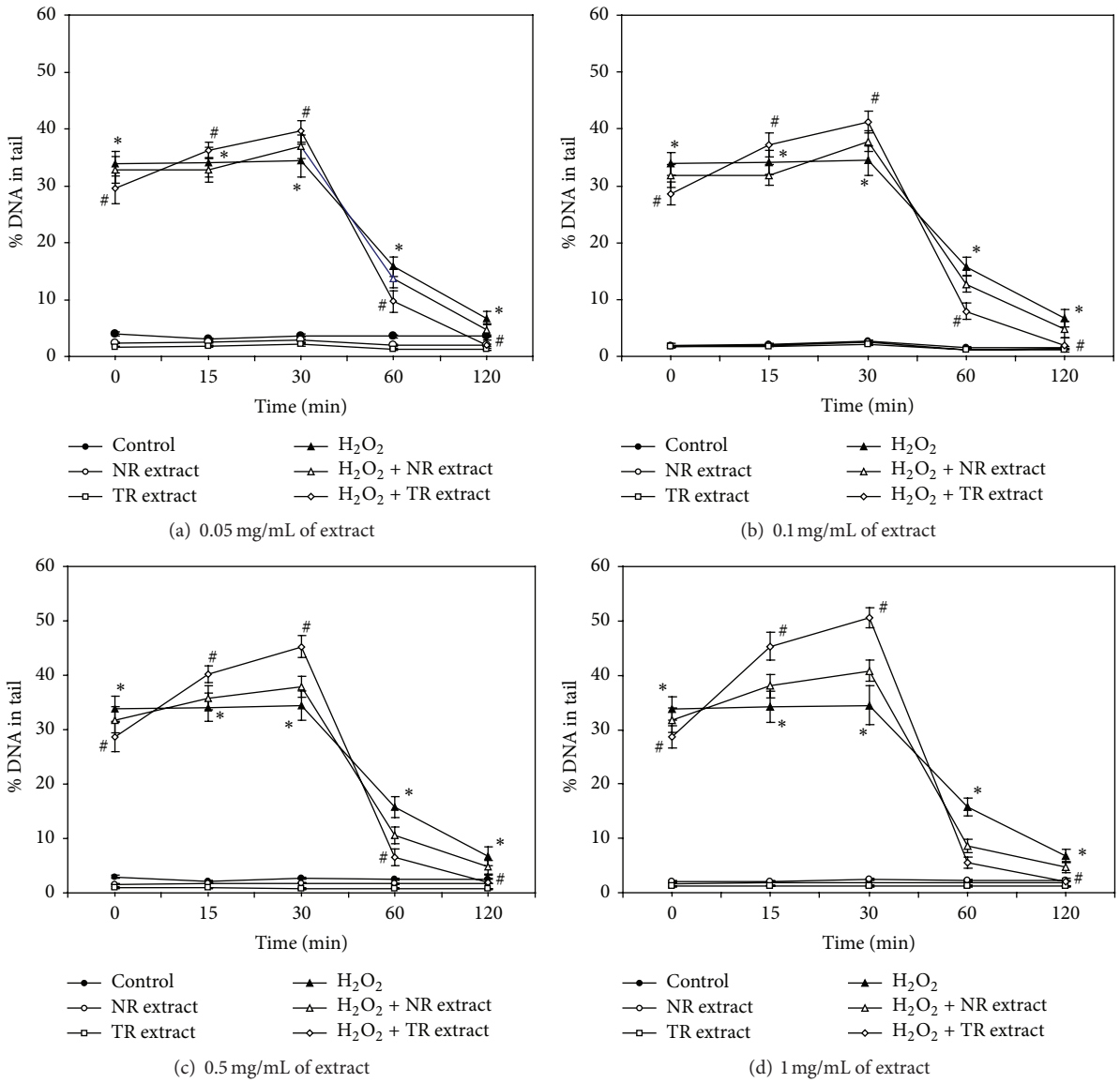

Figure 2: DNA damage and repair in CHO cells with 0.05 mg/mL (a), 0.1 mg/mL (b), 0.5 mg/mL (c), and 1.0 mg/mL (d) of *R. carthamoides* TR and NR extracts. DNA damage was induced by H_2O_2 at a concentration of 50 μM at 4°C on ice and measured as a percentage of the tail DNA in the alkaline comet assay. $^{*}p < 0.001$ as compared to TR or NR extract with H_2O_2 at the appropriate time of incubation. $^{\#}p < 0.001$ as compared to TR or NR extract at the appropriate time of incubation The number of cells scored was 100. Data is expressed as means ± SE of three independent experiments. Control: cells incubated with PBS; H_2O_2: cells incubated with H_2O_2; NR extract: cells incubated with NR extract; TR extract: cells incubated with TR extract; H_2O_2 + NR extract: cells incubated with H_2O_2 and NR extract; H_2O_2 + TR extract: cells incubated with H_2O_2 and NR extract.

