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Antioxidant potential of gamma ray irradiated winged bean (*Psophocarpus tetragonolobus*) seed protein hydrolysate

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ABSTRACT

Background: Winged bean or *Psophocarpus tetragonolobus* (WB) seeds have high protein content and could be applied as a source of antioxidant proteins and peptides. The utilization of gamma rays in plant protein extraction provides consumers with a safe and harmless technology.

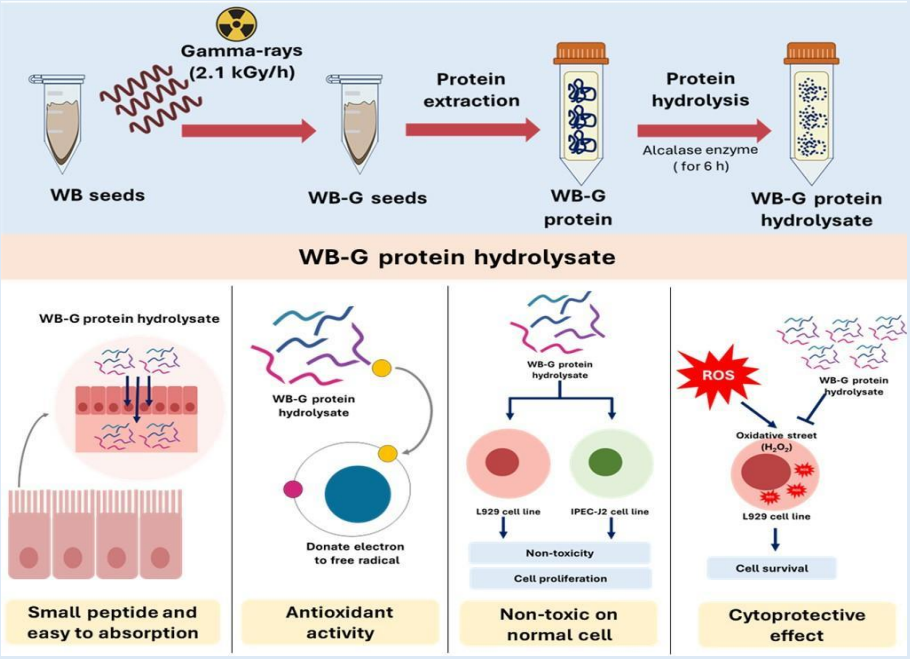
Objective: To determine the efficiency of gamma rays in the protein extraction process of WB seeds and investigate the antioxidant activity, cytotoxicity, cytoprotective effect of the gamma ray irradiated WB (WB-G) seed protein hydrolysate.

Methods: WB seeds were irradiated with gamma ray (WB-G) and total protein were extracted by acid-base extraction method. The total protein was further hydrolyzed with alcalase enzyme to obtain the protein hydrolysate. The antioxidant activity was evaluated by DPPH and ABTS assay. The cytotoxicity and cytoprotective effect were determined by MTT assay. The cellular reactive oxygen species was further analyzed by H2-DCFDA assay.

Results: The protein extracted from WB-G seeds showed higher protein yield and antioxidant activity than that of non-irradiated seeds. After hydrolysis with alcalase enzyme at 55°C for 6 hours, WB-G protein hydrolysate with degree of hydrolysis of 90.00±0.91% exhibited strong antioxidant activity with IC_{50} value of 10.30±0.02 µg/ml and 3.10±0.06 µg/ml as measured by DPPH and ABTS assay, respectively. Moreover, it showed no toxicity toward L929 mouse fibroblast cells and IPEC-J2 cell lines and can reduce the damage caused by H_2O_2. As determined in cellular reactive oxygen species by H_2-DCFDA assay, the WB-G protein hydrolysate can reduce the level of ROS and subsequently the damage of cell by H_2O_2.

Conclusions: This study demonstrated that gamma ray irradiation on the WB seeds can increase the protein yield and its protein hydrolysate exhibited promising antioxidant activity and cytoprotective effect on cells against H_2O_2 damage. It might be developed as an alternative food or feed supplement.

Keywords: *Psophocarpus tetragonolobus*; Gamma rays; Protein hydrolysate; Antioxidant activity; Cytoprotective effect



INTRODUCTION

According to Functional Food Center (FFC), functional foods are defined as natural or processed foods containing biologically active compounds that, when consumed in defined, effective, non-toxic amounts, provide a clinically proven and documented health benefit by utilizing specific biomarkers to promote optimal health and reduce the risk of chronic/viral diseases and manage their symptoms [1]. Plants are sources of functional foods because they provide chemical compounds with biochemical activity, such as antioxidant substrates (flavonoids and phenolic acids) and bioactive peptides [2]. Bioactive peptides are found naturally in plants released when proteins are hydrolyzed with proteolytic enzymes [3] such as alcalase, papain, and bromelain [4]. Various biological activities of protein hydrolysate have been reported; these include antimicrobial, anticancer, angiotensin-converting enzyme inhibitors (ACE inhibitors), anti-inflammatory and antioxidant properties which depend on the type and size of the amino acids [5]. Therefore, protein hydrolysate can be applied in a variety of functional foods.

The winged bean (WB) or (*Psophocarpus tetragonolobus*) seeds are one promising plant protein source due to their high protein content. One hundred grams of winged bean contains 25-30 grams of protein, or one-third of the total mass as protein [6, 7]. WB is easy to cultivate, widespread and has no problems with plant diseases or pests. All components of WB are beneficial for physical health because of their nutritional value and

medicinal properties [8]. Consuming winged beans at least two or three times a week can help prevent the growth of cancer cells such as in breast cancer and has a favorable impact on female hormones. WB has also been discovered to be highly beneficial to the body, especially nourishing the body due to its high protein content and other essential substances, therefore it is ideal for developing healthy food.

Although plants are a good source of protein, there are certain limitations to extracting protein from them, such as complex technology and poor yield or protein content. The use of gamma rays and electron radiation provide safe and harmless technology for consumers [9], can increase the efficiency of production and can be supplemented with other extraction methods. Gamma rays are employed in various aspects of food processing including food preservation, microbiological destruction, and nutritional preservation of food without toxic residues [10, 11]. They have also been used in the extraction of proteins and other essential substances or constituents in food. Irradiation affects water molecules inside the cytoplasm of plant cells by forming the free radicals including hydroxyl radical (OH) and hydrogen ion (H). The two molecules combine to form hydrogen peroxide (H_2O_2), which contributes to the destruction of cell walls, resulting in the extraction of essential compounds within the cell such as proteins, fats, and carbohydrates. Gamma irradiation improves extraction efficiency while not affecting the environment by avoiding the use of hazardous chemicals [12, 13]. The use of gamma rays and electron beam irradiation is another non-thermal approach. It can also aid in the preservation of critical heat-sensitive chemicals.

