

Effect of Pegagan Leaves (*Centella Asiatica*) on mRNA Nrf2 in Hemopoietic Stem Cells with H₂O₂ Exposure: In Vitro Study

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Abstract. Oxidative stress in metabolic diseases, such as diabetes mellitus, poses a significant challenge that necessitates effective therapeutic strategies to mitigate its impact on hematopoietic stem cells (HSCs). Damage caused by oxidative stress leads to premature aging, thereby decreasing the self-renewal and multipotency of HSCs, which can disrupt hematopoietic function. This study aims to assess the effect of Pegagan (*Centella Asiatica*) leaf extract on the expression of Nrf2 mRNA in HSCs exposed to H₂O₂ in vitro. A pure experimental design was employed, comprising five treatment groups: a positive control (without H₂O₂ exposure), a negative control (with H₂O₂ exposure), and three groups treated with Pegagan extract at concentrations of 15%, 30%, and 60%. The analysis results showed that the mean Nrf2 expression levels in the five groups were 0.038 ± 0.005 (negative control), 0.006 ± 0.0009 (positive control), 0.016 ± 0.004 (group treated with 15% Pegagan extract), 0.033 ± 0.009 (group treated with 30% Pegagan extract), and 0.023 ± 0.004 (group treated with 60% Pegagan extract). The results revealed a significant difference ($p < 0.05$) between the Pegagan extract groups and the negative control group. These findings suggest that Pegagan leaf extract may serve as a therapeutic agent to enhance Nrf2 expression and mitigate oxidative stress in HSCs, potentially aiding in the repair of damage and restoration of hematopoietic function in diseases related to oxidative stress.

1 Introduction

Non-communicable diseases, including diabetes mellitus, are on the rise annually, constituting 63% of global mortality [1]. These individuals typically have elevated concentrations of glucose and fatty acids in the bloodstream, resulting in enhanced synthesis of acetyl-CoA. Free fatty acids undergo β -oxidation to generate acetyl-CoA, which subsequently enters the TCA cycle. An elevation in acetyl-CoA can improve energy

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generation via the TCA cycle. Conversely, an overabundance of acetyl-CoA may induce metabolic imbalances, leading to oxidative stress and mitochondrial dysfunction [2].

Oxidative stress adversely affects hemopoietic stem cells (HSCs) in both the bone marrow and the bloodstream. HSCs possess self-renewal capabilities and multipotency, allowing them to differentiate into various types of blood cells. HSCs that undergo aging will diminish their capacity for self-renewal and multipotency, hence impairing the functionality of the blood cells. Oxidative stress induces DNA damage in HSC, activating the production of cell cycle inhibitors, which leads to premature aging, diminished stem cell functionality, and alterations in the HSC cycle [3].

The body contains a Kelch-like ECH-associated protein 1/Nuclear Factor Erythroid 2-related factor 2 (Keap1/NRF2 and Nrf2), which functions as a "master regulator" of antioxidant responses, influencing several genes that govern inflammation, immunity, tissue remodeling, carcinogenesis, and metastasis. This transcription factor positively regulates ARE and modulates the production of antioxidant enzymes, including NAD(P)H and NQO1 [4].

Due to the detrimental impacts of free radicals, a method is required to mitigate or diminish these consequences. An approach involves utilizing a chemical that mitigates oxidative damage and reduces the inflammatory effects of free radicals. A multitude of studies have been undertaken about the creation of antioxidants from natural sources. The Pegagan leaf (*Centella asiatica*) possesses notable antioxidant properties derived from bioactive components like asiaticoside, madecassoside, asiatic acid, and madecassic acid. These chemicals engage with antioxidant enzymes, including superoxide dismutase (SOD) and glutathione peroxidase (GPx), enhancing their activity and stabilizing reactive oxygen species (ROS) [5]. Moreover, *Centella Asiatica* extract exhibited significant free radical scavenging activity across multiple assays, confirming its efficacy in neutralizing free radicals. This study aims to investigate the effect of Pegagan leaves (*Centella Asiatica*) on Nrf2 expression in HSCs exposed to H₂O₂ in vitro.

2 Materials and Methods

2.1 Research design

This study employs an experimental design that utilizes a Post-Test-Only Control Group approach, focusing on HSC cell culture in vitro. It consists of five groups: a positive control group not exposed to H₂O₂, a negative control group exposed to 0.3% H₂O₂, and three treatment groups exposed to 0.3% H₂O₂ along with 15%, 30%, and 60% concentrations of Pegagan leaf ethanol extract. Based on the sample size formula, five replicates were obtained for each group.

