Antioxidant effects of *Geranium nepalense* ethanol extract on H₂O₂-induced cytotoxicity in H9c2, SH-SY5Y, BEAS-2B, and HEK293

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Abstract Oxidative damage leads to many diseases. In this study, we evaluated the antioxidant effects of 70% ethanol extract of *Geranium nepalense* (GNE) on hydrogen peroxide-induced cytotoxicity in cell lines: H9c2, SH-SY5Y, HEK293, and BEAS-2B. We determined the free radical scavenging activity of GNE using 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), and superoxide dismutase-like activities, as well as the polyphenol and flavonoid contents of GNE. The results showed that GNE scavenged DPPH and ABTS radicals in a dose-dependent manner. In addition, it contained abundant contents of total polyphenol and flavonoid contents and strongly suppressed cellular reactive oxygen species, thereby protecting H₂O₂-induced cytotoxicity in H9c2, SH-SY5Y, HEK293, and BEAS-2B cell lines. The powerful antioxidant activity exhibited by GNE, both in vitro and in cell systems, was attributed to its free radical scavenging activity. Therefore, GNE may be useful in preventing oxidative stress-induced diseases including Alzheimer's disease, respiratory inflammatory disease, and chronic kidney diseases.

Keywords Antioxidant · Cytotoxicity · *Geranium nepalense* · Oxidative stress · ROS

Introduction

Oxygen plays an important role in organisms for aerobic life [1]. However, the imbalance of energy production and fuel from biological processes generates free radicals, which induce oxidative stress [1]. Low level of reactive oxygen species (ROS) plays a role in physiological cell processes; however, high levels of ROS in the body can cause oxidative damage to biomacromolecules, such as DNA, proteins, membrane, lipids, and carbohydrates, in addition to the formation of proinflammatory cytokines [2, 3]. H₂O₂ is a main component of ROS and has been widely used as an inducer of oxidative damage in in vitro models [4]. It can lead to many diseases including neurological disorders, cancer, ischemia, diabetes, and asthma [4, 5].

Therefore, maintaining the balance of the free radical production and antioxidant defense is crucial for normal organism functioning [6, 7]. The human body has antioxidant repair systems that progress to protect the body against oxidative damage and help prevent or reduce the incidence of chronic and other diseases. Therefore, the antioxidant defense and repair systems contribute to a better quality of life [8, 9]. However, these systems often fail to completely protect oxidative damage [9]. Therefore, antioxidants that scavenge and block free radicals can be used to supplement the systems in organisms before they attack targets in biological cells [9, 10]. These antioxidants retard not only the progress of many chronic diseases but also lipid peroxidation [11, 12].

Natural antioxidants are used for dietary and functional food purposes, and might help to prevent oxidative damage [1]. Recently, the search for antioxidants from plant sources has received significant attention as they are considered safe and protect from several diseases [3]. *Geranium*

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nepalense (GN) is used for the treatment of diseases such as shigellosis, gastric ulcer, and duodenal ulcer [13]. Previously, a study has reported that the major components of GN are tannin, (-)-epicatechin, kaempferitrin, kaempferol-7-rhamnoside, corilagin, tannin, geraniin, gallic acid, succinic acid, quercetin, quercitrin, and protocatechuic acid, and that it possesses bioactivities [14]. Especially, geraniin is isolated from most of the *Geranium* species [14] and has numerous bioactivities, including antioxidant, anti-hyperglycemic, anti-hypertensive, anti-inflammatory, and anti-cancer activities [15]. *Geranium* showed the cytoprotective effects of geraniin through the scavenging of free radicals and protected cells by regulating antioxidant enzymes (Nrf2) [16]. Although, GN was reported to reduce intracellular ROS levels in H₂O₂-induced cytotoxicity in human keratinocyte, its antioxidant effects in H₂O₂-induced oxidative damage in H9c2 (cardiomyoblasts cell), SH-SY5Y (human neuroblastoma cell line), HEK293 (human embryonic kidney 293 cell line), and BEAS-2B (human bronchial epithelial cells) have not been investigated.

