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Original Article

Protection of Fargesin against Hydrogen Peroxide-induced Cell Death in RAW 264.7 Cells

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ABSTRACT

Background: The imbalance level of Reactive Oxygen Species (ROS) in the body can lead to oxidative stress in cells, resulting in the progression of chronic diseases. Fargesin, a bioactive lignan found in Flos magnoliae, an herb widely used in Chinese herbal medicines, has been identified as a potential compound to mitigate this imbalance. **Materials and Methods:** Hydrogen peroxide (H_2O_2), one of the major ROS, is commonly employed as an experimental model to induce oxidative stress. This study aimed to investigate the protective effect of fargesin against cell death induced by hydrogen peroxide in RAW 264.7 macrophage cells. RAW 264.7 macrophage cells were pre-treated with various concentrations of fargesin before being exposed to toxic dose (1 mM or 2 mM) of hydrogen peroxide. The viability of the cells was assessed using the MTT assay, and the morphological changes were described. **Results and Discussion:** The MTT assay demonstrated that non-toxic doses of fargesin (1.56 µM, 3.13 µM, 6.25 µM, 12.5 µM, and 25 µM) significantly reduced H_2O_2-induced cytotoxicity in the treated cells compared to the control group. **Conclusion:** This research is expected to provide a basis for future studies in the development of a new antioxidant agent for the treatment and prevention of diseases associated with oxidative stress.

Keywords: RAW264.7, Oxidative stress, Hydrogen peroxide, Cell death, Fargesin.

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INTRODUCTION

Reactive Oxygen Species (ROS) play a key role in maintaining intracellular and extracellular body homeostasis. These unstable molecules containing oxygen can aggressively react with other molecules in the body. However, it is crucial to balance the levels of reactive oxygen species, as they can cause oxidative stress to cells when present in high amounts. Oxidative stress occurs when oxidants damage cells, negating the favorable effects of antioxidants. The primary types of reactive oxygen species include superoxide (O_2.-) and hydroxyl (OH.) free radicals, as well as non-radical molecules like hydrogen peroxide (H_2O_2). Enzymatic pathways contribute to their generation, involving superoxide dismutase in the mitochondrial enzyme (MnSOD), NADH oxidase (Nox), Electron Transport Chain (ETC), xanthine and myeloperoxidase (MPO).[1-5] When maintained at an optimal level, reactive oxygen species help regulate cell proliferation and apoptosis by ensuring a proper signaling system. However, an imbalance of reactive oxygen species can have deleterious effects on the body, leading to the progression of chronic diseases. The incomplete removal of excess reactive oxygen species causes oxidative stress in cells and can even result in DNA mutations. The development of cancers such as breast cancer, prostate cancer, and colorectal cancer is a consequence of these mutated genes caused by oxidative stress. Moreover, oxidative stress plays a role in the progression of cardiovascular diseases and atherosclerosis by recruiting more macrophages and endothelial cells, which secrete proinflammatory cytokines. Additionally, an increase in reactive oxygen species and a disruption of antioxidant activity can contribute to the development of diabetes mellitus.[6,7]

In response to infection and inflammation, macrophages derived from monocytes originating from the bone marrow, blood, and spleen are mobilized. The functions of macrophages are varied, from detecting tissue damage and assisting in tissue repair to phagocytosing pathogenic microorganisms.[8] Macrophages

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can be classified into two phenotypes: M1 phenotype, which exhibits high microbicidal activity and aids in pathogen removal by producing pro-inflammatory cytokines, and M2 phenotype, which produces high levels of anti-inflammatory cytokines to reduce inflammation in cells.[9,10] Macrophages phagocytose pathogenic micro-organisms and remove them by producing ROS. However, some microorganisms can promote the macrophages to produce ROS to such high levels that it leads to macrophage death. Therefore, macrophages are often chosen as models of oxidative stress in antioxidant activity tests. As for ROS, hydrogen peroxide is mostly used as a stimulus. Antioxidants can be used to maintain the homeostasis of the free radical thus prevent the deleterious effect of reactive oxygen species. Antioxidants act as neutralizers that become oxidized when reacting with free radicals.[7] They either act in the chain-breaking process or serve as preventive agents.[8] Antioxidants can be produced endogenously and obtained exogenously through foods and supplements. The main endogenous enzymes involved are Superoxide Dismutase (SOD), Catalase (CAT), Glutathione Peroxidase (GPx), and Reductase (GRx).[8-14] There are also non-enzymatic antioxidants and nutrient antioxidants such as coenzyme Q10, L-arginine, melatonin etcetera, vitamin A, C, E and omega-3 and omega-3 fatty acid.

