International Journal of Biological Macromolecules 101 (2017) 126–130

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Induction of mitochondrial-dependent apoptosis in T24 cells by a selenium (Se)-containing polysaccharide from *Ginkgo biloba* L. leaves

Dong Chen, Shaopeng Sun, Dawei Cai, Guangqi Kong*

Department of urological surgery, Beijing Luhe Hospital, Capital Medical University, Beijing 101149, China

ARTICLE INFO

Article history:
Received 23 January 2017
Received in revised form 16 February 2017
Accepted 6 March 2017
Available online 7 March 2017

Keywords:
Ginkgo biloba L.
Selenium-polysaccharides
Human bladder cancer

ABSTRACT

In the present study, a selenium (Se)-containing polysaccharide (Se-GBLP) was isolated and purified from the leaves of *Ginkgo biloba* L. Se-GBLP was further evaluated for its antitumor activity against human bladder cancer T24 cells together with the possible mechanism of action. Our results showed that treatment of T24 cells with Se-GBLP (50, 100 and 200 μg/ml) for 48 h significantly inhibited cell viability and induced apoptosis in a dose- dependent manner. This Se-GBLP-induced apoptosis is associated with an increased protein expression of pro-apoptotic Bax, decreased expression of anti-apoptotic Bcl-2, loss of mitochondrial membrane potential, and cleavage of caspase-9, caspase-3 and PARP, suggesting that Se-GBLP-induced apoptosis occurs through the mitochondria-dependent pathway. Se-GBLP therefore merits further investigation as a promising preventive and/or therapeutic agent against human bladder cancer.

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1. Introduction

Bladder cancer is the fourth and eighth most common urological cancer in men and women, respectively, clinically characterized by a high overall rate of recurrence and poor prognosis once tumors invade the lamina propia [1,2]. Epidemiological studies have demonstrated with sufficient evidence that approximately 75% of bladder cancer occurs in men and 25% in women [3]. Despite recent advances in the treatment of bladder cancer, half of these patients subsequently develop disease recurrence [4]. Even if after surgical and chemotherapeutic procedures, the 5-year survival rate in patients at advanced stage remains very low. Therefore, the development of novel therapeutic approaches is of great importance to control this disease.

Selenium (Se) is an essential trace element responsible for growth and development of animals and humans [5]. A substantial amount of evidence has supported that Se plays an important role in cancer therapy [6,7]. Appropriate Se consumption or supplementation in vivo may contribute to certain human diseases, among which the prevention of cancer and cardiovascular disease is of special interest to most scientists [8]. Unlike inorganic Se, organic Se coupled with other molecules, such as polysaccharides or proteins, has been found in various organisms to exhibit higher biological activities and lower toxicity [9]. Recent studies demonstrated that Se-polysaccharides isolated from medicinal plants exert potent antitumor activity against a wide range of human cancer cells, including breast cancer [10], liver cancer [11], ovarian cancer [12], and so on. As a result, searching for a safe and effective Se-enriched polysaccharide has a great potential value for bladder cancer chemoprevention. *Ginkgo biloba* L. is a valuable herb that has been existed on earth since 200 million years and is regarded as a "living fossil" [13]. *G. biloba* leaf polysaccharides have multiple pharmacological functions, for instance, antioxidant, anti-aging, anti-inflammation, neuroprotective and anticancer effects [14–17]. However, up until now no investigation has been carried out on isolation and identification of Se-polysaccharides from *G. biloba* leaves that could account for its anticancer effect. Therefore, the present study was undertaken to purify the Se-polysaccharide from this plant and first assessed its antitumor potential on the growth of bladder cancer T24 cells by determining its capabilities to induce apoptosis, and then explored its potential mechanism.