H₂O₂-induced oxidative stress in these cell lines has been used for treating ROS-induced diseases. For example, in vitro model of H₂O₂-induced oxidative stress in H9c2, SH-SY5Y, BEAS-2B, and HEK293 is typically used for determining ROS-induced heart disease, neurodegenerative disorder, respiratory inflammatory disease, and kidney injury, respectively. Therefore, in this study, we evaluated GN as a suitable candidate for treating diverse oxidative stress-induced diseases in H9c2, SH-SY5Y, HEK293, and BEAS-2B cell lines.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, penicillin, and streptomycin were purchased from Invitrogen (Grand Island, NY, USA). 2',7'-Dichlorofluorescein diacetate (DCFH-DA) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of GNE

GN was harvested in the Cheonam area (34°47'20"N 126°63'76"E 139 m) of South Korea in March 2015. The samples were identified by professor Yuk Chang Soo (Kyung Hee University, Seoul, South Korea) and are listed in the NIKOM TKM2079 by a code number. The GN with 70% ethanol extracts (GNE) were prepared by boiling at 80 °C with 70% ethanol for 3 h. The mixture was filtered and freeze-dried, and the powder was stored at −4 °C. GNE was dissolved in PBS when used.

Total polyphenol and flavonoid contents

The total phenol content was determined by the modified Folin-Denis colorimetric method [17]. Gallic acid (0–500 µg/mL) was used to construct the standard curve. Flavonoids were determined by a slight modification of the colorimetric method described by Moreno et al. [18]. Rutin (0–500 µg/mL) was used to construct the standard curve.

DPPH and ABTS⁺ scavenging assay

The radical scavenging activity of GNE was determined by the DPPH radical scavenging assay [19] with some modifications. The ABTS method is based on the discoloration that occurs when the radical cation ABTS⁺ is reduced to ABTS [20].

Superoxide dismutase (SOD)-like activity assay

SOD-like activity was determined using commercial kits in accordance with the manufacturer's instructions (SOD-WST kit; Dojindo, Kumamoto, Japan). Twenty microliters of samples (sample and Blank 1; Blank 1 is sample coloring without inhibitor) or double-distilled water (ddH₂O) (Blanks 2 and 3; Blank 2 is the sample blank and Blank 3 is the reagent blank) were mixed with 200 µL of WST working solution. Blanks 2 and 3 were added to 20 µL of ddH₂O, and sample and Blank 1 were added to 20 µL of enzyme working solution. The solutions were thoroughly mixed and incubated at 37 °C. After 20 min, the absorbance was read at 450 nm using a microplate reader. The SOD-like activity was calculated using the following equation.

$$\text{SOD activity (inhibition rate \%)} = \frac{[(\text{Blank 1} - \text{Blank 3}) - (\text{Sample} - \text{Blank 2})]}{(\text{Blank 1} - \text{Blank 3})} \times 100.$$

Cell culture and treatment

Cells were supplied by ATCC and cultured in specific media as recommended by ATCC (EMEM media for HEK293 and SH-SY5Y; DMEM media for H9c2; DMEM + F12 media for BEAS-2B) in a 37 °C, 5% CO₂, and 95% air-humidified atmosphere. To induce oxidative stress, cells were incubated with H₂O₂ (HEK293: 200 µM, SH-SY5Y: 100 µM, H9c2: 250 µM, BEAS-2B: 100 µM) for 24 h. To study the effects of GNE, cells were pretreated with GNE for 2 h, followed by the addition of H₂O₂ to the medium and incubation for an additional 24 h.

Assay of cell viability

The cell viability of the samples was measured using an MTS assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Assay of intracellular ROS

Intracellular ROS generation was determined using DCFH-DA. In brief, 1×10^5 cells/well (in a 96 well plate) were incubated in 10 μ M of DCFH-DA for 30 min at 37 °C.

Table 1 Polyphenol and flavonoid contents and free radical scavenging activity of GNE

	Polyphenol (mg GAE/g)	Flavonoid (mg RE/g)	IC ₅₀ of SOD (μ g/mL)	IC ₅₀ of DPPH (μ g/mL)	IC ₅₀ of ABTS (μ g/mL)
GNE	169.4 $\pm$ 7.84	48.3 $\pm$ 1.34	23.4 $\pm$ 1.25	46.3 $\pm$ 0.84	80.9 $\pm$ 0.77

Each value in the tables is represented as mean $\pm$ SD (n = 9). Values in the same *GNE Geranium nepalense* ethanol extracts, *GAE* gallic acid equivalents, *RE* rutin equivalents

Fig. 1 Cytoprotection of GNE against H₂O₂ in (A) H9c2 cells, (B) SH-SY5Y, (C) BEAS-2B, and (D) HEK293. Cells were pretreated with indicated concentrations of GNE for 60 min and then exposed to H₂O₂ for 24 h. Data are presented as mean $\pm$ SD (n = 9).

