Sea Buckthorn Leaf Extract Inhibits Glioma Cell Growth by Reducing Reactive Oxygen Species and Promoting Apoptosis

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Abstract *Hippophae rhamnoides* L., also known as sea buckthorn (SBT), possesses a wide range of biological and pharmacological activities. However, the underlying mechanism is largely unknown. The present study examined whether SBT leaf extract could inhibit proliferation and promote apoptosis of rat glioma C6 cells. The results revealed that the treatment with SBT leaf extract inhibited proliferation of rat C6 glioma cells in a dose-dependent manner. SBT-induced reduction of C6 glioma cell proliferation and viability was accompanied

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by a decrease in production of reactive oxygen species (ROS), which are critical for the proliferation of tumor cells. SBT treatment not only significantly upregulated the expression of the pro-apoptotic protein Bcl-2-associated X (Bax) but also promoted its localization in the nucleus. Although increased expression and nuclear translocation of Bax were observed in SBT-treated C6 glioma cells, the induced nuclear morphological change was distinct from that of typical apoptotic cells in that most of SBT-treated cells were characterized by convoluted nuclei with cavitations and clumps of chromatin. All of these results suggest that SBT leaf extract could inhibit the rapid proliferation of rat C6 glioma cells, possibly by inducing the early events of apoptosis. Thus, SBT may serve as a potential therapeutic candidate for the treatment of glioma.

Introduction

Brain glioma, which is characterized by rapid proliferation and diffuse invasion, is one of the most common malignant brain tumors in adults [1]. Owing to its infiltrative nature and frequent invasion into the eloquent regions of the brain and spinal cord, surgical treatment to remove all malignant glioma cells is not possible. This consequently results in high morbidity, high mortality, and extremely poor prognosis. Moreover, although several thera-peutic agents are being used for glioma treatment [2, 3], the efficacy of chemotherapy drugs has been shown to be usually poor because of high drug resistance and the blood-brain barrier [4]. Therefore, the development of rationally targeted therapeutics is urgently needed for the treatment of malignant gliomas.

Reactive oxygen species (ROS) are critical signaling molecules that are produced in mitochondria as an indispensable by-product of oxidative phosphorylation [5, 6]. In cancer cells, elevated rates of ROS production and changes in redox status due to altered metabolic pathways have long been demonstrated to drive unregulated cellular proliferation and survival [7, 8]. Accordingly, cancer cells persistently upregulate ROS-triggered signaling pathways, through which proliferation, differentiation, protein synthesis, glucose metabolism, and sur-vival are mediated in cancer cells [7]. As therapeutic strategies targeting these pathways, one approach has been to treat cancer cells with agents that induce excess ROS [5, 8, 9], consequently triggering apoptosis. The other has been to increase ROS scavenging, thereby interfering with H_2O_2 signaling and inhibiting cell growth [8, 10].

In recent years, there has been a wide interest in finding natural compounds that could have antioxidant, antiinflammatory, and anticancer activities [11–13]. Among the various natural resources reported for versatile activities, sea buckthorn also known as *Hippophae rhamnoides* L. has gained much interest as a versatile nutraceutical plant. Sea buckthorn (SBT) is a thorny nitrogen-fixing deciduous shrub that is currently grown in several parts of the world owing to its nutritional and medical potential [14, 15]. All parts of this plant are primarily valued for their abundance of vitamins (A, B1, B12, C, E, K), carotenoids, phytosterols, organic acids, polyunsaturated fatty acids, and some essential amino acids [16–19]. Its berry and leaf extracts have been demonstrated to possess antibacterial, antiviral, antitumor, antioxidant, immuno-modulatory, and antiinflammatory properties [20–24]. However, the molecular mechanisms underlying the various biological and therapeutic activities of SBT are not clear.

Given that rapid proliferation, invasion, and recurrence are common in glioma, preventing proliferation of glioma cells can be a critical therapeutic strategy for treatment. Therefore, in this study, we aimed to investigate the antiproliferative effects of SBT leaf extract on rat C6 glioma cells, which are an experimental model of glioma [25]. Our results demonstrated that SBT leaf extract reduces proliferation and viability of rat C6 glioma cells, which in part results from reduced ROS levels and increased expression and nuclear localization of Bcl-2-associated X (Bax).

