Protective Effect of *Gynura Bicolor* Alcohol Extracts on Rat Kidney Epithelial NRK-52E Cells Induced by Tert-Butyl Hydroperoxide

Shu-Ling Hsieh
National Kaohsiung University of Science and Technology
Chia-Hsuan Wu
Providence University
Jyh-Jye Wang
Fooyin University
Chien-Chun Li
Chung Shan Medical University
Chih-Chung Wu (ccwumail@pu.edu.tw)
Providence University

Research Article

Keywords: G. bicolor alcohol extracts, antioxidative stress and anti-inflammation, apoptosis, kidney protection.

Posted Date: December 30th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-129084/v1

Abstract

Background: *Gynura bicolor* is a common vegetable in the Asia and has many physiological effects. We aimed to investigate the renoprotective effects of *G. bicolor* alcohol extracts (GBAEs).

Methods: Rat kidney epithelial NRK-52E cell culture and kidney damage induction by tert-butyl hydroperoxide (t-BHP) were used as experimental models. In this study, the antioxidative and anti-inflammatory activities, the levels of apoptosis and apoptosis-related molecules were analyzed in NRK-52E cells following t-BHP-induced injury.

Results: The results showed the levels of thiobarbituric acid reactive substances (TBARS) and reactive oxygen species (ROS) decreased by levels by 61-71% and 41-59%, respectively, in NRK-52E cells treated with 0.5, 1, or 5 mg/ml GBAE combined with t-BHP compared with t-BHP-induced cells ($p<0.05$). The GSH levels and GSH-to-GSSG ratio were increased in NRK-52E cells treated with 0.5, 1, and 5 mg/ml GBAE combined with t-BHP compared to t-BHT-induced cells ($p<0.05$). The nitrite (NO), prostaglandin E2 (PGE_2), IL-6, and TNF-α levels of NRK-52E cells treated with 0.5, 1, and 5 mg/ml GBAE combined with t-BHP were decreased compared with t-BHP-induced cells ($p<0.05$). In addition, Bcl-2 levels were increased after treatment with 5 mg/ml GBAE combined with t-BHP, and the levels of Bax and poly (ADP-ribose) polymerase (*PARP*) and the apoptosis percentage were decreased after treatment with 1 or 5 mg/ml GBAE combined with t-BHP induction compared to after t-BHP induction alone ($p<0.05$).

Conclusions: These data showed that 5 mg/ml GBAE can protect against kidney cell damage induced by t-BHP. The mechanism involves reducing cell damage and apoptosis through decreasing oxidative stress and inflammation.

Background

Gynura bicolor (Roxb. and Willd.) DC is usually used as a dietary vegetable in South Asia and the Far East. The top and bottom of *G. bicolor* leaves are dark green and purple, respectively. Chen et al. (2012) reported high contents of sesquiterpene compounds such as beta-caryophyllene, alpha-caryophyllene, and alpha-copaene in *G. bicolor* [1]. Our previous studies showed that *G. bicolor* is rich in plant pigments, such as chlorophyll, gallic acid, β-carotene, rutin, anthocyanidin, myricetin, and morin [2, 3]. Lu et al. (2010) showed that there are abundant plant pigments in leaves that provide *G. bicolor* with its pigmentation and may have physiological effects [4]. *G. bicolor* exhibits neuroprotective [5], liver-protective [6], hypoglycemic [7], antioxidant [8, 9], and anticancer properties [10], promotes iron bioavailability [3], has anti-inflammatory effects [2], and improves atherosclerosis and immune regulate [9, 11]. However, the renoprotective effects of *G. bicolor* are unclear. Moreover, an acute oral toxicity study indicated that a methanol extract of *G. bicolor* has a negligible level of toxicity when administered orally and is regarded as safe in experimental rats [12]. Additionally, in an in vitro hepatotoxic assessment of pyrrolizidine alkaloids in *G. bicolor*, no significant genotoxic toxic effects were detected [13]. *G. bicolor* is

a safe dietary vegetable and traditional herb, and investigating the protective effects of *G. bicolor* on the kidney could be beneficial.

The kidneys have a very important role in various basic physiological functions, including blood pressure control, salt and water homeostasis, acid-base balance, and calcium homeostasis [14]. Once renal dysfunction occurs, it causes multiple pathologies. A variety of ischemic conditions, oxidative stress, bacterial infection, sepsis, and toxicity can lead to kidney cell damage and death, which can result in acute kidney injury (AKI) [15, 16]. In addition to AKI, diabetic complications, hypertension, obesity, and autoimmunity lead to chronic kidney disease (CKD). Moreover, patients with AKI have a high risk of developing CKD within 10 to 15 years [17]. Both AKI and CKD can lead to end-stage renal disease (ESRD). CKD is an increasingly important public health issue in Taiwan and worldwide, with increasing incidence and prevalence [18]. Oxidative stress often occurs with inflammation. Oxidative stress and inflammation are two important factors involved in kidney cell damage [19]. Increasing antioxidative ability, such as by increasing reactive oxygen species (ROS) scavenging and glutathione (GSH) levels, and inhibiting excessive inflammation, such as by decreasing cytokine release and inflammatory molecule levels, are potential ways to prevent and/or treat renal disease. For example, dipyridamole, a clinical kidney therapy drug for proteinuria, reduces the inflammatory response [20] and oxidative damage [21] to treat CKD. In addition, chronic renal failure (CRF) causes kidney cell apoptosis along with kidney fibrosis. Oxidative stress and inflammation are two major causes of apoptosis induction [22]. ROS production and inflammatory molecule release trigger the initiation of apoptosis, leading to cell death and kidney fibrosis [23]. Protecting kidney cells from apoptosis and fibrosis is another approach to protect the kidney.

Previous studies showed that aqueous *G. bicolor* extracts can attenuate H_2O_2-induced injury in artificial rat neurons (PC-12 cells) by decreasing ROS, IL-1β, IL-6, and TNF-α levels and increasing GSH levels and GSH peroxidase activity [5]. Ethanol extracts of *G. bicolor* also protect against UVB-induced photodamage in human skin keratinocytes (HaCaT cells) by inhibiting apoptosis [24]. Whether *G. bicolor* can protect the kidney from damage is unknown.

The aims of this study were to investigate the renoprotective effects of *G. bicolor* alcohol extracts on rat kidney epithelial NRK-52E cells induced by tert-butyl hydroperoxide (t-BHP).

Methods

Preparation of G. bicolor alcohol extract (GBAE)

Fresh *G. bicolor* was purchased from the agricultural production and marketing groups of Yuanshan Village (Ilan, Taiwan). A voucher specimen of *G. bicolor* (TCF13549) has been deposited at National Chung Hsieh University (NCHU, Taichung, Taiwan), and a *G. bicolor* specimen growing in the Department of Forestry, NCHU was identified by Yen Hsueh Tseng, Ph.D. GBAE was prepared according to our previous method with some modifications [3]. After removal and cleaning, the leaves of *G. bicolor* were blended in cold ethanol (4°C, w/v: 1/3). The cold homogenates were extracted by ultrasonication (40

KHerz, Delta Ultrasonic Co., Ltd, New Taipei City, Taiwan) for 30 min twice in a 4°C cold room. The extracts were centrifuged at 250 x g for 10 min at 4°C. The supernatant was concentrated under a rotary evaporator (water bath at 55°C) (Eyela, Tokyo Rikakikai Co., Tokyo, Japan). Finally, the concentrated product was dried in a freeze-dryer (Labconco Co., Kansas City, MO, US) at -43°C. The percent yields of GBAE were 1.1% (w/w). The GBAE was kept at -20°C until use in the study.

Cell culture and treatment

NRK-52E cells, normal rat kidney epithelial cells, were purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan). Cells were cultured on 30-mm or 60-mm culture plates with Dulbecco's modified Eagle's medium containing 42 mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, and 10% (v/v) heat-inactivated fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in an atmosphere of 5% CO_2/95% air at 37°C. To prevent the serum from interfering with oxidative stress induction, DMEM with the same additives as above, but without serum, was used as the experimental medium.