^{abcd}Means not sharing a common letter are significantly different between the groups based on one-way ANOVA with Duncan's multiple-range post hoc test at $p < 0.05$

Fig. 2 Effects of GNE on intracellular ROS accumulation in H9c2 cells. H9c2 were pretreated with DCFH-DA for 30 min, followed by the indicated concentration of GNE and 1 mM of H_2O_2 treatment for 3 h incubation. Levels of intracellular ROS were measured by flow

cytometry. (A) Unstaining, (B) control, (C) only H_2O_2 -treated cells, (D) H_2O_2 treated with 50 $\mu\text{g}/\text{mL}$ GNE (E) H_2O_2 treated with 100 $\mu\text{g}/\text{mL}$ GNE, and (F) H_2O_2 treated with 200 $\mu\text{g}/\text{mL}$ GNE

DCF fluorescence was measured using a BD dual laser FACS Calibur (BD Biosciences, San Jose, CA, USA) with 10,000 events collected for each sample.

Statistical analysis

All data are presented as mean $\pm$ SD. Statistically significant differences between groups were identified by one-way analysis of variance (ANOVA) using SPSS version 21 (Chicago, IL, USA) with Duncan's multiple-range post hoc test. Values were considered to be statistically significant when $p < 0.05$.

Results and discussion

Polyphenol and flavonoid contents and free radical scavenging activity

The antioxidant activity of GNE was determined by measuring its differential radical scavenging activities against DPPH and ABTS radicals and its SOD-like activity (Table 1). The differential radical scavenging activities of GNE against DPPH and ABTS radicals and SOD-like

activities were investigated and may arise because of different mechanisms underlying the antioxidant-radical reaction [21].

Polyphenols are natural phytochemical compounds [22] and secondary metabolites of plants [23]. They are classified into several categories, including tannin, lignin, and flavonoids. Flavonoids are major polyphenols that are crucial components of the human diet [22]. They have many biological effects, such as antiviral, anti-inflammatory, anti-diabetic, anti-obesity, anti-ischemic, and antioxidant activity [24]. The total polyphenol content of GNE was investigated and determined as gallic acid equivalents, whereas the flavonoid content of GNE was determined as rutin equivalents. GNE had the phenolic contents of 169.4 ± 1.48 mg GAE/g extract and 48.3 ± 0.20 mg rutin/g extract, respectively (Table 1).

Many studies reported that GNE has components including tannin, (-)-epicatechin, kaempferitin, kaempferol-7-rhamnoside, brevifolin, corilagin, pyrogallol, ellagitannin, geraniin, gallic acid, succinic acid, quercetin, protocatechuic acid, furosonin, *p*-hydroxybenzoic acid, brevifolin carboxylic acid, and ellagic acid. These compounds belong to the polyphenol and possess many bioactivities [22–25]. For examples, tannin, flavonoids, and

Fig. 3 Effects of GNE on intracellular ROS accumulation in SH-SY5Y cell. SH-SY5Y were pre-treated with DCFH-DA for 30 min, followed by the indicated concentration of GNE and 1 mM of H_2O_2 treatment for 3 h incubation. Levels of intracellular ROS were

measured by flow cytometry. (A) Unstaining, (B) control, (C) only H_2O_2 -treated cells, (D) H_2O_2 treated with 50 $\mu\text{g/mL}$ GNE (E) H_2O_2 treated with 100 $\mu\text{g/mL}$ GNE, and (F) H_2O_2 treated with 200 $\mu\text{g/mL}$ GNE

phenolic acid are major polyphenolic compounds, with many bioactivities such as anti-inflammatory, anti-cancer, anti-obesity, anti-diabetic, antioxidant, hepatoprotective, and neuroprotective effects [25]. Therefore, the antioxidant properties of GNE may be attributed to the presence of many bioactive compounds.

