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ORIGINAL ARTICLE

Transformed Root Extract of *Leonurus sibiricus* Induces Apoptosis through Intrinsic and Extrinsic Pathways in Various Grades of Human Glioma Cells

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Abstract This study determines the influence of transformed root (TR) extract of *Leonurus sibiricus* L. on various grades (I-III) of human glioma cells derived from patients. This plant occurs in southern Asia and Siberia and is widely used as a medicinal plant with various biological activities. Chromatographic profile of TR extract have revealed the presence of various polyphenolic compounds (4-hydroxybenzoic acid, gentisic acid, vanilic acid, 1,3-dicaffeoylquinic acid, α-resorcylic acid). We found TR root extract to have antiproliferative activity on glioma cells after 24 h of treatment. TR root extract induces apoptosis on various grades (I-III) of human glioma cells by the generation of reactive oxygen species (ROS) along with concurrent loss of mitochondrial membrane potential, enhanced S and G2/M phases of the cell cycle, and altered mRNA levels of Bax, Bcl-2, p53, Cas-3, Cas-8 and Cas-9 factors involved in apoptosis. This work for the first time demonstrate that TR extract from *L. sibiricus* root has the potential to activate apoptosis in grade I-III human glioma cells through the intrinsic and extrinsic pathways.

Keywords Apoptosis · Cell cycle · Gene expression · Transformed roots of *Leonurus sibiricus* · Mitochondrial membrane potential · Reactive oxygen species

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Introduction

Nowadays, cancer is one of the leading causes of death due to its profound complex nature. The prognosis of patients with glioma is closely related to the World Health Organization (WHO) tumour grade. Currently, one of the most dangerous brain tumours is glioblastoma multiforme (GBM), WHO IV. With a high recurrence rate, high mortality and low cure rate, it has a very poor prognosis [1–4]. Due to infiltrative nature and frequent involvement in intricate regions of the brain, complete surgical removal is usually impossible, and adjuvant therapies, such as radiotherapy and chemotherapy, are often used as an attempt to control the disease. Consequently, the two-year survival rate is 7.5%, and five-year survival rate only 5% [5]. The discovery of more effective agents to treat glioma is becoming increasingly urgent. In the recent years, natural plant products may be used for the treatment of various cancers, as these natural products could represent a source of secondary metabolites and be used in the development of new chemotherapeutic drugs. A number of studies have demonstrated apoptosis

induction in various cancer cells following treatment with the plant extract [6–8]. *Leonurus sibiricus* L. (Lamiaceae), traditionally known as Chinese motherwort, is a plant which occurs in several regions in Asia, including China, Korea, Japan and Cambodia up to southern Siberia. It has been used widely in folk medicine and possesses various biological activities, including anti-inflammatory, antibacterial, antioxidant, antiviral and anticancer effects [9, 10]. Our previous study found transformed root extract of *L. sibiricus* to have anti-proliferative activity against IV grade glioma [11]. These pharmacological properties are thought to be associated with the polyphenolic compounds contained in transformed roots of *L. sibiricus*, but their mechanism is not yet fully understood. In current reports we hypothesize that polyphenolic compounds content in TR root extract of *L. sibiricus* may induce apoptosis in glioma cells in various grades (I-III) by arresting cell cycle in the S and G2/M phase, enhancing expression of apoptotic genes, increasing ROS and reducing mitochondrial membrane potential. Additionally, in this study we evaluated phytochemical profile of *L. sibiricus* TR root extract.

Materials and Methods

Plant Material and Confirmation of Transformation Roots of *L. sibiricus*

Transformed (TR) and non-transformed (NR) roots of *L. sibiricus* were used in this study. The transformed roots were obtained by infection of five-week-old in vitro shoots of *L. sibiricus* with *Agrobacterium rhizogenes* strain A4. Establishment of transformed (TR) and non-transformed (NR) root cultures has been described previously by Sitarek et al. [11]. TR and NR roots were grown in liquid SH medium [12], either without growth regulators or supplemented with 0.2 mg/L indole-3-butyric acid (IBA), respectively. The roots were cultured in 300 mL Erlenmeyer flasks containing 80 mL of SH medium, 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}. PCR (polymerase chain reaction) performed according to Skała et al. was used to confirm transformation of the *rol*B and *rol*C genes to T-DNA. [13].

Preparing Extracts from Transformed and Non-transformed Roots of *L. sibiricus*

Lyophilized and powdered TR and NR roots (about 10 g dry weight, each) were used for extraction. The roots were extracted 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. Finally, 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 tested extracts were 52.5% for TR extract and 47.3% for NR extract, in terms of initial crude plant material dry weight [11].