Materials and Methods

Preparation of SBT Leaf Extract

Dried SBT leaves (50 g; Baekjangsaeng Co.) were mixed with 300 mL distilled water. SBT leaf extract was prepared as described by Kim et al. [26] with minor modifications. Briefly, the extraction process was performed at 110 °C and 5.69 psi for 15 min in an autoclave (Wiseclave WAC-60, Daihan Scientific). After centrifugation at 6500 rpm and 4° for 10 min, the supernatant was collected and subjected to filtration with a 0.2-μL syringe filter (Minisart, Sartorius). The extract obtained was freeze-dried and stored in the dark until use. The freeze-dried SBT leaf extract (62 mg) was reconstituted in 1 mL of distilled water.

Determination of Total Phenol and Catechin Contents in SBT Leaf Extract

Total phenol content of SBT leaf extract was determined by the Folin-Ciocalteu method [27]. Briefly, 100 μL of SBT leaf extract was mixed with 200 μL of 10% Folin-Ciocalteu phenol reagent (Sigma-Aldrich) by vortexing. The mixture was allowed to react for 1 min at room temperature, and then, 800 μL of 700 mM sodium carbonate (Sigma-Aldrich) was added and mixed well, followed by incubation at 37 °C in the dark for 30 min. The absorbance was measured at 765 nm using a spectrophotometer (Optizen). The results were expressed in milligram of gallic acid equivalents per 1 g of SBT leaf extract.

Vanillin assays were conducted to determine catechin content in SBT leaf extract as described by Price et al. [28]. Briefly, 20 μL of SBT leaf extract was mixed with 100 μL of vanillin reagent containing 1% (w/v) vanillin 8% concentrated HCl in methanol, followed by incubation for 30 min at 30 °C. The absorbance was measured at 500 nm using a spectrophotometer.

Cell Culture, Cell Growth Curves, and Cell Viability Assay

Rat C6 glioma cells and non-cancerous NIH/3T3 cells were obtained from ATCC. Cells were cultured in DMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gemcell) at 37 °C in a CO_2 incubator (Thermo Scientific; 95% air and 5% CO_2).

To examine the effect of SBT leaf extract on proliferation, C6 glioma cells were plated at a density of 4000 cells per well in a 24-well plate. After incubation for 12 h, SBT leaf extract was added to cultures at the final concentrations of 62, 6.2, and 0.62 μg/mL and culture media were used as a vehicle. Cell counts were performed on days 0, 1, 2, and 3 using a hemocytometer after staining with 0.4% (w/v) trypan blue dye (Thermo Fisher).

Cell viability was determined by using the WST-1 assay kit (DoGenBio). Rat C6 glioma cells and non-cancerous NIH/3T3 cells were seeded in 96-well plates, with an average of 10^4 cells/well, in 100 μL of media. Cells were incubated overnight and then treated in triplicate for 24 h at 37 °C in a 5% CO_2 incubator with SBT leaf extract at a final concentration of 62 μg/mL. Ten microliters of EZ cytox cell viability assay reagent was added to each well, followed by incubation for 2 h at 37 °C in a 5% CO_2 incubator. Finally, the optical density was measured at 450 nm using a microplate reader (Sunrise™, Tecan). All experiments were repeated at least three times.

Measurement of the Intracellular Level of ROS

ROS was measured by using fluorogenic dichlorofluorescein diacetate (H_2DCFDA, Invitrogen), which is first deacetylated by intracellular esterase to generate a nonfluorescent compound. This compound is then oxidized by ROS to generate the fluorescent compound dichlorofluorescein. Therefore, the fluorescence signal intensity of dichlorofluorescein reflects the amount of ROS produced by the cells. Briefly, C6 glioma cells were treated with different concentrations of SBT leaf extract for 24 h and were subsequently incubated with 10 μM H_2DCFDA for 30 min at 37 °C in the dark. The cells were harvested, washed with PBS three times, and were analyzed by using a Guava EasyCyte (Millipore). The mean fluorescence intensity (MFI) was determined by FlowJo Version 7.0 (TreeStar). All experiments were repeated at least three times.