2. Materials and methods

2.1. Materials and chemicals

The leaves of *G. biloba* L. were purchased from Chinese Traditional Medicine Store in Beijing City of China. Fetal bovine serum (FBS), medium RPMI-1640, penicillin and streptomycin were purchased from Gibco Life Technologies (Maryland, USA). 3-(4,5-Dimethylthia-zol-2-y1)-2,5-diphenyltetrazolium bromide (MTT), dimethylsulfoxide (DMSO) and rhodamine 123 (Rh-123) were

* Corresponding author.
E-mail address: guangqikong@126.com (G. Kong).

http://dx.doi.org/10.1016/j.ijbiomac.2017.03.033
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obtained from Sigma Chemical Co. (St. Louis, MO, USA). The primary antibodies against cleaved caspase 3, cleaved caspase 9, and cleaved poly (ADPribose) polymerase (PARP) were purchased from Cell Signaling Technology (Beverly, MA, U.S.A.), Antibody for cytochrome c, Bax, Bcl-2, β-actin and horseradish peroxidase (HRP)-conjugated secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.), Annexin V-FITC Apoptosis Detection Kit, an enhanced chemiluminescence (ECL) kit, the caspase-3 or -9 colorimetric assay kit was obtained from BioSource International, Inc. (Camarillo, CA, USA). DEAE-cellulose and Sepharose 6 Fast Flow were purchased from Amersham Pharmacia Co. (Sweden). Other chemicals were analytical grade reagents, bought from Sinopharm Chemical Reagent Company (Shanghai, China).

2.2. Isolation and purification of a water-soluble Se-polysaccharide from G. biloba leaves

The air-dried leaves of *G. biloba* were pulverized into powder, and then exhaustively extracted with 80% ethanol for 24 h. After filtered, the residues were air-dried and extracted twice with distilled water at 90 °C for 3 h each time. The extract solutions were combined, concentrated, stepwise precipitated by10, 20, 30, 40, 50, 60, 70, and 80% of ethanol for 24 h at 4 °C. The maximum fraction (final concentration of ethanol was 50%) was collected by centrifugation, centrifuged, and deproteinated by a combination of proteinase and Sevag method [18], and the supernatant was lyophilized to obtain crude *G. biloba* leaves polysaccharides (cGBLP).

cGBLP was dissolved in distilled water and loaded onto DEAE-cellulose column (2.6 cm × 30 cm), and then eluted successively with distilled water and 0–1 M NaCl at a flow rate of 2 ml/min. One major fraction were collected, monitored with the phenol–sulfuric acid method assay at 490 nm [19], and were further fractioned on a Sepharose 6 Fast Flow column (2.6 cm × 100 cm), eluted with 0.15 M NaCl at a flow rate of 2 ml/min to yield one main fraction, coded as Se-GBLP.

2.3. Chemical component analysis

The protein content was determined according to the method of Bradford using bovine serum albumin as the standard [20]. The total carbohydrate content was estimated by the phenol–sulfuric acid method, with d-glucose as the standard [19]. Uronic acid content was determined colorimetrically by m-hydroxydiphenyl method using d-galacturonic acid as standard [21]. Se in Se-GBLP was quantified using the method of Cheng [22].

2.4. Cell lines and cell culture

The human urinary bladder cancer T24 and normal bladder mucosa HCV29 cell lines were purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences. Cells were grown in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin and l00 U/ml streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. T24 cell line was used as a bladder tumor model to study the anticancer effect of the polysaccharide and HCV29 cell line was used as a normal control.

2.5. MTT assay

The antiproliferative effect of the samples against T24 cell was determined by the colorimetric MTT assay, as described previously [23]. Briefly, T24 cells were plated onto 96-well flat-bottomed plates at a density of 1.0×10^4 cells per well for 24 h and treated with Se-GBLP (50, 100 and 200 μg/ml, final concentration), or vehicle alone for another 24, 48 or 72 h. Then 10 μl MTT solution (5 mg/ml) was added to each well, and incubated at 37 °C for another 4 h, after which, 150ul of DMSO was added to each well to fully dissolve the colored formazan precipitate. The optical density was measured in an ELISA reader at 570 nm. Similar experiments were repeated in triplicates. Cell survival was calculated as the percentage of MTT inhibition, using the following formula: Inhibition rate (%) = [A570 (control)-A570 (sample)]/A570 (control) × 100.