HPLC and LC-MS/MS Analyses

Chemical analysis of TR- and NR-extracts was performed as described earlier by Sitarek et al., [14]. Phenolic compounds (phenolic acids and flavonoids) were identified by LC-MS/MS and their contents were determined by HPLC according to Sitarek et al. [14]. 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 a 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. 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. 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. Standards of HPLC-grade purity (≥96%) were purchased from Sigma-Aldrich (Germany/USA).

Cell Culture

Human glioma primary cells, derived from surgical specimens, were established in the Department of Molecular Genetics. The tumors were obtained from 3 patients; astrocytoma-I grade, astrocytoma-II grade, astrocytoma-III grade treated in the Department of Neurosurgery, Surgery of Spine and Peripheral Nerves, University Hospital WAM-CSW, Medical University of Łódź. Tumor tissue was histopathologically examined and classified as the classic histotype, according to the World Health Organization (WHO) classification of tumors of the central nervous system [15]. The study was approved by the Ethical Commission of the Medical University of Lodz, and informed consent was obtained from the patients. Briefly, the cell culture conditions are described in an earlier study [11]. Normal human astrocytes (NHA) (CC-2565, Lonza) were cultured according to the manufacturer's protocol.

MTT Cell Viability Assay

The MTT assay [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] was used to assess cell viability [16]. Human glioma cell lines and human astrocytes seeded onto 96-well microplates at 1×10^5 cells/100 μL per well were

incubated with TR root extract (0.1–1.5 mg/ml) for 24 and 48 h. The medium was then removed, and the cells were incubated for four hours with 100 μL of MTT solution (5 mg/mL MTT in PBS). The plates were read in a microplate spectrophotometer at 550 nm. The IC_{50} were determined from a dose-response curve created using a range of root extract concentrations (0–1.5 mg/mL). All experiments were performed in triplicate for each concentration.

Analysis by Flow Cytometry of Human Glioma Cells Treated with TR Extract of *L. sibiricus*

Cell Cycle Analysis

The human glioma cells in various grades (4×10^5 cells) were incubated (37° C, 5% CO_2) in 6-well plates with *L. sibiricus* TR extract, while controls were incubated under the same conditions with DMSO vehicle (0.1–0.3%). After treatment with plant extract at IC_{50} concentration for 24 h, cells were stained with propidium iodide, incubated for 30 min in the dark then analyzed by flow cytometry (Becton-Dickinson). Further analyses were performed as described previously by Sitarek et al. [11]. All experiments were performed in triplicate.

Induction of Apoptosis in Glioma Cells

An Annexin V-FITC apoptosis detection kit was used for apoptotic cell quantification by Annexin V-FITC and Propidium iodide (PI) double staining, according to manufacturer's protocols. All cells were seeded in 6-well culture plates at a density of 1×10^5/well, and then incubated with *L. sibiricus* TR root extract (0.25–1.5 mg/ml) for 24 h. Further procedure and analyses were performed as described previously by Sitarek et al. [11]. All experiments were performed in triplicate.

Reverse Transcriptase-PCR Analysis for *Bcl-2, Bax, p53, Caspase 3, Caspase 8 and Caspase 9*

The study examines the expression level of the apoptosis-related genes *Bcl-2, Bax*, *p53, caspase 3, caspase 8 and caspase 9* using reverse transcriptase-PCR (RT-PCR). Various grades of human glioma cells (I-III) at a concentration of 4×10^5 cells in a total volume of 3 mL were seeded in 6-well multi dishes. These were then incubated in the presence of TR root extract for 24 h at 37 °C. The concentration which caused the highest percentage of apoptosis was chosen for further study. The following sequences of primers were used: 5'TCACAGCAAAAGGAGCAGTTT 3′, 5'CGTCAAAG GAAAAGGACTCAA 3′ for the *caspase 3* gene; 5′ CTACCAACTCATGGACCACAG 3′, 5′ GTGACTGG ATGTACCAGGTTC 3′ for the for *caspase 8* gene; 5′ GAGTCAGGCTCTTCCTTTG 3′, 5'CCTCAAAC TCTCAAGAGCAC 3′ for *caspase 9*. Further procedures, PCR conditions and primers for *Bcl-2, Bax*, *p53,* are described in a previous study by Sitarek et al. [11].