Immunofluorescence Microscopy and Western Blot

To examine the localization of Bax, immunofluorescent microscopic analysis was conducted. Rat C6 glioma cells were seeded at 10^5 cells/well into two-chamber slides. Cells were cultured in the presence or absence of SBT leaf extract at a final concentration of 62 μg/mL for 24 h. Cells were washed with PBS three times, fixed with 3.7% formaldehyde solution for 15 min, and permeabilized with 0.1% Triton X-100 for 5 min. The fixed and permeabilized cells were blocked with 2% bovine serum albumin, followed by incubation with anti-Bax antibody (Santa Cruz Biotechnology) for 1 h. Cells were then incubated with the appropriate immunoglobulin G antibody conjugated with Alexa Fluor 488 (Thermo Fisher Scientific). Nuclei were stained with 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma). Fluorescence was visualized using a DMi8 fluorescence microscope (Leica), and images were processed with the LAS X (Leica) and the Photoshop 7.0 program (Adobe).

Western blot analysis was performed as described previously [29] with antibodies for Bax, KDEL to detect both glucose-regulated protein 78 (GRP78) and GRP94 (Novus Biologicals), superoxide dismutase (SOD) 2 (Millipore), and β-actin (Santa Cruz Biotechnology). Protein expression was quantified by densitometry as described previously [30]. At least three separate experiments were conducted for each protein.

Statistical Analysis

Student's t test was performed using Microsoft Excel 2010. Values were considered statistically significant at $P < 0.05$.

Results

Total Phenol and Catechin Contents in SBT Leaf Extract

As a part of chemical composition analysis, total phenol and catechin contents of SBT leaf extracts were determined by Folin-Ciocalteu method and vanillin assay, respectively. The results as given in the Table 1 show that SBT leaf extract (1 g of dry weight) contains approximately 71 mg of total phenol. Total phenol content in SBT leaf extract has been reported to be 28.4 to 93.7 mg/g while depending on extraction methods [31]. Total catechin was shown to be included in SBT leaf extract at a concentration of 455.4 μg/g (Table 1).

SBT Leaf Extract Inhibits Proliferation and Reduces Viability of Rat C6 Glioma Cells

Treatment of rat C6 glioma cells with SBT leaf extract at the indicated concentrations for 4 days was shown to inhibit the growth of C6 glioma cells (Fig. 1a, b). On the second day, inhibition of cell growth was only observed upon treatment with SBT leaf extract at a concentration of 62 μg/mL, and then, by the third day, C6 cell growth was significantly inhibited by 49.5 and 11.0% at concentrations of 62 and 6.2 μg/mL of SBT leaf extract, respectively, while the cell growth was not significantly inhibited at a concentration of 0.62 μg/mL (Fig. 1b).

The effects of SBT leaf extract on the viability of C6 cells and non-cancerous NIH/3T3 cells were evaluated after treating the cells for 24 h with different concentrations of SBT leaf extract. As shown in Fig. 1c, C6 cell viability was significantly inhibited by SBT leaf extract at concentrations of 62, 6.2, and 0.62 μg/mL, while the treatment of NIH/3T3 with SBT leaf extract was not shown to affect cell viability.

SBT Leaf Extract Reduces ROS Production in Rat C6 Glioma Cells

We measured intracellular ROS levels in C6 glioma cells, which were treated with indicated concentrations of SBT leaf extract for 24 h. The cellular oxidation of H_2DCFDA, which is oxidized to green fluorescent DCF by intracellular ROS, was used as a probe. As shown in Fig. 2, the treatment of C6 cells with SBT leaf extract significantly decreased intracellular ROS production by 42.3 and 16.3% at concentrations of 62 and 6.2 μg/mL of SBT leaf extract, respectively, compared with levels in vehicle-treated cells.