Even though protein is regarded as an important food source, it must be digested into short-chain proteins or peptides that can be utilized by the body. Protein hydrolysis with proteolytic enzyme will help in processing of protein into bioactive short-chain proteins or peptides. The objective of this study is to improve the protein content and/or quality of winged bean seeds with gamma ray irradiation followed by protein extraction and hydrolysis to obtain the protein hydrolysate. The antioxidant activity, cytotoxicity, and cytoprotective effect will be further investigated and the peptide will be identified.

MATERIALS AND METHODS

Material: Winged bean (WB) seeds were obtained from Jia Seng Heng Agriculture Co., Ltd, Thailand. Hydrochloric acid (HCl) and ALCALASE® Enzyme, *Bacillus licheniformis* was purchased from Merck, Germany. 2,2-Di(4-tert-octylphenyl)-1-picrylhydrazyl (DPPH) and 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) are from Sigma, USA. Sodium hydroxide (NaOH) is obtained from AppliChem, Germany. MTT (3-(4,5-Dimethylthiazol-2-yl) and 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) were obtained from Invitrogen, US. 1X Dulbecco's Modified Eagle Medium (DMEM), Fetal bovine serum (FBS) and 10,000 U/mL of Penicillin-Streptomycin (Pen-Strep) were purchased from Gibco (Thermo Fisher Scientific), US. Hydrogen peroxide was obtained from QRëC™, New Zealand. Coomassie G-250 staining dye was obtained from Bio-Rad, USA. All chemical reagents used were analytical grade.

Gamma ray irradiation on winged bean seeds: WB seeds were cleaned with distilled water, dried at room temperature and ground into powder with the grinder. They were filtered and irradiated with gamma rays at an intensity of 2.1 kGy/h for 4.7 hours at the National Institute of Nuclear Technology, Nakhon Nayok, Thailand. They were designated as WB-G and kept at room temperature and with no moisture.

Protein extraction by acid-base extraction method: The total protein was extracted using acid-base extraction methods. Ten grams of WB-G were mixed with DI water in a 1:10 (w/v) ratio, and the pH was adjusted to 10.00 with 1 M NaOH. The mixture was stirred for 1 hour at room temperature and centrifuged at 8,820xg for 20 minutes at 4 °C to collect the supernatant. The protein was precipitated by adjusting the pH to 3.80 with 1 M HCl and centrifuged at 10,000xg for 20 minutes at 4 °C. The precipitate was collected, subsequently dissolved in DI water and the solution was adjusted to pH 7.00 with 1 M NaOH [14]. After that, the protein concentration was determined by the Bradford Protein Assay technique using Bovine serum albumin (BSA) as a standard [15]. The extracted protein was kept at -20 °C until use.

Preparation of protein hydrolysate with alcalase enzyme: The protein extracted from gamma ray-irradiated WB seeds was pre-incubated at 55 °C and adjusted to pH 7.00 with 1 M NaOH, and alcalase enzymes were added at a concentration of 2.4 AU/g, then incubated for 0, 5, 10, 15, 30, 45, 60 minutes, 2, 4, 6, 12, 24, and 48 hours. The reaction was then inactivated by incubation at temperature 80 °C for 10 minutes and centrifuged at 8,000 x g at room temperature for 20 minutes [16]. The protein concentration and antioxidant activity of the supernatant was measured with DPPH and ABTS assay techniques for each incubation time.

Determination of the degree of hydrolysis (%): The degree of hydrolysis (%) of protein hydrolysate in WB seeds irradiated with gamma rays at various times (from 0 to 48 hours) was investigated by OPA assay. Five µl of sample were mixed with 215 µl of OPA reagent (containing 25 ml of 100 mM sodium tetraborate, 2.5 ml of 20% (w/w) SDS, 40 mg/ml of OPA dissolved in methanol, and 100 µl of beta-mercaptothion) and incubated for 2 minutes. The absorbance was measured at 340 nm and the (%) degree of hydrolysis was calculated using this equation [17].

$$Degree\ of\ hydrolysis\ (\%) = \left(\frac{A_{free\ nitrogen\ groups}}{A_{total\ nitrogen\ groups}}\right) \times 100$$

Determination of protein pattern by SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with separating gel (15% polyacrylamide gel) and stacking gel (4% polyacrylamide gel) was used to investigate the protein in protein hydrolysate. The samples were mixed with 4X loading dye and denatured by incubating at 95 °C for 5 minutes. The samples were loaded onto SDS gel with 1X running buffer. The running process was powered by a 50-volt power source for 30 minutes before increasing the voltage of the electrical system to 120-volts for 2 hours. The gel was stained with Coomassie G-250 staining dye for the band of protein [18].

Determination of antioxidant activity by 2,2–diphenyl–1–picrylhydrazyl (DPPH) assay: One hundred µL of two-fold diluted sample (0.98 to 1,000 µg/mL) were mixed with 100 µL DPPH reagent and incubated for 30 minutes at room temperature in the dark. After that, the absorbance was measured at 517 nm with a microplate reader, and the obtained absorbance was used to calculate for %DPPH scavenging activity and IC_{50} [19].

$$DPPH\ scavenging\ activity\ (\%) = 1 - \left(\frac{A_{sample}}{A_{Control}}\right) \times 100$$

A_{sample} was the absorbance of protein mixed with DPPH reagent at 517 nm and $A_{control}$ was the absorbance of distilled water mixed with DPPH reagent at 517 nm.

Determination of antioxidant activity by 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) assay: Fifty µL of two-fold diluted sample (0.98 to 1,000 µg/mL)

were mixed with 200 µL ABTS reagent and the absorbance at 734 nm was immediately measured (within 5 minutes) using a microplate reader. The measured absorbance was then used to calculate %ABTS scavenging activity and IC_{50} [20].

$$ABTS\ scavenging\ activity\ (\%) = (\frac{A_{Control} - A_{sample}}{A_{Control}}) \times 100$$

A_{sample} was the absorbance of protein mixed with ABTS reagent at 734 nm and $A_{control}$ was the absorbance of distilled water mixed with ABTS reagent at 734 nm.