2.2 Collection and preparation of Pegagan Leaves extract

The Pegagan plant used in this study was sourced from Pegagan leaves collected in the Banjar Segah area of Asahduren Village, Pekutatan District, Jembrana Regency, Bali. The Pegagan herb is cleaned and then processed into a powder with a particle size of 40 mesh. One thousand grams of Pegagan herb powder are extracted using the maceration method, ensuring that all simplicia are fully submerged in 96% ethanol until complete extraction is achieved. The extraction yields are then concentrated using a Rotary Evaporator, followed by freeze-drying to remove moisture and preserve the quality of the extract.



Figure 1. The process of making the Pegagan leaf extract

The freeze-drying method used on Pegagan herb extract involves removing water through the sublimation of ice crystals from frozen material. This technique is more effective at producing high-quality Pegagan herb extract than traditional drying methods. Freeze drying includes several stages: sample preparation, freezing, primary drying, and secondary drying, resulting in a final product with the desired moisture level. The moisture concentration gradient between the drying front and the condenser drives water removal during the process. The water vapor pressure increases as temperature rises during primary drying. The primary drying temperature must be kept high but below the critical temperature to prevent damage. During freezing, ice crystals form and segregate until the solution reaches its maximum concentration. With further cooling, phase separation between the solute and ice occurs [5].

2.3 Isolation of HSC CD34 from venous blood

The process of isolating HSC from healthy human venous blood, without prior disease or treatment, begins with collecting 5 mL of venous blood in an EDTA tube by gently rotating it. Next, 5 mL of PBS-EDTA-BSA 0.5% (pH 7.2) is added in the same manner. This mixture is then slowly transferred into a 15 mL tube containing 5 mL of Ficoll or Histopaque, ensuring the layers remain separate. Afterward, the mixture is centrifuged at $300 \times g$ for 10 minutes. The middle layer containing the cells is carefully removed, along with the top layer. The remaining layer is then aspirated into a new tube. After adding 10 mL of 0.5% PBS-EDTA-BSA (pH 7.2), the mixture is centrifuged again, and the supernatant is discarded. Next, DCS is labeled with CD34 antibodies by adding 300 μL of 0.5% PBS-EDTA-BSA, 10 μL of FcR blocking reagent, and 10 μL of CD34 microbeads, followed by incubation at 4°C for 30 minutes. Following incubation, 10 mL of 0.5% PBS-EDTA-BSA is added, and the sample is processed again using a centrifuge. Magnetic separation is performed by attaching the column to a magnetic field, washing with PBS, and ensuring all liquid drains completely. The cell mixture is then loaded into the column and washed several times with PBS. Finally, 1 mL of 0.5% PBS-EDTA-BSA is added to the column after it is removed from the magnetic field, and the HSC is dispensed into a 1.5 mL tube by pressing with a plastic plunger [6].

2.4 Quantitative real-time PCR (qPCR)

The expression of *Nrf2* was analyzed using quantitative PCR (qPCR) (Bio-Rad, USA). Genome sequence data for the initial design were obtained from the NCBI human mRNA database, and primers were developed with the Primer3Plus online tool. mRNA was isolated according to the manufacturer's instructions using a Qiagen (Germany) mRNA isolation kit. Then, cDNA was synthesized with a reverse transcriptase kit from the same manufacturer.

Nrf2 gene expression was measured using quantitative PCR (qPCR) with SYBR Premix Ex Taq II (TaKaRa, Japan) according to the manufacturer's guidelines. Gene expression levels were normalized to GAPDH and expressed as relative fold changes. The sequences used are as follows: Forward (F) for target: TGT TTT CCT TTG TGT CAT TCC C; Reverse (R): ATG GAG ATT CAT TGA CGG GAC; GAPDH Forward (F): AAG AAG GTG GTG AAG CAG GC; GAPDH Reverse (R): GTC AAA GGT GGA GGA GTG GG.

2.5 Statistical analysis

Statistical analysis was performed using version 16.0 of the Statistical Package for Social Sciences (SPSS, Inc., Chicago, IL, USA) for Windows. Data are presented as mean ± standard error of the mean. A post-hoc LSD test was applied following the One-Way Anova test. A p-value below 0.05 is considered statistically significant.