2.6. Quantification of apoptosis by flow cytometry

For quantification of apoptosis, T24 cells (2×10^5 cells per well) were seeded in 6-well plates and treated with various concentrations of Se-GBLP for indicated period. After treatment, both adherent and floating cells were trypsinized, washed with PBS, and stained with Annexin V-FITC/PI according to the manufacturer's instructions. After staining, the quantification of apoptotic cells was immediately measured with a Becton-Dickinson FACS-Calibur flow cytometer using Cell Quest software.

2.7. Measurement of caspase-3 and caspase-9 activities

The activities of caspase-3 and caspase-9 were studied with the caspase fluorometric assay kits, as described early [24]. Briefly, after treatment, T24 cells were collected, washed with PBS and lysed in cell lysis buffer. Then 50 μl final reaction buffer (1 ml reaction buffer +10 μl DTT) and 5 μl caspase-3/9 colorimetric substrate (DEVDpNA/LEHD-pNA) were added into 96-well plates and incubated at 37 °C for 4 h in the dark in a CO_2 incubator. Optical density was measured at 405 nm with an ELISA micro-plate reader. Activities of caspase-3 and caspase-9 were expressed relative to theoretical density value (OD).

2.8. Assay for change of mitochondrial membrane potential (Δym)

The uptake of the cationic fluorescent dye Rh-123 has been used for the estimation of mitochondrial membrane potential [25]. After treatment with or without Se-GBLP (50, 100 and 200 μg/ml), T24 cells were harvested and washed twice with PBS buffer (pH 7.4), and centrifuged at 2000 rpm for 5 min. Then the pellet was suspended in 2 ml of fresh incubation medium containing 10 μM Rh-123 for 30 min at 37 °C in the dark. After incubation, cells were washed twice with PBS and re-suspended in 200 μl of PBS, then stained with 2 μg/ml PI prior to flow cytometry. At least 1×10^4 cells were analyzed for each sample.

2.9. Protein extraction and Western blot assays

After treatment, total cell lysates were prepared by incubating cells with cell lysis buffer containing (40 mM Tris pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Na_3CO_4, 1 mM phenylmethanesulfonyl fluoride (PMSF) and 0.01% v/v protease inhibitor cocktail) for 15 min on ice [26]. Following the centrifugation at 12000*g* for 30 min at 4 °C, the amount of protein in the supernatant was quantified by the method of Bradford [20]. For the detection of the release of cytochrome c from mitochondria into the cytosol, cytosolic fractions were made by incubating the cells with lysis buffer containing (75 mM NaCl, 8 mM Na_2HPO_4, 1 mM NaH_2PO_4, 1 mM EDTA, 350 μg/ml digitonin and 0.01% v/v protease inhibitor cocktail) on ice for 10 min. The lysates were centrifuged at 12,000*g* and the supernatant was collected [27]. Cytosolic fraction was stored at −80 °C until ready for Western blot analysis.

The samples containing equal amounts of protein (20 mg) from cell lysate were subjected to 12% of SDS polyacrylamide gel for electrophoresis followed by immunoblotting to nitrocellulose membranes. Then the membranes were blocked with 5% defatted milk, blocking solution (1% BSA in TBS plus 0.1% Tween-20) for 1 h at

room temperature, and then incubated overnight with the appropriate primary antibody specific to Bax, Bcl-2, cleaved caspase-3, cleaved caspase-9, cleaved PARP, cytochrome c, or β-actin, respectively, at dilutions specified by the manufacturer, followed by incubation at room temperature for 1 h with the corresponding HRP-conjugated secondary antibody at 1:1000 dilution in blocking solution. Signals were detected using an enhanced chemiluminescence (ECL) system (Pierce Biotechnology Inc., Rockford, IL, USA).

2.10. Statistical analysis

All values are expressed as means ± standard deviation (SD). Statistical significance was compared between groups using the one-way analysis of variance (ANOVA). A statistically significant difference was considered at the level of $P < 0.05$.

