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Bioactive Enhancement of Fish Sauce Using Black Garlic: Chemical Characterization and In Vitro Antiproliferative Activity

Vuong Thi Huyen Trang[1] , Pham Duc Tri[2]

[1] Vietnam Academy of Science and Technology
Email: vuongthihuyentrang@gmail.com
[2] Ha Tinh High School For The Gifted
Email: phamductri120409@gmail.com
Corresponding email: vuongthihuyentrang@gmail.com

Abstract: *This study was conducted to evaluate the physicochemical changes, antioxidant activity, and ability to inhibit hepatocellular carcinoma (Hep-3B) cells of black garlic under in vitro conditions, while also investigating the process of applying black garlic to the production of functional fish sauce. Fresh garlic was thermally fermented at with humidity for 35 days to convert it into black garlic. The analysis results showed that the fermentation process increased the reducing sugar content by 11 times (from 20 mg/g to 220 mg/g) and increased the antioxidant activity by 5 times compared to fresh garlic, with a value of .In anti-cancer tests, black garlic extract and S-allylcysteine (SAC) at a concentration of (calculated as SAC) showed a marked inhibition of Hep-3B cell line proliferation after 72 hours of exposure , through a mechanism of cell cycle arrest without causing immediate direct toxicity. Based on biological indicators, the study optimized the black garlic fish sauce mixing ratio at 3g black garlic/15mL fish sauce, achieving the highest sensory evaluation score and ensuring microbiological safety standards. These results help prove the value of black garlic as a potential ingredient for functional foods that support cancer prevention and health protection.*

Keywords: Black garlic; Antioxidant activity; S-allylcysteine; Hep-3B cells; Hepatocellular carcinoma; Functional fish sauce; Maillard reaction; Bioactive compounds.

1. Introduction

Garlic (*Allium sativum* L.) has been used as a spice and valuable herb in traditional medicine for thousands of years in many cultures around the world (Vecchi et al., 2025). Modern studies have confirmed that garlic contains many organic sulfur compounds with powerful biological effects, including antibacterial, antioxidant, cardiovascular protection, and blood sugar regulation properties (Colín-González et al., 2012; IJMS, 2024). However, the use of fresh garlic is often limited by its pungent, spicy odor and bitter taste, which can irritate the digestive tract and leave an unpleasant odor on the body. To address these limitations, black garlic has emerged as a breakthrough solution through a thermal fermentation (aging process) under strictly controlled temperature and humidity conditions (Utama et al., 2024; Changes of Physicochemical Properties in Black Garlic).

Black garlic is created from fresh garlic through the Maillard reaction – a series of chemical reactions between reducing sugars and amino groups of amino acids under the influence of high temperatures (Utama et al., 2024). This process not only changes the sensory characteristics of garlic, from white to deep black with a soft, chewy texture and a sweet taste like dried fruit, but also profoundly alters its internal chemical composition (Vecchi et al., 2025; Foods, 2025). Most notably, allicin—a pungent and unstable compound—is converted into water-soluble antioxidants such as S-allylcysteine (SAC) and S-allylmercaptocysteine (SAMC), which are many times more stable and bioavailable (Colín-González et al., 2012; Woo et al., 2022; Antioxidants, 2023).

Recent studies have shown that black garlic possesses significantly higher levels of polyphenols and flavonoids than fresh garlic, leading to superior antioxidant capacity (Yu et al., 2025; Ma et al., 2021; Biochemical Composition and Antioxidant Activity). This ability plays a crucial role in neutralizing free radicals and minimizing oxidative stress – one of the main causes of chronic diseases and tumor formation (Colín-González et al., 2012; Frontiers in Cell and Developmental Biology, 2025). In particular, the anti-cancer potential of black garlic is becoming the focus of many biomedical studies. Liver cancer, with the Hep-3B epithelial cell line, is a major challenge in modern medicine due to its high malignancy and rapid metastasis. The search for natural compounds that can inhibit the growth of liver cancer cells without causing serious side effects is a strategic direction (Ng et al., 2012; Zou et al., 2016; In Vitro Cytotoxic Activity of Quercetin and Silymarin). Beyond its pharmacological value, a modern trend in food technology is to integrate bioactive compounds into everyday foods and spices to create functional products (Applied Sciences, 2020; Applied Sciences, 2025). Fish sauce, an indispensable traditional condiment in the diets of Vietnamese and many Asian countries, serves as an ideal medium for supplementing nutrients from black garlic (Maietti et al., 2025; Sauce Product Development with Black Garlic Addition).