3 Results and Discussion

This study is a purely experimental investigation that compares the expression of NRF2 mRNA in HSCs exposed to hydrogen peroxide (H₂O₂). After stressing the HSCs, 15%, 30%, and 60% of Pegagan leaf extract are administered. Based on phytochemical tests of pegagan leaf extract, the content of active compounds was identified, including saponins, tannins, flavonoids, steroids, terpenoids, and alkaloids (Table 1).

Table 1. Phytochemical Content of Pegagan (*Centella Asiatica*) Leaves

Compounds	Phytochemical Analysis
Saponin	+
Tannins	+
Flavonoids	+
Steroids	-
Terpenoids	+
Alkaloids	+
Vitamin C	+

The analysis results of the average difference in Nrf2 expression in human vein blood across five groups are shown in Table 2. The highest expression was found in the positive control group, which was not exposed to H₂O₂ (0.038 ± 0.005), followed by the group treated with a 30% ethanol extract of Pegagan leaves (0.753 ± 0.183). Notably, administering the 30% Pegagan leaf extract led to a 0.033-fold increase in Nrf2 mRNA expression compared to the reference gene. In contrast, the negative control group, exposed to H₂O₂, showed the lowest Nrf2 expression at 0.006 ± 0.0009, representing a 0.006-fold increase relative to the reference gene.

Table 2. Differences in mean mRNA Nrf2 expression in all five groups

Treatment Groups	Relative quantification of mRNA Nrf2 expression.		
	Median (Min-Max)	Mean±SD	p-value
Positive control	0.04 (0.028-0.042)	0.038±0.005	<0.001*
Negative control	0.006 (0.005-0.008)	0.006±0.0009	
Dose 1: 15% Pegagan leaf extract	0.014 (0.012-0.021)	0.016±0.004	

Dose 2: 30% Pegagan leaf extract	0.029 (0.026-0.042)	0.033±0.009
Dose 3: 60% Pegagan leaf extract	0.024 (0.016-0.026)	0.023±0.004

*Significance $p < 0.05$

The results of the Post Hoc analysis showed a difference in the average expression of Nrf2 mRNA across the five groups (Figure 3). Based on these images, there was a significant difference in the average expression of Nrf2 mRNA between the groups given gotu gotu leaf extract doses of 15%, 30%, and 60% compared to negative controls. These results demonstrate that gotu kola ethanol extract induces higher Nrf2 mRNA expression in the treatment groups compared to the negative control after exposure to H₂O₂.

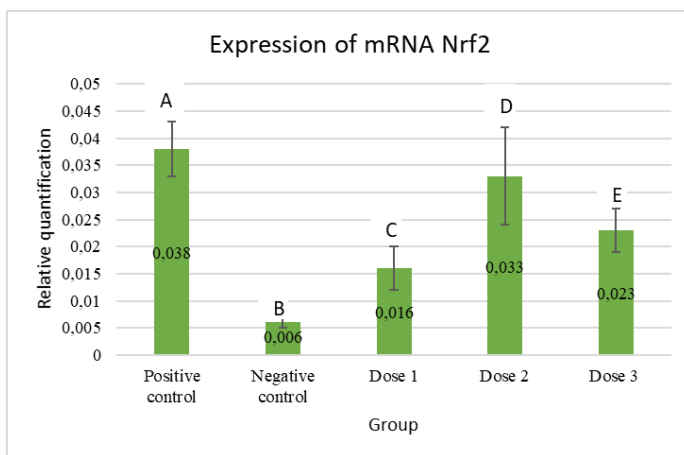


Fig 2. displays the post hoc results for differences in the average expression of Nrf2 across the five groups. A: positive control vs negative control ($p < 0.05$), vs Dose 1 ($p < 0.05$), vs Dose 3 ($p < 0.05$); B: negative control vs positive control ($p < 0.05$), vs Dose 1 ($p < 0.05$), vs Dose 2 ($p < 0.05$), vs Dose 3 ($p < 0.05$); C: Dose 1 vs positive control ($p < 0.05$), vs negative control ($p < 0.05$), vs Dose 2 ($p < 0.05$), vs Dose 3 ($p < 0.05$); D: Dose 2 vs negative control ($p < 0.05$), vs Dose 1 ($p < 0.05$), vs Dose 3 ($p < 0.05$); E: Dose 3 vs positive control ($p < 0.05$), vs negative control ($p < 0.05$), vs Dose 1 ($p < 0.05$), vs Dose 2 ($p < 0.05$).