3. Results and discussion

3.1. Isolation and characterization of Se-polysaccharide Se-GBLP

The crude *G. biloba* leaves polysaccharides cGBLP was precipitated step by step with 10, 20, 30, 40, 50, 60, 70, and 80% of ethanol overnight at 4 °C, among of which 50% ethanol precipitation was further purified by DEAE-cellulose anion-exchange and Sepharose 6 Fast Flow chromatography.

One water-soluble purified polysaccharide Se-GBLP, with its yield being 5.52‰ of the dried material, was successfully prepared for next assays. Se-GBLP was determined to be the polysaccharides containing 93.7% (w/w) of carbohydrate. And based on the results of the Bradford test and absorbance at 280 or 260 nm, the Se-GBLP contained no proteins and nucleic acids. Se-GBLP also contained organic 38.34 μg/g organic Se, indicating that it is the Se-containing polysaccharide.

3.2. Cytotoxic activity of Se-GBLP

The anti-proliferative activity of Se-GBLP on T24 cells was determined by using the MTT reduction assay. After T24 cells were treated with various concentrations of Se-GBLP for indicated periods, their viability was determined by formazan dye uptake and expressed relative to the optical density of untreated control cells, which was set as 100%. In the presence of Se-GBLP, a significant dose-dependent reduction in viable formazan accumulating cells was observed accordingly at all time intervals measured (Fig. 1A). More importantly, it was noticed that the viability of T24 cells was effectively reduced to 56.5%, 43.2% and 30.2% of the untreated cells by 50, 100 and 200 μg/ml of Se-GBLP at 48 h, respectively. No similar inhibitory effects were observed in normal bladder mucosa HCV29 cells (Fig. 1B). These parameters were therefore used for mechanistic studies.

3.3. Se-GBLP treatment induces apoptosis

To evaluate whether the cytotoxic effect observed in T24 cells upon treatment with Se-GBLP was due to the induction of apoptosis, treated or untreated cells were stained with Annexin V-FITC/PI, and then subjected to flow cytometry. The percentage of apoptotic cells was expressed as Annexin V positive cells. As shown in Fig. 2, exposure of the cells to Se-GBLP at the concentrations of 50, 100 and 200 μg/ml for 48 h led to 36.5%, 56.3% and 70.3% apoptotic cell death as compared with the untreated cells, respectively. Thus the annexin V/PI double staining assay confirmed the observation that Se-GBLP-induced apoptosis

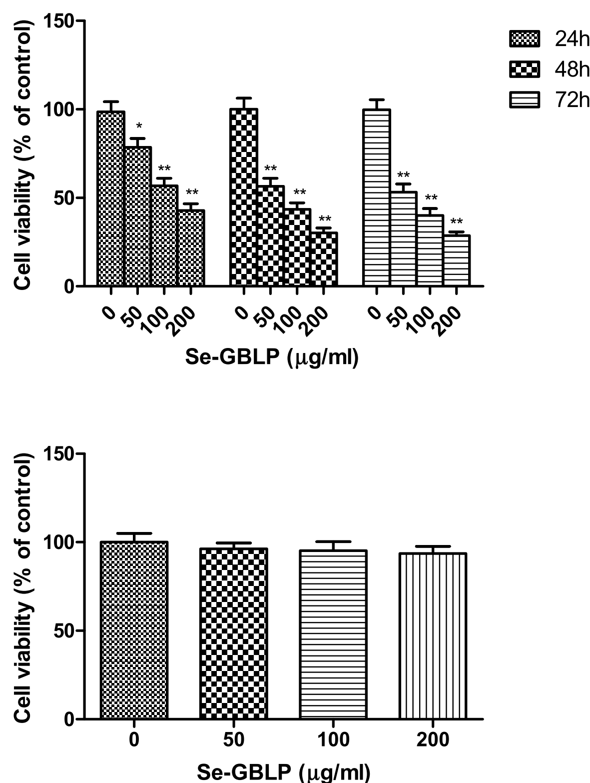


Fig. 1. (A) Effect of Se-GBLP on the viability of T24 cells at 24, 48 and 72 h as determined by MTT assay. (B) Effect of Se-GBLP on the viability of normal bladder mucosa HCV29 cells at 48 h as determined by MTT assay. Data shown represent the mean ± SD of three replicates. *$P < 0.05$, **$P < 0.01$ compared with control group.