The study details experiments on the black garlic fermentation process, analyzes changes in key chemical components (Utama et al., 2024), evaluates the impact of black garlic extract on Hep-3B liver cancer cell lines (Ng et al., 2012), and establishes an optimal production process for black garlic fish sauce in terms of sensory quality and food safety (Maietti et al., 2025).

2. Materials and Methods

2.1. Raw Materials and Chemicals

The raw garlic selected for the study included Hai Duong garlic and Ly Son garlic, purchased from standard cultivation areas to ensure consistency in initial active ingredient content. Fresh garlic was checked for sensory criteria, ensuring large, firm cloves without pests or mechanical damage.

The fish sauce used as a base for the blending process was traditional Muoi Thu fish sauce, which has stable protein content and physicochemical parameters. The bacterial strains used for the antimicrobial testing included *Escherichia coli, Bacillus subtilis,* and *Staphylococcus aureus*, which were supplied by standard microbiology laboratories.

The analytical chemical system includes:

- DPPH (2,2-diphenyl-1-picrylhydrazyl radical) for antioxidant testing.
- Extraction solvents: methanol, ethanol, n-hexane, ethyl acetate.
- Qualitative and quantitative reagents: NaOH, concentrated HCl, magnesium powder, lead acetate, Bertrand reagent.
- Cell culture media: DMEM (Invitrogen), fetal bovine serum (FBS), penicillin/streptomycin.

2.2. Black garlic production process

The black garlic fermentation process is based on the principle of prolonged exposure to heat and humidity without the use of any additives (Utama et al., 2024; Changes of Physicochemical Properties in Black Garlic; Foods, 2025).

1. Cleaning and preparation: Fresh garlic is peeled to remove the outer dirty layer, left whole, and soaked in water for 4–5 hours to allow moisture to penetrate evenly into the skin.
2. Heat incubation: The garlic is wrapped tightly in aluminum foil to maintain stable moisture and placed in a warm cabinet at a temperature of. The humidity in the incubation block is maintained at. This process lasts from 30 to 35 days. During this stage, changes in color and flavor are monitored regularly (Utama et al., 2024; Yu et al., 2025; Vecchi et al., 2025).
3. Drying: After the garlic is ripe (black, chewy, sweet, no longer pungent), it is placed in a warm cabinet at to dry until the moisture content reaches (Changes of Physicochemical Properties in Black Garlic).
4. Storage: The final black garlic product is packaged and stored at for subsequent analysis.

(A) *(B)*

Figure 1. *Fresh garlic and black garlic produced by controlled fermentation. (A) Fresh garlic bulbs and peeled cloves before fermentation. (B) Black garlic bulbs and cloves after fermentation.*

2.3. Chemical and biological analysis methods

2.3.1. Determination of Physical and Chemical Components

Moisture content is determined by drying to constant weight at 115^0 C according to TCVN 4069 standard. Total nitrogen content is measured by the Kjeldahl method. Total sugar and reducing sugar content are determined by the Bertrand method, based on the reducing ability of aldehyde and ketone groups present in sugars.