NRK-52E cells were plated at a density of 1 x 10^5 per 30-mm culture dish or 1 x 10^6 per 60-mm culture dish and were incubated until 80% confluence was reached. The cells were treated with 0.5, 1, or 5 μg/mL GBAE for 24 h and then stimulated with 50 μM t-BHP (Sigma-Aldrich Co., St. Louis, MO, US) for another 24 h. t-BHP was used in this study to induce oxidative stress and inflammation [25]. The group treated with 50 μM t-BHP alone served as the induced control group. GBAE was diluted in 95% ethanol, and cells treated with 95% ethanol alone served as the control group. GBAE treatment (5 μg/mL) without t-BHP treatment for 48 h was used as another control group.

Cell viability assay

The cell viability of NRK-52E cells was determined following the method described by Denizot and Lang (1986) [26] to assess an optimum experimental dose of GBAE in this study. At the end of the experimental treatment, the reaction was stopped by removing the medium. Then, the cells were incubated in DMEM containing 0.5 mg/mL thiazolyl blue formazan (MTT; Sigma-Aldrich Co.) for an additional 3 h, and the medium was removed and extracted by isopropanol for 15 min. The optical density (OD) of the isopropanol fraction was measured at a wavelength of 570 nm in a Microplate Biokinetics Reader (Bio-Tek Instruments, Winooski, VT, USA).

A phase-contrast inverted fluorescence microscope was used to evaluate morphological changes (Olympus IX51, Olympus, Tokyo, Japan).

Lipid peroxidation and ROS level analysis

The lipid peroxide levels of NRK-52E cells were determined according to the method described by Fraga et al. (1988) [27]. At the end of the experimental treatment, the reaction was stopped by removing the medium and washing with cold p*hosphate-buffered saline* (*PBS,* 3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 1.3

mM KCl, 135 mM NaCl, pH 7.4). The cells were removed with a cell scraper with 100 mL of 20 mM phosphate buffer containing 0.5% Triton X-100 and 10 mM butylated hydroxytoluene (BHT, in ethanol) and centrifuged at 10000 x g at 4°C. Using 100 mL of cell upper suspension, 2 mL of 0.1 N HCl, 0.3 mL of 10% phosphotungstic acid, and 1 mL of 0.7% 2-thiobarbituric acid was added to the mixture. The resulting mixture was heated for 30 min in boiling water, and thiobarbituric acid reactive substances (*TBARS*) were extracted into 5 mL of n-butanol. After centrifugation at 3500 x g and 4℃ for 15 min, the fluorescence of the butanol layer was measured with a fluorescence microplate reader (Bio-Tek Instruments). The excitation and emission wavelengths were 515 nm and 555 nm, respectively, in a fluorescence microplate reader (Bio-Tek Instruments). The total protein concentrations of NRK-52E cells were determined as described by Lowry et al. (1951) [28].

At the end of the experimental treatment, the reaction was stopped by removing the medium. The medium removed from each group was collected as the sample for the ROS level assay. The level of ROS in NRK-52E cells was examined by Cellular ROS Assay Kit (ab113851, *Abcam Inc.*, Cambridge, MA, USA) according to the manufacturer's instructions.

GSH level analysis

At the end of the experimental treatment, the reaction was stopped by removing the medium and washing with cold PBS. Samples for GSH (reduced form) and GSSG (oxidized form) determination were prepared by adding 1 mL of 5% perchloric acid, which contained 2 mmol/L 1,10-phenanthroline, to each culture plate. The plates were scraped, and the contents were centrifuged at 10,000 x g for 10 min. The acid-soluble GSH in the hepatocellular supernatant was measured by HPLC [29]. Total protein levels of cultured cells were determined using the method described by Lowry et al. (1951) [28]. The levels of GSH and GSSG are presented as nmol/mg protein.

Nitrite (NO) and *prostaglandin* E_2 (*PGE*$_2$) determination

At the end of the experimental treatment, the reaction was stopped by removing the medium. The medium removed from each group was collected as the sample for the NO and PGE_2 level assays. The nitrate levels of NRK-52E cells were determined following the Griess assay method [30] and were used as an indicator of NO production in cells. The absorbance at 550 nm was measured and calibrated using a standard curve of sodium nitrite prepared in culture medium.

The PGE_2 levels of NRK-52E cells were measured by a competitive enzyme immunoassay (EIA) kit (ADI-900-001, Cayman Chemical, Ann Arbor, MI). The concentrations of the mediator in the samples were calculated according to reference calibration curves of the standards.

IL-1β, IL-6 and TNF-α assessment

At the end of the experimental treatment, the reaction was stopped by removing the medium. The medium removed from each group was collected as the sample for the IL-1β, IL-6 or TNF-α level assays.

In this study, the IL-1β, IL-6 and TNF-α levels in NRK-52E cells were assayed using Rat IL-1β/IL-1F2 DuoSet ELISA (R&D, DY501-05), Rat IL-6 DuoSet ELISA (R&D, DY506-05), and Rat TNF-α DuoSet ELISA (R&D, DY510-05) kits, respectively (*R&D Systems*, *Inc.*, Minneapolis, MN, US), according to the manufacturer's instructions.

Apoptosis analysis

At the end of the experimental treatment, the reaction was stopped by removing the medium and washing with cold PBS, and the cells were harvested and stained with an Annexin V-FITC/PI Apoptosis Detection Kit (CE 1004) according to the manufacturer's instructions.

Immunoblot analysis of apoptosis-regulated protein expression

At the end of the experimental treatment, the reaction was stopped by removing the medium and washing with cold PBS. Then, the cells were harvested in 200 μl of lysis buffer (10 mM Tris-HCl, 5 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, and 20 μg/ml aprotinin at pH 7.4). The total protein levels in NRK-52E cells were determined using the method described by Lowry et al. (1951) [28].

For each sample, 10-20 μg of cellular protein was separated by 10% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis [31] and transferred to polyvinylidene difluoride membranes [32]. The membranes were then incubated with anti-Bax, Bcl-2, and poly (ADP-ribose) polymerase (*PARP*) antibodies at 37°C for 1 h, followed by incubation with a peroxidase-conjugated secondary antibody. The bands were visualized using an enhanced chemiluminescence detection kit (RPN3243, Amersham Life Science, Buckinghamshire, UK). For densitometric analysis, the blots were treated with enhanced chemiluminescence substrate solutions and exposed using a ChemiDoc XRSþ System (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

The data were analyzed using the statistical analysis software SPSS for Windows, version 20.0 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance and Tukey's multiple range tests were used to evaluate the significance of differences between mean values. ^{abcd}Values are significantly different from the other groups. A p-value less than 0.05 indicated a statistically significant difference.

Results

GBAE protects NRK-52E cells from t-BHP-induced damage

The cell viability of NRK-52E cells treated with only t-BHP, (86.4 ± 1.2%) was significantly decreased compared to that of the control cells (100%) ($P < 0.05$) (Fig. 1). However, the cell viability of NRK-52E cells treated with 0.5, 1 or 5 μg/mL GBAE and stimulated with t-BHP (97.6 ± 1.7%, 98.4 ± 1.5% and 98.1 ± 1.4%, respectively) was not significantly different from that of the control group (100%) (Fig. 1). In addition, the

cell viability of the group treated with only 5 μg/mL GBAE for 48 h (98.5 ± 1.9%) did not differ from that of the control group (100%). According to morphological examination by inverted microscopy, the cell number and cell morphology did not significantly differ among the groups treated with 0.5, 1 or 5 μg/mL GBAE and stimulated with t-BHP, the group treated with only GBAE, and the control group. The cell viability of the group treated with only 5 μg/mL GBAE did not differ from that of the control group. Thus, treatment with 0.5, 1, or 5 μg/mL GBAE can protect the cell viability of NRK-52E cells from t-BHP-induced damage, and GBAE is not toxic to NRK-52E cells.

GBAE reduced the oxidative stress of NRK-52E cells induced by t-BHP

According to the TBAR analysis, the treatment of NRK-52E cells with t-BHP significantly increased TBAR levels by 180% ($P < 0.05$) (Fig. 2A). However, after NRK-52E cells were incubated with 0.5, 1, or 5 μg/mL GBAE and then stimulated with t-BHP, the TBAR levels were significantly decreased by 47 to 72% compared with those in the t-BHP-treated group ($p < 0.05$) (Fig. 2A). In addition, the TBAR levels of the group treated with only 5 μg/mL GBAE did not differ from those of the control group.