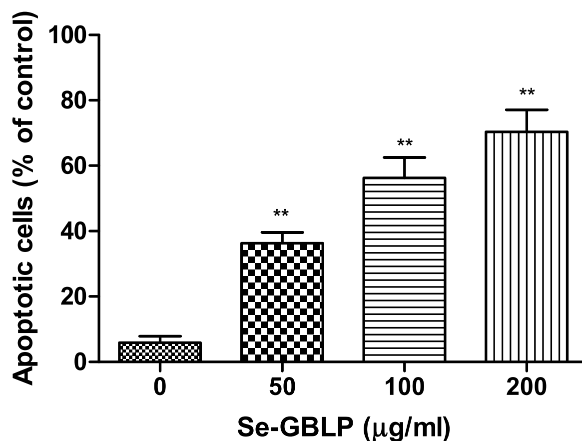
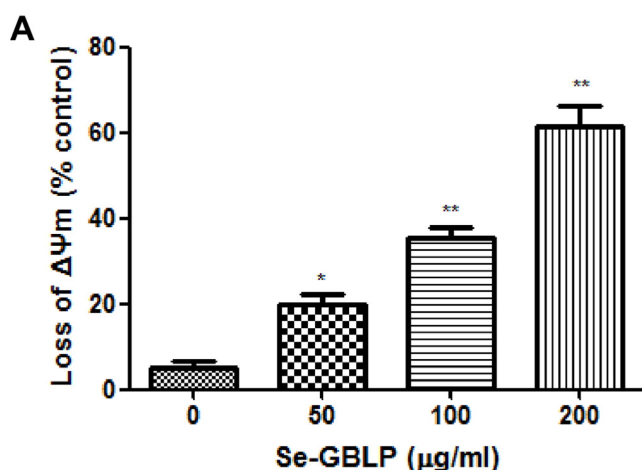


Fig. 2. Flow cytometric analysis of Se-GBLP-induced apoptosis in T24 cells using annexin V-FITC/PI. Data shown represent the mean ± SD of three replicates. **$P < 0.01$ compared with control group.

3.4. Se-GBLP treatment induces the loss of mitochondria membrane potential (Δψm) and release of cytochrome c to the cytosol

Mitochondria have been linked to the propagation of apoptosis. The disruption of Δψm is a critical step occurring in cells undergoing apoptosis [28]. To verify whether Se-GBLP-induced apoptosis is accompanied by the disruption of Δψm, the integrity of mitochondrial membranes of Se-GBLP-treated cells was investigated by measuring their ability to retain Rh123 that is a sensitive cationic fluorescent dye selectively entering mitochondria with an intact membrane potential [29]. As shown in Fig. 3A, the addition of Se-



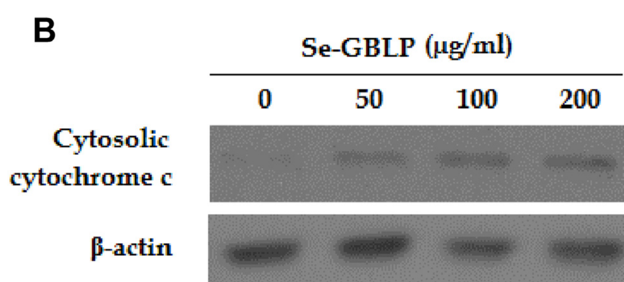


Fig. 3. (A) Effect of Se-GBLP on the loss of mitochondria membrane potential ($\Delta\psi$m) in T24 cells; (B) Effect of Se-GBLP on the release of cytosolic cytochrome c in T24 cells. Data shown represent the mean ± SD of three replicates. *$P < 0.05$, **$P < 0.01$ compared with control group.