Determination of cytotoxicity by 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay: The cell lines (L929 mouse fibroblasts and IPEC-J2, intestinal porcine enterocytes) were cultured in complete medium (Dulbecco's Modified Eagle Medium (DMEM), 10% Fetal bovine serum, and 1% Penicillin/Streptomycin), and cells were seeded in 96-well plates at a concentration of 10,000 cells/well and incubated overnight at 37 °C with 5% CO_2. Two-fold diluted sample (0.98–500 µg/mL) was incubated with cells for 24 hours. Then, 100 µL of MTT reagent (0.4 mg/mL) were added and the combination incubated for 4 hours. Formazan crystals were dissolved in 100 µl of dimethyl sulfoxide (DMSO) and absorbance was measured at 570 nm [21]. The %cell viability was calculated as shown in the equation.

$$Cell\ viability\ (\%) = (\frac{A_{sample}}{A_{Control}}) \times 100$$

A_{sample} is the absorbance of protein samples at concentrations of 500 to 0.98 µg/mL against cell lines and $A_{control}$ is the absorbance of cells tested in DMEM with cell lines.

Determination of cytoprotective effect: The L929 mouse fibroblast cell lines were cultured in complete medium (DMEM medium, 10% fetal bovine serum, and 1% penicillin/streptomycin), and cells were seeded in 96-well plates at a concentration of 10,000 cells/well and incubated overnight at 37 °C with 5% CO_2. The sample (0.98–500 µg/mL) was then added and incubated with cells for 24 hours. Then, hydrogen peroxide at 125 µM (100 µL) was added to each well and further incubated for 3 hours. Finally, the medium was removed and 100 µL of 0.4 mg/mL MTT reagent was added and the sample with MTT reagent was incubated for 4 hours. Formazan crystals were dissolved in 100 µl of dimethyl sulfoxide (DMSO) and the absorbance at 570 nm was measured [22]. The %cell viability was calculated as described above.

Determination of cellular reactive oxygen species by H_2-DCFDA: The L929 mouse fibroblast cell lines were cultured in complete medium (DMEM medium, 10% fetal bovine serum, and 1% penicillin/streptomycin), and cells were seeded in 96-well plates at a concentration of 10,000 cells/well and incubated overnight at 37 °C with 5% CO_2, then incubated with sample (0.98–500 µg/mL) for 24 hours, with hydrogen peroxide at 125 µM (100 µL) for 3 hours. Lastly, the medium was removed and 100 µL of 10 µM 2',7'-dichlorodihydrofluorescein diacetate (H_2-DCFDA) were added and incubated at 37 °C for 30 minutes. The H_2-DCFDA solution was then removed, and the cells were washed with DMEM and 1X phosphate-buffered saline (PBS). The fluorescent intensity was measured using a fluorescent microplate reader with excitation and emission wavelengths of 485 nm and 535 nm, respectively and the cell image was taken under a fluorescence microscope [23].

12. Statistical analysis: All experiments were performed in triplicate and data were determined as mean ± SD.

Statistical analysis was performed using SPSS version 16. Tukey analysis was used to find differences between control and experimental groups at a significant level of 0.05.

RESULTS AND DISCUSSION

Effect of gamma ray irradiation on winged bean seeds protein extraction: The total protein extracted from WB-G by acid-base technique showed a protein concentration of 12.38 ± 1.86 mg/mL with %yield of 4.95 ± 0.74% which is higher than that of normal WB seeds (protein concentration of 8.08 ± 1.60 mg/mL with %yield of 3.23 ± 0.64) (Table 1). These results demonstrated that gamma ray irradiation resulted in higher protein extraction which might be due to the ability of gamma ray to break down the cell wall's inner structure causing the release of protein [24, 25]. Previous research showed gamma rays can break the bond between water molecules and cause water-derived radicals, which react to break down the cell wall [26, 27]. As shown in Fig. 1, the protein of both gamma ray-irradiated and non-irradiated WB seeds showed the same protein pattern with molecular weight ranging from 17-180 kDa.

Table 1. Protein concentration and yield of crude protein extract from WB seeds

Source of protein	Protein concentration (mg/mL)	Total protein (mg)	%Yield
WB seeds	8.08 ± 1.60	323.55 ± 64.26	3.23 ± 0.64
WB seeds irradiated with gamma ray (WB-G)	12.38 ± 1.86*	495.44 ± 74.58*	4.95 ± 0.74*

*Significant difference between non-irradiated and gamma ray irradiated winged bean seed at $p < 0.05$.

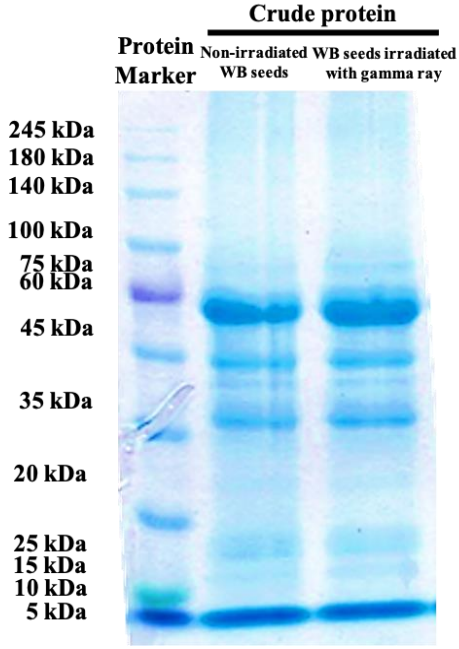


Figure 1. SDS-PAGE analysis of protein extracted from non-irradiated WB seeds and WB seeds irradiated with gamma ray (WB-G). Lane 1: Protein marker (245 – 5KDa); Lane 2: Crude protein from non-irradiated WB seeds; Lane 3: Crude protein from WB-G. The crude protein of gamma ray-irradiated showed the same protein pattern with that of non-irradiated WB seeds.