DNA damage. The level of DNA damage can be measured as the percentage of DNA in the tail of the comet. Figures 2(a)–2(d) show the % DNA in the tail of the CHO cells incubated with NR and TR extracts (0.05–1.0 mg/mL) immediately after incubation, as well as during repair incubation time (15, 30, 60, and 120 min). Before treatment with plant extracts, the cells were exposed to 50 μM H_2O_2. Our preliminary experiments showed that 50 μM of H_2O_2 was a suitable concentration to induce a high level of DNA damage and was chosen for further experiments. The percentage of DNA in the tail, at this H_2O_2 concentration, was about 35% (0–30 min) (Figure 2). After 2 h, the % DNA in the tail was about 10%. CHO cells exposed to H_2O_2 and subsequently treated with the TR extract at all tested concentrations demonstrated more efficient DNA repair than those treated only with hydrogen peroxide or NR extract, especially in early stage of repair (incision of damaged bases) (Figures 2(a)–2(d)). The level of DNA damage induced by 50 μM of H_2O_2 was decreased by exposure to the plant extracts and corresponded with increasing concentrations of extracts. The efficacy of DNA repair, measured as the relative decrease in DNA damage, was significantly higher after treatment

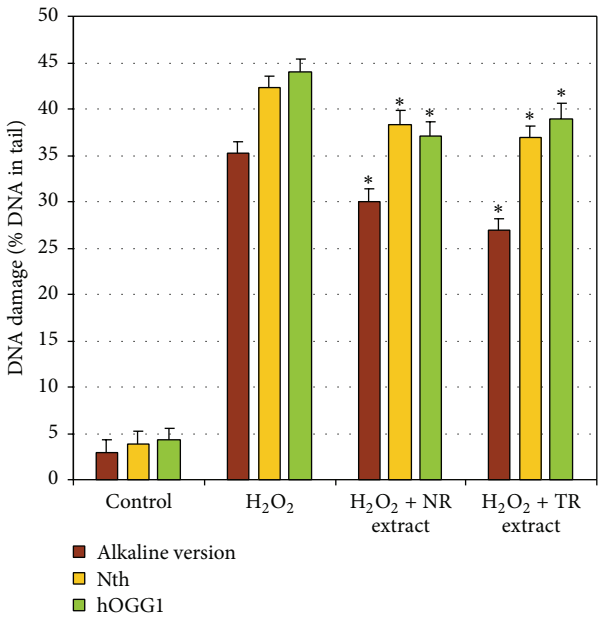

Figure 3: Oxidative DNA damage as percent of DNA in the comet tails in CHO cells. DNA damage was induced by H_2O_2 at a concentration of 50 μM at 4°C on ice. After treatment with H_2O_2, CHO cells were treated for 24 h with 1.0 mg/mL of *R. carthamoides* TR and NR extracts. $^*p < 0.001$ as compared to TR and NR extract with H_2O_2. The number of cells scored was 100. Data is expressed as means ± SE of three independent experiments. Alkaline version: strand breaks and alkali-labile sites; Nth: oxidative DNA damage recognized by Nth; hOGG1: oxidative DNA damage recognized by hOGG1.

by the TR than the NR extract. After 15 and 30 min of incubation, the levels of DNA damage were significantly increased to 35–50% for all tested concentrations of plant extracts (Figures 2(a)–2(d)), indicating that DNA incision followed by nucleotide excision took place in these DNA repair incubation times. The BER pathway is initiated by one of many DNA glycosylases, which recognize and catalyze the removal of damaged bases. Subsequently, downstream enzymes carry out strand incision, gap-filling, and ligation. The process was found to be most effective after using the transformed root extract compared with the roots of the soil-grown plant extract and H_2O_2 used alone (Figures 2(a)–2(d)). After 60 min, the percentage of DNA in the tail in CHO cells exposed to H_2O_2 (positive control) was about 18%. At the same time, the level of DNA damage in CHO cells exposed to H_2O_2 and subsequently treated with 1.0 mg/mL TR and NR extracts decreased to 5% and 8%, respectively (Figure 2(d)).

Also, the oxidative DNA damage level in CHO cells pretreated with H_2O_2 was evaluated (Figure 3). The extent of oxidative DNA damage was estimated with a modified comet assay based on 2 glycosylases: Nth removing oxidized pyrimidines and hOGG1 excising oxidized purines. The oxidative damage level was higher in the cells treated only with H_2O_2 compared with those incubated with the plant extracts (Figure 3).

Subsequently, the protective effects of *R. carthamoides* root extracts (NR and TR extracts) on H_2O_2-treated CHO cells were examined. The cells were preincubated with TR and NR extracts (0.05, 0.1, 0.5, or 1.0 mg/mL) for 24 h prior to the addition of 50 μM H_2O_2. Pretreatment with TR and NR extracts resulted in a protective effect against oxidation in CHO cells which correlated with increasing concentrations of extract (Figures 4(a)–4(d)). The TR extract showed the most protective abilities on CHO cells at a concentration of 1.0 mg/mL (Figure 4(d)). The percentage of DNA in the tail was about 18%. The level of DNA damage in CHO cells treated only with H_2O_2 was about 35% (Figure 4).

