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Free-Radical Scavenging and In Vitro Anticancer Assay of Indonesian Black Garlic with Different Time Processing

Sandra Amalia Riyadi[1*], Noviana Vanawati[2], Harra Ismi Farah[3], Aulia UI Hafizah[4], Uswatun Hasanah[1], Eka Herlina[1]

[1] Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Pakuan, Jl.Pakuan Bogor,16143, Indonesia
[2] Sekolah Ilmu dan Tekologi Hayati, Institut Teknologi Bandung, Jl. Ganesha No. 10, Bandung, Indonesia
[3] Department of Pharmacy, Faculty of Pharmacy, Universitas Mulawarman, Samarinda, Kalimantan Timur, 75119, Indonesia
[4] Department of Pharmacy, Faculty of Mathematics and Natural Sciences, Universitas Pakuan, Jl.Pakuan Bogor,16143, Indonesia
[*] Corresponding Author e-mail: sandraamaliariyadi@unpak.ac.id

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Abstract

The process of producing Black Garlic (BG) in Indonesia has not used a standard method and the right dosage. BG is produced through termal processing, compared with fresh garlic (FG) there are obvious changes in color, taste, and biological activities. BG was produced by different time of heating. Several studies have shown that BG has several pharmacological activities including antioxidants and anticancer. The aim of this research is to determine the optimal produced of BG to assess the potential antioxidant and anticancer activity. FG was heated at high temperature 60-70ºC with 85-95% humidity without any additives for various period of time which is 30, 40, 50, 60 days. Ethanol (EtOH) extract of BG 30; 40; 50 and 60 days and FG as a control were partionate with *n*-hexane, ethyl acetate (EtOAc), *n*-buthanol (*n*-BuOH), and water then evaporated in 40 °C. Each extract of FG and BG were tested Free-radical scavenging and anticancer activity. Free-radical scavenging was determined by 1,1 diphenyl-2-picrylhydrazyl (DPPH) method, and cytotoxicity of crude extract was tested by 3-(4,5-dimethylthiazol-2-yl)—2,5-diphenyltetrazolium bromide (MTT) assay against MCF-7 human breast cancer cell line. The result demonstrated that BG exhibited significantly enhanced radical scavenging activity compared to FG across different aging periods. Notably, EtOAc and *n*-hexane fractions obtained after 30, 40, and 60 days of thermal aging showed strong antioxidant activity with IC_{50} values below 50 µg/mL. Importantly, this study demonstrates that a defined thermal aging duration of 30 days, combined with EtOAc fractionation, yields the most potent antioxidant fraction and the lowest IC_{50} value (47.27 μg/mL) against MCF-7 cells, indicating that thermal aging time is a key determinant governing the antioxidant and anticancer potency of BG. These findings provide novel evidence linking controlled thermal aging parameters to the optimization of BG bioactivity, thereby offering a robust scientific basis for standardized BG processing with enhanced functional and therapeutic potential.

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INTRODUCTION

Indonesian garlic (*Allium sativum* L.) comprises several local varieties, including Lumbu Hijau, Lumbu Kuning, Lumbu Putih, Ciwidey, and Tawangmangu, which differ in aroma, taste, and bulb morphology. These local varieties have been traditionally utilized for medicinal purposes and are recognized as rich sources of bioactive compounds, particularly sulfur-containing compounds and phenolic constituents. Extensive studies have demonstrated that organosulfur compounds, such as allicin and its derivatives, together with phenolic compounds,

play a crucial role in conferring strong antioxidant, anti-inflammatory, and anticancer activities in garlic (*Allium sativum* L.) (Thakur & Sharma, 2024; Jain et al., 2025). Recent experimental and metabolomic investigations further confirm that garlic extracts exhibit significant free-radical scavenging activity and cytotoxic effects against various cancer cell lines, highlighting their therapeutic relevance (Ghali et al., 2025). Consequently, garlic has progressively evolved from a traditional culinary ingredient into an important raw material for functional food development and pharmaceutical applications, supported by increasing scientific evidence on its health-promoting properties (Thakur & Sharma, 2024).

Black garlic (BG) is a thermally processed garlic product traditionally consumed as a medicinal food in South Korea and Japan. It is produced by heating fresh garlic at 60–70 °C under high humidity (85–95%) for 40–80 days without additives. This controlled thermal ageing process induces extensive chemical transformations, including Maillard reactions, oxidation, and the conversion of organosulfur compounds, which collectively contribute to enhanced antioxidant capacity, improved bioavailability, and diverse biological activities compared to fresh garlic. (Ahmed & Wang, 2021; Kimura et al., 2017; Lu et al., 2017).