The antioxidant activity of crude protein was further investigated by DPPH and ABTS methods. As shown in Fig. 2, the IC_{50} of protein extracted from WB-G was 47.08 ± 1.75 ug/mL and 49.39 ± 8.91 µg/mL as determined by ABTS and DPPH assay, respectively. This antioxidant activity was higher than that of non-irradiated WB seeds which was 67.32 ± 0.71 µg/mL and 49.39 ± 8.91 µg/mL as determined by ABTS and DPPH assay, respectively. Glutathione was used as a positive control. The protein extracted from WB-G seeds has greater antioxidant activity than that of non-irradiated seeds. The gamma rays can break down disulfide and hydrogen bonds in proteins resulting in smaller proteins or peptides [28] which might be involved with higher antioxidant activity. The low molecular weight protein was reported to have high antioxidant activity as it is easy to interact with free radicals [29]. Both direct and indirect effects of gamma radiation on proteins are possible. In the direct effect, radiation is taken up directly by protein molecules, changing the protein molecule. In the indirect effect, radiation first affects water molecules, creating active species such hydroxyl radicals and hydrated electrons that then interact with protein molecules to change the structure of the proteins [27]. Proteins are affected by gamma radiation in a way that affects their structure and causes covalent bond to break [30]. Moreover, total phenols, flavonoids, amino acids, and antioxidant enzymes in seed plants were all induced by gamma radiation [31]. It has been reported that gamma ray irradiation of sunflower protein resulted in changes of the protein structure and increased the antioxidant activity [32].

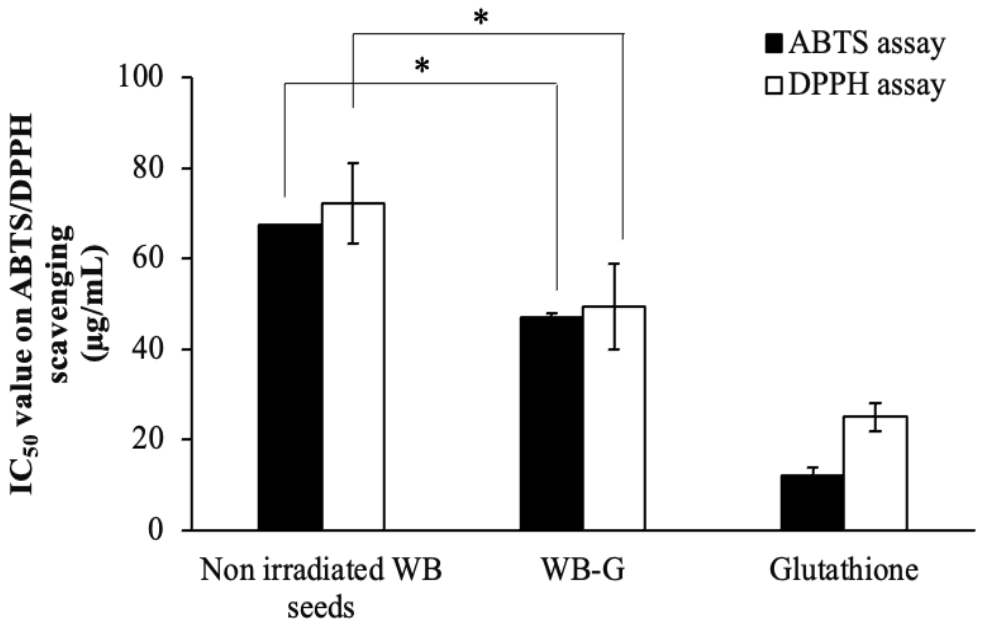


Figure 2. The antioxidant activity of protein extracted from non-irradiated WB seeds and WB-G seeds determined by ABTS and DPPH assays. * Indicates significantly different at $p < 0.05$. The protein extracted from WB-G seeds showed higher antioxidant activity than that of non-irradiated seeds.

The degree of hydrolysis and antioxidant activity of WB-G protein hydrolysate: The isolated protein was hydrolyzed for different durations to determine the optimum time needed for complete hydrolysis. The

duration ranges from 5 min to 48 hours. As shown in Fig. 3, the total protein isolated from WB-G was completely hydrolyzed by alcalase enzyme into shorter proteins and peptides with molecular weight ranging from 5-10 kDa within 5 min until 48 hours of hydrolysis. More than 90% degree of hydrolysis was observed at 5 min until 48 hours after hydrolysis (Fig. 4). The degree of hydrolysis is 90.42±0.68 at 5 min of hydrolysis and 92.02±1.35% at 48 hours after hydrolysis (Fig. 4). The alcalase enzyme can hydrolyze protein at a specific site and is used to convert long protein into short peptides to increase the yield of peptides [33, 34, 35]. Furthermore, alcalase enzymes are widely used in the hydrolysis of proteins to produce low-allergenic infant foods [36].

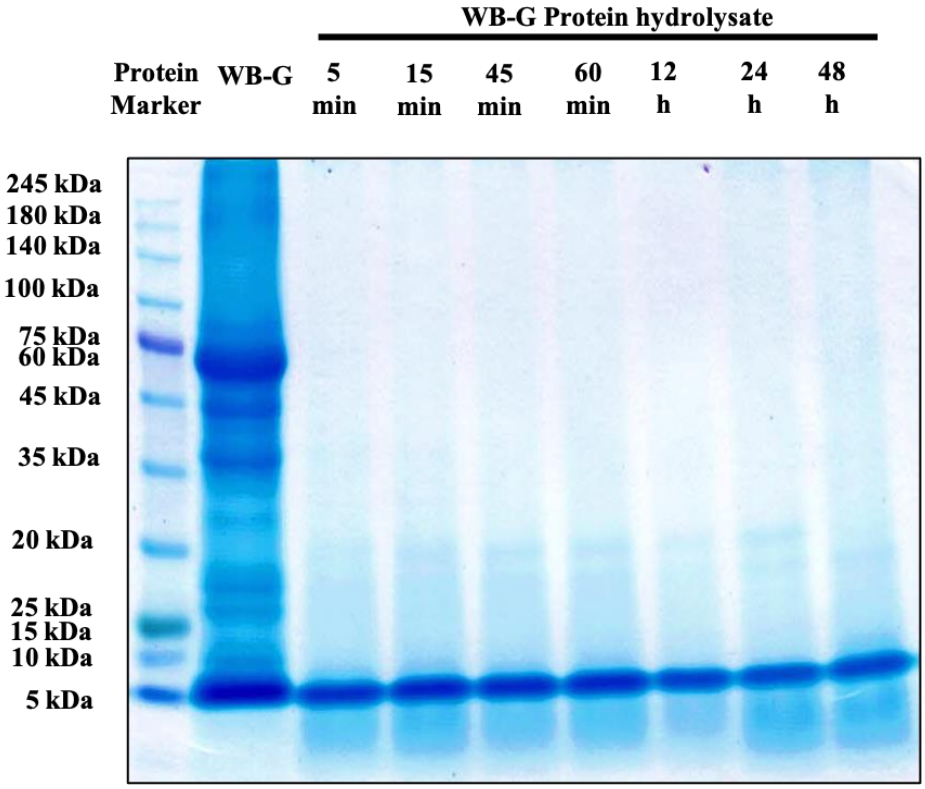


Figure 3. SDS-PAGE analysis of WB-G protein hydrolysate at different time of hydrolysis (0–48 h). Lane 1: Protein marker (5-245 kDa); Lane 2: Crude protein of WB-G; Lane 3-9: WB-G Protein hydrolysate at different hydrolysis time. The crude protein isolated from WB-G was completely hydrolyzed by alcalase enzyme within 5 min until 48 hours of hydrolysis.