Examination of Mitochondrial Membrane Potential Using JC-1 Staining

The human glioma cells (grades I-III) were seeded on 6-well plates at a density of 1×10^5 cells/well in 2 mL medium in 10% FBS. After incubation for 24 h, the cells were treated with TR root extract for 24 h. The cells (1×10^5) in each group were "collected" after trypsinization with 0.25% trypsin and were incubated with 10 mg/mL JC-1 (1,1,3,3-tetraethylbenzimidazolcarbocyanine iodide) dye (Invitrogen) at 37 °C and 5% CO_2 for 30 min. To examine the loss of mitochondrial membrane potential, the cells were stained with fluorescent JC-1 dye. The JC-1 dye is a cationic dye that indicates mitochondrial potential during the apoptosis of cells by shift of fluorescence emission from green to red [17]. The resulting fluorescence was measured on a Fluoroskan Ascent plate reader with filter pairs of 530 nm/590 nm and 485 nm/538 nm.

ROS Formation Assay

Reactive oxygen species (ROS) concentration was determined using a fluorescent probe, dichlorodihydrofluorescein diacetate (H_2DCF-DA), as previously described [18]. H_2DCF-DA diffuses through the cell membrane, where it is enzymatically hydrolysed by intracellular esterases and oxidized to the fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of ROS. The intensity of fluorescence is proportional to the levels of intracellular oxidant species. Briefly, glioma cells were seeded in 96-well plates and incubated with TR extract for 24 h. The cells were then incubated with 5 μM H_2DCF-DA at 37 °C for 30 min and ROS fluorescence (DCF) was measured using a Fluoroskan Ascent FL microplate reader.

Statistical Analysis

Statistical analysis was performed using STATISTICA 12.0 software (StatSoft Poland), with results reported as means ± SD of at least triplicate experiments. Significant differences between the treated groups and control were evaluated by performing a one-way analysis of variance (ANOVA). Where ANOVA showed significant differences, it was followed by a Mann-Whitney U-test or Student's t-test for comparison. $p \leq 0.05$ was considered to be statistically significant.

Results

Phenolic Acid and Flavonoids in Non-transformed (NR) and Transformed (TR) Root Extracts of *L. sibiricus*

Our previous study reported that phenolic acids and flavonoids are the main groups of the secondary metabolites in non-transformed and transformed *L. sibiricus* roots [11]. The content of phenolic compounds ranged from 8.5 to 18 mg/g DW, respectively. In this work we identified additionally five new phenolic acids: vanilic acid (5), 4-hydroxybenzoic acid (3), α-resorcylic acid (1), gentisic acid (4), 1,3-dicaffeoylquinic acid (6) and two flavonoids: (+)-catechin (2) and rutin (7) in *L. sibiricus* 80% aqueous methanol root extracts (Table 1). The HPLC examination revealed statistically significant differences between transformed and non-transformed roots regarding the phenolic acid and flavonoid concentrations ($p<0.05$) (Table 1): The total phenolic acid concentrations were 19.4 mg/g DW in the non-transformed and 34 mg/g DW in the transformed root extracts (Table 1) [11]. Chlorogenic acid (4–10 mg/g DW) [11] and vanilic acid (2.5–3.4 mg/g DW) (Table 1) were a major component in both extracts. Moreover, flavonoid production was 1.5-times higher in transformed roots (5.1 mg/g DW) than the non-transformed roots (3.7 mg/g DW). Rutin was a dominant compound of flavonoids.

Cytotoxic Effect and Induction of Apoptosis by Cell Cycle Arrest in S and G2/M Phase after Treatment of TR Root Extract in Various Grades (I-III) of Gliomas

As the content of active compounds contained in the TR extract was higher than that of the NR extract, the TR extract was chosen for further study. Grade I-III human glioma primary cultures were treated with TR extract at increasing concentrations (0–1.5 mg/mL) for 24 or 48 h (Fig. 1a). A dose-dependent relationship was observed between the applied extract and glioma cell grade: although a cytotoxic effect was observed for all grades of glioma primary cells, significantly higher cytotoxicity was observed for grades I and II. In comparison to the control cells, 24-h treatment with the TR extract decreased the viability of glioma primary cells for grades I and II. As no significant difference as observed between longer incubation times (24 and 48 h), the 24-h period was chosen for subsequent experiments (Fig. 1a).

TR extract did not affect the viability of normal astrocyte cells (NHA) when treated with TR root extract at 0–1.5 mg/mL for 24 h and 48 h. The dependency on dose and progression grade obtained from the MTT assay is given in Fig. 2a.