In NRK-52E cells treated with only t-BHP, the ROS levels (100%) were significantly higher than those in the control cells (20.4 ± 3.4%) ($P < 0.05$) (Fig. 2B). The ROS levels of the group treated with only 5 μg/mL GBAE did not differ from those of the control group. However, the ROS levels of NRK-52E cells did significantly decrease in the groups treated with 0.5, 1, or 5 μg/mL GBAE stimulated with t-BHP (approximately 40.5–58.7%) ($P < 0.05$). These results demonstrate that GBAE reduces the oxidative stress of NRK-52E cells induced by t-BHP.

To determine the antioxidative effects of GBAE on NRK-52E cells in this study, the intracellular GSH levels of NRK-52E cells were analyzed. Figure 3C shows that the GSH levels of the group treated with only t-BHP (26.9 ± 3.3 nmol/mg protein) were significantly lower than those of the control group (51.7 ± 4.6 nmol/mg protein) ($P < 0.05$) (Fig. 2C). The GSH levels of NRK-52E cells treated with 1 or 5 μg/mL GBAE and stimulated with t-BHP (44.3 ± 3.7 and 51.0 ± 7.2 nmol/mg protein, respectively) were significantly higher than those in the group treated with only t-BHP (26.9 ± 3.3 nmol/mg protein) ($P < 0.05$). In addition, when NRK-52E cells were treated with 0.5, 1, or 5 μg/mL GBAE and stimulated with t-BHP (7.4 ± 0.6, 10.7 ± 0.7 and 11.3 ± 0.5, respectively) (Fig. 3D), the GSH/GSSG ratio was significantly higher than that of the group treated with only t-BHP (4.5 ± 0.5) ($P < 0.05$). These results showed that GBAE can decrease oxidative stress and increase intracellular GSH levels in NRK-52E cells induced by t-BHP.

GBAE alleviated the inflammation of NRK-52E cells induced by t-BHP

Figure 3A and B show that compared with the control group, the t-BHP-only group had significantly increased levels of NO and PGE_2($P < 0.05$). The levels of NO and PGE_2 in NRK-52E cells are presented in Fig. 3A and 3B. The treatment of NRK-52E cells with 1 or 50 μg/mL GBAE combined with t-BHP stimulation significantly decreased NO levels by 20 and 22% and reduced PGE_2 levels by 23 to 33% compared with those in the t-BHP-treated group (100%) (Fig. 3A, B). These results showed that GBAE can alleviate the inflammatory response of NRK-52E cells under t-BHP induction.

When the inflammatory response was induced in NRK-52E cells by t-BHP, the IL-6 level was significantly higher than that in the control group ($p < 0.05$). However, the IL-6 levels of NRK-52E cells were significantly decreased after stimulation with 0.5, 1, or 5 μg/mL GBAE combined with t-BHP (54.9 ± 4.7, 54.1 ± 12.4, and 69.1 ± 1.8%, respectively) compared with those after stimulation with only t-BHP (100%) ($P < 0.05$) (Fig. 3C). Similar to the changes in IL-6 levels, the TNF-α levels of NRK-52E cells were significantly decreased by 4–7% after 1 or 5 μg/mL GBAE treatment compared with after t-BHP stimulation ($P < 0.05$) (Fig. 3D). Figures 3C and 3D also show that the IL-6 and TNF-α levels of the group treated with only 5 μg/mL GBAE did not significantly differ from those of the control group. In addition, IL-1β levels were not different among the control group, the t-BHP-treated group, the groups treated with various concentrations of GBAE and t-TBHP, and the GBAE-treated group (data not shown).

GBAE reduces the apoptosis and apoptosis-related molecule levels of NRK-52E cells induced by t-BHP

As shown in Fig. 4A, the levels of Bax in NRK-52E cells treated with 0.5 or 1 μg/mL GBAE and stimulated with t-BHP were significantly decreased by 78.5 ± 6.8 and 80.0 ± 4.4% compared to the group treated with only t-BHP ($p < 0.05$). The Bcl-2 protein expression level of NRK-52E cells treated with 1 μg/mL GBAE and t-BHP was significantly increased by 134% compared with that of the cells treated with only t-BHP ($p < 0.05$) (Fig. 4B). The PARP protein expression level of NRK-52E cells treated with 0.5, 1, or 1 μg/mL GBAE and stimulated with t-BHP was significantly increased by 10–21% compared with that of the t-BHP-treated cells ($p < 0.05$). (Fig. 4C). In addition, when NRK-52E cells were treated with 0.5, 1, or 5 μg/mL GBAE and stimulated with t-BHP, the apoptosis percentage was significantly decreased by 21–22% compared with that of the t-BHP-treated group ($p < 0.05$) (Fig. 4D). These results show that GBAE can significantly reduce the levels of apoptosis by regulating Bax, Bcl-2, and PARP expression.

Discussion

The present study showed that GBAE exhibits the potential to protect against the damage to the rat kidney epithelial cell line, NRK-52E, induced by t-BHP. This protective mechanism is involved in the decreased apoptosis of kidney epithelial cells by reducing oxidative stress and the inflammatory response.

Yoshitama et al. (1994) and Hayashi et al. (2002) showed that *G. bicolor* is rich in three major anthocyanidins, namely, pelargonidin, delphinidin, and malvidin [33, 34]. Our previous studies showed that various extracts of *G. bicolor* are rich in chlorophyll, flavonoid, and carotenoid family molecules, three major plant pigment families, including chlorophyll, gallic acid, β-carotene, rutin, anthocyanidin, myricetin, and morin [2, 3]. Recently, high contents of sesquiterpene compounds such as beta-caryophyllene, alpha-caryophyllene, and alpha-copaene were found in *G. bicolor* [1]. These plant pigment constituents of *G bicolor* are related to its physiological effects [4]. Previous studies have shown that these plant pigments and phytochemical components have anti-inflammatory, antioxidative stress, and proapoptotic effects. Such as, Arjinajarn et al. (2017) showed that an anthocyanin-rich riceberry bran extract can prevent the liver dysfunction and damage induced by gentamicin through its antioxidation, anti-inflammatory, and

antiapoptotic effects [35]. In addition, β-caryophyllene, a sesquiterpene compound, attenuates oxidative stress, inflammation, and apoptosis and prevents doxorubicin-induced acute cardiotoxicity in rats [36]. In addition, pretreatment with β-carotene can protect against the nephrotoxicity induced by bromobenzene by reducing oxidative stress and proinflammatory and proapoptotic factors [37]. In the present study, GBAE reduced apoptosis in NRK 52E cells induced by t-BHP, protecting against kidney cell damage. The phytochromes of GBAE, such as quercetin, rutin, gallic acid, chlorophyll, β-carotene, anthocyanidin, myricetin, and morin, may play an important role in reducing apoptosis in NRK-52E cells through antioxidative stress and anti-inflammatory responses.

Inflammation is a normal immune response to stimuli, such as pathogens, damaged cells, toxic compounds, or irradiation [38]. However, a chronic inflammatory response can lead to many chronic diseases, such as cardiovascular disease, diabetes mellitus, cancer, and CKD [39]. Reduced chronic or excessive inflammatory responses protect from, decrease, or alleviate these chronic diseases [40]. Cytokines, such as IL-1β and TNF-α, released during the inflammatory response trigger a series of physiological effects, including increased ROS levels [41]. ROS not only help protect against extracellular pathogens but also induce cell damage and cell death, i.e., apoptosis [42, 43]. Inflammation is one of the major causes of kidney injury, and chronic inflammation will lead to CKD, dialysis, and kidney failure. Suppression of acute, chronic, or overexpression of inflammation is a way to prevent kidney injury. In the present study, GBAE significantly decreased the levels of the cytokines IL-6 and TNF-α, and these inflammatory responses mediated the levels of NO and PGE_2. This is a very beneficial effect in reducing kidney cell inflammation and injury under oxidative stress. Previous studies have shown that the water extract of *Hedyotis diffusa* (equal to 5.0 g/kg body weight) obviously protects against lipopolysaccharide (LPS)-induced kidney inflammation and kidney damage by suppressing the levels of TNF-α, IL-1β, IL-6, and MCP-1 and promoting the levels of IL-10 in the serum of Kunming mice [44]. In kidney structural and functional disorders in a CKD experimental model, quercetin attenuated LPS-stimulated apoptosis by reducing the production of IL-6 and TNF-α in HK-2 cells [45]. Our previous study showed that GBAE can reduce inflammation by reducing NO and PGE_2 levels and iNOS and COX-2 expression in RAW264.7 cells induced by LPS [2]. The above results showed that *G. bicolor* has a renoprotective effect by reducing the inflammatory response.

