**Effects of *Citrus limon* Extract on Oxidative Stress-Induced Nitric Oxide Generation and Bovine Teeth Bleaching**

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**Background:** We aimed to investigate the effect of *Citrous limon* extract (CLE) on oxidative stress–induced cytotoxicity and nitric oxide (NO) generation and the tooth bleaching effect of CLE as a substitute for hydrogen peroxide (H$_2$O$_2$) and determine the feasibility and application of CLE as a safe and effective natural tooth bleaching agent.

**Methods:** The protective effect of CLE on H$_2$O$_2$-induced cytotoxicity in Raw264.7 macrophages was investigated by the MTT assay. The inhibitory effect of CLE on the generation of H$_2$O$_2$-induced NO was confirmed by the NO assay, and the changes in inducible nitric oxide synthase (iNOS) protein expression were confirmed by western blotting. Stained bovine teeth were treated with/without 15% and/or 35% CLE and H$_2$O$_2$, 15% sodium bicarbonate (NaHCO$_3$) for 3 hours, and were irradiated with/without bleaching light (BL) for 15 minutes. The color change of the treated bovine tooth surface was measured using a colorimeter.

**Results:** The viability of Raw264.7 cells treated with each concentration of CLE and 500 μM H$_2$O$_2$ significantly increased as CLE increased, and NO generation and iNOS protein expression were significantly reduced in cells treated with 300 μg CLE+/500 μM H$_2$O$_2$+ and 300 μg CLE+/500 μM H$_2$O$_2$+/150 μg NaHCO$_3$+. The bleaching effect of 35% CLE+ was higher than that of 15% CLE+ and 15% NaHCO$_3$+, and the effect was similar to that of 15% H$_2$O$_2$+. The 35% CLE+/15% NaHCO$_3$+ showed the greatest bleaching effect and was higher than that of the groups irradiated with the BL. The greatest bleaching effect was observed with 35% CLE+/15% NaHCO$_3$+, followed by 35% H$_2$O$_2$+/BL+.

**Conclusion:** CLE inhibited oxidative stress–induced cytotoxicity and NO generation in Raw264.7 cells and could replace H$_2$O$_2$, which causes side effects and risks in teeth bleaching treatment. It showed greatest teeth bleaching effect when combined with NaHCO$_3$. CLE is an effective and safe natural tooth bleaching substitute.

**Key Words:** *Citrus limon*, Hydrogen peroxide, Nitric oxide, Oxidative stress, Tooth bleaching

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**Introduction**

With an increasing interest in beauty treatments, concurrent with economic growth, patients visiting dental clinics have increased chances of receiving not only simple treatment for dental caries and periodontal diseases, but also tooth bleaching treatment for esthetic improvement.$^{1,2}$ Tooth discoloration is caused by intrinsic and extrinsic factors. Intrinsic factors are associated with a pathology of the pulp, drugs, such as tetracycline and fluoride, certain systemic diseases, and tooth dysplasia, while extrinsic factors are associated with externally colored substances, such as cigarettes, coffee, and wine.$^{3}$ As a treatment for discoloration of teeth, tooth bleaching is mainly used to preserve the tooth body and improve the tone of the teeth.$^{3}$ There are two types of tooth bleaching: in-office bleaching using 30 to 35% high concentration hydrogen peroxide (H$_2$O$_2$), performed at dental clinics, and home bleaching using a low concentration of 10 to 15% H$_2$O$_2$.$^{4-6}$ In-office bleaching involves the application of a bleaching agent for a short period of time by a dentist in a dental clinic, and the dentist applies a bleaching agent...
to teeth after protecting the soft tissue of the patient and induces additional activity with heat or light\(^7\). In office tooth bleaching involves a strong light capable of inducing photolysis of \(\text{H}_2\text{O}_2\) with high-frequency and special wavelengths of less than 248 nm and heat, which activates thermocatalysis increasing the breakdown of \(\text{H}_2\text{O}_2\) to hydroxyl radicals\(^8,9\). Home bleaching involves the application of a low-concentration bleaching agent to the teeth at home under the guidance of a dentist and has the advantages of convenience and low cost\(^10\). \(\text{H}_2\text{O}_2\) and carbamide peroxide are mainly used as tooth bleaching agents\(^11\). \(\text{H}_2\text{O}_2\) is a strong oxidizing agent that decomposes water and unstable free radicals\(^12\). Free radicals react with the staining material to exert tooth bleaching effects\(^7,13\). However, \(\text{H}_2\text{O}_2\) changes the enamel surface to become rough and porous\(^14,15\) and induces side effects such as dentin hypersensitivity, swollen gingiva, inflammation, and bleeding\(^16,17\). Therefore, it is necessary to develop a safe tooth bleaching agent to replace \(\text{H}_2\text{O}_2\).