2.3.2. Quantification of Flavonoids

Flavonoids are fractionally extracted from black garlic using solvents of increasing polarity (n-hexane, ethyl acetate, ethanol, warm water). The total flavonoid content (F%) is calculated by the gravimetric method based on the formula: Where is the mass of flavonoids obtained, is the initial sample mass, and is the moisture content of the raw material.

$$F(\%) = \frac{M \times 100}{M_0 \times (100 - W)} \times 100$$

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M is the mass of flavonoids obtained, M_0 is the mass of the initial sample, and W is the moisture content of the raw material.

2.3.3. Evaluation of antioxidant activity (DPPH)

Black garlic extract was mixed with DPPH solution and incubated in the dark at 30°C for 30 minutes. Optical absorption was measured at a wavelength of 517 nm. Antioxidant activity was expressed as the value IC_{50} (50% free radical inhibition concentration), which was directly compared with Vitamin C.

2.3.4. Antimicrobial testing

The agar well diffusion method was used to measure the antibacterial zone diameter (D – d) of the garlic extract against indicator bacterial strains.

2.4. Anticancer Test on Hep-3B Cells

2.4.1 Cell culture

The human hepatocellular carcinoma cell line Hep-3B was cultured in DMEM (Invitrogen, Carlsbad, CA) add 10% (FBS) and 1% penicillin + streptomycin under standard conditions 37 °C and 5% CO_2. Cells were regularly medium-changed and tested when they reached an appropriate density (Ng et al., 2012).

2.4.2 Trypan Blue Assay

To assess direct cytotoxic effects, cells were incubated with black garlic extract and SAC at various concentrations for 1 hour. Subsequently, cells were stained with Trypan Blue; dead cells stained blue due to damaged cell membranes, allowing observation under an inverted microscope (Ng et al., 2012).

2.4.3 MTT Assay

The principle of the MTT assay is based on the ability of dehydrogenase enzymes in the mitochondria of living cells to convert tetrazolium salts into insoluble purple formazan crystals. The assay was performed at 48 and 72 hours after sample addition. Optical density is measured at 590 nm using a SmartSpec Plus spectrophotometer. The experiment was repeated 3 times, and the optical density was measured at a wavelength of 590 nm (Ng et al., 2012).

Preparation of cell samples:

Blank sample: Only standard culture medium.

Test sample: Add black garlic extract and SAC at the expected concentrations.

Preparation of black garlic extract: Black garlic is diluted test solutions with corresponding SAC concentrations: C1 = 0.0625 mM, C2 = 0.125 mM, C3 = 0.25 mM, C4 = 0.5 mM, C5 = 1.0 mM.

Preparation of SAC: Dilute the test SAC solutions with SAC concentrations of 50 mM or 100 mM.

2.4.4 Data Processing

Data was processed using Statview 5.01E and STATA 7.0 software employing statistical algorithms. Comparison of two proportions and two means were performed. Differences were statistically significant when $p < 0.05$.

2.5. Application in black garlic fish sauce production

2.5.1. Blending and Optimization

The amount of black garlic added is calculated based on the body's minimum daily antioxidant requirement (equivalent to 150mg of Vitamin C) and the salt concentration in fish sauce. Six samples of black garlic fish sauce with different ratios (2g; 2.5 g; 3 g; 3.5 g; 4 g; 4.5 g black garlic in 15 mL fish sauce) were prepared for sensory evaluation.

2.5.2. Sensory evaluation and statistics

A preference test was conducted by a panel of 60 people to determine overall preference. Data were processed using the Friedman test and LSRD (Least Significant Rank Difference) value with confidence level. In addition, a descriptive test with a panel of 13 experts was used to analyze in depth the attributes of color, smell, taste, and texture of the product.

2.5.3. Microbiological safety testing

The coliform content in black garlic fish sauce was quantified using the MPN method (TCVN 4882 - 2007) to ensure compliance with the Ministry of Health's regulations on biological contamination limits.

3. Results and Discussion

3.1. Changes in physicochemical composition during black garlic production

The 35-day fermentation process significantly altered the physicochemical properties of the garlic. This change resulted from complex biochemical reactions occurring under the influence of temperature and humidity.