Fargesin can be found in Flos magnoliae, an herb widely used in Chinese herb medicine. This herb is native to the Asian region and is currently cultivated worldwide. It is commonly utilized as an anti-inflammatory agent for conditions such as allergic rhinitis, sinusitis, and headaches.[15] Several studies have highlighted the diverse pharmacological actions of fargesin, including its ability to prevent inflammation, allergic reactions, anti-angiogenesis, anti-proliferation, reduce hypertension, act as a calcium ion blocker, exhibit anti-microbial properties,[16-21] and potential inhibitors of brain-specific CYP46A1, therapeutic target for Alzheimer disease.[22] Furthermore, it has been shown to suppress oxidative stress, apoptosis, and improve lipid and glucose metabolism.[23] However, limited research has been conducted on the antioxidant properties of fargesin. Thus, this *in vitro* study aims to investigate whether fargesin exhibits protective effects against cell death caused by hydrogen peroxide in RAW 264.7 macrophage cells through employing various assay methods such as cell viability and morphological studies.

MATERIALS AND METHODS

Materials

Fargesin was purchased from Wuhan ChemFaces Biochemical Co., Ltd. (Wuhan, Hubei, China). Dulbecco's Modified Eagle's Medium (DMEM) and Fetal Bovine Serum (FBS) were bought from Life Technologies Limited (United Kingdom). TrypLE Express was purchased from Life Technologies (Denmark). Penicillin-Streptomycin mixed solution was bought from Nacalai Tesque, Inc. (Kyoto, Japan). Phosphate Buffer Solution (PBS) was purchased from Sigma-Aldrich Co. (United States). Other chemicals, MTT reagent 3-(4,5-dimethylthiazol-2-yl)2,5-dip henyltetrazolium bromide, Dimethylsulfoxide (DMSO), and hydrogen peroxide were obtained from a commercial supplier.

Methods

Cell Culture

RAW 264.7 (ATCC®TIB-71™) macrophage cells were originally procured from American Type Culture Collection (ATCC) (Virginia, United States). They were grown in DMEM medium, 10% fetal bovine serum and 100 unit/mL of penicillin, 100 µg/mL of streptomycin serum. The cells were maintained in a humidified incubator at 37°C with 5% carbon dioxide until they reached 80% confluence. They were sub-cultured according to the ATCC protocol.

Determination of Cytotoxicity of Hydrogen Peroxide

RAW 264.7 macrophage cells were exposed to various concentrations of hydrogen peroxide to identify the lethal dose of hydrogen peroxide for the cells. The concentrations tested were 0.125 mM, 0.25 mM, 0.5 mM, 1.0 mM and 2.0 mM of hydrogen peroxide. Prior to the test, the medium in the 96-well culture plate was gently removed after overnight incubation. Next, 100 µL of different concentrations of hydrogen peroxide were treated to the RAW 264.7 macrophage cells and incubated for 4 hr. Cell viability was then assessed using the MTT assay.

Determination of Effective Concentration of Fargesin

Under an identical setup, RAW 264.7 macrophage cells were pre-treated with various concentrations of fargesin for 24 hr before exposure to a lethal dose of hydrogen peroxide (1 mM or 2mM) for the next 4 hr. The influence of fargesin's activity was assessed using the MTT assay.