3.3. Phenolic Compounds in NR and TR Extract. The results of the present study indicate that the *R. carthamoides* TR and NR extracts demonstrate differences in DNA repair and DNA protection activity against oxidative-induced DNA damage. CHO cells stimulated with TR extract demonstrated significantly stronger properties than the cells treated with NR extract. Our previous study [14] reported that the caffeoylquinic acid derivatives are the main group of biologically active constituents of *R. carthamoides* root extracts (NR and TR extracts). Both extracts contained 3-caffeoylquinic acid, 5-caffeoylquinic acid, 4-caffeoylquinic acid, 1,3-dicaffeoylquinic acid, 3,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, 1,5-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid, and tricaffeoylquinic acid derivatives. Additionally, in TR extract, the presence of 1,4,5-tricaffeoylquinic acid has been reported. Total phenolic content ranges from 1908 mg/100 g DW to 3520 mg/100 g DW in TR extract and NR extract, respectively [14]. The results of our earlier work showed also predominance of monocaffeoylquinic acid derivatives in NR extract with chlorogenic acid (about 1826 mg/100 g DW) as the main constituent, whereas the major fraction in TR extract was the derivatives of tricaffeoylquinic acid (800 mg/100 g DW in TR extract *versus* 300 mg/100 g DW in NR extract) [14]. The percentage of mono-, di-, and tricaffeoylquinic acid derivative contents in NR and TR extracts of *R. carthamoides* is shown in Figure 5. It is possible that the higher activity of TR extract may be due to higher overall content of tricaffeoylquinic acid derivatives. Many studies indicate that tricaffeoylquinic acid derivatives possess greater biological activity, for example, antioxidant activity, than mono- and dicaffeoylquinic acid derivatives [25–27]. The radical-scavenging activity of caffeoylquinic acids may be proportional to the number of caffeoyl groups at the molar concentration in quinic acid. The increased number of caffeoyl groups might have positive effects on the single electron transfer reaction [25–28]. Moreover, only transformed roots of *R. carthamoides* were able to produce small amount (about 300 mg/100 g DW) of the flavonoid glycosides (quercetagetin, quercetin, luteolin, and patuletin hexosides) [14]. Flavonoids are well known in respect to their strong antioxidant properties [29–31]. The phenolic compounds presented in the *R. carthamoides* transformed and normal root extracts can donate hydrogen atoms to trap deleterious free radicals and form less reactive phenoxy radicals, thereby protecting DNA [32]. However, the reduction of DNA dysfunction arising from ROS attack

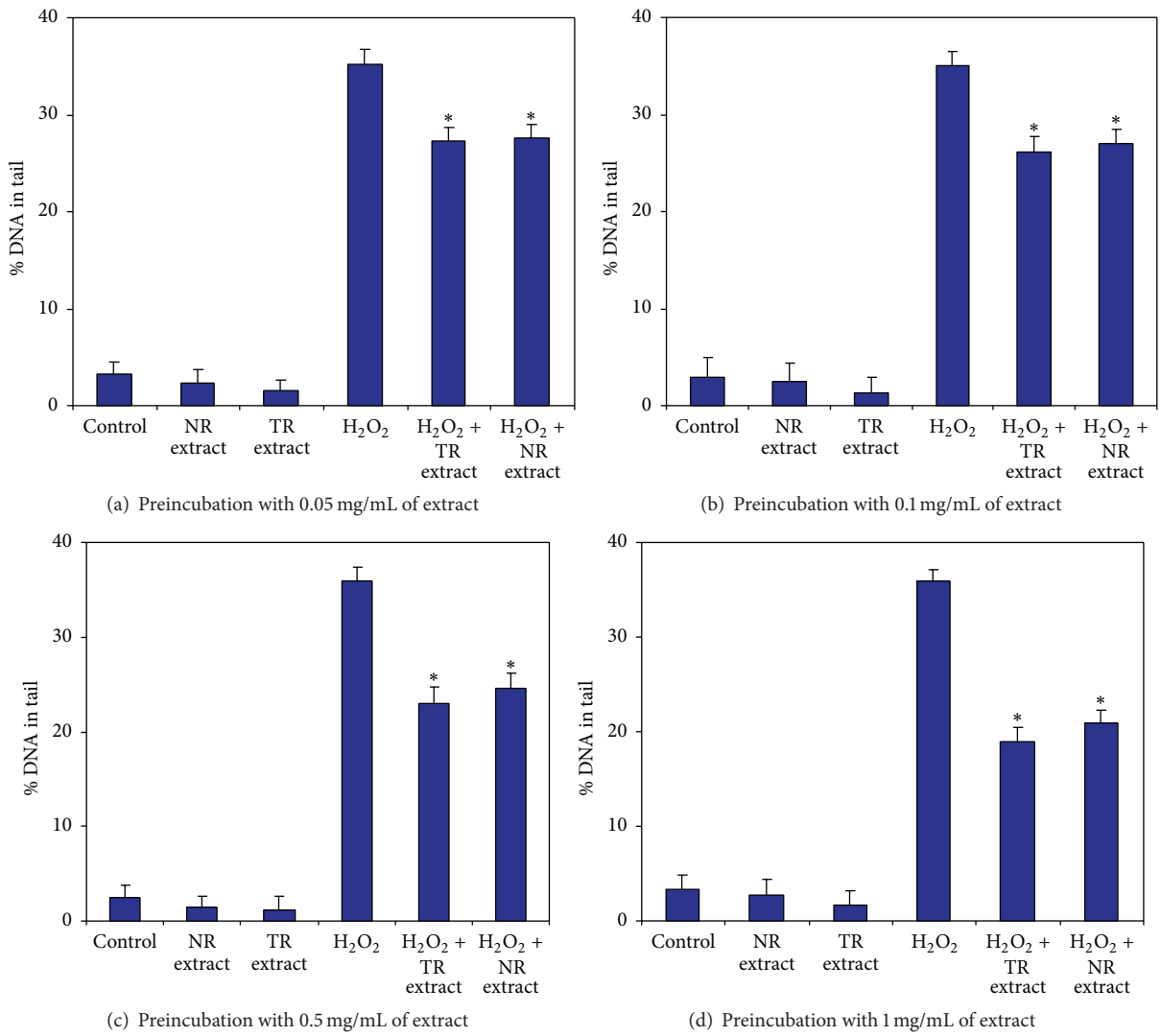

Figure 4: The protective effect of 0.05 mg/mL (a), 0.1 mg/mL (b), 0.5 mg/mL (c), and 1.0 mg/mL (d) of *R. carthamoides* TR and NR extracts on H_2O_2 DNA damaged in CHO cells after 24 h. DNA damage was measured as a percentage of the tail DNA in the alkaline comet assay. $^*p < 0.001$ as compared to TR and NR extract with H_2O_2. The number of cells scored was 100. Data is expressed as means ± SE of three independent experiments. Control: cells incubated with PBS; H_2O_2: cells incubated with H_2O_2; NR extract: cells preincubated for 24 h with NR extract; TR extract: cells preincubated for 24 h with TR extract; NR extract + H_2O_2: cells treated with H_2O_2 after 24 h preincubation with NR extract; TR + H_2O_2: cells treated with H_2O_2 after 24 h preincubation with TR extract.