Flow cytometric analysis was used to investigate the effect of TR extract on cell cycle progression for grade I to III glioma cells. Greater occurrence of S phase and G2/M phase was found in all grades (I-III) after 24-h treatment with the TR extract ($p < 0.05$), with this being more apparent at grades I and II than grade III (Fig. 1b).

It was further verified that TR root extract induces apoptosis by using flow cytometry to examine the number of apoptotic cells (Fig. 1c) after staining the different grades (I-III) of glioma cells with FITC-Annexin V and PI. After treatment with TR extract, the proportions of both early and late apoptotic cells for all grades (I-III) ranged from 52 to 55%, and were dependent on grade progression and dose (Fig. 1c). When grade I and II glioma cells were treated with TR root extract, the proportion of apoptotic cells increased in a dose-dependent manner. The same percentages of apoptotic cells were observed at higher extract concentrations for the grade III glioma cells (Fig. 1c).

Down-Regulation or Up-Regulation of Genes Involved in Apoptosis in Various Grades (I-III) of Glioma Cells after Treatment of *L. sibiricus* Root Extract

To investigate the molecular mechanism of TR-induced apoptosis in various grades (I-III) glioma cells, the expression of several apoptosis-related genes were examined following after 24-h exposure. The most effective concentration (IC_{50}) of TR root extracts was chosen based on the results of a previous study. The mRNA level of anti-apoptosis gene Bcl-2 (Fig. 2a, b) decreased and the levels of pro-apoptosis genes Bax and p53 (Fig. 2a, b) were up-regulated in glioma cells treated with TR root extract after 24 h. Caspase-9 and -3 and caspase-8 were also up-regulated, which could contribute to apoptosis. Densitometry analyses showed the level of expression depending on progression grade of glioma. The increase of expression for grade I and II and mRNA levels was observed. All samples were unified using *GAPDH* as an intrinsic control.

Increased of ROS and Loss of Potential Mitochondrial in Various Grades (I-III) of Glioma Cells after Treatment of *L. sibiricus* Root Extract

Fluorescence (DCF) analysis was used to determine the level of ROS production and $\Delta\Psi m$ level induced in various grades of glioma cells (I-III) after treatment with TR root extract (Fig. 3a). ROS are generated in and around the mitochondria and are regarded as the by-products of normal cellular oxidative processes. Our findings indicate a significant increase (about 4-fold) in intracellular ROS in cells treated with TR extract after 24 h in all three grades of tested glioma cells in comparison to controls. In addition, no significant differences were observed between tested glioma cells. Mitochondria play an important role in the propagation of apoptosis and are believed to be responsible for 90% of the energy needed for cell function. Additionally, after 24 h, the TR extract was found to significantly reduce the level of $\Delta\Psi m$ in all tested grades (I-

Table 1 Compounds identified in 80% aqueous methanol extracts from non-transformed (NR) and transformed roots (TR) of *L. sibiricus* and contents of phenolic compounds in NR and TR extracts using HPLC-ESI-MS/MS.

Peak No.	Phenolic compounds	λ_{max} (nm)	RT (min)	Molecular (precursor) ion $[M-H]^-$ (m/z)	NR mg/g DW	TR mg/g DW
1	α-resorcylic acid	226, 248, 306	12.05	153	1.025 ± 0.0311^a	1.410 ± 0.0511^b
2	(+)-Catechin	230, 278	15.73	289	0.551 ± 0.0224^a	1.581 ± 0.0689^b
3	4-Hydroxybenzoic acid	211, 253	16.35	137	1.533 ± 0.0622^a	2.231 ± 0.088^b
4	Gentisic acid	213, 239, 328	16.83	153	1.125 ± 0.0325^a	1.566 ± 0.0612^b
5	Vanilic acid	257, 290	18.56	167	2.496 ± 0.098^a	3.399 ± 0.225^b
6	1,3-Dicaffeoylquinic acid	241, 294, 231	20.04	515	0.935 ± 0.0196^a	4.255 ± 0.312^b
7	Rutin	255, 265, 352	27.26	609	3.288 ± 0.200^a	4.255 ± 0.312^b
8	**Sum of phenolic acids**	-	-	-	7.594 ± 0.2332	11.548 ± 0.5463
9	**Sum of flavonoids**	-	-	-	3.288 ± 0.122	4.255 ± 0.112

Different superscript letter within the rows indicate significant differences in the mean values at $p < 0.05$

III) (by about 2-fold) in comparison to controls (Fig. 3b). This result confirms that TR extract induced apoptosis through the disruption of mitochondrial membrane potential. This reduction in mitochondrial membrane potential may well initiate the apoptotic cascade in the cells treated with TR extract.