In addition to the anti-inflammatory effects of GBAE, it can also significantly decrease ROS and TBARS levels and increase GSH levels to prevent the oxidative stress that leads to cell damage under t-BHP induction. Oxidative stress is one of the more important factors in kidney damage. For example, the metabolism of food and medicine, physiological antioxidants, and antioxidant enzyme deficiency are sources of ROS and reactive nitrite species (RNS). Under conditions of antioxidative deficiency, kidney cells experience inflammation, damage, fibrosis, and death. Previous studies have also shown that gravinol, a proanthocyanidin from grape seeds, has polyphenolic properties and potently inhibits lipid peroxidation and decreases total RNS, O(2), NO, and ONOO(-) levels; its protective effect against high glucose-induced kidney tubular epithelial cell damage attenuates diabetic nephropathy [46]. The acetone and hydroethanol extracts of *Phyllanthus phillyreifolius*, a plant species indigenous to Reunion Island,

protect against oxidative stress induced by H_2O_2 in the human embryonic kidney 293 (HEK293) cell line by reducing ROS levels, increasing GSH levels, and activating SOD, catalase, and GPX activity [47]. The ethanol extract of the root bark of *Illicium henryi* can decrease TNF-α, IL-1β, and IL-6 levels and increase SOD activity and GSH levels in kidney tissue after lipopolysaccharide (LPS)-induced AKI in BALB/c mice [48]. Previous studies have shown that *G. bicolor* aqueous extracts can attenuate H2O2-induced injury in artificial rat neurons (PC-12 cells) by decreasing ROS, IL-1β, IL-6, and TNF-α levels and increasing GSH levels and GSH peroxidase activity [5]. In the present study, GBAE significantly enhanced cell antioxidation ability and prevented kidney cell damage, indicating potential potential for kidney protection.

Apoptosis usually plays an important role in the pathological process of kidney injury, CKD, and related diseases, such as glomerulonephritis, acute and chronic kidney failure, diabetic nephropathy, and polycystic kidney disease [23]. Especially in kidney epithelial cells or mesangial cells, some apoptotic triggers, such as chemicals, drugs, oxidative stress, etc., induce apoptosis through extrinsic or intrinsic pathways, leading to kidney cell death and fibrosis [22, 49]. Therefore, reducing apoptosis may be one of the therapeutic approaches for kidney disease. Previous studies have shown that colchicine has antifibrotic and antiapoptotic effects in obstructed kidneys. An optimal dose of colchicine could reduce apoptosis to prevent kidney disease progression [46]. Zeaxanthin can decrease MDA levels, increase SOD activity, inhibit apoptosis, decrease ROS levels in mesangial cells in a high-sugar environment and improve diabetic kidney disease [50]. In addition, cryptotanshinone (CTS), a component of *Salvia miltiorrhiza* Bunge, can ameliorate ischemia/reperfusion injury in HK-2 cells, human proximal tubular cells, in vitro partly by regulating Bax, Bcl-2, and caspase-3 expression to decrease apoptosis [51]. Li et al. (2019) showed that an ethanol extract of *G. bicolor* can protect against UVB-induced apoptosis of skin HaCaT cells by reducing Bax and caspase-3 mRNA expression and increasing Bcl-2 mRNA expression [24]. In the present study, GBAE significantly decreased the apoptosis of kidney epithelial NRK-52E cells induced by t-BHP by decreasing the level of ROS and the expression of Bax, and PARP and increasing the level of Bcl-2. This represents an important new finding that the potential renoprotective effect of *G. bicolor* may be due to a decrease in apoptosis.

As shown in Fig. 5, GBAE can protect NRK52E kidney epithelial cells from damage induced by t-BHP. The major mechanism is that GBAE can reduce oxidative stress by increasing intracellular GSH levels and reducing peroxidation. GBAE can also inhibit inflammation by reducing TNF-α, IL-6, NO, and PGE2 production. After GBAE treatment, reduced oxidative stress and anti-inflammation suppressed apoptosis in NRK-52E cells by increasing Bcl-2 levels and decreasing Bax and PARP expression.

Conclusion

In summary, the present results showed that 5 μg/ml GBAE can protect against kidney cell damage induced by t-BHP. The protective antioxidative and anti-inflammation mechanism of GBAE involves a reduction in cell damage and apoptosis. The molecular regulatory mechanisms of these antioxidative, inflammation and apoptosis-related molecules require further investigation.

Abbreviations

GBAE
Gynura bicolor alcohol extracts; t-BHP:tert-butyl hydroperoxide; TBARS:thiobarbituric acid reactive substances; ROS:reactive oxygen species; NO:nitrite; PGE_2:prostaglandin E2; PARP:poly (ADP-ribose) polymerase; AKI:acute kidney injury; CKD:chronic kidney disease; GSH:glutathione

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author.

Competing interests

The authors declare that they have no competing interests.

Funding

The authors thank the Ministry of Science and Technology of Taiwan for support of this work through grants (grant No. MOST 108-2813-C-126-021-B; MOST 108-2320-B-126 -004 -).

Authors' contributions

Chih-Chung Wu and Shu-Ling Hsieh conceived and designed the study and were major contributors to writing and critically revising the article. Chia-Hsuan Wu performed the experiments and analyzed the data. Jyh-Jye Wang and Chien-Chun Li provided advice on the experiments and technical assistance. Chih-Chung Wu supervised the study. All Authors read and approved the article and agree to be accountable for all aspects of the research and ensure that the accuracy or integrity of any part of the work is appropriately investigated and resolved.

Acknowledgements

We acknowledge the assistance in the identification of G. bicolor and a voucher specimen of G. bicolor (TCF13549) by Yen Hsueh Tseng, Ph.D. in Department of Forestry, National Chung Hsieh University (NCHU, Taichung, Taiwan).