Table 1. *Physicochemical characteristics and antioxidant activity of fresh garlic and black garlic.*

Analytical parameters	Fresh garlic (FG)	Black garlic (BG)	Change
Moisture content (%)	61.73 ± 0.50	45.39 ± 0.43	Decreased by 26.5%
Total sugars (mg/g)	92.5 ± 0.72	260 ± 1.27	2.8-fold increased
Reducing sugars (mg/g)	20 ± 0.36	220 ± 2.1	11-fold increased
Total nitrogen (%)	3.95 ± 0.05	3.32 ± 0.08	Slight decrease
Antioxidant activity (IC_{50}, µg/mL)	646.3	125.04	Significant reduction (~5-fold) in IC_{50}

Experimental data shows that the reducing sugar content skyrocketed from 20 mg/g to 220 mg/g. This explains why black garlic has a rich sweet taste instead of the pungent taste of fresh garlic. The increase in reducing sugars is due to the hydrolysis of polysaccharides, particularly fructans, into simple sugars (glucose, fructose) under the influence of heat. Reducing sugars are also the main agents involved in the Maillard reaction with amino acids to form melanoidins – compounds that give the product its characteristic black color.

Total nitrogen content decreased slightly from 3.95% to 3.32% g, reflecting that some nitrogen-containing compounds (such as proteins and free amino acids) participated in browning and flavor-forming reactions. Although total nitrogen decreased, supplementary studies showed that the content of S-allylcysteine e (SAC)—a metabolite of alliin—increased sixfold, playing an important role in enhancing the pharmacological value of black garlic.

3.2. Evaluation of Biological Activity

3.2.1. Superior Antioxidant Activity

Antioxidant activity is one of the most important indicators for evaluating black garlic quality. DPPH test results show that black garlic's free radical scavenging capacity is five times stronger than fresh garlic (black garlic's value is compared to fresh garlic).

This increase can be explained by two main mechanisms:

1. Increased polyphenol and flavonoid content: The thermal process releases phenolic compounds from bound to free forms, while also creating intermediate products with strong antioxidant activity.

2. Formation of Melanoidins: These are products of the Maillard reaction, which have been proven to be highly effective at trapping free radicals.

Table 2. *Qualitative and quantitative analysis of flavonoids in fresh and black garlic.*

Test samples	Flavonoid activity (Qualitative)	Total Flavonoid Content (mg/g)
Fresh Garlic (FG)	+ (Light yellow)	6
Black garlic (BG)	++ (Dark yellow/Distinct spots)	49

Flavonoid quantification showed an increase from 6 mg/g in fresh garlic to 49 mg/g in black garlic (an approximately 8-fold increase). Qualitative results with specific reagents also showed that the reaction color of black garlic extract was significantly darker than that of fresh garlic, confirming black garlic as a rich source of antioxidants.

3.2.2. Changes in antimicrobial activity

In contrast to its antioxidant activity, the antibacterial activity of black garlic is significantly reduced compared to fresh garlic. Fresh garlic extract produces a wide antibacterial zone with *E. col*i (21 mm), *Staphylococcus* (23 mm), and *B. subtilis* (25 mm). In contrast, black garlic only produces a small, indistinct zone with *E. coli* and has no effect on the other two strains.

This reduction is due to the fact that during thermal fermentation, allicin—the strongest natural antibiotic compound in garlic—is broken down and converted into other sulfur compounds such as SAC and SAMC. Although black garlic loses some of its direct antibacterial activity, the increase in stable antioxidant compounds provides greater long-term health benefits in preventing chronic diseases.

3.3. Anticancer effects on Hep-3B cell lines

3.3.1. Cytotoxicity Results (Trypan Blue)

(A) Control *(B) SAC (100 µM)* *(C) Black garlic extract (1.0 mM)*

***Figure 2**. Trypan Blue staining of Hep-3B cells after 1 h treatment.*

Observation under a microscope after 1 hour of incubation with black garlic extract and SAC did not reveal a significant increase in Trypan Blue-stained cells. This indicates that at the tested concentrations (up to 1mM based on SAC), black garlic extract does not cause cell death through direct cell membrane destruction. This is an important characteristic, indicating that black garlic has the potential to be a safe therapeutic agent that does not cause toxic shock to healthy tissues.

