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Thermal Aging of Black Garlic Enhances Cellular Antioxidant Potential Through Nrf2-Mediated Pathway Activation

Running title: Enhanced Antioxidant Black Garlic: Nrf2 Activation

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SUMMARY

Research background. Oxidative stress plays a crucial role in various diseases, including chronic hepatitis, cirrhosis, and liver cancer, which are significant causes of mortality worldwide. Liver cell

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injury resulting from oxidative stress contributes to the development of these diseases. Garlic is known for its diverse physiological activities, and black garlic, produced through thermal aging of raw garlic, has gained attention for its biological properties.

Experimental approach. This study explores the hepatoprotective potential of black garlic prepared using an electric cooker. The investigation covers weight loss, brown index, free amino acids, free-reducing sugar content, total phenolic compounds, and DPPH radical scavenging ability. Additionally, sensory assessment indicates a preference for a black garlic sample. The study also examines Nrf2-ARE pathway activation in HepG2-C8 cells and evaluates protective effects against H_2O_2-induced damage.

Results and conclusions. The findings indicated that black garlic lost weight, possibly due to water loss and the Maillard reaction, which led to an increase in brown index and decreased free amino acids. However, the free-reducing sugar content increased. After 14 and 21 days, black garlic showed an increase in total phenolic compounds and a better ability to scavenge DPPH radicals. Significant activation of the Nrf2-ARE pathway was observed in HepG2-C8 cells. The sensory evaluation showed a preference for the 14-day-aged black garlic. The Nrf2 pathway can be effectively activated in HepG2 cells by 14-day aged black garlic extract, resulting in protection against H_2O_2-induced damage.

Novelty and scientific contribution. Our research reveals the significant impact of thermal aging on black garlic, highlighting its enhanced antioxidant properties. A straightforward approach has been established to prepare black garlic that is more potent and healthier, with potential applications in liver protection and oxidative stress-related diseases.

Keywords garlic; Maillard reaction; liver; Nrf2; oxidative stress; antioxidant properties

INTRODUCTION

An imbalance of reactive oxygen species (ROS) in cells or tissues causes Oxidative stress, which results in disruption of physiological activities (*1*). High levels of reactive oxygen species, including superoxide radicals, hydrogen peroxide, hydroxyl radicals and single oxygen, can damage the cellular structure or DNA, compromising normal cellular functions (*2*). The transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) regulates cellular redox reactions and controls the expression of various genes of the antioxidant and detoxification enzyme. It acts as a defense system in the body, protecting normal cells from oxidative stress injury and toxic substances (*3*). In the cytoplasm, Nrf2 is bound to Keap1 (kelch-like ECH-associated protein 1) under normal conditions, resulting in an inactive state. Nrf2 is dissociated from Keap1 and translocated into the nucleus to form a heterodimer with Maf proteins when exposed to oxidative stress (*4*). The complex binds to antioxidant response elements (AREs) and activates genes that encode various antioxidant and detoxification enzymes, such as heme oxygenase 1 (HO-1), NAD(P)H:quinone oxidoreductase-1 (NQO1), and UDP-glucuronosyltransferases (UGTs) (*5*). The role of these enzymes is crucial in mitigating cellular damage caused by toxic substances and free radicals, while also exerting inhibitory effects on the carcinogenic process (*6*).

The liver is the most important digestive organ in the human body and is responsible for various functions of detoxification and metabolism, including the metabolism of nutrients, the homeostasis of lipids and cholesterol, and the regulation of endocrine activities (*7*). Exposure to substances like drugs, alcohol, or toxins can cause oxidative stress in liver cells, which could lead to the development of liver diseases, such as alcoholic fatty liver, non-alcoholic fatty liver, and chronic hepatitis (*8*). Peroxides generated by mitochondria and peroxisomes may induce lipid peroxidation and activate hepatic stellate cells or cause death of hepatocytes (*8*). However, activating Nrf2 can enhance the activity of cellular antioxidants and protect liver cells from oxidative stress (*6*). Phytochemicals, like the various phenolic compounds found in plants, have a variety of physiological effects, which include anti-

inflammatory, antioxidant, and anti-cancer activities (*9*). Some studies have shown that phytochemicals can protect the liver through activation of the Nrf2 pathway (*10*).

Garlic (*Allium sativum*) is a plant that belongs to the Amaryllidaceae family and has a long history of being used as both a culinary ingredient and a medicinal plant. Organosulfur compounds, saponins, and phenolics are the bioactive compounds that are valued for their range of health benefits, including anti-inflammatory, antioxidant, and anticancer properties (*11*). However, garlic is generally thought to be healthy, it can be challenging for some individuals to tolerate its pungent taste. Black garlic is a processed food product that is derived from fresh garlic and undergoes an enzyme-free browning reaction called the Maillard reaction under high temperature and humidity conditions (*12*). The aroma of black garlic is more subdued and it contains bioactive compounds, including phenolic compounds, flavonoid compounds, and sulfur compounds (*13*). The sulfur compounds found in fresh garlic are γ-glutamyl-*S*-alk(en)yl-L-cysteine and *S*-alk(en)yl-L-cysteine sulfoxides, the primary precursors of *S*-allylcysteine (*14*). *S*-allylcysteine and hydroxymethylfurfural have been found in black garlic (*15*). Fresh garlic is placed in a high-temperature and high-humidity environment to produce black garlic, which results in a non-enzymatic browning reaction called the Maillard reaction (*16*). Garlic cloves turn brown-black as a result of this process, resulting in a soft texture and a slightly sugary, tangy taste that resembles dried fruit.