Discussion

Gliomas are still treated by means of possibly radical surgery followed by radiotherapy and chemotherapy. Nevertheless, conventional drugs, such as temozolomide, are known to have therapeutic benefits in prolonging the survival of patients [19]. However, the use of these drugs is further complicated by their heterogeneity, poor prognosis, poor penetration through the blood-brain barrier, and unsuccessful chemotherapy and radiotherapy regimes. Hence, there is a strong demand for developing effective and alternate therapeutic strategies and reagents. Interest in the use of phytocompounds to develop safe and more effective therapeutic agents for cancer treatment has been growing over recent years [20, 21]. In the last few decades, many traditional Chinese herbs with anti-tumor effects have drawn a great degree of attention due to their efficiency, lack of drug resistance, and low levels of toxicity and side effects [22]. One such plant, *L. sibiricus*, has a long history of use as a popular folk medicine. Following on from our previous studies, which showed that extract of *L. sibiricus* induced apoptosis in IV grade glioma [11], the aim of the present work is to further clarify the anti-proliferative potential of treatment with TR extract on various grades (I-III) of patient-derived glioma cells by increased expression of apoptosis genes. The study investigates several parameters associated with the intrinsic and extrinsically-mediated apoptosis pathways in glioma cells to clarify the potential mechanism of apoptosis induction employed by TR extract in glioma cells: (a) apoptosis mediated by cell-cycle arrest, (b) the genes involved in apoptosis, (c) apoptosis mediated by mitochondria-involved signalling and (d) reactive oxygen species (ROS) induced apoptosis. Apoptosis may induced by sequence communication between the caspases, genes and death receptors of intrinsic and extrinsic pathways [23]. As the side effects of *L. sibiricus* TR extract are modest and well tolerated, it may be suitable for use in chemotherapy and cancer treatment.

The MTT analysis confirms that after 24-h treatment, the extract has a cytotoxic effect on all grades (I-III) of glioma cells at lower concentrations than observed for grade IV [11] and this effect is dependent on tumor grade. The IC_{50} concentration for the tested TR extract against grade IV glioma cells was previously found to be about 2 mg/ml [11]. In present study, the IC_{50} concentration decrease to 1 mg/ml for grades I and II, and to 1.5 mg/ml for grade III. Additionally, no cytotoxic effect was found on normal astrocytes cells at the same tested concentrations: the IC_{50} concentration for these cells being above 5 mg/ml.

In our previous study, the chromatographic profiles of *L. sibiricus* TR extract revealed a high presence of polyphenolic compounds such as ellagic acid, caffeic acid, chlorogenic acid and verbascoside. Additionally, in present work we identified other phenolic acids and flavonoids (catechin, gentisic acid, vanilic acid, α-resorcylic acid dicaffeoylquinic acid and rutin), with the total phenolic compound content being around 17 mg/g DW. This high total phenolic content within the TR extract may be responsible for the induction of apoptosis in glioma cancer: A number of bioactive molecules, including polyphenolic compounds, are known to have anti-proliferative properties [9]. The high anticancer activity of phenolic extract can be attributed to a synergistic effect between different compounds present in the extract [24]. Such synergistic anticancer effects have previously been observed between two or more phenolic or