The antioxidant activity of WB-G protein hydrolysate at different times during 48 hours of hydrolysis was further investigated. (Fig. 4). At 6 hours, WB-G protein hydrolysate showed a higher antioxidant activity than that of the total protein. It exhibited the highest antioxidant activity as determined by DPPH scavenging activity (61.75±1.32%) while there was no significant difference in antioxidant activity determined by ABTS assay. The alcalase enzyme hydrolyzes proteins resulting in the formation of novel peptides with novel activities [37, 38]. The antioxidant activity of protein hydrolysate was varied according to the amino acid sequence, hydrophobic capacity, and peptide composition [39, 40]. In addition, the size of the peptide has an effect on its antioxidant activity; the low molecular weight peptide (less than 10 kDa) easily interacts with free radicals and is good for adsorption in cells [41, 42].

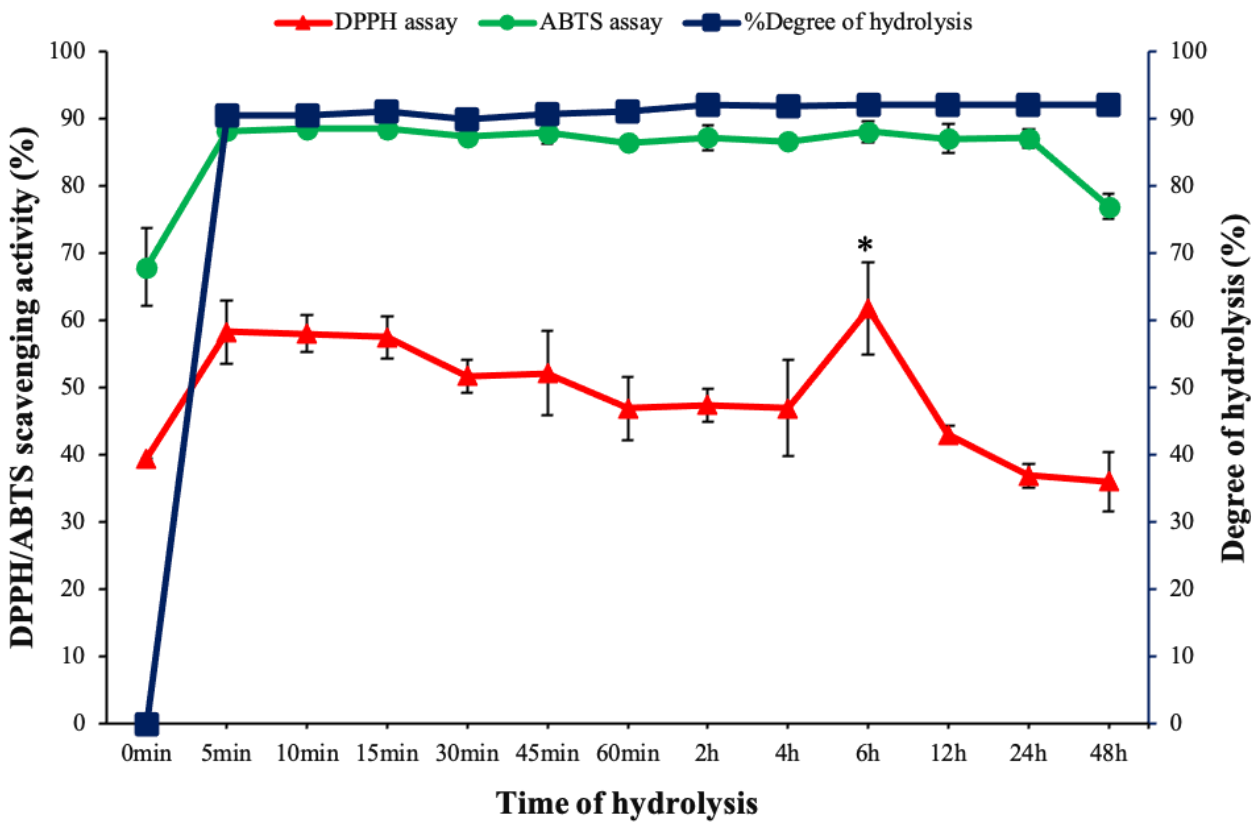


Figure 4. Degree of hydrolysis (%) and antioxidant activity of WB-G protein hydrolysate at 0-48 h after hydrolysis. Values are the mean ± SD from tropical determinations (n = 3). * Significantly different than DPPH/ABTS scavenging activity (%) between protein hydrolysate from WB-G at different time of hydrolysis reaction (0-48 h), $p < 0.05$. At 6 hours, WB-G protein hydrolysate showed higher antioxidant activity than that of total protein as determined by both DPPH and ABTS assay.

The toxicity of WB-G protein hydrolysate: As shown in Fig. 5, WB-G protein hydrolysate showed no toxicity toward both mouse fibroblast (L929) and intestinal porcine enterocyte cell lines (IPEC-J2) even at the highest concentration tested of 500 µg/mL. Moreover, a low concentration of WB-G protein hydrolysate can induce cell proliferation. The protein isolated from WB-G showed no toxicity toward both cell lines at 0.98-62.50 µg/mL, however at higher concentration (125-500 µg/mL), it showed slight toxicity that was significantly different from that of the control group. L929 cell lines were normal cells widely used to test cell toxicity [43] while IPEC-J2 cell lines are intestinal porcine enterocytes isolated from piglets which are widely used as a model for determination of biological activity *in vitro* [44]. For many years, *P. tetragonolobus* or winged bean has been widely utilized in traditional medicine as it contains several bioactive compounds. It is a member of *Fabaceae* family and is high in protein, oils, vitamins, and carbohydrates [45]. It has been reported that winged bean seed hydrolysate is non-toxic and can stimulate cell proliferation in NIH/3T3 mouse fibroblast cells [46] and winged bean extract was non-toxic to RAW264.7 macrophage cells [47]. These studies reveal the health-promoting activity and safety of this legume.