could be significant at all different constituent levels of damaged DNA [33]. We suggest that possible mechanism of *R. carthamoides* root extracts is related to stimulation of CHO cells to efficient DNA repair via activation of DNA glycosylases (higher level of DNA damage at the early step of repair), involved in base excision repair, which recognize and catalyze the removal of damaged base. The antioxidant activity mechanism of *R. carthamoides* root extracts on CHO cells is not clear yet and more detailed examination is required. Perhaps, this mechanism is correlated with synergistic effect of phenolic compounds presented in extracts. Moreover, these compounds may stimulate factors involved in regulation of expression of genes encoding antioxidant proteins such as *SOD* or *CAT*.

3.4. Gene Expression of SOD2 and CAT. The mRNA levels of two antioxidant genes, *SOD2* and *CAT*, in CHO cells incubated with 1.0 mg/mL of *R. carthamoides* NR and TR extracts after 24 h of incubation were also determined. Before treatment with the plant extracts, the cells were exposed to H_2O_2 at 50 μM for 10 min. The levels of expression of *SOD2* and *CAT* were also evaluated in nontreated cells (control), cells treated only with H_2O_2, and cells treated only with plant extracts. CHO cells incubated for 24 h with NR and TR extracts show increased expression level of *SOD2* and *CAT* genes (Figure 6). Optical density (OD) was significantly higher for the plant extracts than for H_2O_2. We confirmed our data on protein level of both genes (SOD2 and CAT) (Figure 7). Therefore, the plant extract may express its antioxidant activity to protect and stimulate the cells

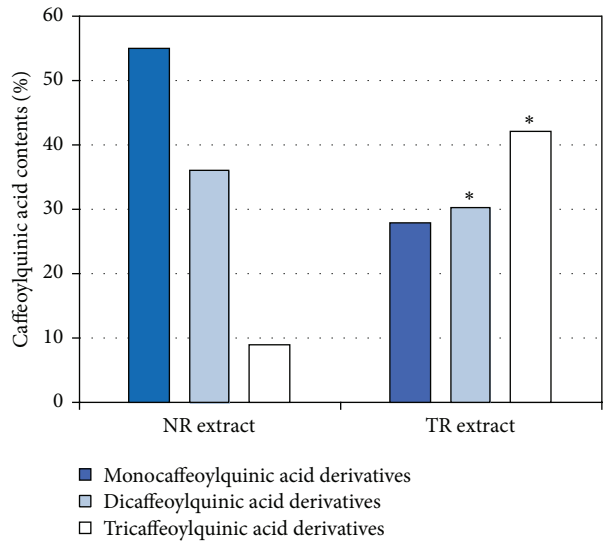

Figure 5: Percentage of caffeoylquinic acid contents in aqueous methanol extract from transformed roots (TR) and roots of soil-grown plants (NR) of *Rhaponticum carthamoides*. $^{*}p < 0.05$ by comparing TR extract with NR extract.