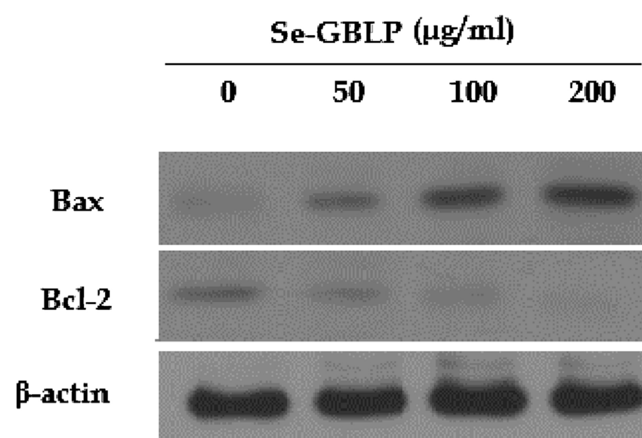


Fig. 4. Effect of Se-GBLP on Bax and Bcl-2 protein expression in T24 cells.

GBLP led to increasing percentages of the uptake of Rh123 from 97.3% in control cells to 83.5% at 50 μg/ml, 68.5% at 100 μg/ml, and 36.8% at 200 μg/ml, respectively. These results suggested that the involvement of disruption of $\Delta\psi$m in Se-GBLP-induced apoptosis in T24 cells.

A decrease in $\Delta\psi$m disrupts the outer mitochondrial membrane, and then triggers the release of cytochrome c from the mitochondria to the cytosol, which, in turn, results in the activation of caspase-9 and subsequently leads to apoptotic cell death [30,31]. In this respect, the cytosolic fractions were prepared from T24 cells upon treatment with or without Se-GBLP (50, 100 and 200 μg/ml) for 48 h. The results from Western blot analysis revealed that a dose-dependent increase in cytosolic cytochrome c was observed in T24 cells (Fig. 3B), thus confirming the loss of $\Delta\psi$m in T24 cells following Se-GBLP treatment.

3.5. Se-GBLP treatment up-regulates of Bax and down-regulats of Bcl-2 protein expression

Bcl-2 family proteins as a crucial role in the mitochondrial-mediated apoptosis pathway have been identified as major regulators in controlling the release of mitochondrial cytochrome c [32]. Bax (pro-apoptotic) induces apoptosis by desintegrating the outer mitochondrial membrane and causing the release of cytochrome c [33]. In contrast, Bcl-2 (anti-apoptotic) inhibits apoptosis by blocking cytochrome c efflux into the outer mitochondrial membrane [34]. The appearance of change of $\Delta\psi$m and the release of cytochrome c from the mitochondria to the cytosol suggested the involvement of Bcl-2 family in T24 cells after exposure to Se-GBLP. Therefore, we analyzed the level of the pro-apoptotic Bax and anti-apoptotic Bcl-2 proteins upon treatment of T24 cells with Se-GBLP for 48 h. As seen in Fig. 4, Se-GBLP treatment of T24 cells resulted in an increased level of Bax expression in a dose-dependent manner, whereas the level of the anti-apoptotic protein Bcl-2 became weak under the same conditions, leading to an increase in the pro-apoptotic/anti-apoptotic Bcl-2 ratio. These results suggest that Se-GBLP-induced apoptosis in T24 cells is via alteration of the Bax/Bcl-2 ratio.