Black garlic has been found to contain various antioxidant and anti-inflammatory components, including phenolic compounds (*17*). Studies have found that black garlic extract has the potential to regulate lipid metabolism, decrease insulin resistance, and treat chronic diseases (*18*). Black garlic production involves using a constant-temperature incubator set above 75 °C, as mentioned in most research papers (*17,19*). Scientific research on the preparation of black garlic using an electric cooker, which typically maintains a central temperature of 50 to 72 °C, is lacking. The aim of this study was to use an electric cooker to create black garlic and evaluate the changes in the composition of the

samples after thermal aging for multiple days. We assessed the DPPH radical scavenging capacity and the potential of black garlic extract to activate cellular ARE-luciferase activity using HepG2-C8 cells. We also examined whether black garlic extract could enhance the expression of Nrf2 and downstream antioxidant genes, which could potentially alleviate H_2O_2-induced hepatic cellular damage.

MATERIALS AND METHODS

Materials

Trolox, 2,4,6-trinitrobenesulfonic acid (TNBS), dimethyl sulfoxide, Folin-Ciocalteu reagent, glucose, hydrochloric acid (HCl), L-leucine, sodium bicarbonate ($NaHCO_3$), sodium carbonate (Na_2CO_3), sodium chloride, trizma base, and phenylmethanesulfonylfluoride were purchased from Sigma-Aldrich (St. Louis, MO, USA). RIPA buffer was obtained from Cell Signaling (Danvers, MA, USA). Penicillin-streptomycin solution, and trypsin were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Dulbecco's modified eagle (DMEM) and fetal bovine serum (FBS) were obtained from Gibco (Dublin, Ireland). DPPH was purchased from Alfa Aesar (Haverhill, MA, USA). Gallic acid (GA) was from Tokyo Chemical Industry (Tokyo, Japan). 3,5-Dinitrosalicylic acid (DNSA) and methanol were obtained from ECHO Chemical (Miaoli, Taiwan).

Black garlic preparation

The black garlic was sourced from the market in Xiluo Township, Yunlin County, Taiwan. The thermal aging processing method of black garlic is shown in the photograph of Fig. S1. After peeling the fresh garlic cloves, a bamboo rack was used to elevate the bottom layer and prevent direct contact with the bottom in the electric cooker (KH-WA10T, SAMPO, Taoyouan City, Taiwan) (Fig. S1a). Paper towels were placed to prevent staining on the bamboo rack and to ensure that the garlic was evenly

distributed without falling into the gaps. Another layer of paper towels was placed on top, and the garlic cloves were stacked inside the electric stove. The top layer was covered with kitchen paper towels to prevent condensation from dripping onto the cloves during the aging process. The electric cooker was then closed with the lid and set to the warm setting. After aging for 7, 14 and 21 days, black garlic was collected into sealed bags for storage, respectively. The appearance of fresh (day 0) and black garlics are shown in Fig. S1b.

$$w(\text{mass loss})/(\text{g}/100\ \text{g}) = ((m_{\text{initial}} - m_{\text{final}})/m_{\text{initial}}) \cdot 100 \qquad /1/$$

where m_{initial} is the initial mass of the sample; m_{final} is the final mass of the sample after processing.

Water content determination

To determine the water content of fresh or black garlic, approximately 1.0 gram of the sample was placed in an aluminum dish and dried in a preheated oven at 105 °C (MOV-212F-PK, Panasonic, Osaka, Japan). The sample was removed every 2 h and immediately transferred into a desiccator for cooling, then weighed at ambient temperature. The drying and weighing steps were repeated until a consistent weight is achieved (ME2002, Metteler Toledo, Columbus, OH, USA). The moisture content was calculated using the following formula:

$$w(\text{water})/(\text{g}/100\ \text{g}) = ((m_{\text{initial}} - m_{\text{final}})/m_{\text{initial}}) \cdot 100 \qquad /2/$$

where m_{initial} is the initial mass of the sample before drying; m_{final} is the final mass of the sample after drying.

Browning index analysis

The measurement of the browning index has been modified from the published study (*20*). Fresh and black garlics were homogenized with ddH_2O in a ratio of 1:10 (homogenizer Bioprep-24, Allsheng, Hangzhou City, China). Subsequently, 1.0 mL of the homogenized sample was transferred to

microcentrifuge tubes and centrifuged at 17,000 xg for 10 min at 25 °C (centrifuge Fresco 17, Thermo Fisher Scientific). After transferring 200 μL of the supernatant to a 96-well plate, the absorbance at 420 nm and 550 nm was measured using a BioTek microplate reader (Synergy HT, Winooski, VT, USA). The browning index refers to the difference between the absorbance at 420 nm and 550 nm.

Free reducing sugar determination

According to the method described in the published study (*21*), the homogenized fresh and black garlics were prepared using ddH_2O at a ratio of 1:10 (homogenizer Bioprep-24). Subsequently, 1 mL of the sample or glucose standard solution, along with 150 μL of ddH_2O and 250 μL of DNSA reagent, were sequentially added and thoroughly mixed. The reaction mixture was then incubated at 100 °C in a water bath for 10 min. After cooling to room temperature, 200 μL of the reaction mixture was carefully transferred to a clear 96-well plate, and the absorbance at 540 nm was measured using a microplate reader (Synergy HT). The relative content of reducing sugars in the samples was determined using the regression equation derived from the standard curve.

Free amino acid determination

The homogenized fresh and black garlics were dissolved in a solution containing 1 % sodium dodecyl sulfate (SDS) according to the method (*21*). In a microcentrifuge tube, 20 μL of the sample or L-leucine standard solution was added, followed by the addition of 160 μL of 0.2 M phosphate buffer (pH=8) and 160 μL of 0.1 % TNBS solution. The mixture was well mixed and incubated at 50 °C in a water bath for 60 min. After cooling to room temperature, 320 μL of 0.1 N HCl was added to the mixture. Finally, 200 μL of the test sample was transferred to a 96-well plate, and the absorbance at 340 nm was measured using a microplate reader (Synergy HT). The relative content of amino acids in the samples was calculated using the regression equation obtained from the standard curve.