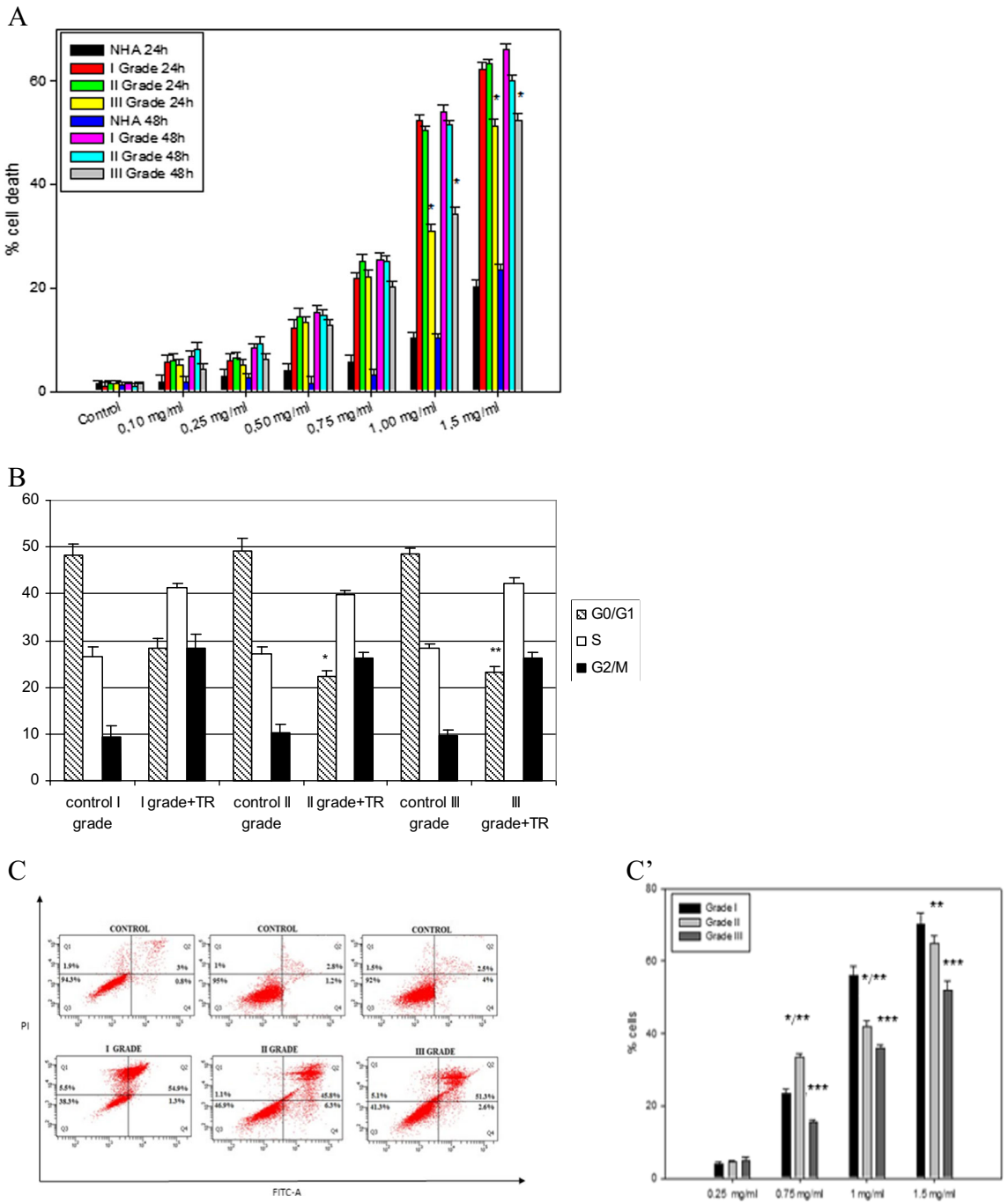

Fig. 1 **a** MTT assay of *L. sibiricus* TR root extract in glioma cells (grades I-III) and normal human astrocytes (NHA). Cells were treated with TR root extract at various concentrations 0.10, 0.25, 0.50, 0.75, 1 and 1.5 mg/ml for 24 and 48 h. The data represent the means ± SD of three independent experiments. *$p < 0.05$ compared grade I with grade III in appropriate time (24 and 48 h). **b** Flow cytometry analysis on cell cycle progression in various grades (I-III) of glioma cells were carried out after incubation with TR root extract of *L. sibiricus* for 24 h. The data represent the means ± SD of triplicate independent experiments. *$p < 0.05$ compared grade I with grade II. **$p < 0.05$ compared grade I with III grade. **c** Representative histogram for flow cytometry analysis. Lower histograms show induction of early and late apoptosis by TR root extract of *L. sibiricus* indicating the percentage of early apoptotic, late apoptotic and necrotic cells after 24 h of treatment for IC_{50} concentration for each grades (I-III) glioma cells. Upper histograms show the cells treated with vehicle DMSO (control). The early and late apoptotic events are depicted in the lower right and upper left quadrant of each panel. C′ percentage of early and late apoptotic cells for various grades in cells after treatment of different concentration of TR extract. The data represent the means ± SD of three independent experiments.*$p < 0.05$ compared I grade with II grade.**$p < 0.05$ compared II grade with III grade. ***$p < 0.05$ compared I grade with III grade

flavonoid compounds present in plant extracts. For example, Mertens-Talcott et al. [25] demonstrate that quercetin and ellagic acid synergistically reduced viability and triggered apoptosis in human leukaemia MOLT-4 cells. Furthermore,