Pegagan leaves contain active compounds, including triterpenoid saponins such as asiaticoside, centelloside, madecassoside, and asiatic acid. Pegagan leaves contain additional components such as volatile oils, flavonoids, tannins, phytosterols, amino acids, and carbohydrates. The antioxidant compounds present in Pegagan leaves include polyphenols, flavonoids, carotene, tannins, vitamin C, and triterpenoids [5]. Multiple prior studies have investigated the antioxidant properties of Pegagan leaves concerning liver tissue and DNA damage [7, 8]. Free radicals are molecules that can induce DNA damage. When free radicals extract electrons from proteins, fats, and nucleic acids within a cell, the structural integrity of these components is altered, leading to a disruption in the cell's function. Nucleic acids, including DNA, can undergo alterations when exposed to free radicals, specifically converting the base Guanine (G) to 8-OHdG, which may lead to mutations. Free radicals may lead to organ damage and dysfunction, potentially initiating cancer development. An imbalance between oxidants and antioxidants leads to lipid peroxidation, protein oxidation, and DNA damage, disrupting cellular functions and contributing to disease pathogenesis and the aging process [8].

Antioxidants are essential compounds that inhibit or mitigate oxidation processes, safeguarding cells from damage induced by ROS and free radicals. Antioxidants are classified into three categories according to their mechanisms of action: primary, secondary, and tertiary. Primary antioxidants, also known as chain breakers, function by sequestering free radicals and transforming them into more stable entities, thereby inhibiting the propagation of oxidative damage. Notable examples include polyphenols and flavonoids present in fruits and vegetables. Secondary antioxidants neutralize singlet oxygen, decompose peroxides, and bind to prooxidative metals, thereby reducing overall oxidative stress. Plant-derived secondary metabolites, including phenolics and terpenes, play a significant role in this process. Tertiary antioxidants, though often overlooked, play a crucial role in repairing oxidative damage after it occurs, encompassing enzymes and compounds that uphold cellular integrity [9].

Nuclear factor erythroid 2-related factor 2 is a crucial transcription factor that plays a central role in the cell's defense against oxidative stress. Activation of Nrf2 begins when it binds to Keap1 in the cytosol, which targets it for degradation by the proteasome. However, when oxidative stress occurs, Nrf2 is released from the Keap1 complex, moves to the cell nucleus, and binds to antioxidant response elements (ARE) within DNA, leading to the transcription of various genes involved in antioxidant and detoxification mechanisms. Nrf2 regulates the expression of more than 2000 genes, many of which contribute to redox balance and protection against oxidative damage, including genes that encode phase II detoxification enzymes and antioxidant proteins such as heme oxygenase-1 (HO-1) and glutathione-related enzymes. By increasing the expression of this antioxidant gene, Nrf2 helps reduce oxidative damage, thereby protecting cells from apoptosis and carcinogenesis. The interaction between Nrf2 and Keap1 is essential for the regulation of Nrf2 activity; under homeostatic conditions, Keap1 promotes Nrf2 degradation, but oxidative stress interferes with this interaction, allowing for Nrf2 accumulation and activation of target genes. In addition, Nrf2 also modulates metabolic processes and inflammatory responses, affecting critical pathways such as the pentose phosphate pathway, NADPH production, and glucomineralysis, which are essential for cell proliferation and survival, particularly in cancer cells. Targeting the Nrf2 pathway shows great potential; Nrf2 activators, both from natural phytochemicals and synthetic compounds, are being explored for their ability to enhance antioxidant defenses and protect against diseases related to oxidative stress. Nrf2 activity tends to decrease with age, contributing to increased oxidative stress and aging phenomena; therefore, increased Nrf2 activity may potentially reduce age-related oxidative damage and accompanying pathologies [10, 11].

This study involved exposing HSC to hydrogen peroxide (H_2O_2). Hydrogen peroxide is a compound that can induce oxidative stress conditions. This study revealed a significant difference in Nrf2 expression between the groups administered Pegagan leaf extract and the negative control group. Pegagan leaves are acknowledged for their notable antioxidant properties, attributed to the diverse bioactive compounds present in the plant. The antioxidant activity of *Centella Asiatica* is contingent upon the extraction method employed. The drying method influences antioxidant activity; samples dried by freezing exhibited the highest radical scavenger activity ($93.97 \pm 0.45\%$) and the lowest IC_{50} value (0.05 mg/mL) [5]. Natural products have a significant influence on gene expression regulation through multiple mechanisms. Natural products can influence gene expression at the transcriptional level by altering DNA-protein interactions. Recent studies demonstrate the significance of post-transcriptional mechanisms that modify mRNA stability and translational efficiency. These processes have a profound impact on gene expression and its subsequent biological consequences. Moreover, exposure to specific phytochemicals may induce reversible epigenetic modifications, such as alterations in DNA methylation and histone modifications. These alterations may result in substantial changes in gene expression