3.6. Se-GBLP treatment induces caspases-3 or -9 activation, cleavage of caspases -3 or -9 and PARP

Caspases are important regulators in the intrinsic apoptotic pathway [27]. Cytochrome c released into cytosol recruits and activates procaspase-9, thus leading to the cleavage of caspase-9 and caspase-3. Subsequently, cleavage of caspase-3 induces apoptotic cell death through cleavage of a broad spectrum of cellular target proteins including PARP [35–38]. Therefore, we investigated the involvement of caspase-3, caspase-9, and PARP in Se-GBLP-induced apoptosis. T24 cells were left untreated or treated with 50, 100 and 200 μg/ml of Se-GBLP, and caspase activities were determined. As expected in Fig. 5A, by comparison of the control, after 48 h of treatment, the fluorometric protease assay showed the activities of caspase-3, and -9 in Se-GBLP-treated cell lysates, were increased significantly. In line with this result, Western blot analysis revealed that the cleavage of caspase-3 and -9 was evident in a dose-dependent manner, as well as a cleavage of PARP, compared with the cells which were not treated with Se-GBLP (Fig. 5B). The activated caspase-3, caspase-9 and the cleavage of PARP detected in the results further clearly indicated that Se-GBLP-induced apoptosis was dependent on caspase activation.

4. Conclusions

Here we report for the first time that a Se-enriched polysaccharide Se-GBLP from *G. biloba leave* inhibited the growth of human urinary bladder cancer T24 cells in a dose-dependent manner and that this reduction in cell viability resulted from apoptotic cell death. We also provide evidences that Se-GBLP-induced apoptosis is associated with the alterations in the protein expression of Bcl-2 family. These changes in the protein expression of the Bcl-2 family may be responsible for disintegrating the outer mitochondrial membrane, and then cause release of cytochrome c from the mitochondria to cytosol. Further, this effect of Se-GBLP arouse downstream of the apoptosis cascade, namely the dose-dependent cleavage of caspase-9, caspase-3 and PARP. In summary, these data

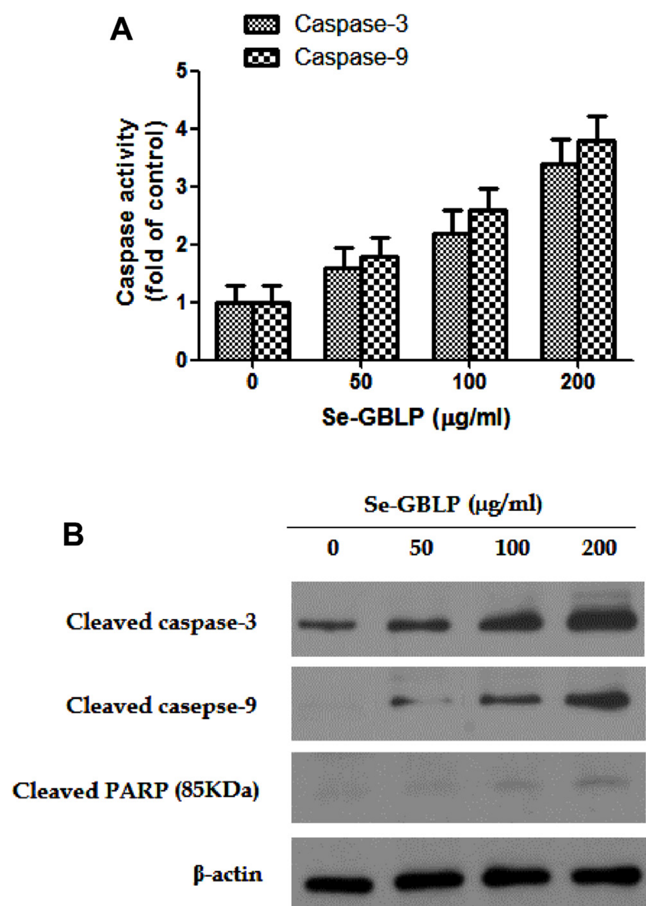


Fig. 5. (A) Effect of Se-GBLP on caspase-3 and caspase-9 activities in T24 cells. (B) Effect of Se-GBLP on the cleavage of caspase-9, caspase-3 and PARP protein expression in T24 cells. Data shown represent the mean ± SD of three replicates. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control group.

offered solid evidence of the induction of mitochondria-related apoptosis in T24 cells, however other pathways may also have a role and that remains to be found out.

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