Determination of Cell Viability using MTT Assay

Tetrazolium bromide salt (0.5 mL/mL of stock in PBS) was used in this experiment for the MTT assay. The hydrogen peroxide-treated RAW 264.7 macrophage cells were added with 100 µL MTT solution and incubated for 3 hr. The formazan formed at the end of incubation was solubilized in 100 µL of DMSO. The percentage of cell viability was assessed using the MTT assay. The cell viability was quantified by measuring absorbance at 570 nm using a Multiwell micro-plate reader (Synergy HT, Bio-Tek Instruments, Inc. Vermont, USA). Untreated sets, which served as blank controls, were also run under the same conditions. The percentage of cell viability was calculated using the following equation: Cell viability (%)=[($OD_{(test)}$-$OD_{(blank)}$) / ($OD_{(untreated)}$-$OD_{(blank)}$)×100.

Morphological Observation

The morphological structure of the RAW 264.7 macrophage cells treated with / without various concentrations of fargesin and hydrogen peroxide was observed using a digital microscope, Olympus CKX 53, at 100x magnification, with the Toup View application.

Statistical Analysis

The results were expressed as mean±standard deviation from at least three independent experiments. The Student's t-test was used to compare the values between the treatment batches and the control batches. The analysis was performed using Statistical Product and Service Solutions (SPSS) to determine the significant difference ($p<0.05$).

RESULTS

Cytotoxicity Assessment of Fargesin

Fargesin ($C_{21}H_{22}O_6$) is categorised under lignans with a molecular weight of 370.4 g/mol (Figure 1A). Its cytotoxicity was tested in RAW 264.7 macrophage cells by exposing them to several concentrations of fargesin for 24 hr. The MTT assay showed a statistically significant reduction ($p<0.05$) in the percentage of cell viability of RAW 264.7 macrophage cells when exposed to 50 µM for 24 hr (Figure 1B). Treatment of the cells with the compound at 1.56, 3.13, 6.25, 12.5, and 25 µM showed non-significant reduction ($p>0.05$) of cell viability or no cytotoxicity. Thus, these doses were chosen for the next experiment. To examine the morphological changes of the RAW 264.7 macrophage cells, they were observed using a digital microscope, Olympus CKX 53. As shown in Figure 1c, some of the 50 µM fargesin-treated cells exhibited typical morphological characteristics of dying cells, which are smaller, shrunken, and appearance of membrane bleb, as compared to the control. No obvious difference in cell morphological characteristics was observed between the 1.56 µM and 25 µM fargesin-treated cells as well as the control.

Cytotoxicity Assessment of Hydrogen Peroxide

Using an identical setup, RAW 264.7 macrophage cells were exposed to various concentrations of hydrogen peroxide (0.125 mM, 0.25 mM, 0.5 mM, 1.0 mM, and 2.0 mM). As shown in Figure 2(A), the cell viability of RAW 264.7 macrophages decreased in a dose-dependent manner when exposed to hydrogen peroxide, indicating that the higher the concentration of hydrogen peroxide exposure, the lower the percentage of cell viability. This assessment was conducted to determine the half-maximal inhibitory concentration (IC_{50}) of hydrogen peroxide, which was found to be 1 mM after a 24 hr treatment in RAW 264.7