Table 1 Extraction of sea buckthorn leaves and determination of total phenol and catechin content

Extraction temperature (°C)	Extraction pressure (psi)	% of Yield	Total phenol (mg/g) ± SD[a]	Catechin (μg/g) ± SD[a]
110	5.69	37.20	70.68 ± 4.11	455.38 ± 21.22

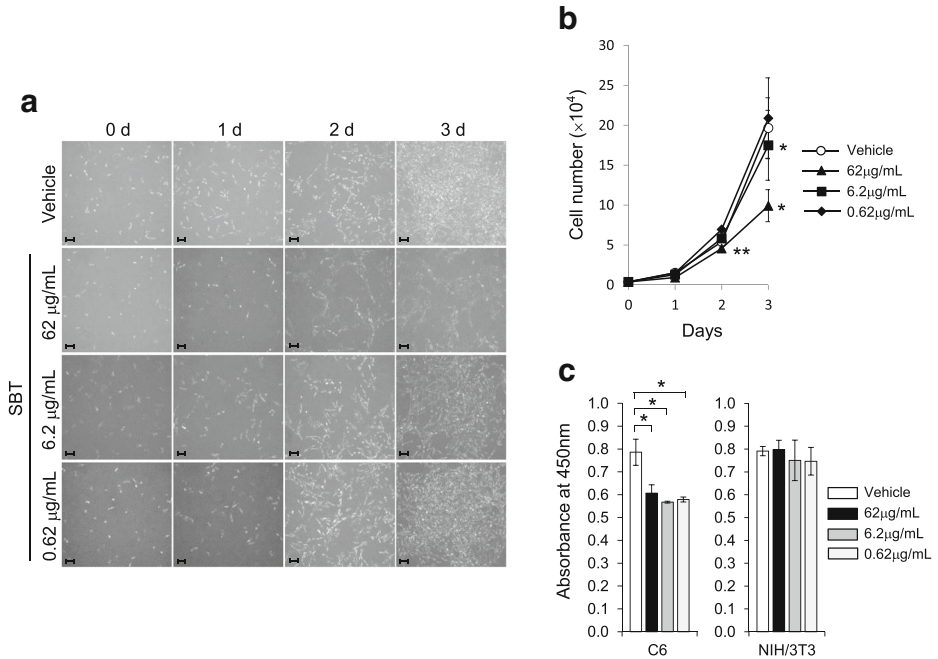[a] Data expressed as mean ± standard deviation (SD) of three replicates

Fig. 1 Effects of SBT leaf extract on proliferation and viability of rat C6 glioma cells. **a** Representative figures of SBT-treated C6 glioma cells, which were visualized by microscopy (×40, *scale bar* = 100 μm); the overall shape of SBT-treated cells under microscopy was normal without any sign of blebbing. **b** Growth curve of C6 glioma cells treated with SBT leaf extract at indicated concentrations for the indicated times ($n = 3$). **c** Viability of non-cancerous NIH/3T3 cells did not decrease with SBT leaf extract treatment. Cell viability was determined by WST-1 assay. Three independent experiments were conducted. Data represents the mean ± SEM ($n = 3$). $*P < 0.05$, $**P < 0.01$
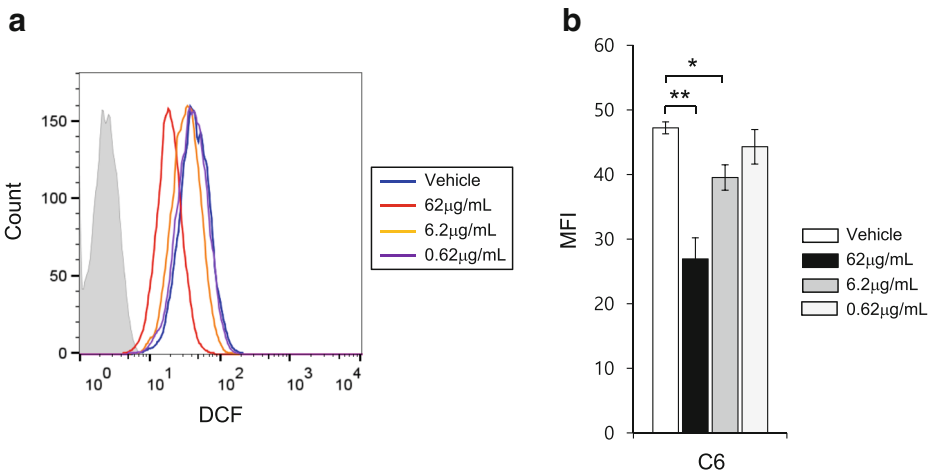