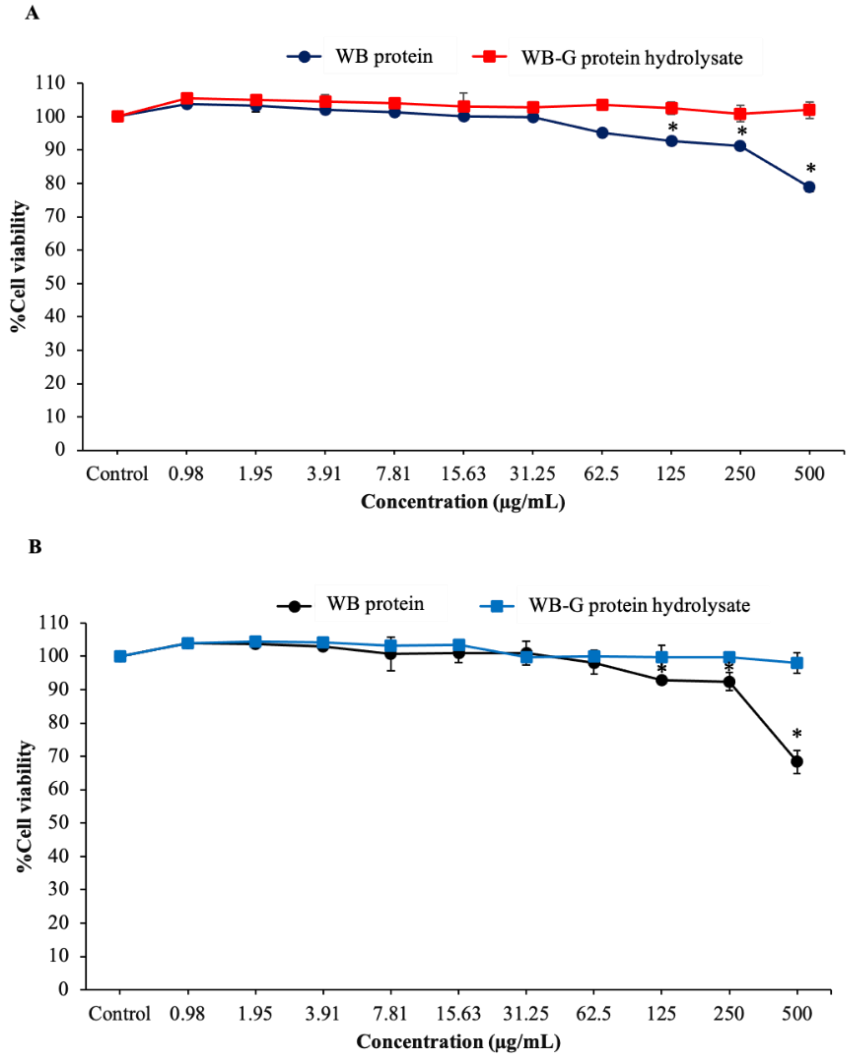


Figure 5. The cytotoxicity of crude protein and protein hydrolysate of WB-G on L929 cell lines (A) and IPEC-J2 cell lines (B). WB-G protein hydrolysate showed no toxicity toward both cell lines.

The cytoprotective effect of WB-G protein hydrolysate: The L929 mouse fibroblast cell lines were pre-incubated with WB-G protein hydrolysate for 24 hours. After that, the oxidative stress was induced by exposure with H_2O_2 for 3 hours and cell viability was determined using the MTT assay. As shown in Fig. 6, the WB-G protein hydrolysate at a concentration range of 0.98-500 µg/mL could protect cells from the damage of H_2O_2 when compared to that of cells exposed with H_2O_2 only. The cell viability ranged from 92.76±2.49% to 102.86±3.01% which is comparable to that of positive control (glutathione). Hydrogen peroxide (H_2O_2) generates reactive oxygen species (ROS) in cells and breaks DNA causing cell death [48]. The cytoprotective experiment mimics the oxidative stress induced by H_2O_2 and the *in vivo* protective effect of WB-G protein hydrolysate against H_2O_2 damage. WB-G protein hydrolysate was shown to have high antioxidant activity and can donate electrons to free radicals or ROS to decrease the damage of H_2O_2 [49]. In addition, the molecular weight of protein hydrolysate from WB-G was smaller and could more easily interact with free radicals and halt the chain

reaction to prevent damage to essential chemicals [50]. Watermelon seed protein hydrolysate with low molecular weight proteins or peptides (5–10 kDa) showed a cytoprotective effect against H_2O_2 in the HepG2 cell line [42]. Soybean protein hydrolysate protected Caco-2 cells against H_2O_2-induced oxidative damage by downregulating ROS in the cell [51].