Total phenolic compounds determination

Fresh and black garlics were homogenized using a 1:10 ratio of 95 % methanol (homogenizer Bioprep-24). After centrifugation at 17,000×*g* for 5 min (centrifuge Fresco 17), the supernatant was collected. Gallic acid (GA) was used as a standard. For the assay, 50 µL of the supernatant and 100 µL of 10 % Folin-Ciocalteu reagent were mixed in a microcentrifuge tube. Subsequently, 400 µL of 700 mM Na_2CO_3 was added to the mixture, thoroughly mixed, and left at room temperature for 1 h. Following this, 200 µL of the reaction mixture was transferred to a transparent 96-well plate, and the absorbance at 765 nm was measured using a microplate reader (Synergy HT). The gallic acid content was calculated as milligrams of gallic acid equivalent (GAE) per 100 grams using the formula:

$$w(\text{GAE})/(\text{mg}/100\ \text{g}) = ((A - b)/s) \times (V_{\text{total}}/m_{\text{sample}}) \cdot 100 \quad /3/$$

where A is the absorbance of the sample; b is the intercept of the standard curve; s is the slope of the standard curve; V_{total} is the total volume of the extract prepared from the garlic sample; m_{sample} is the mass of the garlic sample used for extraction.

DPPH radical scavenging assay

The method used in this study was based on earlier work (*22*). Fresh and black garlics were homogenized using a 1:10 ratio of 95 % methanol (homogenizer Bioprep-24). Then, 0.5 mL of the centrifuged supernatant was mixed with 1.2 mL of ethanol and 0.3 mL of a 0.5 mM DPPH solution. The mixture was thoroughly mixed and allowed to react for 20 min. Trolox was used as a positive control. The absorbance of the reaction mixture was measured at a wavelength of 517 nm using a microplate reader (Synergy HT). The formula for the DPPH radical scavenging activity is:

$$\text{DPPH radical scavenging activity } (\%) = ((A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}) \cdot 100\ \% \quad /4/$$

where A_{control} is the absorbance of the control (DPPH solution without the sample). A_{sample} is the

absorbance of the reaction mixture containing the sample.

Preparation of Black garlic water extracts for cell culture

Thirty grams of fresh or black garlic were weighed and mixed with 500 ml of distilled water. The mix was blended until it was well crushed. A magnetic stirrer bar was placed in the beaker containing the mixture, and stirring was performed for 1 h (stirring hot plate PC-420D, Corning, Corning, NY, USA). The resulting filtrate was collected and subjected to lyophilization for water removal (freeze dry system 7806031, Labconco, Kansas, MO, USA). The yields of water extracts from fresh garlic and black garlic aged for 7, 14, and 21 days were 28.4, 28.3, 29.3, and 30.2 g/100 g, respectively.

Cell culture

Human hepatocellular carcinoma HepG2 cells (ATCC HB-8065) and HepG2-C8 cells stably transfected with the ARE-luciferase plasmid were cultured in DMEM medium supplemented with 10 % FBS, 3.7 g/mL $NaHCO_3$, 50 units/mL penicillin, and 50 µg/mL streptomycin. The cells were incubated in a 5 % CO_2 humidified incubator (MCO-170AIC, Panasonic) at 37 °C to maintain optimal conditions. The cells were used at passages 5–10 to ensure consistency in experimental results.

ARE-luciferase activity evaluation

The ability of the samples to induce ARE-luciferase activity was assessed using HepG2-C8 cells that had stable transfection with the ARE-luciferase plasmid, following the method (*23*). HepG2-C8 cells were seeded at a density of $1 \cdot 10^5$ cells/mL in a 12-well plate and incubated for 24 h. The cells were then treated with a culture medium containing 100 µg/mL of fresh garlic or black garlic extract for another 24 h. The concentration of 100 µg/mL was chosen because of previous studies that demonstrated how this dose effectively modulates ARE-luciferase activity in liver cells (*24*).

Sulforaphane (SFN) was used as a positive control. After removing the cell culture medium, 55 μL of lysis buffer was added to the cells, which were subsequently stored at -20 °C for 24 h. Cell lysate supernatant was obtained by centrifuging at 17,000 xg for 10 min at 4 °C (centrifuge Fresco 17). The fluorescence intensity was measured using a microplate reader (Synergy HT) according to the Luciferase Assay System manufacturer's instructions (Promega, Madison, WI, USA). To calculate the relative ARE-luciferase activity, the following formula was used:

$$\text{Relative ARE-luciferase activity}=(A_{sample}/\gamma_{sample})/(A_{control}/\gamma_{control}) \quad /5/$$

where A_{sample} and $A_{control}$ are the fluorescence intensities of the sample and control, respectively. γ_{sample} and $\gamma_{control}$ are the protein concentrations of the sample and control, respectively, which were determined using the Bicinchoninic Acid (BCA) Assay Kit (G-Bioscience, Taipei, Taiwan).

Sensory evaluation

The public's preference and acceptance of black garlic with different aging times was assessed through sensory evaluation in this study. A total of 51 individuals participated in the evaluation. The group consisted of 2 individuals younger than 20, 41 individuals between 21 and 25, 4 individuals between 26 and 30, 2 individuals between 31 and 35, 1 individual between 51 and 55, and 1 individual who was 60 or older. Among the participants, 23 were female and 28 were male. A random number was assigned to the samples of fresh and dark garlic. The participants were asked to taste the garlic samples and answer an anonymous questionnaire. On a 9-point scale, they evaluated the color, flavor, texture, and overall impression of the garlic samples to express their preference. Lemon water was provided to cleanse the palate during sampling. Following evaluation of all four garlic samples, participants were asked to rank their personal preference for the samples.

H_2O_2 induced liver cellular damage elucidation

HepG2 cells were seeded at a density of 2×10^4 cells/100 µL in a 96-well plate and incubated for 24 h (incubator MCO-170AIC). Subsequently, the cells were treated with culture medium containing various concentrations of 14-day-aged black garlic water extract (BGE), which was filtered through a 0.22 µm filter, for 48 h. After removing the old culture medium, the cells were further treated with culture medium containing 1 or 2 µM H_2O_2 for 8 h. The CellTiter 96 Aqueous One Solution Cell Proliferation (MTS) assay kit (Promega) was used to measure the absorbance at 490 nm (microplate reader Synergy HT) after 1 h of reaction. Absorbance was recorded to calculate cell viability (% of control group) for each treatment group.