Fig. 2 Effect of SBT leaf extract on intracellular ROS levels in rat C6 glioma cells. Cells were treated with vehicle and SBT leaf extract for 24 h. Cells were then loaded with the ROS indicator dichlorofluorescein diacetate (H_2DCFDA, 10 μM), and fluorescence intensity was assessed by flow cytometry. **a** Representative flow cytometric profiles of ROS levels in rat C6 glioma cells treated with SBT leaf extracts (62, 6.2, and 0.62 μg/mL) and vehicle. **b** Quantification of DCF fluorescence change in rat C6 glioma cells after SBT leaf extract treatment. Data represents the mean ± SEM ($n = 3$). $*P < 0.05$, $**P < 0.01$

SBT Leaf Extract-Reduced Intracellular ROS Production Induces Apoptosis

The expression level of the pro-apoptotic protein Bax was measured by western blot. After SBT leaf extract treatment, Bax was upregulated in a dose-dependent manner (Fig. 3a, b). Meanwhile, the expression of GRP94, GRP78, and SOD2 was not shown to be affected by SBT leaf extract treatment.

SBT Leaf Extract Treatment Alters Nuclear Morphology and Bax Localization

When C6 glioma cells were treated with SBT leaf extract at a concentration of 62 μg/mL of SBT leaf extract for 24 h, more than 80% of the cells presented heavily convoluted nuclei, together with numerous cavitations or partially condensed nuclei, suggesting that the chromatin structure was altered in a different manner from typical apoptotic nuclei (Fig. 3c, DAPI staining). Furthermore, patches of partially condensed chromatin were found to be localized along the inner part of the nuclear membrane. The overall morphology of SBT leaf extract-treated C6 cells was not observably different from that of vehicle-treated C6 cells as viewed under a light microscope, with no signs of blebbing (data not shown).
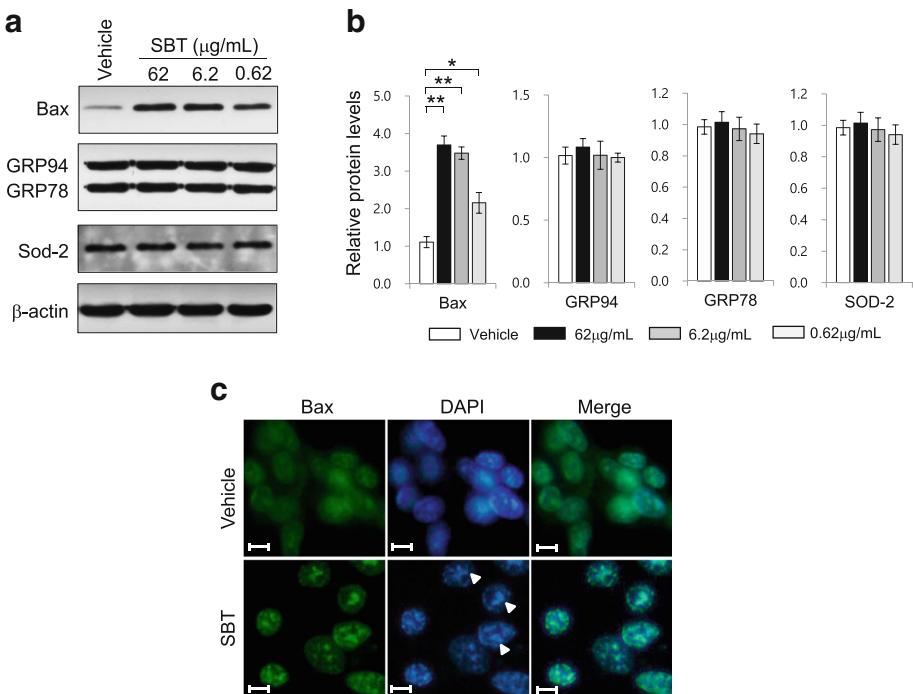