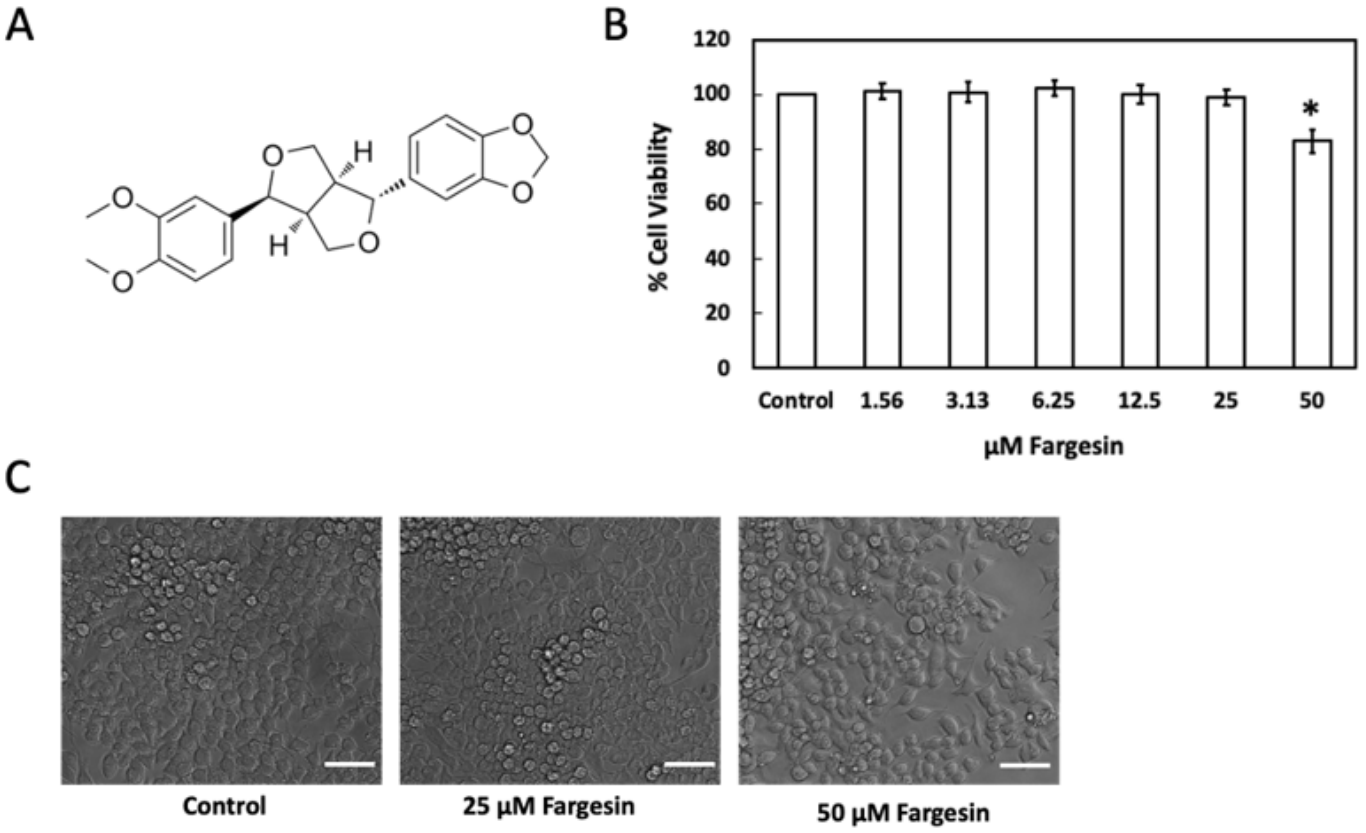

Figure 1: Fargesin cytotoxicity in RAW 264.7 cells. (A) Fargesin molecular structure. (B)% Cell viability of RAW264.7 cells incubated with different concentrations of fargesin. (C) Morphological changes of the cells after treatment with fargesin. Data are presented as mean±SD values from three replications. *$p<0.05$ indicates significant differences from the control group value.