References

1. Chen J, Adams A, Mangelinckx S, Ren BR, Li ML, Wang ZT. Investigation of the volatile constituents of different *Gynura* species from two Chinese origins by SPME/GC-MS. Nat Prod Commun. 2012;7:655–57.
2. Wu CC, Lii CK, Liu KL, Chen PY, Hsieh SL. Antiinflammatory activity of *Gynura bicolor* (Hóng Fèng Cài) ether extract through inhibits nuclear factor kappa B activation. J Tradit Complement. 2013;3:48–52.
3. Wu CC, Chang WL, Lu CH, Chang YP, Wang JJ, Hsieh SL. Effects of extracts from *Gynura bicolor* (Roxb. & Willd.) DC. on iron bioavailability in rats. J. Food Drug Anal. 2015;23(3):425–32.
4. Lu H, Pei Y, Li W. Studies on flavonoids from *Gynura bicolor* DC. Zhongguo Xian Dai Ying Yong Yao Xue. 2010;27:613–14.
5. Yang YC, Wu WT, Mong MC, Wang ZH. *Gynura bicolor* aqueous extract attenuated H_2O_2 induced injury in PC12 cells. Biomedicine (Taipei). 2019;9(2):12. doi: 10.1051/bmdcn/2019090212.
6. Yin MC, Wang ZH, Liu WH, Mong MC. Aqueous extract of *Gynura Bicolor* attenuated hepatic steatosis, glycative, oxidative, and inflammatory injury induced by chronic ethanol consumption in mice. J Food Sci. 2017;82(11):2746–51.
7. Pai PY, Mong MC, Yang YC, Liu YT, Wang ZH, Yin MC. Anti-diabetic effects of *Gynura bicolor* aqueous extract in mice. J Food Sci. 2019;84(6):1631–37.
8. Krishnan V, Ahmad S, Mahmood M. Antioxidant potential in different parts and callus of *Gynura procumbens* and different parts of *Gynura bicolor*. Biomed Res Int. 2015;2015:147909. doi.org/10.1155/2015/147909.
9. Hsieh SL, Tsai PJ, Liu YC, Wu CC. Potential effects of antioxidant and serum cholesterol-lowering effects of *Gynura bicolor* water extracts in Syrian Hamster. Evid. Based Complementary Altern. Med. 2020;2020(7). doi.org/10.1155/2020/2907610
10. Teoh WY, Tan HP, Ling SK, Wahab NA, Sim KS. Phytochemical investigation of *Gynura bicolor* leaves and cytotoxicity evaluation of the chemical constituents against HCT 116 cells. Nat Prod Res. 2016;30(4):448–51.
11. Wu CC, Chang YP, Wang JJ, Liu CH, Wong SL, Jiang CM, Hsieh SL. Dietary administration of *Gynura bicolor* (Roxb. Willd.) DC water extract enhances immune response and survival rate against *Vibrio alginolyticus* and white spot syndrome virus in white shrimp *Litopeneaus vannamei*. Fish Shellfish Immun. 2015;42(1):25–33.
12. Teoh WY, Sim KS, Richardson JSM, Wahab NA, Hoe SZ. Antioxidant capacity, cytotoxicity, and acute oral toxicity of *Gynura bicolor*. Evid Based Complementary Altern Med. 2013;2013(7):958407. doi.org/10.1155/2013/958407
13. Chen J, Lü H, Fang LX, Li WI, Verschaeve L, Wang ZT, Kimpe ND, Mangelinckx S. Detection and Toxicity Evaluation of Pyrrolizidine Alkaloids in Medicinal Plants *Gynura bicolor* and *Gynura*

divaricata Collected from Different Chinese Locations. Chem. Biodivers. 2017;14(2). doi: 10.1002/cbdv.201600221.

14. Imig JD, Ryan MJ. Immune and inflammatory role in renal disease. Compr Physiol. 2013;3(2):957–76.
15. Mocker A, Hilgers KF, Cordasic N, Wachtveitl R, Menendez-Castro C, Woelfle J, Hartner A, Fahlbusch B. Renal chemerin expression is induced in models of hypertensive nephropathy and glomerulonephritis and correlates with markers of inflammation and fibrosis. Int J Mol Sci. 2019;20:6240. doi: 10.3390/ijms20246240.
16. Baer PC, Koch B, Geiger H. Kidney Inflammation, Injury and Regeneration. Int J Mol Sci. 2020;21:1164. doi: 10.3390/ijms21031164.
17. Ratliff BB, Abdulmahdi W, Pawar R, Wolin MS. Oxidant mechanisms in renal injury and disease. Antioxid Redox Signal. 2016;25(3):119–46.
18. Balasubramanian S. Progression of chronic kidney disease: Mechanisms and interventions in retardation. Apollo Med. 2013;10:19–28.
19. Ruiz S, Pergola PE, Zager RA, Vaziri ND. Targeting the transcription factor Nrf2 to ameliorate oxidative stress and inflammation in chronic kidney disease. Kidney Int. 2013;83(6):1029–41.
20. Noji T, Sato H, Sano J, Nishikawa S, Kusaka H, Karasawa A. Treatment with an adenosine uptake inhibitor attenuates glomerulonephritis in mice. Eur J Pharmacol. 2002;449:293–300.
21. Hong Y, Liao WS, Mo RX. Dipyridamole preconditioning protects against ischemia/reperfusion injury of rat liver. Zhongguo Wei Zhong Bing Ji Jiu Yi Xue. 2006;18:425–7.
22. Sanz AB, Santamaría B, Ruiz-Ortega M, Egido J, Ortiz A. Mechanisms of renal apoptosis in health and disease. J Am Soc Nephrol. 2008;19(9):1634-42.
23. Ortiz A. Apoptotic regulatory proteins in renal injury. Kidney Int. 2000;58:467–85.
24. Li L, Han X, Gao Y, Diao Q, Xiao Y. Ethanol extract of *Gynura bicolor* (GB) protects against UVB-induced photodamage of skin by inhibiting P53-mediated Bcl-2/BAX/Caspase-3 apoptosis pathway. Arch Dermatol Res. 2019;312:41–9.
25. Liu H, Mao P, Wang J, Wang T, Xie CH. Azilsartan, an angiotensin II type 1 receptor blocker, attenuates tert-butyl hydroperoxide-induced endothelial cell injury through inhibition of mitochondrial dysfunction and anti-inflammatory activity. Neurochem Int. 2019;94:48–56.
26. Denizot F, Lang R. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J Immunol Methods. 1986;189:271–7.
27. Fraga CG, Leibovitz BE, Tappel AL. Lipid peroxidation measured as thiobarbituric acid-reactive substances in tissue slices: Characterization and comparison between homogenates and microsomes. Free Radic Biol Med. 1988;4:155–61.
28. Lowry O, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with Folin phenol reagent. J Biol Chem. 1951;193:265–75.

29. Reed DJ, Babson JR, Beatty PW, Brodie AE, Ellis WW. High-performance liquid chromatography analysis of nanomole levels of glutathione, glutathione disulfide, and related thiols and disulfides. Anal Biochem. 1980;106:55–62.
30. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [15N]-nitrate in biological fluids. Anal Biochem. 1992;126:131–8.
31. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970;227:680–5.
32. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA. 1979;76:4350–6.
33. Yoshitama K, Kaneshige M, Ishikura N, Araki F, Yahara S, Abe KA. Stable reddish purple anthocyanin in the leaf of *Gynura aurantiaca* cv, 'Purple Passion'. J Plant Res. 1994;107:209–14.
34. Hayashi M, Iwashita K, Katsube N, Yamaki K, Kobori M. Kinjiso (*Gynura bicolor* DC.) colored extract induces apoptosis in HL60 leukemia cells. Nippon Shokuhin Kagaku Kogaku Kaishi. 2002;49:519–26.
35. Arjinajarn P, Pongchaidecha A, Chueakula N, Kao KJ, Chatsudthipong V, Mahatheeranont S, Norkaew O, Chattipakorn N, Lungkaphin A. Riceberry bran extract prevents renal dysfunction and impaired renal organic anion transporter 3 (Oat3) function by modulating the PKC/Nrf2 pathway in gentamicin-induced nephrotoxicity in rats. Phytomedicine. 22016;3(14):1753–1763.
36. Al-Taee H, Azimullah S, Meeran MFN, Almheiri MKA, Jasmi RAA, Tariq S, Khan MA, Adeghate E, Ojha S. β-caryophyllene, a dietary phytocannabinoid attenuates oxidative stress, inflammation, apoptosis and prevents structural alterations of the myocardium against doxorubicin-induced acute cardiotoxicity in rats: An in vitro and in vivo study. Eur J Pharmacol. 2019;858:172467. doi: 10.1016/j.ejphar.2019.172467.
37. Akkara PJ, Sabina EP. Pre-treatment with Beta carotene gives protection against nephrotoxicity induced by bromobenzene via modulation of antioxidant system, pro-inflammatory cytokines and pro-apoptotic factors. Appl Biochem Biotechnol. 2020;190(2):616–33.
38. Medzhitov R. Inflammation 2010: new adventures of an old flame. Cell. 2010;140:771–6.
39. Chen L, Deng H, Cui H, Fang J, Zuo Z, Deng J, Li Y, Wang X, Zhao Y. Inflammatory responses and inflammation-associated diseases in organs. Oncotarget. 2017;9(6):7204–1708.
40. Laveti D, Kumar M, Hemalatha R, Sistla R, Naidu VGM, Talla V. Anti-inflammatory treatments for chronic diseases: a review. Inflamm. Allergy Drug Targets. 2013;12(5):349–61.
41. Mittal M, Siddiqui MR, Tran K, Reddy SP, Malik AB. Reactive oxygen species in inflammation and tissue injury. Antioxid Redox Signal. 2014;20(7):1126–67.
42. Simon HU, Haj-Yehia A, Levi-Schaffer F. Role of reactive oxygen species (ROS) in apoptosis induction. Apoptosis. 2000;5(5):415–8.
43. Forrester SJ, Kikuchi DS, Hernandes MS, Xu Q, Griendling KK. Reactive oxygen species in metabolic and inflammatory signaling. Circulation Res. 2018;122:877–902.