Cell viability (% of control)=($A_{sample}/A_{control}$)·100 % /6/

where A_{sample} is the absorbance of the treatment group; $A_{control}$ is the absorbance of the control group.

Protein expression

HepG2 cells were seeded at a density of 2×10^6 cells/10 mL/dish in cell culture dishes with 10 % FBS and incubated for 24 h (incubator MCO-170AIC). They were then treated with various concentrations of 14-day BGE for 48 h. After removing the culture medium, the cells were collected, washed, and lysed using RIPA buffer. The cell lysates were sonicated (ultrasonic grinder Ultrasonic 250, Hoyu, Taipei, Taiwan) and centrifuged (centrifuge Fresco 17), and the supernatant containing 25 µg of protein was used for SDS-PAGE (Mini Gel Tank, Thermo Fisher Scientific) and subsequently transferred to a PVDF membrane (Trans-Blot® SD semi-dry transfer cell, Bio-Rad, Hercules, CA, USA). The PVDF membrane with proteins was blocked and incubated with primary antibodies overnight. Nrf2 and NQO1 primary antibodies were purchased from ABclonal Technology (Woburn, MA, USA), UGT1A primary antibody was from GeneTex (Irvine, CA, USA), and HO-1 and GAPDH primary antibodies were obtained from Proteintech (Rosemont, IL, USA). The PVDF membrane was then incubated with goat anti-mouse or rabbit IgG-HRP secondary antibodies (Croyez Bioscience,

Taipei, Taiwan), and the protein bands were visualized using a chemiluminescent substrate in an optical system (Fusion Solo 6S, Vilber, Collégien, France). Image analysis was performed using ImageJ software (NIH, USA) (*25*), and GAPDH was used as a standard control for normalization and quantification.

Statistical analysis

The experimental data were statistically analyzed using the SAS computer software (*26*). One-way analysis of variance (ANOVA) was performed, followed by Duncan's new multiple range test, to assess significant differences among the data.

RESULTS AND DISCUSSION

Thermal aging process leads to changes in the composition of black garlic in an electric cooker

Black garlic has become more popular in the market, leading to a growing interest among individuals in preparing it at home. However, due to the absence of large-scale and professionally controlled ovens necessary for the traditional production method, households face limitations. Therefore, the aim of this study is to investigate the alterations in the chemical composition that occur during the preparation of homemade black garlic utilizing an electric cooker with a consistent temperature of approximately 60 °C (Fig. S1 and Fig. 1). Our findings indicate a significant weight loss in black garlic during the aging process. Specifically, after 7 days of aging, the weight loss was observed to be significant, and it reached a maximum of approximately 60 % after 14 and 21 days ($p<0.05$) (Fig. 1a). Additionally, the water content in fresh garlic decreased significantly from 72.5 g/100 g to approximately 22.5–36.1 g/100 g after 7, 14, and 21 days of aging ($p<0.05$) (Fig. 1b). These results suggest that the observed weight loss in black garlic primarily stems from the evaporation of

water. However, it is important to note that the thermal aging process may also contribute to changes in composition and subsequent weight changes in black garlic through the involvement of heat-sensitive volatile components and chemical reactions. According to previous research, the production of black garlic involves subjecting fresh garlic to high temperatures (60–90 °C) and high relative humidity (80–90 %) for a specific duration (*13*). It has been observed that temperatures exceeding 90 °C can result in bitterness, while temperatures below 60 °C led to incomplete fermentation, resulting in a taste and color that is intermediate between raw garlic and black garlic (*13*). Optimal water content for black garlic, achieved after completing the aging process, ranges from 40–50 g/100 g, providing the desired texture and elasticity for consumption (*15*). Water content below 35 g/100 g can lead to dryness and inferior palatability (*15*). In this study, black garlic aged for 14 days using an electric cooker had a water content of approximately 36.1 g/100 g (Fig. 1b). The water content of the homemade black garlic falls within a close range to the recommended values, suggesting that the aging process in the electric cooker may contribute to achieving the desired water content for optimal taste and texture. The findings indicate that homemade black garlic can have the same water content as commercially produced black garlic.

The Maillard reaction is a nonenzymatic browning process that occurs when sugars and amino acids react at high temperatures, producing unique flavors, colors, and Maillard reaction products. In this reaction, Amadori rearrangement results in the condensation of sugars and amino acids, followed by the dehydration of sugars and degradation of amino acids (*27*). Nitrogen-containing heterocyclic compounds can be generated by subsequent condensation reactions, which could influence the final product's color and aroma (*28*). The antioxidant properties of Maillard reaction products have also been recognized (*27*). Black garlic's characteristic black-brown color and unique aroma are caused by the Maillard reaction during the aging process (*29*). In this study, we found a significant increase in the browning index with longer aging periods during the thermal aging of black garlic ($p<0.05$),

indicating the occurrence of a Maillard reaction (Fig. 1c). The Maillard reaction could be responsible for the development of black garlic's distinctive color and flavor profile during the aging process.

The Maillard reaction in garlic is triggered by high-temperature and high-humidity conditions, resulting in an increase in the levels of intermediates and flavonoids that can be responsible for the enhanced antioxidant properties of black garlic (*29*). We examined the changes in reducing sugar content in this study and observed a consistent increase with longer aging times (Fig. 1d). The reducing sugar content reached approximately 93.2 and 98.3 mg glucose/100 g, respectively, after 14 and 21 days of aging ($p<0.05$). Conversely, the free amino acid content showed a decreasing trend during the period, reaching about 7.2 and 6.2 g leu/100 g after 14 and 21 days of aging, respectively (Fig. 1e). According to these findings, using an electric cooker to aging black garlic results in an increase in sugar reduction and a reduction in free amino acid. Moreover, we evaluated the total phenolic compound content of black garlic at various aging durations (Fig. 1f). The total phenolic compound content increased significantly after 7 days of aging ($p<0.05$), resulting in levels of approximately 81.8 mg GAE/100 g. Subsequently, total phenolic content continued to rise substantially after 14 and 21 days of aging, reaching approximately 185.5 and 181.7 mg GAE/100 g, respectively ($p < 0.05$). It is suggested by these findings that the aging process of black garlic in an electric cooker results in the accumulation of phenolic compounds.