3.3.2. Cell proliferation inhibition (MTT assay)

The effect of black garlic on Hep-3B liver cancer cells is clearly evident over time.

Table 3. *Effects of black garlic extract and S-allylcysteine (SAC) on Hep-3B cell viability.*

Treatment Group	48 h (OD ± SD)	72 h (OD ± SD)	Significance (p vs. control)

Negative control (culture medium)	0,164 ± 0,023	0,200 ± 0,027	-
SAC (100 mM)	0,160 ± 0,037	0,170 ± 0,037	p < 0,05
Black garlic extract (C5 = 1.0 mM)	0,170 ± 0,041	0,177 ± 0,010	p < 0,05
Black garlic extract (C4 = 0.5 mM)	0,165 ± 0,046	0,144 ± 0,027	p < 0,05
Black garlic extract (C3 = 0.25 mM)	0,193 ± 0,030	0,157 ± 0,033	p < 0,05

At 48 hours, there was no statistically significant difference in optical density among the groups.1However, after 72 hours, the groups treated with black garlic extract at a concentration of C3, C4, C5 and SAC 100 mM showed a significant reduction in cell density compared to the control group ($p<0.05$). Observation under an optical microscope at 72 hours also confirmed that cell density was significantly lower in the treated groups.

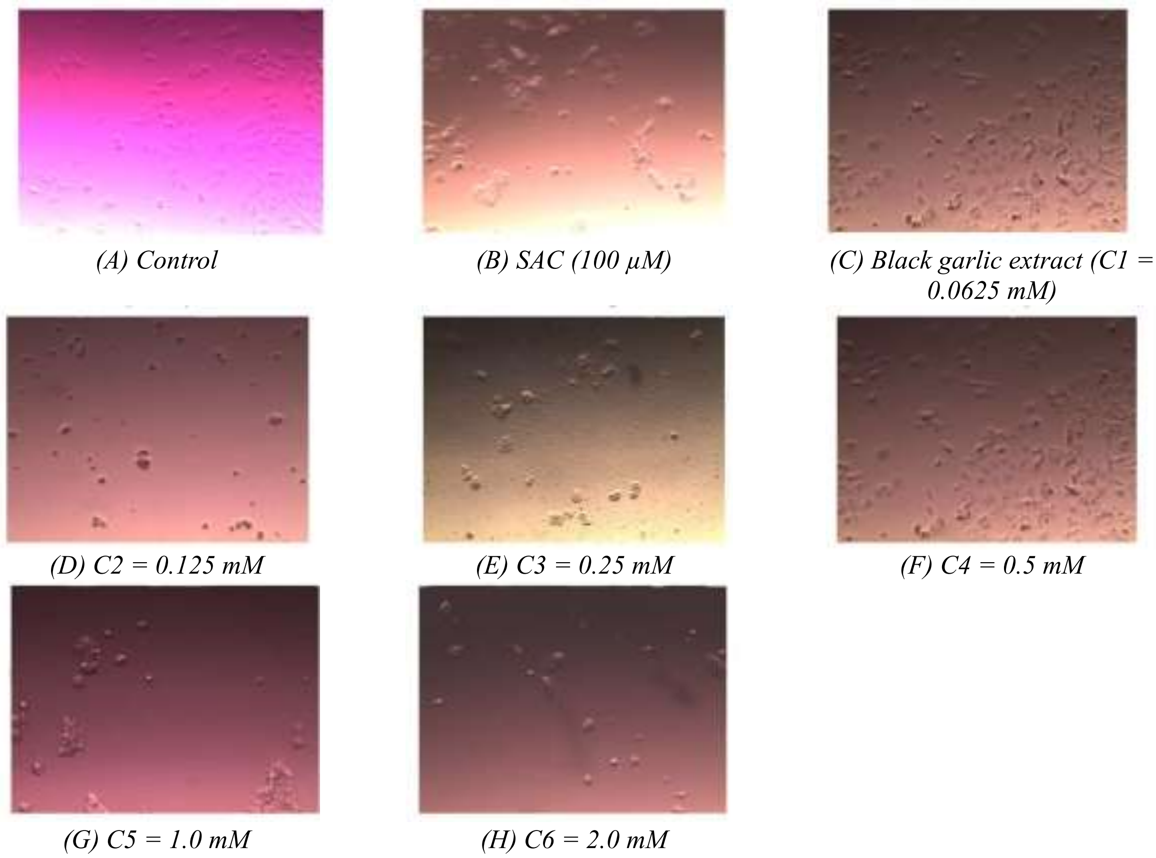

***Figure 3**. Morphological changes of Hep-3B cells after 72 h treatment with black garlic extract and S-allylcysteine (SAC) (200× magnification).*

These results suggest that black garlic extract inhibits the proliferation of liver cancer cells through mechanisms that interfere with the cell cycle or activate slow apoptosis. Related studies have shown that sulfur compounds in garlic, such as SAC, can enhance histone acetylation, inhibit histone deacetylase (HDAC) enzymes, leading to the activation of tumor suppressor genes. Additionally, black garlic reduces the expression of metastasis-promoting genes like MMP-2 and MMP-9, while inhibiting the PI3K/Akt and NF-κB signaling pathways, which are crucial for cancer cell survival.

3.4. Applications in production and optimization of black garlic fish sauce

3.4.1. Establishing the optimal mixing ratio