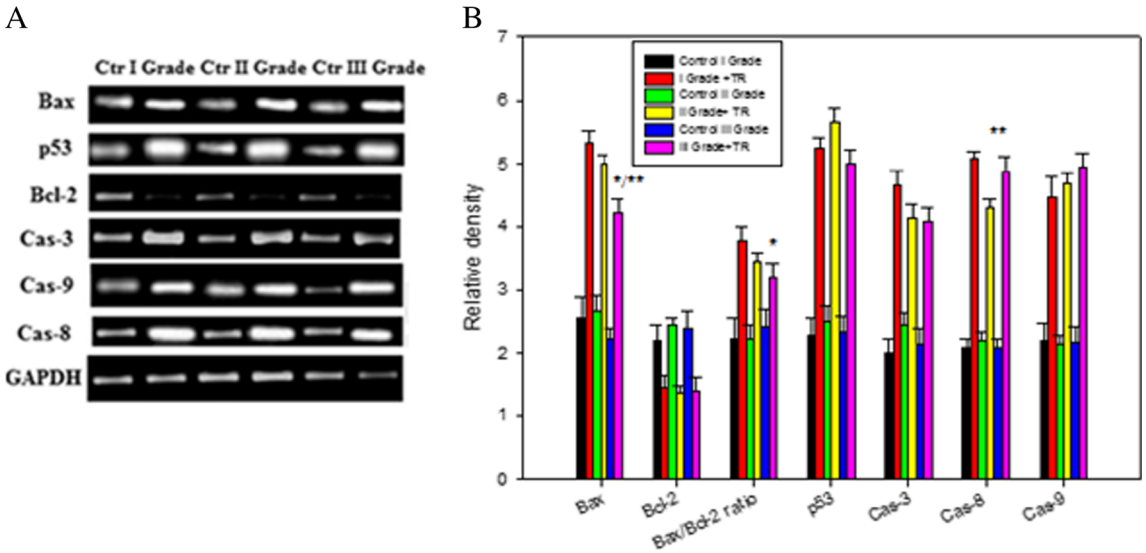

Fig. 2 Impact of TR root extract of *L .sibiricus* on gene expression. Glioma cells (grade I-III) were treated with IC_{50} concentration of TR for 24 h. A Expression of mRNA levels for apoptotic genes. B The bar graph quantitates the relative density. mRNA values are mean ± SD, $n = 3$. * $p < 0.05$ compared grade I with grade III, ** $p < 0.05$ grade II with grade III

Shimizu et al. [26] report that catechins inhibited the growth of human colon cancer HT-29 cells and triggered apoptosis [26]. Our studies suggest that polyphenolic compounds contained in the TR root extract of *L. sibiricus* have an anti-proliferative effect on all grades of glioma cells.

Apoptosis is believed to be deregulated in cancer, and so an ideal therapeutic aim, already used by existing antitumor drugs, is to stimulate the apoptotic pathway to selectively trigger death in tumour cells. Therefore, drugs that restore the apoptotic pathways have the potential for effective treatment of tumours. The mitochondrial apoptotic pathways and death receptor pathways are the two major pathways that have been characterized in mammalian cells. Mitochondria play a central role in regulating the caspase cascade and apoptosis [27] by releasing cytochrome c, which leads to the activation of procaspase-9 and then caspase-3 [27]. In addition, the main executors of apoptosis are the effector caspase, caspase-3, and the initiator caspases, caspase-8 and caspase-9 [28].

Our findings indicate that p53–mediated apoptosis [29] may be associated with the activation of caspases −3, −8 and −9 by the activation of both the extrinsic and intrinsic apoptotic pathways. The release of mitochondrial cytochrome c through Bax/Bcl-2 activation might activate the effector machinery engaged by p53 to mediate teratogen-induced apoptotic pathways. To gain a closer insight into molecular mechanism involved in the activation of apoptosis caused by *L*