44. Ye JH, Liu MH, Zhang XL, He JY. Chemical profiles and protective effect of *Hedyotis diffusa* Willd in lipopolysaccharide-induced renal inflammation mice. Int J Mol Sci. 22015;16(11):27252-69.
45. Guo S, Sun J, Zhuang Y. Quercetin alleviates lipopolysaccharide-induced inflammatory responses by up-regulation miR-124 in human renal tubular epithelial cell line HK-2. Biofactors. 2020;46(3):402 – 10.
46. Kim YJ, Kim YA, Yokozawa T. Attenuation of oxidative stress and inflammation by gravinol in high glucose-exposed renal tubular epithelial cells. Toxicol. 270(2–3):106 – 11.
47. Grauzdytė D, Pukalskas A, Viranaicken W, Kalamouni CE, VenskutonisI PR. Protective effects of *Phyllanthus phillyreifolius* extracts against hydrogen peroxide induced oxidative stress in HEK293 cells. PLoS ONE. 2018;13(11): e0207672.
48. Islam MS, Miao L, Yu H, Han Z, Sun H. Ethanol extract of *Illicium henryi* attenuates LPS-induced acute kidney injury in mice via regulating inflammation and oxidative stress. Nutrients. 2019;11(6):1412–30.
49. Elmore S. Apoptosis: a review of programmed cell death. Toxicol Pathol. 2007;35(4):495–516
50. Ying C, Chen L, Wang S, Mao Y, Ling H, Li W, Zhou X. *Zeaxanthin ameliorates* high glucose-induced mesangial cell apoptosis through inhibiting oxidative stress via activating AKT signalling-pathway. Biomed Pharmacother. 2017;90:796–805.
51. Zhu R, Wang W, Yang SJ. Cryptotanshinone inhibits hypoxia/reoxygenation-induced oxidative stress and apoptosis in renal tubular epithelial cells. Cell Biochem. 2019;120(8):13354–60.

Figures

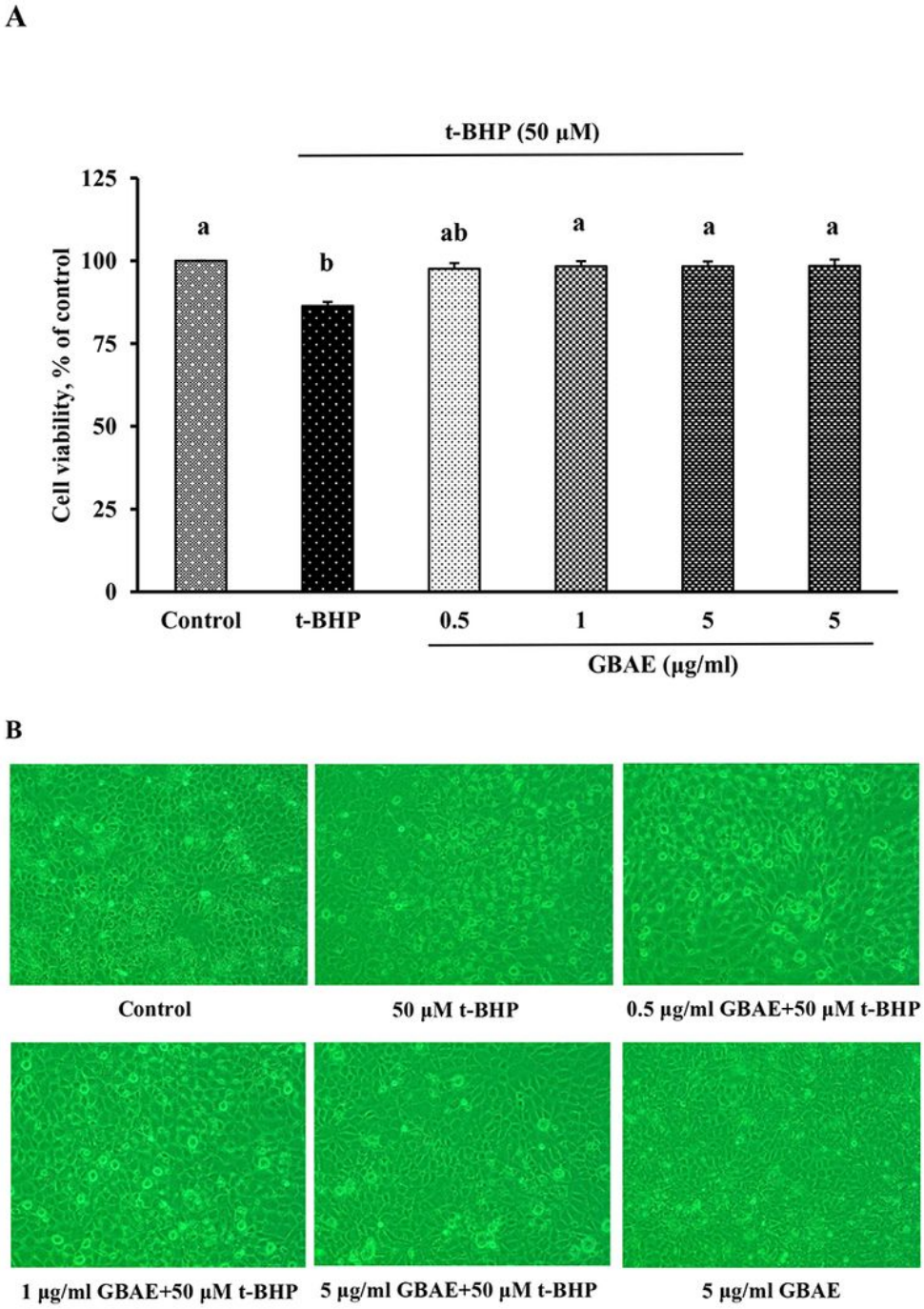

Figure 1

Figure 1

Effect of GBAE on the viability of NRK-52E cells induced by t-BHP. NRK-52E cells (1 × 105 cells/30-mm plate) were seeded and cultured overnight. NRK-52E cells were treated with 0.5, 1, or 5 μg/mL GBAE for 24 h and then induced with or without 50 M t-BHP for another 24 h. The group treated with 50 μM t-BHP alone served as an induced control group. GBAE was diluted in 95% ethanol, and cells treated with 95% ethanol alone served as the control group. Cells treated with 5 μg/mL GBAE without t-BHP treatment for

48 h were used as another control group. Cell viability (A) and morphological changes were examined. Values are presented as the mean±SD (n=3). Values are significantly different from the other groups, as determined by Duncan's test ($p<0.05$).