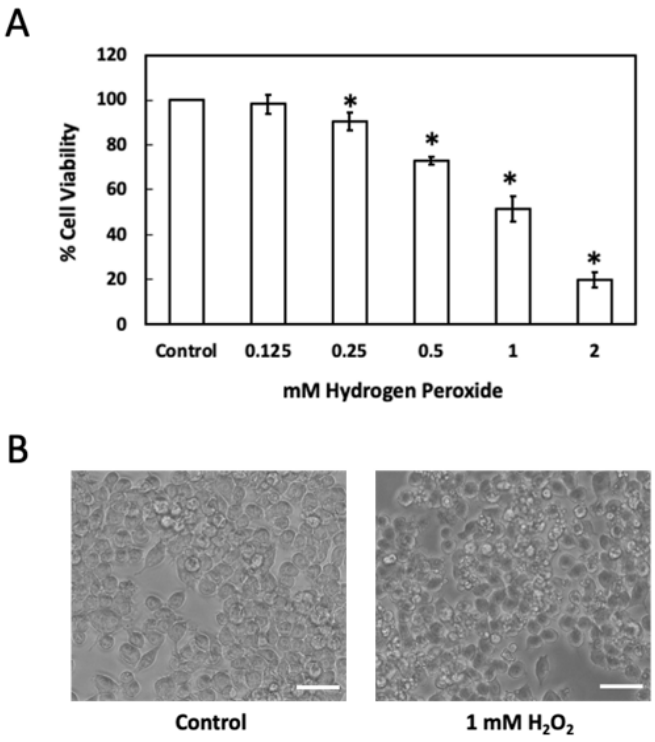

Figure 2: Hydrogen peroxide cytotoxicity in RAW 264.7 cells. (A) Percentage of cell viability of RAW264.7 cells incubated with different concentrations of hydrogen peroxide. (B) Morphological changes of the cells after treatment with hydrogen peroxide. Data are presented as mean ± SD values of three replications. *p<0.05 indicates significant differences from the control group value.

cells. Hydrogen peroxide at 2 mM inhibited 80% of the cells compared to the control cells. The hydrogen peroxide-treated cells displayed typical morphological characteristics of apoptosis, with membrane-bound apoptotic bodies and cell shrinkage, as shown in Figure 2b. In contrast, the untreated control cells (0 mM) showed a normal morphological structure with elongated pseudopods.

Pre-treatment of Fargesin against Hydrogen Peroxide

The final results highlight the protective activity of fargesin against a lethal dose of hydrogen peroxide (1 mM) in RAW264.7 macrophages. This protective effect was assessed using the MTT assay, as shown in Figure 3(A). The cells were pre-treated with non-toxic doses of fargesin for 24 hr before a 4 hr treatment with 1mM hydrogen peroxide. Consistent with the previous data (Figure 2(A)), treatment with 1mM hydrogen peroxide reduced cell viability. However, pre-treatment of the cells with 6.25, 12.5, and 25 µM fargesin significantly increased (p<0.05) cell viability compared to the non-pre-treated group. To confirm this effect, we increased the concentration of hydrogen peroxide to 2 mM using the same experimental setup, and the results showed a similar trend. This indicates that fargesin does exhibit protective activity in the cells after pre-treatment. The morphological changes of the control group, 1 mM hydrogen peroxide-treated cells with and without fargesin pretreatment, are displayed in Figure 3c. Consistent with the previous data, cells treated with 1mM hydrogen peroxide showed morphological characteristics of dying cells. However, pretreatment with 6.25, 12.5, and 25 µM fargesin reversed the alterations caused by hydrogen peroxide, indicating its protective effect on cell death.

DISCUSSION

Generally, exposure to a high concentration of hydrogen peroxide (H_2O_2) can cause oxidative stress in RAW 264.7 macrophages.[24,25] Our data align with these findings, as we observed cell death in a dose-dependent manner upon hydrogen peroxide exposure. Numerous research studies have reported the dose-dependent nature of hydrogen peroxide-induced oxidative stress.[26,27] This oxidative stress is associated with various diseases, including cancer, diabetes mellitus, and cardiovascular diseases.[6,7] Apart from that, elevation of ROS particularly hydrogen peroxide is also part of inflammatory responses by macrophages and plays important roles in pathogen killing. However, excessive accumulation and duration of hydrogen peroxide in macrophages can cause detrimental effects.[28] A study conducted by Robert N. Goddu *et al*. suggested that chronic exposure of RAW 264.7 macrophage cells to hydrogen peroxide enables them to survive oxidative stress due to the elevated expression of the endogenous enzyme catalase.[25] Therefore, we assume that macrophages can adapt to progressive environmental stress but may not survive sudden acute changes in the environment.

We also assessed the cytotoxicity activity of fargesin in this study. The findings revealed that high concentrations of the compound (more than 25 µM) reduced the percentage of cell viability in macrophages. This result is consistent with our previous study, where we reported an IC_{50} of 173.5µM for fargesin in RAW264.7 cells.[29] Previous research has also reported that fargesin isolated from Z. planispinum roots exhibited moderate cytotoxic activity against Hela cancer cells, with an IC_{50} of 15.00±1.50 µg/mL.[30] These cytotoxic properties, supported by previous studies, are promising for the treatment of cancer through the inhibition of ERK1/2 kinases, which retard the release of inflammatory mediators such as interleukin-6 (IL-6), Tumour Necrosis Factor-α (TNF-α), and Reactive Oxygen Species (ROS).[31,32]