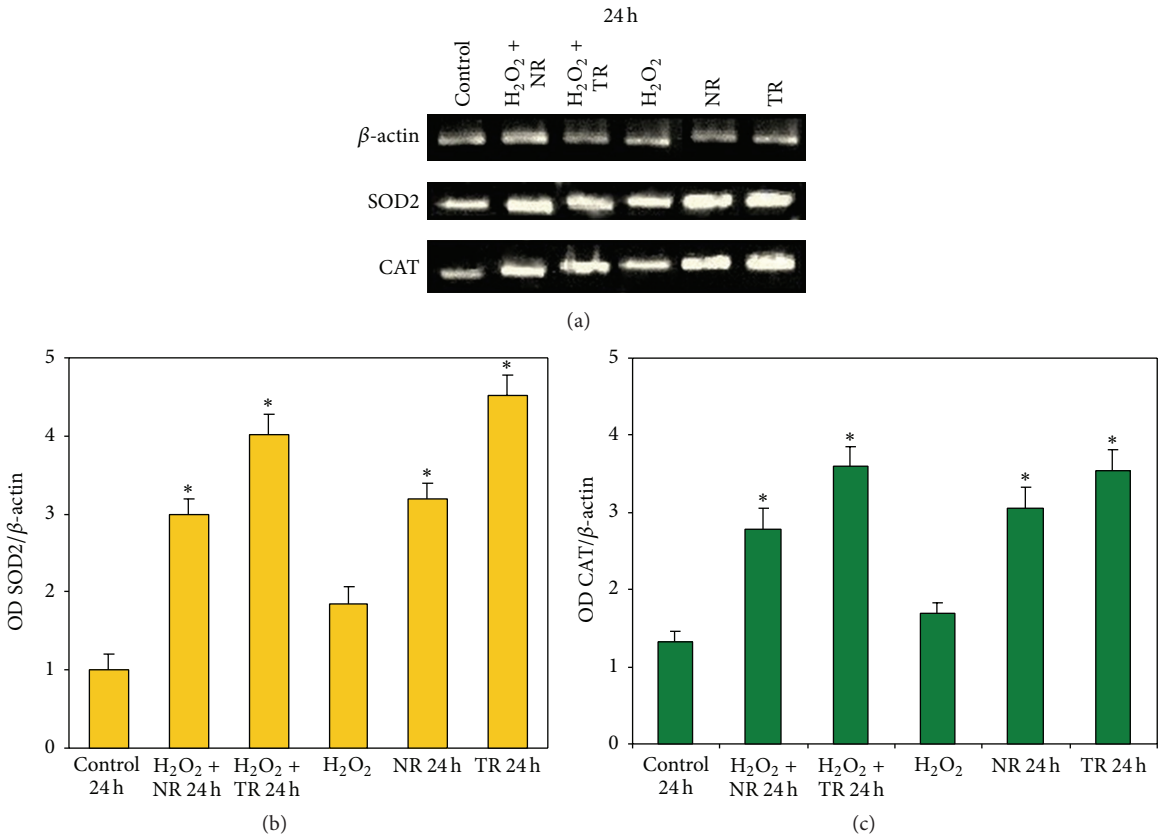

Figure 6: The *SOD2*, *CAT*, and *β-actin* mRNA levels in the CHO cell line, incubated in the presence of 1.0 mg/mL *R. carthamoides* TR and NR extracts (a) for 24 h. The expression of each target gene was standardized with an internal control gene (*β-actin*) and represented as a ratio. Representative gels from three independent experiments are shown. Before treatment with plant extracts, the cells were exposed to H_2O_2 at 50 μM for 10 min. The bar graph shows a semiquantitative comparison of *SOD2* (b) and *CAT* (c) to *β-actin* optical density ratio. Results were represented as means ± SE from three independent experiments. $^{*}p < 0.001$ as compared to TR and NR extract with control (cells incubated with PBS). Control 24 h: cells incubated for 24 h with PBS; H_2O_2: cells incubated with H_2O_2; NR 24 h: cells incubated for 24 h with NR extract; TR 24 h: cells incubated for 24 h with TR extract; H_2O_2 + NR 24 h: cells treated with H_2O_2 before 24 h incubation with NR extract; H_2O_2 + TR 24 h: cells treated with H_2O_2 before 24 h incubation with TR extract.

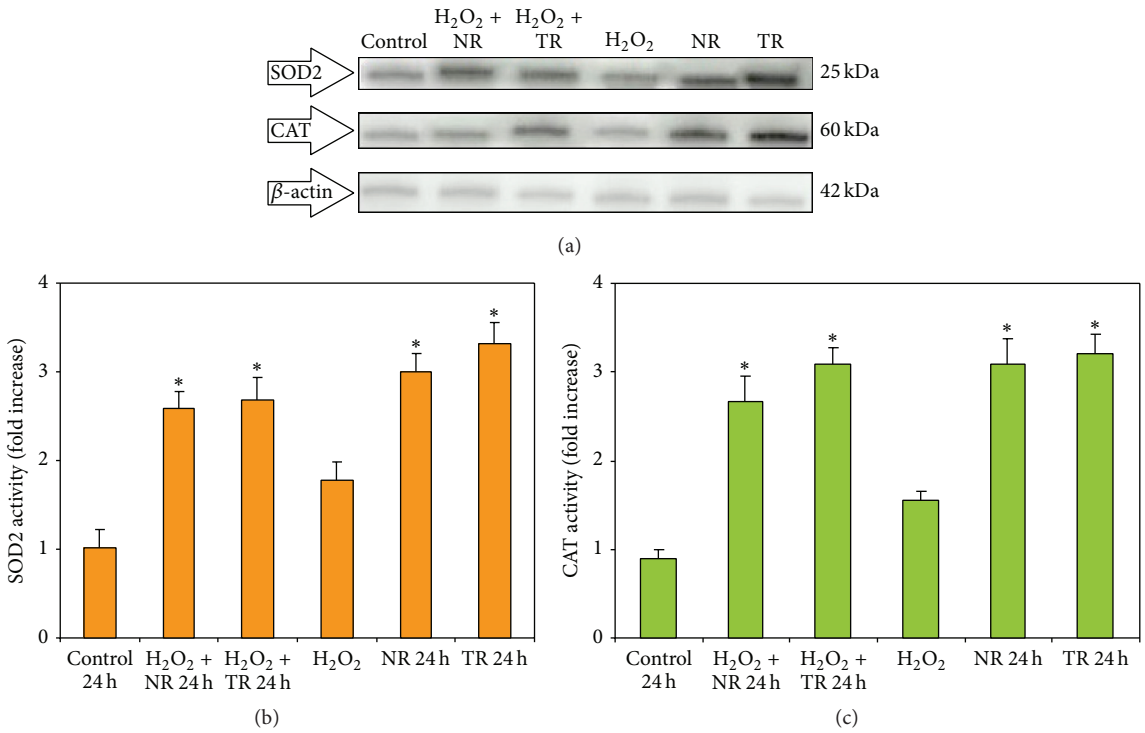

Figure 7: Effect of 1.0 mg/mL *R. carthamoides* TR and NR extracts (a) for 24 h on protein expression in the CHO cells. Before treatment with plant extracts, the cells were exposed to H_2O_2 at 50 μM for 10 min. (a) Western blot analysis was performed using SOD2 and CAT antibodies. Representative gels from three independent experiments are shown. The bar graph shows a semiquantitative comparison of SOD2 (b) and CAT (c) to β-actin optical density ratio. Results were represented as means ± SE from three independent experiments. $^*p < 0.001$ as compared to TR and NR extract with the control (cells incubated with PBS). Control 24 h: cells incubated for 24 h with PBS; H_2O_2: cells incubated with H_2O_2; NR 24 h: cells incubated for 24 h with NR extract; TR 24 h: cells incubated for 24 h with TR extract; H_2O_2 + NR 24 h: cells treated with H_2O_2 before 24 h incubation with NR extract; H_2O_2 + TR 24 h: cells treated with H_2O_2 before 24 h incubation with TR extract.