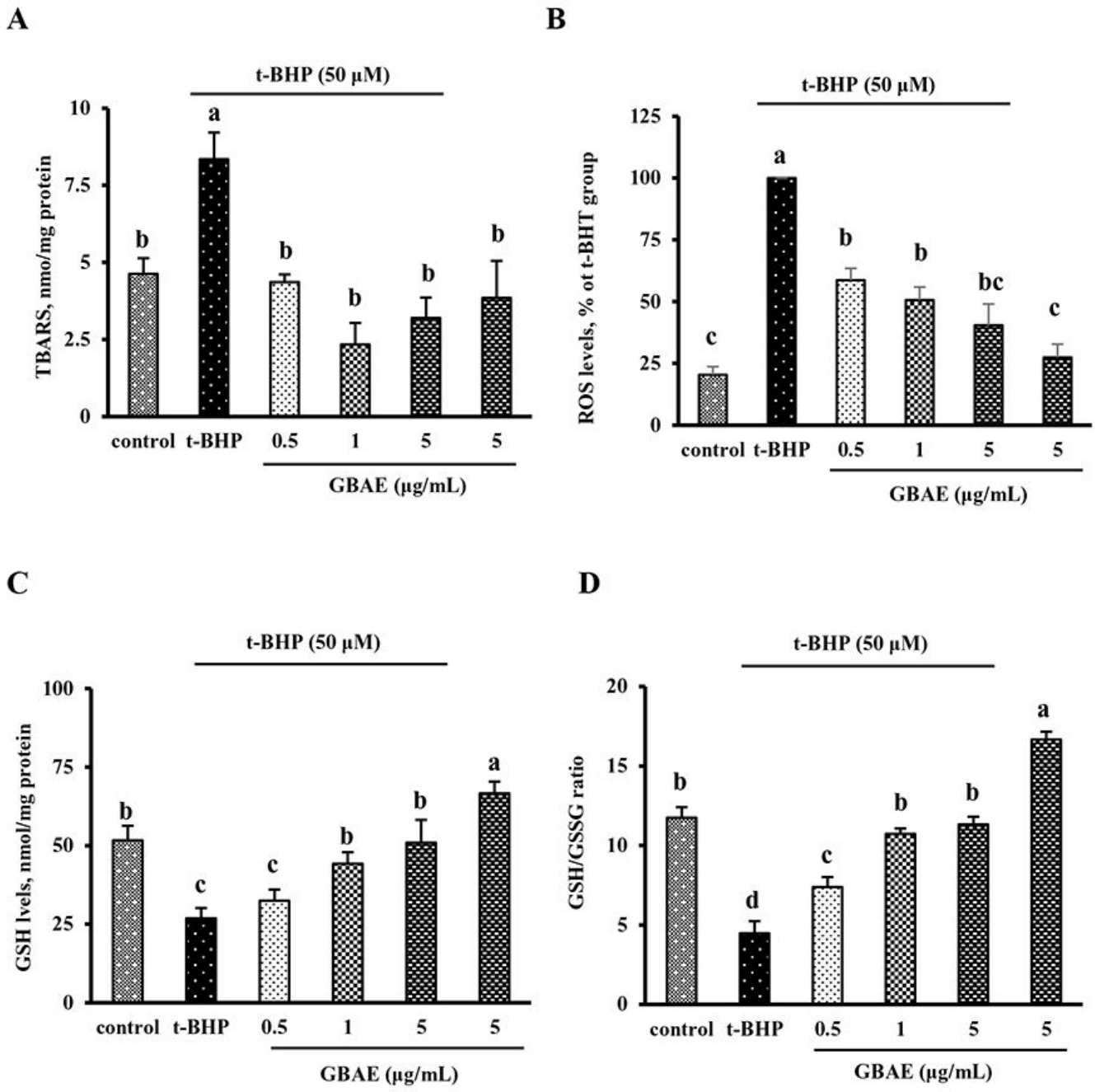

Figure 2

Figure 2

Effect of GBAE on the oxidative stress of NRK-52E cells induced by t-BHP. NRK-52E cells (1 × 105 cells/30-mm plate) were seeded and cultured overnight. NRK-52E cells were treated with 0.5, 1, or 5

μg/mL GBAE for 24 h and then induced with or without 50 □M t-BHP for another 24 h. The group treated with 50 μM t-BHP alone served as an induced control group. GBAE was diluted in 95% ethanol, and cells treated with 95% ethanol alone served as the control group. Cells treated with 5 μg/mL GBAE without t-BHP treatment for 48 h were used as another control group. The levels of TBARS (A), ROS (B), and GSH (C) and the GSH-to-GSSG ratio (D) were analyzed. Values are presented as the mean±SD (n=3). Values are significantly different from the other groups, as determined by Duncan's test ($p<0.05$).

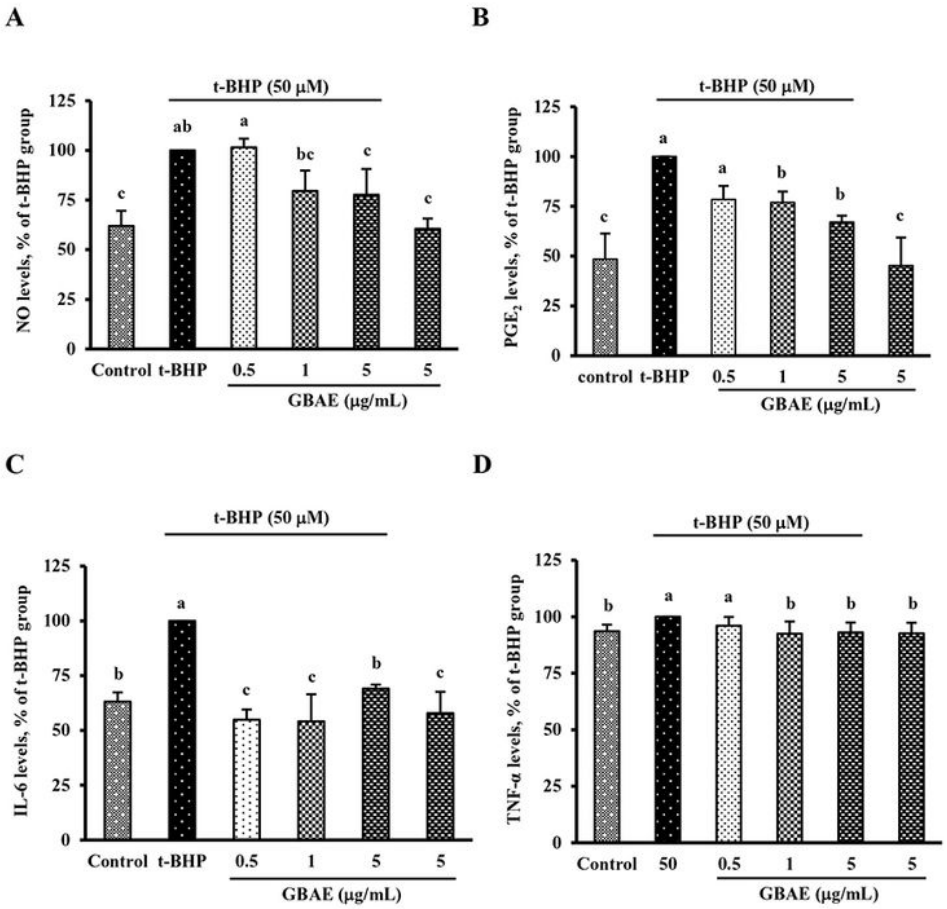

Figure 3

Figure 3

Effect of GBAE on the inflammatory response in NRK-52E cells induced by t-BHP. NRK-52E cells (1 × 105 cells/30-mm plate) were seeded and cultured overnight. NRK-52E cells were treated with 0.5, 1, or 5 μg/mL GBAE for 24 h and then induced with or without 50 □M t-BHP for another 24 h. The group treated with 50 μM t-BHP alone served as an induced control group. GBAE was diluted in 95% ethanol, and cells treated with 95% ethanol alone served as the control group. Cells treated with 5 μg/mL GBAE without t-BHP treatment for 48 h were used as another control group. The levels of NO (A), PGE2 (B), IL-6 (C) and TNF-α (D) were analyzed. Values are presented as the mean±SD (n=3). Values are significantly different from the other groups, as determined by Duncan's test ($p<0.05$).