GNE protection of H9c2, SH-SY5Y, BEAS-2B, and HEK293 cells from H_2O_2 -induced cytotoxicity

Oxidative stress is an important mediator of heart failure, ischemia–reperfusion injury [26], neurodegenerative diseases [27], pulmonary disorder [28], and kidney-related diseases [29]. In this study, GNE-protected cells were tested against oxidative stress-induced cell cytotoxicity in H9c2, SH-SY5Y, BEAS-2B, and HEK293 by MTS assay. First, H9c2 cells were exposed to H_2O_2 (0–1000 μM) for 24 h, and then, the cell viability was assessed by the MTS reduction assay. However, the H_2O_2 -induced cytotoxic effects decreased in the presence of GNE and the pretreatment with GNE affected the cell survival (Fig. 1A). H_2O_2 showed a significant cytotoxic effect in SH-SY5Y and BEAS-2B cells

when tested with at least 100 and 80 μM of H_2O_2 , respectively (data not shown). However, pretreatment with different concentrations of GNE significantly increased the cell viability in a dose-dependent manner (Fig. 1B, C). GNE also significantly inhibited cell death induced by H_2O_2 in HEK293 cells, thus increasing the cell viability (Fig. 1D). These data suggest that GNE exerts inhibitory effects against oxidative stress-induced cell death.

GNE inhibits production of ROS from H_2O_2 -induced cytotoxicity in H9c2

H9c2 cell line is a cardiomyoblast, with cardiomyocyte property. H_2O_2 and/or other ROS-induced oxidative stress in cardiomyocytes significantly affect the pathogenesis of heart failure and ischemia–reperfusion injury [26]. Therefore, in this study, the cardio-protective effect of GNE was investigated against exposed H_2O_2 in H9c2 cells, which is a well-established model and widely used for determining heart functions.

To determine whether GNE prevents H9c2 cell death by inhibiting ROS production, the intracellular levels of ROS were measured. ROS levels dramatically increased in the

Fig. 4 Effects of GNE on intracellular ROS accumulation in BEAS-2B cell. BEAS-2B were pre-treated with DCFH-DA for 30 min, followed by the indicated concentration of GNE and 1 mM of H_2O_2 treatment for 3 h incubation. Levels of intracellular ROS were

measured by flow cytometry. (A) Unstaining, (B) control, (C) only H_2O_2 -treated cells, (D) H_2O_2 treated with 50 $\mu\text{g/mL}$ GNE, (E) H_2O_2 treated with 100 $\mu\text{g/mL}$ GNE, and (F) H_2O_2 treated with 200 $\mu\text{g/mL}$ GNE

H_2O_2 group compared to the control group, whereas GNE at the concentrations of 50, 100, and 200 $\mu\text{g/mL}$, administered 30 min prior to H_2O_2 exposure, decreased ROS (Fig. 2). Our results also showed that GNE reduced the oxidative stress induced by H_2O_2 by scavenging ROS in H9c2 cells. These results demonstrate that the cardio-protective effects of GNE against H_2O_2 -induced oxidative damage might be involved in the inhibition of intracellular ROS.

GNE inhibits the production of ROS from H_2O_2 -induced cytotoxicity in SH-SY5Y

Oxidative stress is an important mediator of neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease, and stroke [27]. Human neuroblastoma SH-SY5Y cells are frequently utilized as an in vitro model in neuroscience research. Therefore, the neuroprotective effects of GNE against H_2O_2 -induced oxidative stress were evaluated in SH-SY5Y cells. To evaluate whether GNE prevents neuronal cell death by inhibiting ROS production, the intracellular ROS fluorescence intensity was measured by the DCFH-DA assay. The treatment with 1 mM H_2O_2

dramatically increased the intracellular ROS levels, while GNE at the concentrations of 50, 100, and 200 $\mu\text{g/mL}$, administered 30 min prior to the H_2O_2 exposure, decreased the ROS levels to $\sim 12.6\%$ (Fig. 3). These data indicated that GNE can inhibit H_2O_2 -induced cell death. Therefore, the inhibition of neuronal cell death by GNE might be mediated by a reduction in the intracellular ROS production.

GNE inhibits production of ROS from H_2O_2 -induced cytotoxicity in BEAS-2B

BEAS-2B, the origin of human bronchial epithelial cells grown in tissue culture, is used to understand the mechanism of oxidative stress-induced pulmonary disorder. Cho et al. [28] reported on oxidative stress-induced asthma using an in vitro model of H_2O_2 -induced cytotoxicity in BEAS-2B. Therefore, the antioxidant and protective pulmonary disorder of GNE H_2O_2 -induced oxidative injury was investigated in BEAS-2B.