Recently, there has been increasing interest in revealing the functionality of natural products with fewer side effects and safety, and the use of natural products for the prevention and treatment of various diseases\(^18\). *Citrus limon* extract (CLE) is a citrus fruit that is consumed worldwide and contains various phenolic compounds and flavonoids\(^19\). More than 90% of the ingredients are bioflavonoids, including hesperidin (vitamin P), neohesperidin, naringin, and the structure of more than 60 of its ingredients has been revealed\(^20\). These ingredients not only have various physiological activities, including antioxidant activity, hyperlipidemia suppression, tooth caries prevention effect, antibacterial effect, blood pressure strengthening effect, and anti-ulcer effect\(^21,22\) but are also known for their potential use in tooth bleaching\(^23\). However, studies on the effects of tooth bleaching and application methods are still insufficient. The purpose of this study was to therefore investigate the effect of CLE on oxidative stress-induced cytotoxicity and nitric oxide (NO) generation and the tooth bleaching effect of CLE as a substitute for \(\text{H}_2\text{O}_2\) which causes side effects, and to determine the possibility and application of CLE as a safe and effective natural tooth bleaching agent.

### Materials and Methods

#### 1. Cell culture

Raw264.7 macrophages (Korean Cell Line Bank, Seoul, Korea) were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and 1% antibiotic-antimycotic solution (all from WelGENE Inc., Daegu, Korea) in a 5% CO\(_2\) incubator at 37°C.

#### 2. Preparation of *Citrus limon* extract and bleaching agents

Freeze-dried CLE (Sanmaeul Co., Ltd., Changnyeong, Korea) was dissolved in D.W. at an appropriate concentration and filtered through a filter paper (Whatman Inc., Maidstone, UK) and syringe filter (Minisart\(^\circledR\); Sartorius, Göttingen, Germany), before use. Fifteen% sodium bicarbonate (NaHCO\(_3\), Arm & Hammer Baking soda; Yuhan Corp., Seoul, Korea) and 15% \(\text{H}_2\text{O}_2\) (Sigma-Aldrich Co., St. Louis, MO, USA) were used as positive controls for the comparison of the bleaching effect. The bovine teeth were treated with CLE and the bleaching agents for 3 hours, and the bleaching light (BL) (Light Radiator; KMG, Busan, Korea) was applied twice for 15 minutes.

#### 3. Assessment of cell viability

The effect of CLE on cytotoxicity was assessed using the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay. Raw264.7 macrophages were seeded on 96 well plates (1×10\(^3\) cells/well) and treated with various concentrations of CLE for 1 hour before treatment with 500 \(\mu\text{M H}_2\text{O}_2\) for 24 hours. MTT (Sigma-Aldrich Co.) was added to the prepared cells and incubated in the dark for 3 hours. After removal of the MTT solution, the cells were treated with 200 \(\mu\text{L of 5% dimethylsulfoxide (Sigma-Aldrich Co.)}\). Viable cells were assayed at 540 nm using an enzyme-linked immunosorbent assay (ELISA) microplate reader (Molecular Devices, Sunnyvale, CA, USA).

#### 4. Nitric oxide assay

Raw264.7 macrophages were treated with 300 \(\mu\text{g CLE and/or 150 }\mu\text{g NaHCO}_3\) (ThermoFisher Scientific Inc., Waltham, MS, USA) for 1 hour before treatment with 500...
μM H₂O₂ for 24 hours. Supernatants from the prepared cells were treated with Griess reagent (Sigma-Aldrich Chemical Co.) for 10 minutes at room temperature. NO concentrations were measured at 540 nm using the ELISA reader.

5. Protein isolation and western blot analysis
Total protein was isolated and quantified according to a previously described method. For the western blot analysis, the prepared total protein was electrophoresed on 10% sodium dodecyl sulfate polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Schleicher & Schuell, Keene, NH, USA). The membranes were probed with 1:1,000 anti-mouse inducible nitric oxide synthase (iNOS, BD Bioscience, San Jose, CA, USA) and 1:1,000 anti-mouse actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) primary antibodies. After washing, the membranes were blotted with horseradish peroxide-conjugated secondary antibodies (dilution, 1:500; goat anti-rabbit IgG-HRP; Santa Cruz Biotechnology Inc.). The protein bands were detected with ECL solution (Merck Millipore) and measured using a Science Lab Image Guage (FUJI FILM, Tokyo, Japan).