Thermal aging black garlic enhances the DPPH radical scavenging ability and cellular antioxidant activity

Black garlic is recognized for its diverse physiological activities and antioxidant potential (*13*). Processing methods can influence the antioxidant activity of garlic by converting alliin, a stable compound, into *S*-allylcysteine during the aging process (*30*). Moreover, oral administration of 100 mg/kg black garlic in male Sprague-Dawley rats with ethanol-induced oxidative liver damage reduced

the activities of AST, ALT, ALP, and LDH in the blood, while increasing the expression of CYP2E1 in the liver, indicating hepatoprotective effects (*31*). In this study, our aim was to investigate the enhanced biological activity of homemade black garlic with antioxidant potential. Our findings suggest that homemade black garlic, prepared using an electric cooker, undergoes changes in the content of bioactive compounds, especially polyphenols, during the aging process. These changes may contribute to its physiological activities, including antioxidant capacity (Fig. 2). The results demonstrated that black garlic aged for 14 and 21 days may exhibit higher levels of total phenolic compounds and better DPPH radical scavenging ability (Fig. 2a). The concentrations (IC_{50}) required for black garlic aged for 7, 14, and 21 days to scavenge 50 % of DPPH radicals were 0.4312, 0.2445, and 0.2211 mg/mL, respectively.

To assess the activation of cellular Nrf2 antioxidant/anti-inflammatory mechanisms, we examined the ARE-luciferase activity in HepG2-C8 cells treated with water extracts of black garlic (Fig. 2b). HepG2-C8 cells, which express the ARE-luciferase sequence plasmid, are commonly used for evaluating Nrf2 pathway induction (*23*). Sulforaphane (SFN), a known inducer of the Nrf2 pathway, served as a positive control (*32*), and our study produced similar results. Water extracts (100 μg/mL without affecting cell growth) from black and fresh garlics had a significant increase in ARE-luciferase activity compared to the control group ($p<0.05$). While specific phenolic compounds were not analyzed in this study, the increased content of phenolic compounds in black garlic is likely to contribute significantly to its antioxidant capacity. Future research should focus on analyzing the specific phenolic compounds to gain a more comprehensive understanding of their role in the physiological activities of black garlic.

Black garlic aged for 14 days received the highest overall preference

The sensory assessment involved evaluating black garlic samples from different aging times based on color, aroma, texture, and overall liking. Analyzing the statistical results (Table 1), although no significant differences were observed in liking for color, aroma, or texture, participants tended to prefer black garlic aged for 14 days in terms of overall liking (score of 5.88). This preference was also reflected in the ranking, with the 14-day-aged black garlic ranking first. The 14-day aged black garlic was praised by the participants for its soft texture, sweet and sour flavor, and unique taste. At first, some participants disliked the aroma, but they found it more acceptable after giving it a second chance. Regarding the sensory comments on black garlic samples with other aging times, fresh garlic was described as very spicy and pungent, while the 7-day-aged black garlic received comments such as a soy sauce-like taste and a less pronounced sweetness. The 21-day aged black garlic was described as having sourness, softness, sweetness, and resembled dried fruits. Some participants mentioned that they prefer the aroma after the initial taste.

Considering the total phenolic content, DPPH radical scavenging ability, and activation of the Nrf2 antioxidant pathway in this study, black garlic aged for 14 and 21 days showed potential for enhancing intracellular antioxidant capacity. However, based on the sensory evaluation results, the preference was for black garlic aged for 14 days. Further research should focus on exploring the potential protective mechanisms and effects of the water extract from black garlic aged for 14 days on liver cells against oxidative stress-induced damage.

Black garlic water extract (BGE) exhibits protective effects against H_2O_2-induced damage in HepG2 cells through the activation of the Nrf2 pathway

Hepatoprotective potential of natural compounds is often assessed using an H_2O_2-induced liver cell damage model (*33*). Additionally, selenium-enriched black garlic extract has been shown to protect against LPS/D-GalN-induced acute liver failure in rats by restoring metabolic balance,

regulating intestinal flora, and exerting antioxidant effects (*34*). In this study, we evaluated the protective effect of the water extract from black garlic aged for 14 days (BGE) against H_2O_2-induced HepG2 cell damage (Fig. 3). Fig. 3a and Fig. 3b show that H_2O_2 at concentrations of 1.0 and 2.0 μM significantly reduced cell viability to approximately 49 % and 35 %, respectively, compared to the control group ($p<0.05$). However, pretreatment of HepG2 cells with 100 mg/mL BGE for 48 h, followed by exposure to H_2O_2 for 8 h, significantly improved cell viability and protected against cell damage caused by 1.0 or 2.0 μM H_2O_2 ($p<0.05$). These findings indicate that BGE can protect against H_2O_2-induced oxidative stress and cell damage in HepG2 cells.