against oxidative damage. The SOD and CAT proteins induce the activation of the endogenous antioxidant defense system against free radicals [34]. It is known that SOD2 catalyzes the dismutation of the superoxide radical into oxygen and hydrogen peroxide [35]. The toxic hydrogen peroxide is subsequently converted to water and nonreactive oxygen species by CAT thus preventing the generation of hydroxyl radicals and protecting the cell from oxidative damage [2, 36]. One catalase molecule can convert approximately 6 million molecules of hydrogen peroxide to water and oxygen per minute [37]. Hydrogen peroxide is not reactive enough to introduce damage to the lipid chain, but when combined with superoxide radical products, which are highly reactive, it is able to initiate the lipid peroxidation [38]. This lipid peroxidation damages the cell membrane, resulting in the development of several physiological and pathological disorders.

4. Conclusions

Our study suggests that both transformed root extract and extract of soil-grown plant roots may stimulate CHO cells to repair oxidative DNA damage and protect DNA from lesions by increased antioxidant gene and protein expression levels (SOD2 and CAT) regulating intracellular antioxidant capacity. Additionally, the extract from the transformed root demonstrated stronger properties than the soil-grown plant roots extract. The mechanisms underlying this interaction may be complex and need further research.

Conflict of Interests

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

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References

[1] A. B. Robertson, A. Klungland, T. Rognes, and I. Leiros, "Base excision repair: the long and short of it," *Cellular and Molecular Life Sciences*, vol. 66, no. 6, pp. 981–993, 2009.

[2] P. Chelikani, I. Fita, and P. C. Loewen, "Diversity of structures and properties among catalases," *Cellular and Molecular Life Sciences*, vol. 61, no. 2, pp. 192–208, 2004.

[3] B. R. Goyal, S. Mahajan, R. Mali, G. K. Ramesh, and M. A. Anita, "Beneficial effect of *Achyranthes apsers* Linn. in toluene diisocynate induced occupational asthma in rats," *Global Journal of Pharmacology*, vol. 1, no. 1, pp. 6–12, 2007.

[4] B. N. Ames, M. K. Shigenaga, and T. M. Hagen, "Oxidants, antioxidants, and degenerative diseases of aging," *Proceedings of the National Academy of Sciences*, vol. 90, no. 17, pp. 7915–7922, 1993.

[5] M. Stankovic, M. Topuzovic, S. Solujic, and V. Mihailovic, "Antioxidant activity and concentration of phenols and flavonoids in the whole plant and plant parts of *Teucrium chamaerdys* L. var. Glanduliferum Haussk," *Journal of Medicinal Plants Research*, vol. 4, no. 20, pp. 2092–2098, 2010.

[6] A. Gugliucci and D. H. M. Bastos, "Chlorogenic acid protects paraoxonase 1 activity in high density lipoprotein from inactivation caused by physiological concentrations of hypochlorite," *Fitoterapia*, vol. 80, no. 2, pp. 138–142, 2009.

[7] L. Kokoska and D. Janovska, "Chemistry and pharmacology of *Rhaponticum carthamoides*: a review," *Phytochemistry*, vol. 70, no. 7, pp. 842–855, 2009.

[8] N. P. Timofeev, A. A. Lapin, and V. N. Zelenkov, "Quality assessment of *Rhaponticum carthamoides* (Willd.) Iljin as medicinal raw material by the bromic antioxidant capacity," *Journal Chemistry and Computation Simulation: Bulterov Communications*, vol. 8, no. 2, pp. 5–40, 2006.

[9] G. Miliauskas, T. A. van Beek, P. de Waard, R. P. Venskutonis, and E. J. R. Sudhölter, "Identification of radical scavenging compounds in *Rhaponticum carthamoides* by means of LC-DAD-SPE-NMR," *Journal of Natural Products*, vol. 68, no. 2, pp. 168–172, 2005.

[10] G. Miliauskas, P. R. Venskutonis, and T. A. van Beek, "Screening of radical scavenging activity of some medicinal and aromatic plant extracts," *Food Chemistry*, vol. 85, no. 2, pp. 231–237, 2004.

[11] E. Biskup and E. Lojkowska, "Evaluation of biological activities of *Rhaponticum carthamoides* extracts," *Journal of Medicinal Plants Research*, vol. 3, no. 12, pp. 1092–1098, 2009.

[12] E. Biskup, B. Szynklarz, M. Golebiowski, K. Borsuk, P. Stepnowski, and E. Lojkowska, "Composition and biological activity of *Rhaponticum carthamoides* extracts obtained from plants collected in Poland and Russia," *Journal of Medicinal Plants Research*, vol. 7, no. 11, pp. 687–695, 2013.