Our findings indicate that fargesin exhibits a protective effect against cell death induced by hydrogen peroxide, possibly due to its anti-inflammatory and antioxidant activities. Previous studies have shown that, beside ROS, hydrogen peroxide can induce cell death through activation of the inflammatory pathway via the MAPK pathway and the expression of genes related to inflammation, including interleukins, TNF-α, and NF-κB. Research suggests that fargesin is a bioactive lignan with anti-inflammatory properties.[32] Recent studies have shown that fargesin exerts anti-inflammatory actions on human monocytic cells (THP-1) by preventing NF-kB signaling activation.

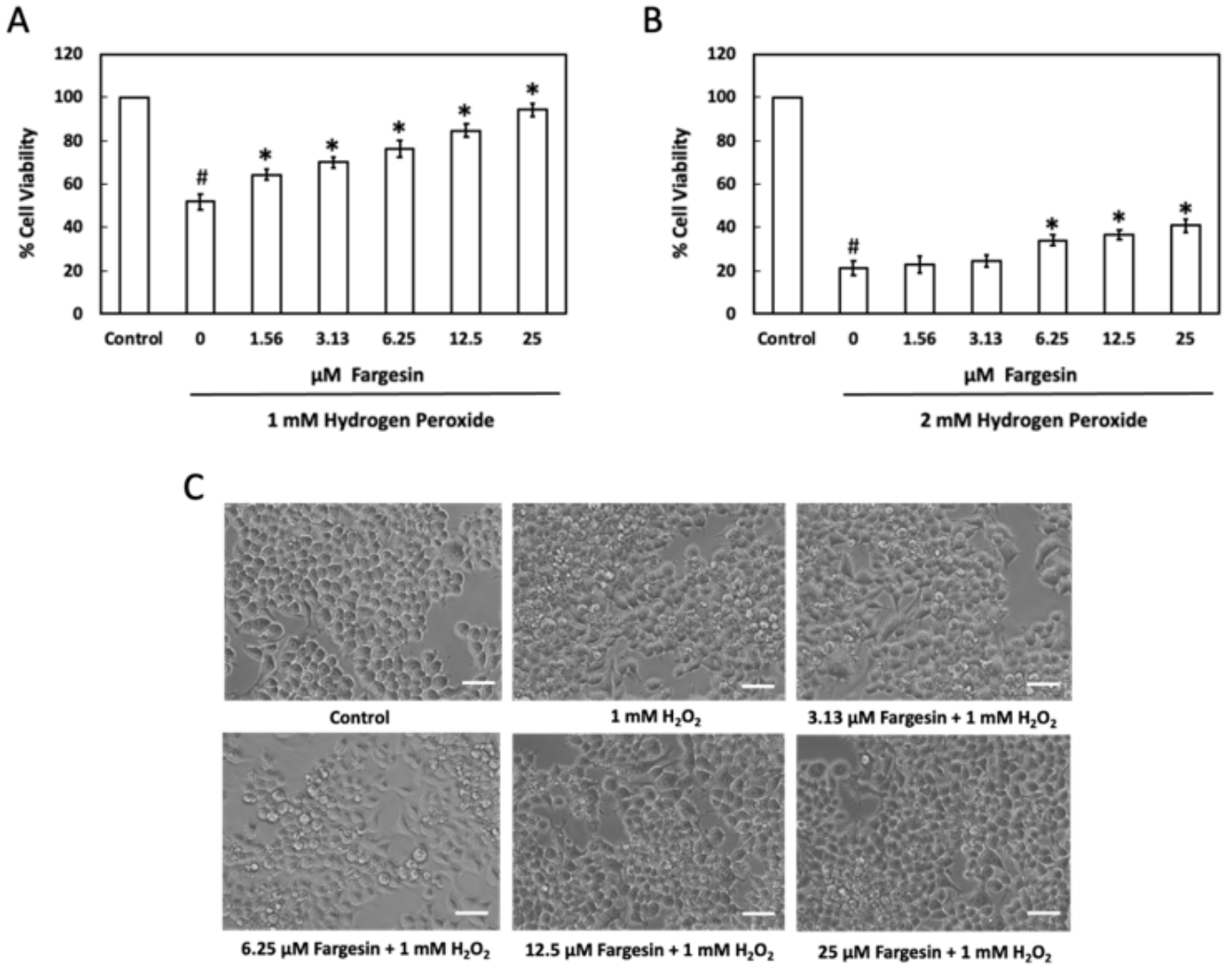

Figure 3: Figure 3: Cell death protective activity of fargesin in RAW 264.7 cells. Effects of fargesin on % cell viability in (A) 1mM and (B) 2 mM H2O2-induced RAW264.7 cells. (C) Morphological changes of the cells after treatment with fargesin and hydrogen peroxide. Data are presented as mean SD values of three replications. *p<0.05 indicates significant differences from the hydrogen peroxide alone group value. #p<0.05 indicates significant differences from the control group value.

NF-kappa B plays a key role in the transcriptional induction of pro-inflammatory mediator genes. When activated, NF-kappa B translocate from the cytosol to the nucleus, facilitating the transcription of inflammatory cascades. Therefore, treatment with dose-dependent fargesin prevents the translocation of the NF-kappa B transcription factor.[23] Additionally, an *in vitro* study on the anti-inflammatory activity of lignans from M. fargesii demonstrated that the compound suppresses NF-kappa B, reducing the expression of induced Nitric Oxide Synthase (iNOS) and Cyclooxygenase-2 (COX-2). The overproduction of nitric oxide stimulates the production of prostaglandins by activating Cyclooxygenase-2 (COX-2).[33] Prostaglandins are potent inflammatory mediators derived from the conversion of arachidonic acid. Hence, the inhibition of induced Nitric Oxide Synthase (iNOS) and Cyclooxygenase-2 (COX-2) by the lignans from M. fargesii has been shown to reduce inflammation in treated cells.[34] Other studies have also demonstrated fargesin's anti-inflammatory effect in chemically induced inflammatory bowel disease in mice, significantly reducing the expression of pro-inflammatory cytokines IL-1B, IL-15, TNF-α, and IFN-γ, while increasing the expression of the anti-inflammatory cytokine IL-10 in the colon.[15]