Numerous studies have found that natural compounds possess hepatoprotective effects, potentially through the activation of the Nrf2 pathway, including the upregulation of downstream antioxidant enzymes NQO1, HO-1, and UGT1A (*24*). In addition, research on industrial garlic peel waste demonstrated its sustainability potential, with an increase in antioxidant and anti-inflammatory activities and activation of the Nrf2/HO-1/NQO1 pathway (*35*). In this study, it was initially observed that black garlic water extract (BGE) effectively induced Nrf2-ARE-luciferase activity in HepG2-C8 cells (Fig. 2b). Therefore, further investigations were conducted to examine whether the water extract from black garlic aged for 14 days (BGE) could increase the protein expression levels of Nrf2 and its downstream antioxidant enzymes (Fig. 4a). Compared to the control group (treated with 0 μg/mL concentration), it was found that 100 μg/mL of BGE significantly increased the protein expression level of Nrf2 ($p < 0.05$) (Fig. 4b). Regarding UGT1A, both 50 and 100 μg/mL BGE enhanced the protein expression level of UGT1A significantly compared to the control group (treated with 0 μg/mL concentration) ($p < 0.05$) (Fig. 4e). Although there were some increases in the expression levels of NQO1 and HO-1 by BGE, the differences were not significant (Fig. 4c and Fig.4d). These results suggest that cells *in vitro* need longer treatment durations, like 48 h, to activate antioxidant responses through mechanisms like epigenetic modifications, leading to enhanced expression of Nrf2 target

genes (*24*). Overall, these results indicate that black garlic water extract can activate the Nrf2 pathway and upregulate the expression of downstream antioxidant enzymes. This is the first study to find that black garlic may exhibit an hepatoprevetive potential via activating Nrf2-mediated anti-oxdiative mechanisms.

However, the study's significant findings are accompanied by limitations. The bioavailability and stability of garlic's active compounds during thermal aging were not measured, and normal liver cells were not used to compare them. In the future, it is important to explore these aspects and pinpoint the specific phenolic compounds that are responsible for the observed antioxidant and hepatoprotective effects.

CONCLUSIONS

Based on the findings of this study, it is demonstrated that the duration of thermal aging significantly affects the composition of black garlic, resulting from the Maillard reaction in fresh garlic. After 14 and 21 days of thermal aging, black garlic showed notable increases in total phenolic compounds and free radical scavenging capacity. Sensory evaluation indicated that the 14-day aged black garlic was the most preferred among participants. Further investigations revealed that the 14-day aged garlic water extract (BGE) upregulated the expression of Nrf2 and antioxidant-related enzymes, including NQO1 and UGT1A. This activation of the Nrf2 pathway may enhance intracellular antioxidant capacity, leading to a reduction in H_2O_2-induced oxidative stress, liver cell damage, and cell death. These findings emphasize the potential of homemade black garlic prepared with an electric cooker as a functional food with enhanced antioxidant properties and hepatoprotective potential.

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COMPETING INTERESTS

The authors declare no conflict of interest.

SUPPLEMENTARY MATERIAL

Supplementary materials are available at: www.ftb.com.hr.

AUTHORS' CONTRIBUTIONS

S.S. Yu was responsible for conceptualizing the study, conducting the investigation, and contributing to the methodology design, validation, visualization, and drafting of the original manuscript. Y.L. Chu participated in the critical revision. Y.C. Tung supplied resources and assisted in the validation process. Z.Y. Su was involved in supervision of the study, managing funding acquisition, project administration, methodology design, validation, and data curation, and also contributed to writing through review and editing. The final manuscript was reviewed and approved by all authors.

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Table 1. Sensory analysis of black garlic

Sample random number	*t*(aging)/day	Sensory evaluation[a]				Ranking[b]
		Color	Aroma	Taste	Overall	
182	0	6.25±1.51	5.92±2.05	5.61±2.25	5.43±2.27	2.63±1.33
410	7	5.37±1.51	5.76±1.54	5.25±1.86	5.45±1.71	2.31±0.91
696	14	5.51±1.69	5.96±1.70	5.59±2.15	5.88±1.91	2.71±1.14
092	21	5.12±1.82	5.80±2.04	5.04±2.22	5.24±2.08	2.20±0.98

[a] This sensory evaluation is performed within a 9-point scale for the sample, with higher scores indicating a higher preference. In each analysis, there were no significant differences between any samples ($p > 0.05$).

[b] After assessing the four samples, the impression scores were ranked from 1 to 4, with higher scores indicating a greater preference

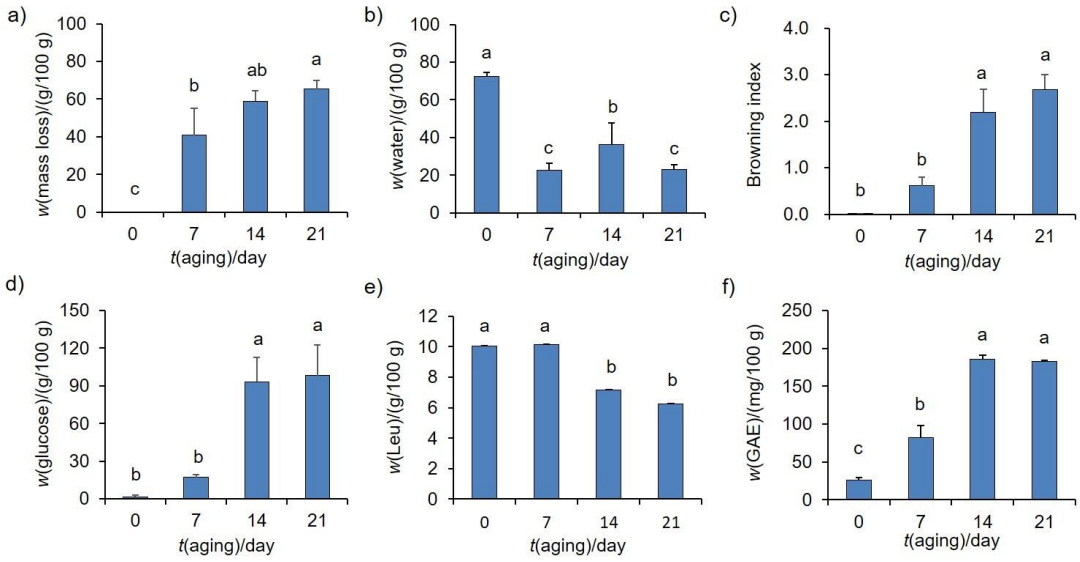

Fig. 1. Effects of thermal aging processing on weight loss, water content, browning index, reducing sugar content, free amino acid content, and total phenolic content of the garlics. a, b) After thermal aging processing on days 7, 14, and 21, 1.0 g of garlic samples were dried at 105℃ in an oven until the weight stabilized. Weight loss and water content of black garlic were calculated based on the original material weight, respectively. c) A homogenized solution of 25.0 g garlic in 5.0 mL distilled water was filtered, and the absorbance of the resulting solution was measured at wavelengths of 420 nm and 550 nm. d) The reducing sugar content in black garlic was determined at 540 nm wavelength with glucose as the standard. e) The changes in free amino acids were determined at 340 nm wavelength with leucine as the standard. f) The total phenolic content was assessed at 765 nm wavelength using gallic acid (GA) as the standard. Different letters indicate significant differences ($p<0.05$) between the groups. The data are expressed as mean±SD from three independent experiments.