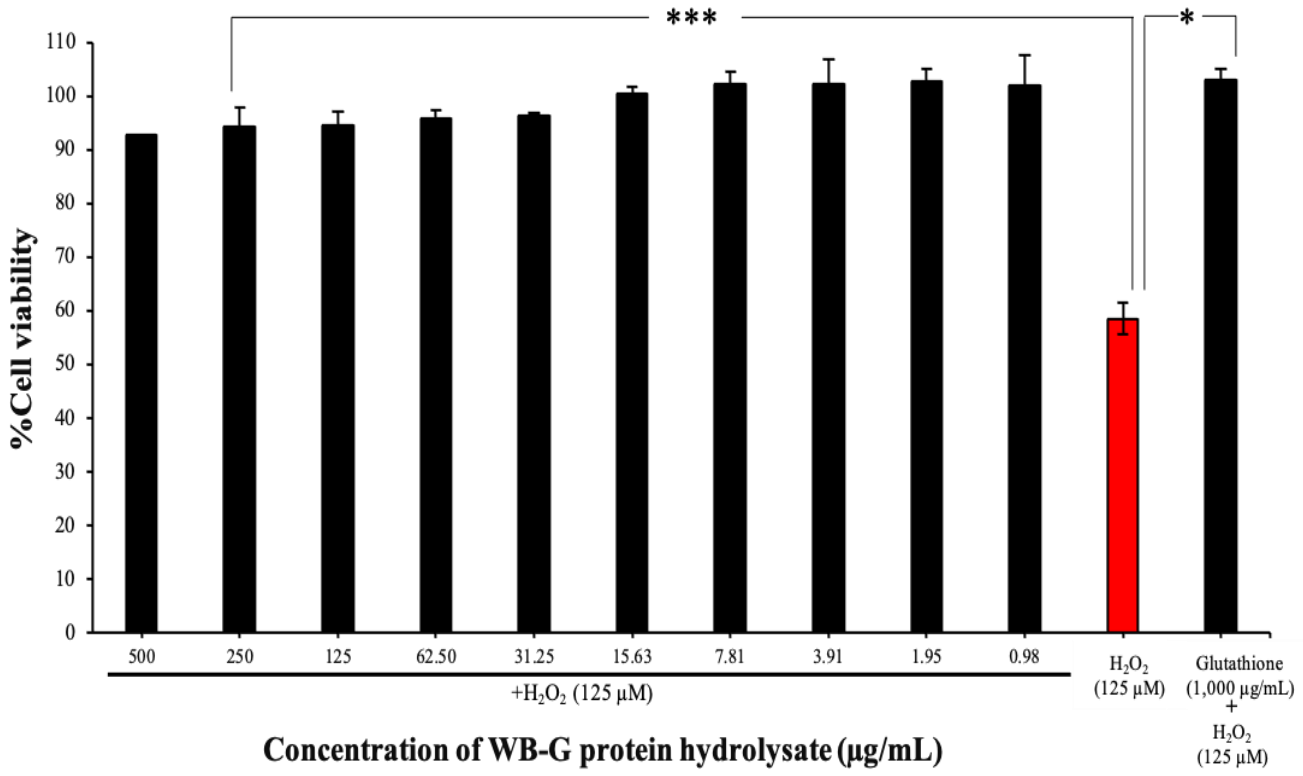


Figure 6. The cytoprotective effect of WB-G protein hydrolysate in L929 cell lines. *** indicates significant difference in %cell viability between cells pre-incubated with WB-G protein hydrolysate and non-treated cells at $p < 0.05$. *indicates significant difference in %cell viability between positive control and H_2O_2 -treated group at $p < 0.05$. WB-G protein hydrolysate could protect cells from the damage of H_2O_2.

The reactive oxygen species in cells after treatment with WB-G protein hydrolysate: 2',7'-dichlorodihydrofluorescein diacetate (H_2-DCFDA) is the cell-permeable chemically reduced form of fluorescein used as an indicator of reactive oxygen species (ROS) in cells [23]. The nonfluorescent H_2-DCFDA is turned into fluorescent 2', 7,-dichlorofluorescein (DCF) by intracellular esterases and oxidation. It can be used to measure ROS generation after chemical or sample treatment. After pre-incubating L929 cell lines with various concentrations of WB-G protein hydrolysate (0.98-500 µg/mL) for 24 hours, and subsequently treating the cells with H_2O_2 at 125 µM for 3 hours, DCF fluorescent intensity was measured by a microplate reader. The result showed that WB-G protein hydrolysate decreases the intensity of DCF in a dose-dependent manner (from 39.03±0.67 to 67.55±0.64) when compared to that of cells treated with H_2O_2 only (76.50±2.12) (Fig. 7).

Glutathione, a common antioxidant reagent that can reduce the damage of free radicals in cells [52], also reduces the fluorescent intensity of DCF (27.50±3.53). The WB-G protein hydrolysate can reduce ROS or hydrogen peroxide in the L929 cell line, resulting in a decrease of DCF fluorescent intensity.

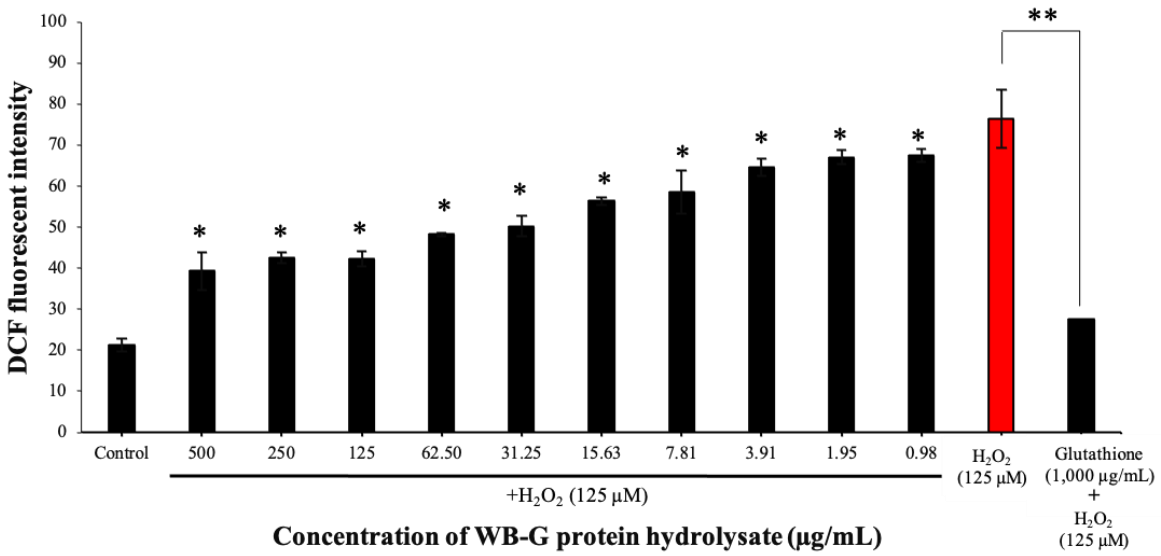


Figure 7. The DCF fluorescent intensity of H_2O_2-damaged L929 cells after pre-incubation with different concentrations of WB-G protein hydrolysate. * Indicates significant difference in fluorescent intensity between samples pre-incubated with WB-G protein hydrolysate and non-treated cells at $p < 0.05$. ** Indicates significant difference in fluorescent intensity between glutathione and H_2O_2 -treated cells (125 µM), $p < 0.05$. WB-G protein hydrolysate decreases the intensity of DCF in a dose-dependent manner.