profiles. Natural products that contain polyphenols, terpenoids, and alkaloids can induce Nrf2 expression by disrupting the Nrf2-Keap1 complex. This disruption facilitates the translocation of Nrf2 to the nucleus, where it binds to antioxidant response elements (ARE) and initiates the transcription of detoxification and antioxidant genes [12].

Centella Asiatica demonstrates significant impacts on the Nrf2 transcription factor pathway. The activation of the Nrf2 pathway enhances several critical functions, including cognitive and mitochondrial improvements in aged mice, through the use of *Centella asiatica* water extract. The activation is essential for the mental benefits derived from *Centella Asiatica* administration, which are absent in Nrf2 knockout mice. The ethanol extract of *Centella Asiatica* activates the Nrf2/HO-1 signaling pathway, resulting in a significant increase in the expression of Nrf2 and HO-1 proteins. This activation reduces oxidative stress associated with LPS-induced production of NO and PGE2 in RAW 264.7 macrophage cells. Furthermore, acetic acid, a significant constituent of *Centella Asiatica*, enhances the expression of the Nrf2 protein, thereby offering protection against organ toxicity induced by doxorubicin through the reduction of oxidative stress in the heart, liver, and kidneys. The administration of *Centella Asiatica* in diabetic mice resulted in increased levels of Nrf2 and other antioxidant enzymes, thereby mitigating oxidative and inflammatory stress in the brain. The action mechanism of *Centella Asiatica* and its constituents has been demonstrated to enhance the expression of Nrf2 and antioxidant target genes, including HO-1, NQO1, and GPX4 [13].

The highest expression of Nrf2 mRNA was observed at a dosage of 30% Pegagan leaf extract. This dosage may achieve the optimal concentration of bioactive compounds in Pegagan that act as Nrf2 activators. At lower concentrations, the compounds may fail to elicit a maximal response, whereas at higher concentrations, toxic or inhibitory effects may arise. Additionally, Pegagan extract comprises several bioactive compounds, including flavonoids, saponins, and terpenoids. A 30% dosage may provide a balanced mixture of these compounds, facilitating synergistic interactions that promote Nrf2 activation. Conversely, elevated doses (60%) may induce adverse reactions or cellular toxicity, potentially impairing cellular responses to oxidative stress and subsequently diminishing Nrf2 gene expression. The activation of Nrf2 can initiate a negative feedback regulation mechanism, whereby excessive activation may inhibit further expression to preserve cellular homeostasis. A 30% dose may effectively enhance antioxidant activity while avoiding excessive oxidative stress, which can adversely affect cellular function and reduce gene expression [14].

Our comprehensive literature review reveals a notable absence of studies investigating the impact of *Centella Asiatica* on hematopoietic stem cells subjected to oxidative stress. A study investigating the effects of *Centella Asiatica* on human mesenchymal stem cells in vitro revealed that this plant can facilitate the differentiation of human mesenchymal stem cells into Schwann cells and various other types of nerve cells. *Centella Asiatica* has also shown promise in inducing neurodifferentiation from mesenchymal stem cells [15].

4 Conclusion

This research demonstrated that Pegagan (*Centella asiatica*) leaf extract significantly enhanced Nrf2 mRNA expression in HSCs subjected to H₂O₂ exposure. Significant differences in Nrf2 expression were observed between groups treated with varying doses of Pegagan extract (15%, 30%, and 60%) and the negative control group exposed to H₂O₂. The elevation of Nrf2 expression may facilitate the mitigation of oxidative stress damage, enhance HSC functionality, and diminish the impacts of premature ageing on these cells. Due to its potent antioxidant properties, Pegagan may serve as a viable therapeutic alternative to chemical drugs in managing conditions related to oxidative stress. Future research is required to investigate the mechanism of action and effectiveness of Pegagan extract in

clinical settings, assess its long-term safety, and directly compare it with conventional drugs to determine its applicability and advantages in the treatment of degenerative and inflammatory diseases.

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