Fig. 3 Effects of SBT leaf extract on expression and localization of the pro-apoptotic protein Bax. a Western blot analysis for Bax, GRP78, GRP94, and SOD2 in rat C6 glioma cells treated for 24 h with SBT leaf extract at indicated concentrations. Three independent experiments were conducted, and representative western blots are shown. b The relative Bax, GRP78, GRP94, and SOD2 protein levels were quantified by densitometry analysis. Vehicle (*white bar*), 62 μg/mL (*black bar*), 6.2 μg/mL (*dark gray*), 0.62 μg/mL (*light gray*). Data represents the mean ± SEM ($n = 3$). *$P < 0.05$, **$P < 0.001$. c Nuclear localization of Bax in rat C6 glioma cells after treatment for 24 h with SBT leaf extract (*scale bar* = 20 μm). Images of representative cells with altered Bax localization are shown. SBT-mediated apoptosis-related morphological changes were examined by DAPI staining; clumps of chromatin were observed in most SBT-treated cells (*arrowheads*)

As shown in Fig. 3c, Bax expression was highly localized in the nuclei of SBT leaf extract-treated C6 cells, whereas Bax expression was evenly distributed in the cytoplasm of vehicle-treated C6 cells.

Discussion

This study showed that the proliferation of C6 glioma cells was inhibited by SBT leaf extract, which was prepared by a high temperature and pressure method, in a dose-dependent manner. Extraction from plant tissues has usually been conducted by different extraction techniques such as solid-liquid extraction employing methanol, ethanol, and acetone and subcritical water extraction. Recently, there has been an interest in developing rapid, reliable, reproducible, and environmentally clean extraction methods that are able to obtain bioactive compounds with high quality and high activity [31]. In this context, we adopted the subcritical water extraction technique using hot water under pressure to prepare SBT leaf extract. As a result, approximately 37.2% extraction yield was achieved with subcritical water extraction method without any toxicity related to solvents. Further study is however necessary to demonstrate that subcritical water extraction is comparable to solvent-based extraction techniques in terms of quality and activity of bioactive compounds.

We demonstrated that SBT treatment significantly increased the proportion of early apoptotic cells, accompanied by chromatin morphological changes. This proved that SBT leaf extract inhibited rat C6 glioma cell proliferation by inducing apoptosis. We further demonstrated that SBT treatment induced a significant decrease in intracellular ROS production, upregulation of Bax expression, and nuclear translocation of Bax from cytosol. These results indicated that a ROS-mediated mitochondrial pathway was involved in the mechanism of SBT-induced apoptosis of rat C6 glioma cells.

SBT has been recognized as a herbal medicine that possesses a wide spectrum of pharmacological effects including antioxidant, antiinflammatory, anticancer, hepatoprotective, antistress, cardioprotective, and antiatherogenic activities [20, 21, 23, 24, 32]. Particularly, SBT leaves have been known to be abundant in phenolics including leucoanthocyanidins, (−)-epicatechin, (+)-gallocatechin, (−)-epigallocatechin, and gallic acid [33], most of which have important effects on cancer chemoprevention and chemotherapy [34]. In vitro cell culture studies have demonstrated that SBT leaf extract can inhibit proliferation and promote apoptosis in cancer cells such as SGC7901 gastric carcinoma, L1200 lymphatic leukemia, HT29 colon cancer, MCF-7 breast cancer, and HL-60 leukemia cells [22, 23, 32, 35, 36]. In addition, Padmavathi et al. [37] reported that SBT inhibits carcinogen-induced stomach and skin tumorigenesis via stimulation of antioxidant enzymes such as glutathione-S-transferase, superoxide dismutase, catalase, and glutathione reductase in the mouse liver. Based on these observations, we first examined the effect of different doses (62, 6.2, and 0.62 μg/mL) of SBT leaf extract on the growth rate and viability of rat C6 glioma cells via cell counting and WST-1 assay, respectively. Our results demonstrate that 62 μg/mL of SBT leaf extract most effectively inhibited the growth and viability of rat C6 glioma cells. Meanwhile, the viability of SBT-treated non-cancerous NIH/3T3 cells, relative to that of vehicle-treated cells, did not decrease, suggesting that SBT might only inhibit the proliferation and viability of tumor cells. As a follow-up study, in vivo experiments should be conducted to investigate whether dietary administration of SBT leaf extract could inhibit the growth of tumors and whether the effects of SBT leaf extract observed in vitro are relevant to the situations in vivo. Li et al. [38]