Fargesin has been shown to increase the activities of some endogenous antioxidants, including superoxide dismutase, catalase, and glutathione peroxidase, while suppressing Malondialdehyde (MDA), which is a marker for oxidative stress. This reduction in MDA indicates a decrease in the release of intracellular reactive oxygen species.[35] These findings explain the possible pathways of fargesin's protective activities and its anti-inflammatory effect. Superoxide Dismutase (SOD), Catalase (CAT), Glutathione Peroxidase (GPx), and Reductase (GRx) are the main antioxidant enzymes involved in the neutralization of reactive oxygen species. SOD acts as the first-line defense against

ROS, as it catalyzes the dismutation of the superoxide anion radical into hydrogen peroxide through a reduction process.[36] Subsequently, hydrogen peroxide is transformed into water and diatomic oxygen (O_2) by Catalase (CAT) or Glutathione (GPx). The GPx enzyme then removes hydrogen peroxide by catalyzing its oxidation to the reduced form of glutathione, GSH, converting it to its oxidized form, GSSH.[8-14]

CONCLUSION

In conclusion, the RAW 264.7 macrophage cells were showed to be vulnerable against the hydrogen peroxide. However, the pre-treatment with fargesin have shown to exhibit protective mechanism in macrophages cell from hydrogen peroxide-induced cell death in the MTT assay. The data provided can be used in further investigation by other researchers on the protective effect of fargesin *in vivo* and *in vitro*.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

MTT: (3-4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **H_2O_2:** Hydrogen peroxide; **DMSO:** Dimethylsulfoxide; **NF-k**B: Nuclear factor kappa-light-chain-enhancer of activated B cells; **MAPK:** Mitogen-activated protein kinase; **iNOS:** induced nitric oxide synthase; **COX-2:** Cyclooxygenase-2; S**OD:** Superoxide dismutase; **CAT:** Catalase; GPx: Glutathione peroxidase; **GRx:** Glutathione reductase; **ERK:** Extracellular signal-regulated kinase.

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