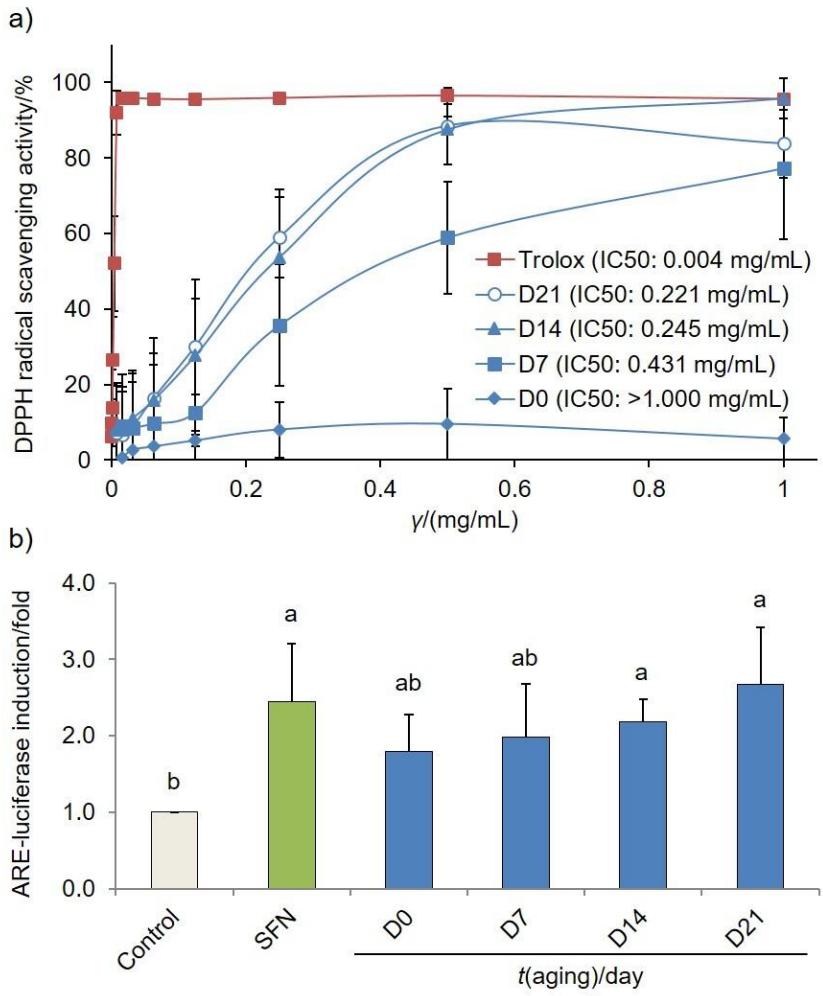

Fig. 2. Effect of thermal aging processing on DPPH radical scavenging and ARE-luciferase induction activities of black garlics. a) DPPH radical scavenging activity of black garlic was measured at a wavelength of 517 nm. Trolox was used as a positive standard. b) ARE-luciferase induction activity of black garlic extracts (BGEs) was examined. HepG2-C8 cells (1.0×10^5 cells/1.0 mL/well) were seeded in a 12-well plate in DMEM medium for 24 h, followed by incubation in new medium containing water extracts of black garlic after thermal aging for an additional 24 h. The ARE-luciferase activity was determined and calculated based on protein concentrations. Significant differences ($p<0.05$) between the groups are indicated by different letters based on the data (mean±SD, n=3).

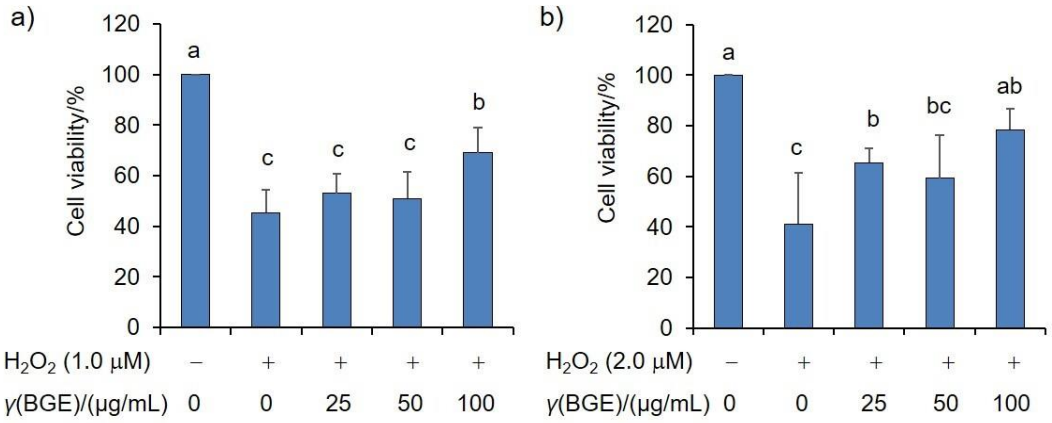

Fig. 3. Effect of black garlic extract (BGE) on the growth of HepG2 cells against oxidative stress caused by H_2O_2. HepG2 cells (2.0×10^4 cells/100 μL/well) were seeded in a 96-well plate with DMEM and incubated for 24 h. Subsequently, the cells were treated with various concentrations of BGE (thermal aging on day 14) in new DMEM for an additional 48 h. a, b) The medium with BGE was then replaced with fresh DMEM containing 1.0 μM and 2.0 μM of H_2O_2, respectively, and treated for 8 h. Cell viability was determined using the MTS assay. Significantly different results ($p<0.05$) are indicated by different letters among the groups based on the data (mean±SD, n=4).