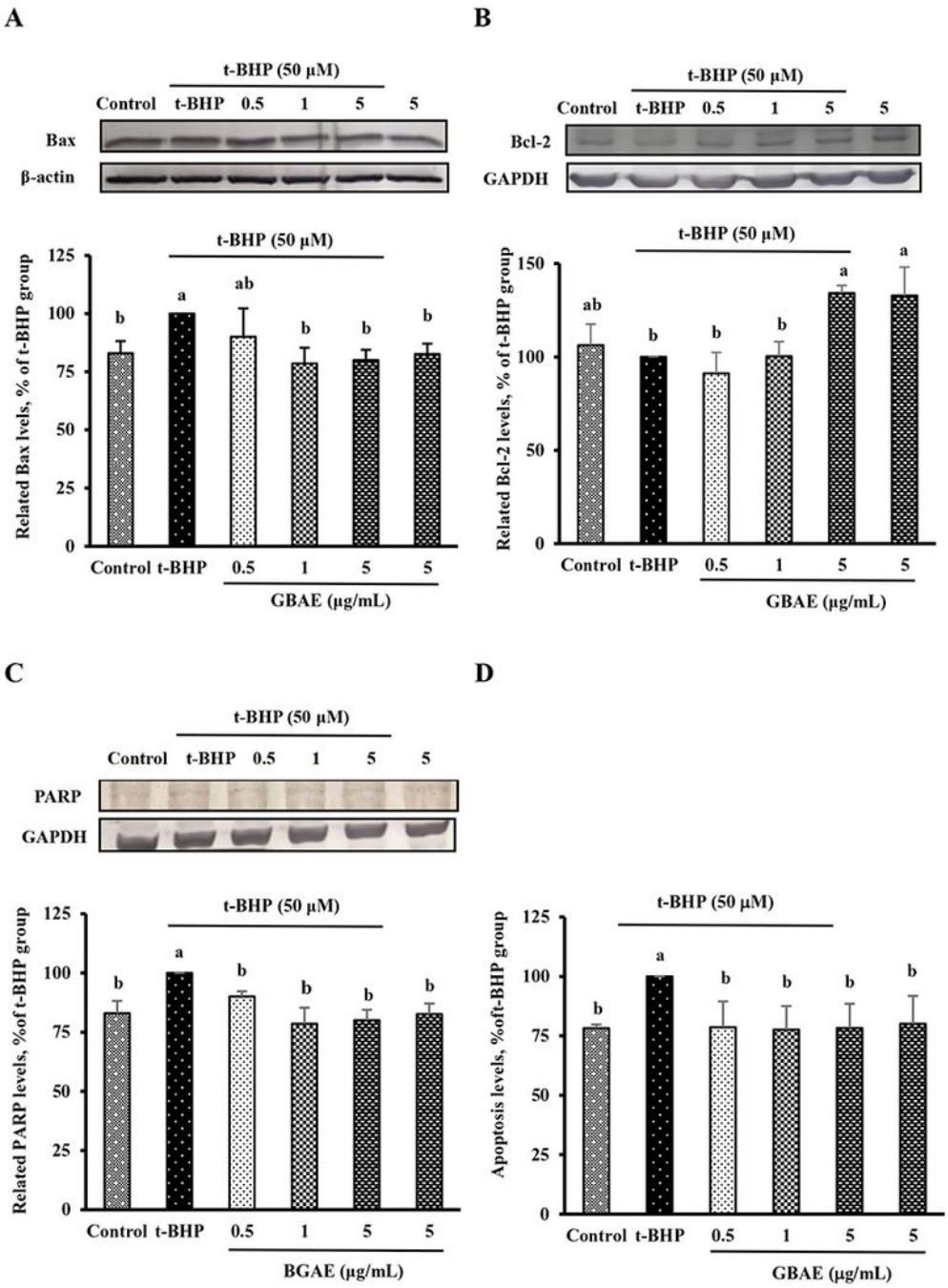

Figure 4

Figure 4

Effect of GBAE on apoptosis and the expression of apoptosis-related proteins in NRK-52E cells induced by t-BHP. NRK-52E cells (1 × 106 cells/60-mm plate) were seeded and cultured overnight. NRK-52E cells were treated with 0.5, 1, or 5 μg/mL GBAE for 24 h and then induced with or without 50 M t-BHP for another 24 h. The group treated with 50 μM t-BHP alone served as an induced control group. GBAE was diluted in 95% ethanol, and cells treated with 95% ethanol alone served as the control group. Cells treated

with 5 μg/mL GBAE without t-BHP treatment for 48 h were used as another control group. Immunoblot assays were performed to determine the expression levels of Bax (A), Bcl-2 (B), and PARP (C) in NRK-52E cells and were quantified using densitometry. The percentage of apoptosis (D) in NRK-52E cells was analyzed. Values are presented as the mean±SD (n=3). Values are significantly different from the other groups, as determined by Duncan's test ($p<0.05$).

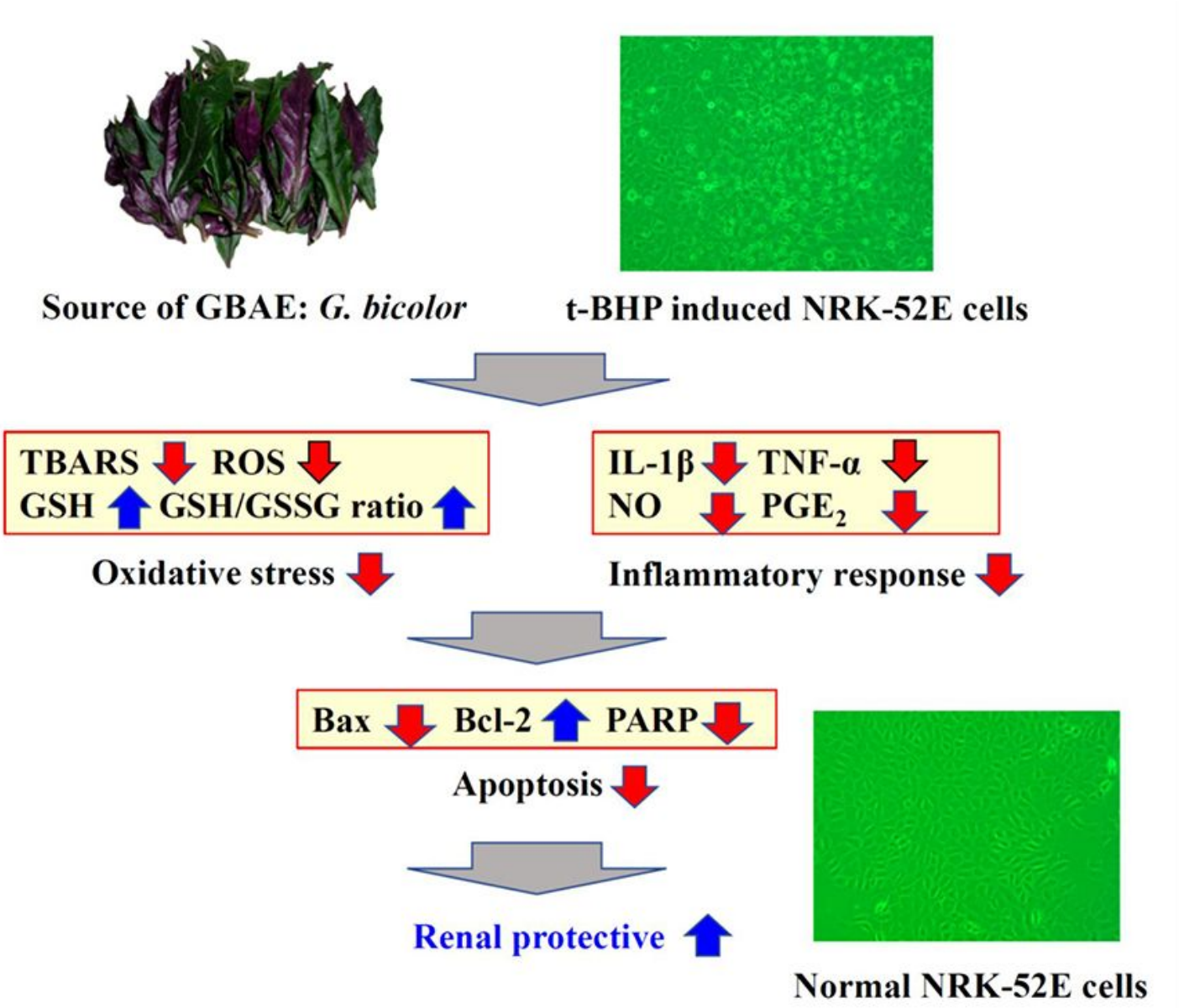

Figure 5

Figure 5

Possible mechanisms by which GBAE protects kidney epithelial NRK-52E cells from damage induced by t-BHP.