The effect of WB-G protein hydrolysate on ROS production was further observed under the microscope and fluorescent microscope (Fig. 8). There were changes in cells' morphology after treatment with H_2O_2 as seen by the cell breakdown and lysis while the cells preincubated with WB-G protein hydrolysate at concentrations of 125-250 µg/mL showed normal cell morphology of L929 cells after exposure to H_2O_2, indicating the ability of WB-G protein hydrolysate to protect the cells from damage by H_2O_2. Fluorescent microscopy revealed that cells treated with H_2O_2 showed higher intensity of DCF as demonstrated by the green light indicating high ROS or free radicals in the cells [53]. Cells preincubated with WB-G protein hydrolysate at concentration of 125-250 µg/mL and exposed to H_2O_2 exhibited a reduction in green fluorescence indicating the protective ability of WB-G protein hydrolysate. The protein hydrolysate of tree peony seeds showed a cytoprotective effect as demonstrated by a decrease in DCF fluorescent signal in HepG2 cell lines because it can decrease the level of malonaldehyde (MDA) and lactate dehydrogenase (LDH), and induce the levels of antioxidant enzymes (superoxide dismutase and catalase) [42]. The protein hydrolysate of soybeans, a product of the alcalase enzyme, showed a decrease in H_2-DCFDA fluorescent signal indicating the inhibition of intracellular ROS production in CaCo2 cell lines [54]. A daily dose of 600 mg of squalene, a natural oil with antioxidant activity, for 84 days increases

catalase and superoxide dismutase activity while decreasing hydrogen peroxide levels. Squalene can help regulate and reduce the repercussions of diabetes induced by changes in the oxidant/antioxidant balance [55]. This highlighted the beneficial effect of antioxidant compound. Further study on health benefit of WB-G protein hydrolysate should be conducted.

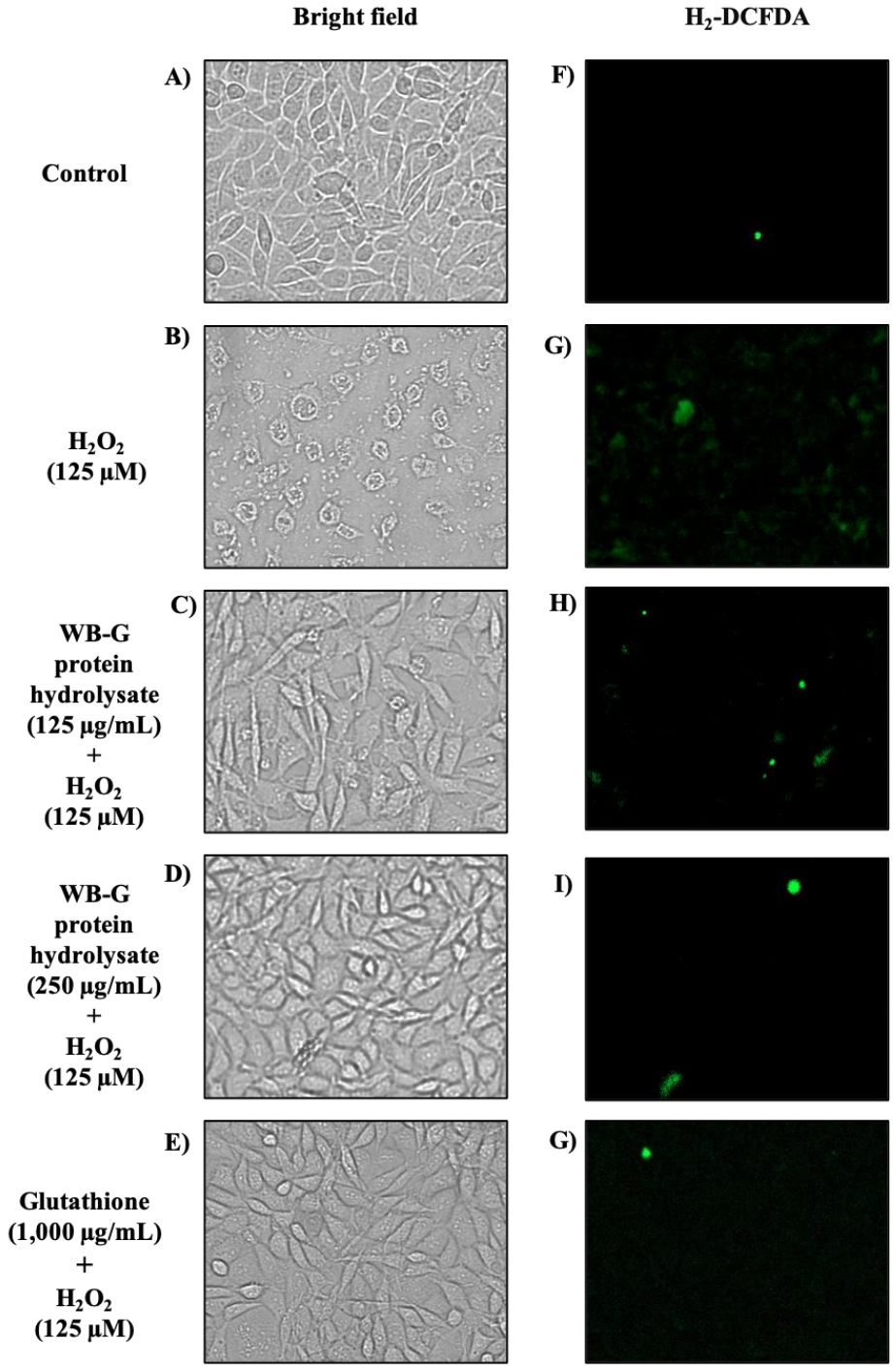


Figure 8. The bright field and fluorescent image of H_2O_2-damaged L929 cells after pre-incubation with WB-G protein hydrolysate. WB-G protein hydrolysate could reduce the level of ROS or free radical in H_2O_2 treated cells.

Bright field: A) Control: Untreated L929 cell lines; B) L929 cells treated with H_2O_2; C-D): L929 cells pre-incubated with WB-G protein hydrolysate (125 and 250 µg/mL) and exposed to H_2O_2; E): L929 cells pre-incubated with glutathione (1,000 µg/mL) and exposed to H_2O_2 (positive control)

Fluorescence: F) Control: Untreated L929 cell lines; G) L929 cell treated with H_2O_2; H-I): L929 cells pre-incubated with WB-G protein hydrolysate (125 and 250 µg/mL) and exposed to H_2O_2; G): L929 cells pre-incubated with glutathione (1,000 µg/mL) and exposed to H_2O_2 (positive control)

CONCLUSIONS

The protein hydrolysate of winged bean or *Psophocarpus tetragonolobus* (WB) seeds from gamma irradiated seeds represented a valuable source of natural antioxidant proteins and peptides with cytoprotective effect against H_2O_2 damage. It might be developed as an alternative food or feed supplement with safety and efficacy.

Abbreviations: WB: Winged bean; WB-G: Winged bean seeds irradiated with gamma ray; DPPH: 2,2–diphenyl–1–picrylhydrazyl; ABTS: 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid.

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