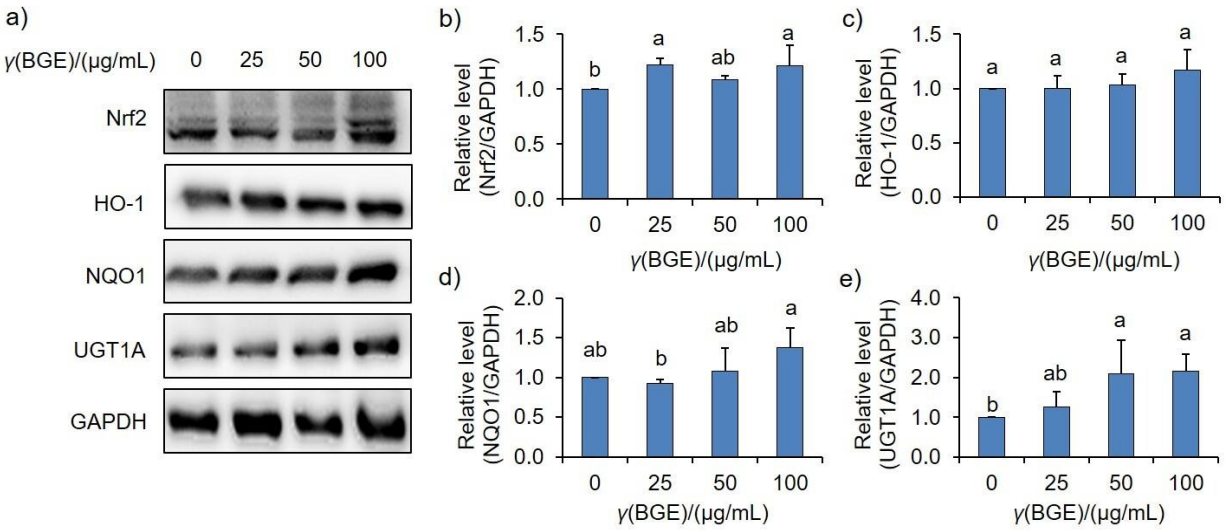

Fig. 4. Effect of black garlic extract (BGE) on the protein expressions of Nrf2-associated antioxidant enzymes in HepG2 cells. HepG2 cells (2.0×10^6 cells/10 mL/dish) were seeded in a 10 cm dish with DMEM and incubated for 24 h. Subsequently, the cells were treated with various concentrations of BGE (thermal aging on day 14) in DMEM for an additional 48 h. a, b, c, d, e) Protein expressions of Nrf2-associated antioxidant enzymes (HO-1, NQO1, and UGT1A) were analyzed. Significant differences ($p<0.05$) among the groups are indicated by different letters based on the data (mean±SD, n=3).

Supplementary Material

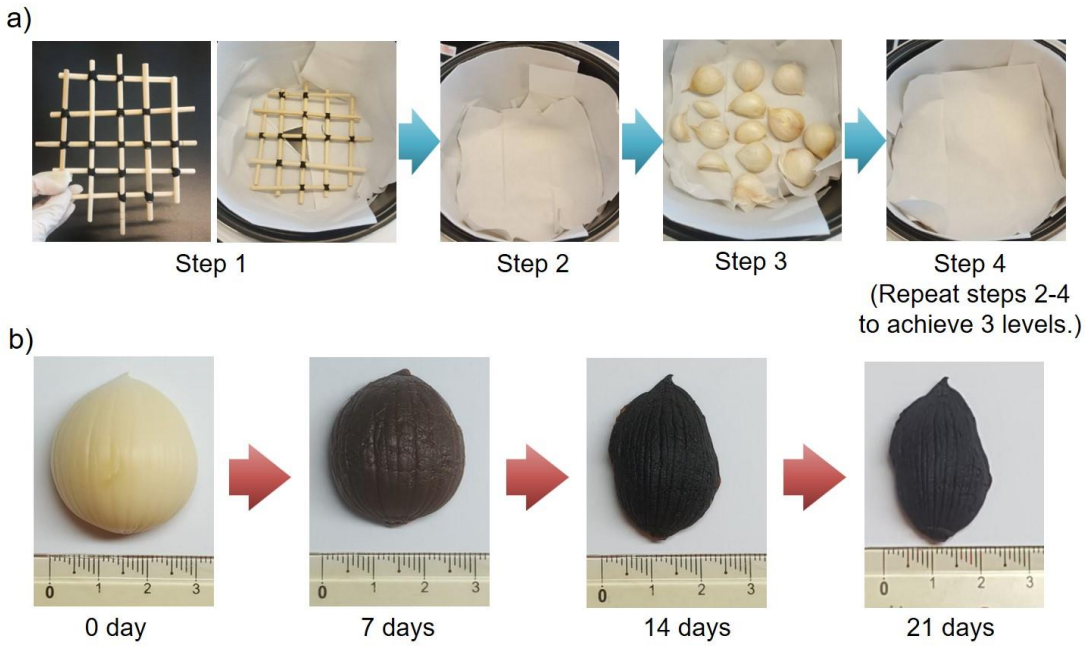

Fig. S1. (A) Thermal aging processing to prepare black garlic. (B) Effect of thermal aging processing on appearance of garlics. Step 1: Elevate the bottom layer using a homemade bamboo rack. Step 2: Spread kitchen paper towels on top. Step 3: After peeling the garlic cloves, evenly spread them out. Step 4: Cover with another layer of kitchen paper towels. Stack up to 3 layers, and place kitchen paper towels on the top layer. Finally, cover the pot with the lid of the electric cooker and keep it in the warm setting. After aging for 7, 14, and 21 days respectively, remove the black garlic.