The development of black garlic fish sauce aims to provide daily antioxidants for consumers. Based on the NaCl salt concentration in fish sauce (199.67 g/L) and salt consumption recommendations (3-6g/day), the average fish sauce consumption volume was determined to be .

Using the IC_{50} value (125.04 µg/mL) of black garlic and comparing it with the body's minimum Vitamin C requirement, the research team calculated that approximately 3g of black garlic is needed in 15mL of fish sauce to provide health benefits.

3.4.2. Sensory Evaluation Results (Preference and Description)

Six samples of black garlic fish sauce were evaluated for preference through a paired comparison test.

***Table 4**. Overall sensory scores and statistical differences of garlic–fish sauce formulations at different mixing ratios ($p < 0.05$).*

Sample (Garlic : Fish Sauce Ratio)	Overall Rating	Significance Level ($p<0.05$)
Sample 1 (2 g : 15mL)	144	Average group
Sample 2 (2.5 g : 15mL)	275	Good group
Sample 3 (3 g : 15mL)	314	Most preferred (C)
Sample 4 (3.5 g : 15mL)	277	Fair group
Sample 5 (4 g : 15mL)	156	Average group
Sample 6 (4.5 g : 15mL)	93	Least preferred

Statistical results using the Friedman test (F_calculated = 190,81 > F_critical = 11,07) confirm a significant difference in preference levels among the samples. Sample 3, with a ratio of 3g of black garlic in 15mL of fish sauce, achieved the highest score. Descriptive analysis shows that sample 3 has superior characteristics: deep yellow color, harmonious aroma between fish sauce and black garlic, moderate saltiness combined with the sweet aftertaste of garlic, smooth texture, and no separation after 10 minutes of standing.1 Conversely, samples with excessively high garlic ratios (such as sample 6) became thick and viscous, overly sweet in taste, and had an overpowering garlic aroma that masked the fish sauce aroma, thereby diminishing the product's traditional seasoning characteristics.