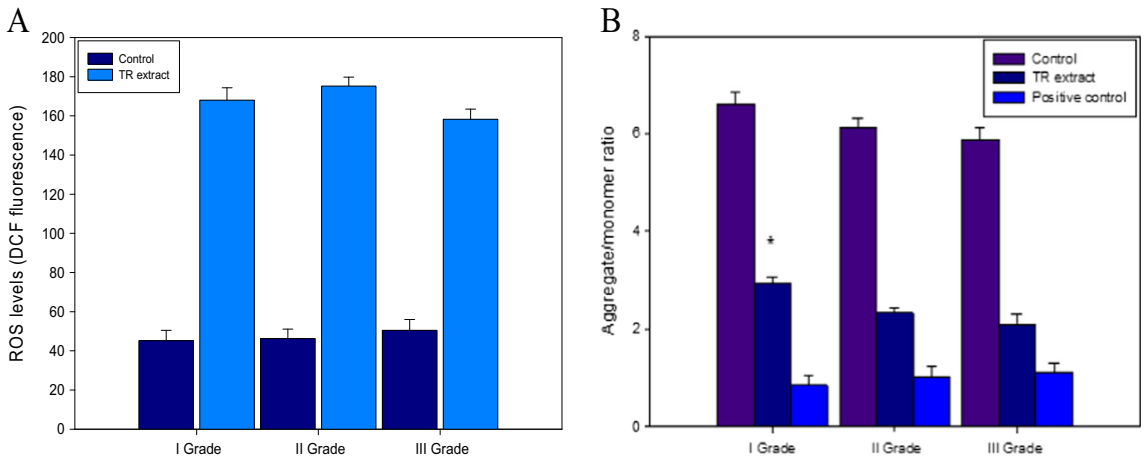

Fig. 3 **a** Effect of TR root extract of *L. sibiricus* on ROS generation in various grades (I-III) of human glioma cells. Representative bar chart indicated dose for IC_{50} elevation in ROS formation in treated glioma cells after 24 h. The data represents the means ± SD of three independent experiments. No significant differences were observed. **b** Mitochondrial membrane potential ($\Delta\Psi m$) of glioma cells in various grades. Cells were treated with concentrations for IC_{50} for each grade for 24 h. Untreated cells were used as controls. As a positive control was used Camptothecin. All of the experiments were performed at least three times from three independent experiments. The symbol * indicates a significant difference between grade I and grade III ($p < 0.05$)

sibiricus root extract, the expression of the apoptosis-related genes Bcl-2 and Bax, caspases −3, −8 and −9 and p53 levels were assessed in various grades of glioma cells. RT-PCR analyses indicate significantly increased mRNA levels for Bax, p53, caspase −3, −8 and - 9 for grades I-III after TR extract, as well as significantly decreased mRNA levels of Bcl-2 in glioma cells. These results are consistent with those of our previous study [11].

Flow cytometric analysis revealed that TR root extract induced apoptosis in glioma cells depending on concentration and grade. Although our previous findings indicate that TR root extract induces apoptosis in various grades of glioma cells depending on dose and progression grade, a greater percentage of apoptotic cells was observed in the present study: about 30% for grade IV, as noted previously [11], compared to 57% for grade I, 52% for grade II and 54% for grade III in the present study.

Several studies have reported that apoptosis involves the disruption of mitochondrial membrane integrity, which is decisive for the cell death process [27, 28, 30], and the depolarization of mitochondrial membrane potential, which is a characteristic feature of apoptosis. The measurement of mitochondrial membrane potential ($\Delta\psi m$) in glioma cells in the present study identified a roughly three-fold loss of fluorescence intensity. This result further confirms that mitochondria are one of the most important organelles in cells which play critical roles in the mitochondrial apoptosis signal transduction pathway [31–33]. After the reduction of membrane potential and the release of mitochondrial cytochrome c, a critical step is the formation of apoptosomes [33]. An increase in ROS has been implicated in the induction of apoptosis through its ability to promote the release of cytochrome c to facilitate caspase activation [34]. In the present study, glioma cells demonstrated increased ROS production (about four-fold) after treatment with *L. sibiricus* TR extract. Many studies have reported that elevated levels of ROS act as secondary messengers in apoptosis following induction by anti-cancer and chemopreventive agents [35]. The proposed mechanism of apoptosis demonstrates that, under the influence of the tested TR root extract, ROS accumulation is observed in glioma cells, resulting in the stimulation of Bax expression and reduction of Bcl-2. Furthermore, greater mitochondrial membrane potential activates caspase-9 and caspase-3, inducing apoptosis at all examined grades of glioma cells (I-III).

In conclusion, our findings suggest that TR root extract might penetrate into cells and directly target mitochondria to increase membrane permeability, decrease $\Delta\psi m$ accompanied, elevate ROS production, and induce apoptosis by modulating the expression of caspase-3, 8, 9, p53, Bax and Bcl-2. This can be seen in grades I to III of human glioma cells. Although, presented reports are consistent with those of our previous studies, a relationship between grade progression stage (I-IV) and the dose-dependent manner of TR extract are the novel findings. However, further investigations of *L. sibiricus* root extract based on in vivo study are necessary before it can be used as a promising chemotherapeutic agent.

Compliance with Ethical Standards

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

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