6. Preparation of the bovine teeth and measurement of the color of the bovine teeth surface
Bovine teeth without caries, fracture and cracks were selected and manufactured in 4×4 mm using a diamond bur and high-speed handpiece, and stained with coffee for 24 hours. Before and after treatment with CLE and bleaching agents, the bovine teeth were washed twice with DW, and after removing excess moisture, changes in the color of the bovine teeth were measured using a colorimeter (Konica minolta, Tokyo, Japan) 3 times in the same environment and conditions. Color values were determined using the previously reported CIE L*A*B* colorimetric method. In the CIE L*A*B* value, L* is the contrast, a* is the red-green color, and b* is the yellow-blue color, and the following equation was used to measure the amount of color change:

\[
\Delta E = \sqrt{(L^*)^2 + (a^*)^2 + (b^*)^2}
\]

7. Statistical analysis
All experiments were performed at least in triplicate. Data are reported as means±standard deviation, calculated using SPSS (version 12.0; SPSS Inc., Chicago, IL, USA). Significant differences (*p < 0.05, **p < 0.01, ***p < 0.001) were determined by independent samples t-test.

Results

1. Protective effect of Citrus limon extract against H₂O₂-induced cytotoxicity in Raw264.7 macrophage
The protective effect of CLE on the oxidative stress-induced cytotoxicity of 500 μM H₂O₂ in Raw264.7 macrophages was investigated by the MTT assay. CLE treatment with each concentration did not show cytotoxicity in Raw264.7 cells, but 500 μM H₂O₂ was cytotoxic and the cell viability was significantly reduced.

![Fig. 1. Protective effect of Citrus limon extract (CLE) against H₂O₂-induced cytotoxicity in RAW264.7 macrophage.](image-url)
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Fig. 3. Whitening effect of Citrus limon extract (CLE) and bleaching agents on bovine teeth. (A) Bovine teeth treated with CLE and bleaching agents. Before treated with (a) D.W., (c) 15% CLE, (e) 35% CLE, (g) 15% H₂O₂, (i) 15% NaHCO₃, (k) 35% CLE+15% NaHCO₃. After treated with (b) DW, (d) 15% CLE, (f) 35% CLE, (h) 15% H₂O₂, (j) 15% NaHCO₃, (l) 35% CLE+15% NaHCO₃. (B) Results of quantitative analysis of whitening effect of CLE and bleaching agents. The results are represented the mean±standard deviation of independent three times experiments. **p<0.01, ***p<0.001.

Fig. 2. Inhibitory effect of Citrus limon extract (CLE) on H₂O₂-induced nitric oxide (NO) generation in RAW264.7 macrophage. (A) Raw264.7 cells were treated with/without 300 μg CLE, 500 μM H₂O₂ and/or 75 μg NaHCO₃ for 24 hours, and the levels of NO were measured by nitric oxide assay. The results are represented the mean±standard deviation of independent three times experiments. (B) The cellular proteins were prepared, and inducible nitric oxide synthase (iNOS) and actin protein levels were measured by Western blot analysis. Actin was used as internal control for the Western blot analysis. The results are represented the mean±standard deviation of independent three times experiments. ***p<0.001 compared with the H₂O₂+ group.

2. Inhibitory effect of Citrus limon extract on H₂O₂-induced nitric oxide generation in RAW264.7 macrophage

NO is a representative pro-inflammatory mediator induced by lipopolysaccharide, H₂O₂, and ultraviolet irradiation, and its production is regulated by the synthesizing enzyme, iNOS. The effects of CLE and NaHCO₃ on NO generation and iNOS protein expression were confirmed in Raw264.7 cells (Fig. 2). Treatment with 300 μg CLE and 150 μg NaHCO₃ did not affect NO generation or iNOS protein expression in Raw264.7 cells but 500 μM H₂O₂ significantly increased NO generation and iNOS protein expression. NO generation and iNOS protein expression in Raw264.7 cells treated with 300 μg CLE+/500μM H₂O₂+ were significantly reduced, indicating that CLE has an inhibitory effect on oxidative stress induced by H₂O₂.
Table 1. Effect of CLE and Bleaching Agents on Bovine Teeth according to the Application of BL