3.4.3. Microbiological quality and antioxidant activity of the product

The black garlic fish sauce product maintained microbiological safety standards after blending. The measured coliform count was 3CFU/ml, unchanged after 10 days of storage at room temperature and within the limits set by the Ministry of Health ($<10^2$ CFU/mL). Regarding biological activity, the antioxidant activity of black garlic fish sauce (IC_{50} = 76.64 µg/mL) is significantly higher than that of pure fish sauce (IC_{50} = 186.09 µg/mL). This demonstrates that antioxidants from black garlic have blended and enriched the nutritional value of fish sauce, creating a functional seasoning.

4. Discussion

4.1. The role of the Maillard reaction and sulfur compounds

The Maillard reaction in black garlic is not merely a coloring process but also produces beneficial compounds (Utama et al., 2024; Vecchi et al., 2025). The 11-fold increase in reducing sugar content is an important indicator of the breakdown of complex carbohydrate structures, making nutrients more easily absorbed (Utama et al., 2024; Changes of Physicochemical Properties in Black Garlic). Meanwhile, the conversion from allicin to SAC is a turning point in terms of medicinal properties (Colín-González et al., 2012; Woo et al., 2022; Antioxidants, 2023). SAC has been proven to be highly water-soluble and stable in the acidic environment of the stomach, helping it achieve high concentrations in the blood plasma after consumption (Colín-González et al., 2012). This active ingredient contributes to the liver-protective and anti-cancer effects of black garlic (Ng et al., 2012; The Protective Effects of an Aged Black Garlic Water Extract).

4.2. Significance of the liver anti-cancer results

In black garlic extract, besides SAC, there are also flavonoids, polyphenols, and other Maillard products (Vecchi et al., 2025; Biochemical Composition and Antioxidant Activity). The combination of these active groups can have multiple effects on cancer cells, from preventing the formation of new blood vessels (by reducing VEGF expression) to inhibiting the invasion process through MMP enzymes (Ng et al., 2012; Zou et al., 2016). This reinforces the view that using raw black garlic as food or whole extract provides higher biological benefits than using a single active compound alone (Vecchi et al., 2025; Frontiers in Cell and Developmental Biology, 2025).

4.3. The feasibility of black garlic fish sauce in dietary regimens

Fish sauce is a particularly effective nutritional carrier in East Asian culinary culture. Optimizing the ratio of 3 g garlic : 15 mL fish sauce is based not only on sensory perception but also on scientific calculations of protective dosage (Maietti et al., 2025; Sauce Product Development with Black Garlic Addition). Daily consumption of black garlic fish sauce helps the body maintain a stable level of antioxidants, thereby supporting the immune system and minimizing DNA damage caused by oxidative agents (Colín-González et al., 2012; Antioxidants, 2023). Furthermore, black garlic reduces pungent flavors, making this product accessible to sensitive groups such as children or the elderly, who need to boost their immunity but find it difficult to consume fresh garlic (Utama et al., 2024; Foods, 2025).

5. Conclusion

The study confirmed that black garlic is a functional food ingredient with high pharmacological value, with the following key conclusions:

1. Optimal fermentation process: Fermenting garlic at a temperature of 70^0 C , humidity 80-90% for 35 days maximizes the conversion of bioactive compounds, increasing the reducing sugar content by 11 times and the antioxidant activity by 5 times compared to fresh garlic.

2. Potential for liver cancer resistance: Black garlic extract and SAC can inhibit the proliferation of Hep-3B cell lines at low concentrations without causing direct cell toxicity, demonstrating potential for safe liver cancer treatment support.

3. Functional seasoning products: The black garlic fish sauce formula with a ratio of 3g black garlic : 15mL fish sauce has been established as optimal, achieving the highest sensory harmony and enhancing the antioxidant activity of traditional seasoning products.

These results open up prospects for the industrial production of black garlic-based products, not limited to fish sauce but also expandable to soy sauce and functional sauces, contributing to increasing the added value of Vietnamese agricultural products and protecting public health. Future studies should focus on evaluating the efficacy of black garlic fish sauce in live animal models (in vivo) to fully confirm its biological benefits before widespread commercialization.

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