Oxidative stress has been attributed to the airway and lung damage, which is directly linked to several respiratory

Fig. 5 Effects of GNE on intracellular ROS accumulation in HEK293 cell. HEK293 were pre-treated with DCFH-DA for 30 min, followed by the indicated concentration of GNE and 1 mM of H_2O_2 treatment for 3 h incubation. Levels of intracellular ROS

were measured by flow cytometry. (A) Unstaining, (B) control, (C) only H_2O_2 -treated cells, (D) H_2O_2 treated with 50 $\mu\text{g/mL}$ GNE, (E) H_2O_2 treated with 100 $\mu\text{g/mL}$ GNE, and (F) H_2O_2 treated with 200 $\mu\text{g/mL}$ GNE

inflammatory diseases. Aberrant production of ROS can lead to the pathogenesis of asthma [28]. Furthermore, environmental antigen-induced allergic asthma may increase the endogenous ROS formation in the respiratory system as well as antioxidant defense because of oxidative stress. Jiang et al. [30] reported that the use of redox-based therapy to decrease the levels of ROS affords a potential target to alleviate oxidative stress-induced airway inflammation in patients with asthma. Moreover, recent research suggests that the reduction of exposure to environmental ROS and/or the strengthening of antioxidant defense can be beneficial for asthmatic patients. Therefore, the inhibition of ROS formation is believed to be a good approach for the treatment of respiratory inflammatory disease. In this study, intracellular ROS productions were dramatically increased in the only H_2O_2 -treated group compared with the control group, while GNE at the concentration of 50, 100, 200 $\mu\text{g/mL}$, administered 30 min prior to H_2O_2 exposure, decreased ROS (Fig. 4). Therefore, this study demonstrates that GNE may be effective against asthma by protecting against H_2O_2 -induced cytotoxicity and reducing the intracellular ROS levels.

GNE inhibits production of ROS from H_2O_2 -induced cytotoxicity in HEK293 cells

The kidney maintains homeostasis by retaining or excreting multiple substances according to the specific needs of the body [30]. However, metallic compounds, for example, lead, cadmium, and platinum, contribute to renal injury. Oxidative stress also leads to kidney-related diseases such as acute kidney injury or chronic kidney diseases. HEK293 cells in the human organism are derived from embryonic kidney cells and have been broadly used in biological studies from a long time, because they can be easily grown and transfected. A recent study also reported the antioxidant effect using H_2O_2 -induced HEK293 models [30]. Therefore, we performed studies to examine the resistance effects of GNE as a defensive strategy to combat oxidative stress-induced disorders in the H_2O_2 -induced oxidative-damaged HEK293 model.

In this study, GNE-protected cells were tested against oxidative stress-induced cell death in HEK293 by FACS analysis. The treatment with 200 μM H_2O_2 significantly increased the intracellular ROS generation to 13% of the

control, but the increase significantly reduced to 3.1, 2.3, and 2.0% of the control value by GNE at 50, 100, and 200 $\mu\text{g/mL}$, respectively (Fig. 5). These results suggest that GNE can decrease the intracellular ROS levels and cell death and increase the cell viability. Therefore, the pre-treatment with GNE was highly capable of inhibiting H_2O_2 -induced oxidative damage in HEK293 cells. Furthermore, it can be used as a good strategy to combat oxidative stress-induced kidney disorders.

Geranium species have been reported to enhance the activity of antioxidant enzymes such as catalase and SOD in H_2O_2 -induced oxidative stress in in vitro models [31, 32]. Consequently, Geranium species reduced the levels of Malondialdehyde (MDA) by enhancing antioxidant enzymes. MDA is one of the ROS. Our study showed that the ROS levels of the GNE-treated group decreased compared to the only H_2O_2 -treated group. GNE also directly increased the SOD-like activity, suggesting that GNE exerts its antioxidant properties by down-regulating the intracellular ROS levels, thus probably enhancing the SOD activity.

In conclusion, GNE contains several polyphenols and flavonoids and confers antioxidant activities via free radical scavenging. It significantly inhibited the H_2O_2 -induced oxidative stress in diverse normal cells including H9c2, SH-SY5Y, HEK293, and BEAS-2B. It decreased the ROS accumulation and increased the cell viability in these cells. Therefore, it can be recommended as an effective strategy for preventing and/or treating diverse ROS-induced disease and disorders such as heart failure, Alzheimer's disease, Parkinson's disease, and asthma. Further investigations are needed to elucidate the effects of GNE on the antioxidant and diverse disease metabolism-related signaling pathways.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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