| Group                              | Number of patients | Before          | After           | Bleaching rate (% of 15% H2O2) |
|------------------------------------|--------------------|-----------------|-----------------|---------------------------------|
| D.W.                               | 5                  | 1.85±0.65       | 2.08±0.40       | 14.50                           |
| 15% CLE                            | 9                  | 1.48±0.84       | 2.48±0.90       | 62.89                           |
| 35% CLE                            | 9                  | 1.25±0.82       | 2.95±1.30       | 106.92                          |
| 15% H2O2 (Hydrogen peroxide)       | 9                  | 2.04±0284       | 3.63±0.9        | 100.00                          |
| 15% NaHCO3 (Sodium bicarbonate)    | 9                  | 3.02±0.82       | 3.66±1.30       | 40.25                           |
| 35% CLE+/15% NaHCO3+               | 5                  | 1.48±0.71       | 4.45±1.00       | 186.79                          |
| 35% H2O2+/BL+                      | 9                  | 2.07±0.98       | 4.07±1.24       | 126.00                          |
| 35% CLE+/BL+                       | 9                  | 2.83±1.06       | 3.66±1.03       | 52.00                           |
| 35% CLE+/15% NaHCO3+/BL+           | 9                  | 2.15±0.99       | 3.57±1.03       | 89.00                           |

Values are presented as mean±standard deviation.
CLE: *Citrus limon* extract, BL: bleaching light.

Discussion

Stimulation by excessive oxidative stress in macrophages, monocytes, hepatocytes, bone marrow cells, and smooth muscle cells induces inflammation and immune responses and increases iNOS expression\(^{19}\). As a result, NO, an indicator of the inflammatory response, is produced\(^{25}\). NO has various physiological activities such as the induction of vascular regulation, immune responses and signaling function but large amounts of NO cause inflammation, tissue damage, and genetic mutation\(^{26}\). Therefore, there is increasing interest in substances with inhibitory activities against excessive oxidative stress and inflammatory mediators during bleaching treatment using H2O2. *Citrus*
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**Fig. 5.** Bleaching rate of *Citrus limon* extract (CLE) and bleaching agents on bovine teeth according to bleaching light (BL) irradiation. The results are represented the mean±standard deviation of independent three times experiments.

*limon* is consumed as a food in many countries worldwide and has numerous physiological activities. Among the components of CLE, citric acid has a fatigue recovery effect, ascorbic acid has a scurvy prevention effect, bioflavonoids have an antioxidant effect, and hesperidin has a blood vessel strengthening effect. In this study, the cell viability of Raw264.7 cells treated with 500 μM H₂O₂ was significantly increased as the concentration of CLE increased, and NO generation and iNOS protein expression were significantly reduced with the use of 300 μg CLE+/500 μM H₂O₂ (Fig. 2). These results indicate that CLE is a safe, natural product with inhibitory effects on oxidative stress-induced cytotoxicity and NO generation. Previous studies have also reported that CLE has antioxidant and anti-inflammatory effects, which is consistent with our results.

NaHCO₃, commonly known as baking soda or bicarbonate of soda, is a non-toxic chemical compound used as a fermenting agent for baking and cooking, for pH control, and as a medicine for indigestion and heartburn. In particular, NaHCO₃ has anti-caries properties and is used as an ingredient in mouthwash and toothpaste. Although excessive NaHCO₃ treatment has been reported to increase the generation of NO and tumor necrosis factor α in macrophages, NaHCO₃ did not affect iNOS and NO generation, and 300 μg CLE+/500 μM H₂O₂+/150 μg NaHCO₃+ inhibited iNOS and NO production (Fig. 2). Therefore, NaHCO₃ can be used together without affecting the activity of CLE. In this study, 35% CLE+ showed a bleaching effect similar to that of 15% H₂O₂+, used for home bleaching, and 35% CLE+/15% NaHCO₃+ showed a greater bleaching effect than 35% H₂O₂+/BL+ for in-office bleaching (Fig. 3~5, Table 1). This indicates that CLE can be applied as a substitute for H₂O₂ and treatment of 35% CLE and 15% NaHCO₃+ is an effective and safe application method that can replace conventional tooth bleaching treatment.

As per the above results, CLE inhibited oxidative stress-induced cytotoxicity and NO generation in Raw264.7 cells, replacing H₂O₂ in terms of side effects and risk of teeth bleaching treatment, and increased the teeth bleaching effect by treatment with NaHCO₃. Therefore, CLE is an effective and safe natural tooth bleaching substitute.

**Notes**

**Conflict of interest**

No potential conflict of interest relevant to this article was reported.

**Ethical approval**

This article is not necessary for IRB screening.

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Acknowledgements
This work was supported by Youngsan University Research Fund of 2020.

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