[13] V. Koleckar, L. Opletal, E. Brojerova et al., "Evaluation of natural antioxidants of *Leuzea carthamoides* as a result of a screening study of 88 plant extracts from the European Asteraceae and Cichoriaceae," *Journal of Enzyme Inhibition and Medicinal Chemistry*, vol. 23, no. 2, pp. 218–224, 2008.

[14] E. Skała, A. Kicel, M. A. Olszewska, A. K. Kiss, and H. Wysokinska, "Establishment of hairy root cultures of *Rhaponticum carthamoides* (Willd.) Iljin for the production of biomass and caffeic acid derivatives," *BioMed Research International*, vol. 2015, Article ID 181098, 11 pages, 2015.

[15] G. B. Lloyd and B. H. McCown, "Commercially-feasible micropropagation of mountain lamel *Kalmia latifolia* by use of shoottip culture," *Combined Proceedings—International Plant Propagators' Society*, vol. 30, pp. 421–427, 1980.

[16] M. B. Hansen, S. E. Nielsen, and K. Berg, "Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill," *Journal of Immunological Methods*, vol. 119, no. 2, pp. 203–210, 1989.

[17] J. Blasiak, E. Synowiec, J. Tarnawska, P. Czarny, T. Poplawski, and R. J. Reiter, "Dental methacrylates may exert genotoxic effects via the oxidative induction of DNA double strand breaks and the inhibition of their repair," *Molecular Biology Reports*, vol. 39, no. 7, pp. 7487–7496, 2012.

[18] M. Bjørås, L. Luna, B. Johnsen et al., "Opposite base-dependent reactions of a human base excision repair enzyme on DNA containing 7,8-dihydro-8-oxoguanine and abasic sites," *The EMBO Journal*, vol. 16, no. 20, pp. 6314–6322, 1997.

[19] S. Boiteux and J. P. Radicella, "Base excision repair of 8-hydroxyguanine protects DNA from endogenous oxidative stress," *Biochimie*, vol. 81, no. 1-2, pp. 59–67, 1999.

[20] M. Dizdaroglu, J. Laval, and S. Boiteux, "Substrate specificity of the *Escherichia coli* endonuclease III: excision of thymine- and cytosine-derived lesions in DNA produced by radiation-generated free radicals," *Biochemistry*, vol. 32, no. 45, pp. 12105–12111, 1993.

[21] Z. Hatahet, Y. W. Kow, A. A. Purmal, R. P. Cuuuingham, and S. S. Wallace, "New substrates for old enzymes. 5-hydroxy-2′-deoxycytidine and 5-hydroxy-2′-deoxyuridine are substrates for *Escherichia coli* endonuclease III and formamidopyrimidine DNA N-glycosylase, while 5-hydroxy-2′-deoxyuridine is a substrate for uracil DNA N-glycosylase," *Journal of Biology Chemistry*, vol. 269, no. 29, pp. 18814–18820, 1994.

[22] N. P. Singh, M. T. McCoy, R. R. Tice, and E. L. Schneider, "A simple technique for quantitation of low levels of DNA damage in individual cells," *Experimental Cell Research*, vol. 175, no. 1, pp. 184–191, 1988.

[23] M. Klaude, S. Eriksson, J. Nygren, and G. Ahnström, "The comet assay: mechanisms and technical considerations," *Mutation Research—DNA Repair*, vol. 363, no. 2, pp. 89–96, 1996.

[24] P. Sitarek, E. Skała, H. Wysokińska et al., "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," *Oxidative Medicine and Cellular Longevity*, vol. 2016, Article ID 5738193, 11 pages, 2016.

[25] M. S. Islam, M. Yoshimoto, and O. Yamakawa, "Distribution and physiological functions of caffeoylquinic acid derivatives in leaves of sweetpotato genotypes," *Journal of Food Science*, vol. 68, no. 1, pp. 111–116, 2003.

[26] S. M. Kim, J.-S. Jeon, S. W. Kang, Y.-J. Jung, L. N. Ly, and B.-H. Um, "Content of antioxidative caffeoylquinic acid derivatives in field-grown *Ligularia fischeri* (Ledeb.) Turcz and responses to sunlight," *Journal of Agricultural and Food Chemistry*, vol. 60, no. 22, pp. 5597–5603, 2012.

[27] T. Matsui, S. Ebuchi, T. Fujise et al., "Strong antihyperglycemic effects of water-soluble fraction of Brazilian propolis and its bioactive constituent, 3,4,5-tri-*O*-caffeoylquinic acid," *Biological and Pharmaceutical Bulletin*, vol. 27, no. 11, pp. 1797–1803, 2004.

[28] M. Yoshimoto, S. Yahara, S. Okuno, M. S. Islam, K. Ishiguro, and O. Yamakawa, "Antimutagenicity of mono-, di-, and tricaffeoylquinic acid derivatives isolated from sweetpotato (*Ipomoea batatas* L.) leaf," *Bioscience, Biotechnology and Biochemistry*, vol. 66, no. 11, pp. 2336–2341, 2002.

[29] P.-G. Pietta, "Flavonoids as antioxidants," *Journal of Natural Products*, vol. 63, no. 7, pp. 1035–1042, 2000.

[30] R. J. Nijveldt, E. van Nood, D. E. C. van Hoorn, P. G. Boelens, K. van Norren, and P. A. M. van Leeuwen, "Flavonoids: a review of probable mechanisms of action and potential applications," *American Journal of Clinical Nutrition*, vol. 74, no. 4, pp. 418–425, 2001.

[31] G. Agati, E. Azzarello, S. Pollastri, and M. Tattini, "Flavonoids as antioxidants in plants: location and functional significance," *Plant Science*, vol. 196, pp. 67–76, 2012.