performed a comparative study in vivo and in vitro to demonstrate the pro-oxidative activities of (−)-epigallocatechin-3-gallate (EGCG), a major polyphenol in green tea. They found that dietary EGCG administration dose dependently increased the EGCG level in animals, and the effective concentrations of EGCG observed in cell culture systems are higher than the physiological concentrations measured in animals.

We then studied the possible mechanism by which SBT leaf extract inhibits the proliferation and viability of rat C6 glioma cells. It has been known that excessive amounts of ROS cause cell damage and physiological dysfunction, causing various pathologies including neurodegenerative diseases, cancer, and premature aging [39]. ROS are highly reactive molecules that play essential roles in the proliferation and differentiation of living organisms, and maintaining ROS homeostasis is critical for normal cell growth and survival [8]. Many studies have demonstrated that almost all cancers have increased levels of intracellular ROS compared with their normal counterparts [7, 40], indicating that the demand for ROS in cancer cells is higher than that in normal cells. If the intracellular ROS amounts were not sufficient to maintain a cellular response, cancer cells would not proliferate and survive normally [8]. Liu et al. [10] demonstrated that a significant decrease of intracellular ROS production can inhibit the growth of human hepatocellular carcinoma cells and significantly induce apoptosis. In this study, we determined intracellular ROS amounts by using H_2DCFDA after treatment with SBT leaf extract, consequently showing that a sharp decline in the production of ROS was observed when C6 glioma cells were treated with SBT leaf extract at a concentration of 62 μg/mL. This result suggested that ROS plays a crucial role in rat C6 glioma cell growth and SBT treatment might have disrupted the balance of redox reactions in these cells, resulting in decreased cell proliferation. However, given that the expression of SOD2 as a ROS scavenger was not significantly altered by SBT, further study is required to confirm that the balance between ROS production and antioxidants could be disturbed by SBT treatment. It has been described that hydrogen sulfide has physiological functions, including regulation of neuronal activity, vascular tension, and protection of the heart, kidney, and brain from ischemic injury [41] Moreover, Shibuya et al. [42] demonstrated a novel pathway for the production of hydrogen sulfide from D-cysteine in mammalian cells, proposing a new therapeutic approach to deliver hydrogen sulfide to tissues or organs. It might be interesting to examine the effect of SBT leaf extract on the hydrogen sulfide-producing activity in rat glioma cells or animal models.

Apoptosis is an essential biological process during cell development, tissue homeostasis, and aging and has been implicated in the pathogenesis of various diseases such as cancer and degenerative neuronal diseases [43, 44]. Two major apoptotic pathways have been well established. One pathway is the cell death receptor-mediated pathway (extrinsic), and the other apoptotic pathway is mediated by mitochondria (intrinsic). The latter is mainly regulated by Bcl-2 family members, including the pro-apoptotic protein Bax and the antiapoptotic proteins Bcl-2 and Bcl-XL [45]. In normal conditions, Bax is localized in the cytoplasm and changes its subcellular location and dimerization pattern in response to apoptotic stimuli. Mounting evidence suggested that nuclear accumulation of Bax occurs early on during apoptosis [46, 47], preceding chromatin condensation. Our data showed that treatment with SBT leaf extract not only increased the expression of Bax but also altered the subcellular localization of Bax primarily into the nucleus. Furthermore, the nuclear morphology of SBT-treated C6 glioma cells was distinct from that of vehicle-treated cells, in that convoluted nuclei with cavitations and clumps of chromatin were observed in most SBT-treated cells. These results indicate that treatment with SBT leaf extract induces pre-apoptosis in rat C6 glioma cells.

Conclusion

We conclude that SBT leaf extract inhibits proliferation via downregulation of intracellular ROS and induction of pre-apoptosis in rat C6 glioma cells. This suggests that SBT may serve as a potential therapeutic candidate for the treatment of glioma.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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