[32] L. O. Dragsted, "Antioxidant actions of polyphenols in humans," *International Journal for Vitamin and Nutrition Research*, vol. 73, no. 2, pp. 112–119, 2003.

[33] Y. M. Shi, W. F. Wang, C. Y. Huang, S. D. Jia, and R. L. Zheng, "Fast repair of oxidation DNA damage by phenylopropanoid glycosides and their analogues," *Mutagenesis*, vol. 23, no. 1, pp. 19–26, 2008.

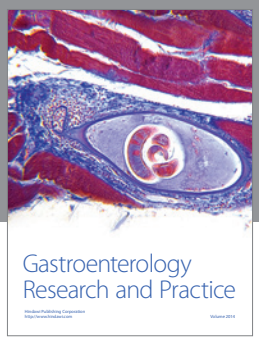
[34] F. J. Menvielle-Bourg, "Superoxide dismutase (SOD), a powerful antioxidant, is now available orally," *Phytothérapie*, no. 5, pp. 1–4, 2005.

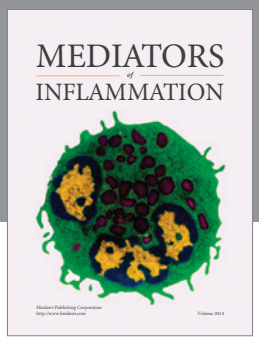
[35] I. Fridovich, "Superoxide radical and superoxide dismutases," in *Oxygen and Living Processes, An Interdisciplinary Approach*, D. L. Gilbert, Ed., pp. 250–272, Springer, Berlin, Germany, 1st edition, 1981.

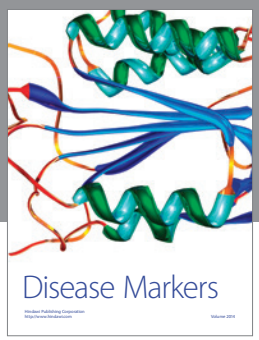
[36] M. Zámocký and F. Koller, "Understanding the structure and function of catalases: clues from molecular evolution and in vitro mutagenesis," *Progress in Biophysics & Molecular Biology*, vol. 72, no. 1, pp. 19–66, 1999.

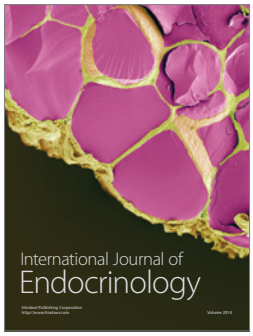
[37] K. Rahman, "Studies on free radicals, antioxidants, and co-factors," *Clinical Interventions in Aging*, vol. 2, no. 2, pp. 219–236, 2007.

[38] B. Lubec, M. Hayn, W. Denk, and G. Bauer, "Brain lipid peroxidation and hydroxy radical attack following the intravenous infusion of hydrogen peroxide in an infant," *Free Radical Biology and Medicine*, vol. 21, no. 2, pp. 219–223, 1996.









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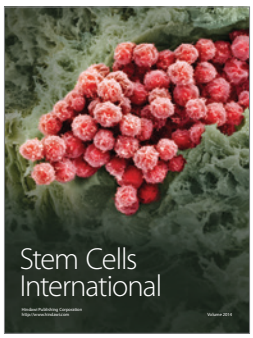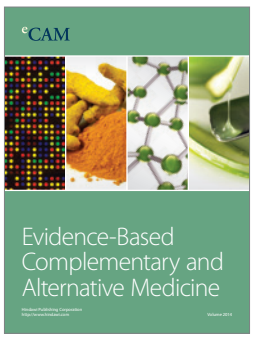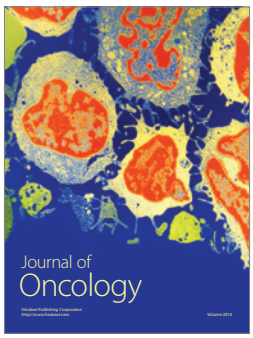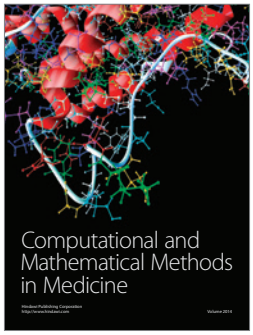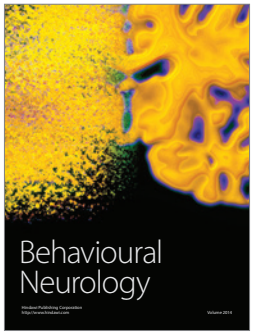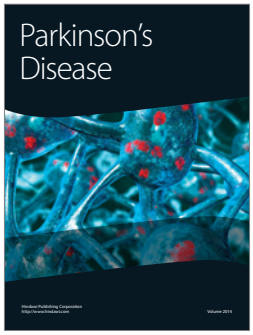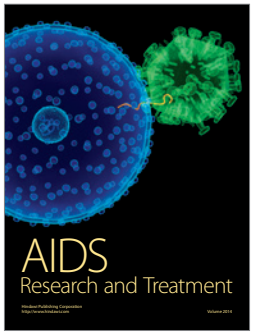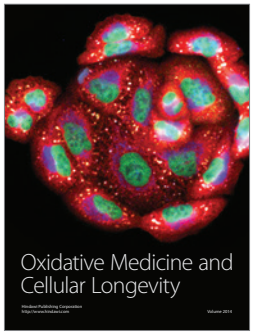
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