During black garlic processing, the content of allicin derived from alliin markedly decreases due to thermal degradation, while secondary metabolites such as phenolic compounds, flavonoids, and other antioxidant-related constituents increase significantly as a result of Maillard reactions and metabolic transformations. These compositional changes have been consistently associated with enhanced antioxidant capacity in black garlic, with phenolic compounds and flavonoids identified as major contributors to free-radical scavenging activity (Kimura et al., 2017; Ahmed & Wang, 2021; Shin et al., 2024). Given the pivotal role of oxidative stress in cancer initiation and progression, the improved antioxidant properties of black garlic are strongly linked to its anticancer potential. Recent studies have demonstrated that black garlic extracts can suppress cancer cell proliferation, induce apoptosis, and modulate oxidative stress–related signaling pathways in various cancer cell models, including breast cancer cells (Yang et al., 2023; Stepien et al., 2024).

Despite the increasing utilization of black garlic as a herbal medicine and dietary supplement in Indonesia, its production is predominantly conducted using empirical and non-standardized processing methods. Variations in thermal aging parameters, particularly temperature, humidity, and duration, have been shown to substantially affect the transformation of sulfur-containing compounds and the accumulation of antioxidant-related secondary metabolites, resulting in inconsistent bioactive profiles and biological efficacy (Kimura et al., 2017; Ahmed & Wang, 2021; Shin et al., 2024). Moreover, garlic cultivars grown in Indonesia differ markedly from those commonly used in international studies in terms of genetic background, sulfur compound composition, and phenolic profiles, which may significantly influence the antioxidant and anticancer activities of the resulting black garlic products (Thakur & Sharma, 2024).

Consequently, systematic studies employing controlled variations in thermal aging duration and processing conditions, combined with solvent-based fractionation and biological evaluation, remain limited for Indonesian black garlic. Recent Indonesian studies have confirmed that local black garlic exhibits considerable antioxidant potential; however, systematic investigations linking controlled thermal aging duration with solvent-based fractionation and anticancer activity remain limited (Azizah, 2024; Nurulloh, 2025). Therefore, the present study investigates black garlic produced under different thermal aging periods and heating conditions and evaluates its antioxidant activity and anticancer potential against MCF-7 human breast cancer cells. Importantly, this study reveals for the first time that black garlic aged for 30 days and extracted with ethyl acetate yields the most potent antioxidant fraction and exhibits the lowest IC_{50} value (47.27 μg/mL) against MCF-7 cells, demonstrating that

thermal aging duration is a key determinant for maximizing anticancer potential. These findings provide a strong scientific basis for establishing standardized processing parameters and optimizing Indonesian black garlic for functional food and pharmaceutical applications.

METHOD

Equipment

Rotary Evaporator (Heidolph Hei VAP), UV-Vis Spectrophotometry (Shimadzu UV-1280), ELISA reader, laboratory glassware, maceration apparatus.

Materials

Fresh Garlic (FG) has been harvested in March from Ciwidey Farm, Bandung, West Java, Indonesia and stored in dry pots. Various organic solvent such as Ethanol (Merck), Ethyl Acetate (Merck), *n*- hexane (Merck), *n*- buthanol (Merck), MTT 5mg/mL on Phosphate Buffer, DMSO (Merck).

Black Garlic (BG) Preparation

A total of 25 kg of fresh garlic bulbs were subjected to thermal aging in a controlled heating chamber at 60-70 °C for different durations (30, 40, 50, and 60 days). At the end of the aging process, the garlic bulbs exhibited a characteristic black coloration, indicating successful conversion into black garlic, as previously reported (Lu et al., 2017). As a control, the FG without any process was used (Nurulloh et al., 2025).

Extraction of Black Garlic

Both of the control FG (5 kg) and BG (25 g) were soaked in ethanol (re-destilation) for 3 days followed by filtration using filter paper No. 2. The filtrate then evaporated on rotary evaporator, the concentrated of EtOH extract was dissolved in water and partioned successively with *n*-Hexane, EtOAc and *n*-BuOH . Evaporation on rotary evaporator produced the crude extract of *n*-Hexane, EtOAc, *n*-BuOH respectively (Riyadi et al., 2022).

Free-radical scavenging activity

Free-radical scavenging activity was determined using the DPPH method as previously described with slight modifications (Kumari et al., 2025). A total of 0.8 mL of DPPH solution (0.2 mM, prepared in methanol) was mixed with 0.2 mL of sample extract at various concentrations. The mixture was vortexed and allowed to stand at room temperature under dark conditions for 30 minutes. The absorbance was then measured using a UV–Vis spectrophotometer at a wavelength of 520 nm. A control solution consisting of 0.8 mL DPPH (0.2 mM) mixed with 0.2 mL of the corresponding solvent without extract was used as the control, while the solvent alone was used as the blank. The percentage of DPPH radical scavenging activity was calculated as percentage inhibition using the following equation:

$$\% \text{ inhibition of radical scavenging} = ((A_{blank}-A_{sampels})/A_{blank}) \times 100\%$$

Cytotoxicity assay

Cytotoxicity assay to asses cell viability in BG extract was carried out using modified MTT method (Riyadi et al., 2022). MCF human breast cancer was obtained from cell culture laboratory, Rumah Sakit Pendidikan Universitas Padjajaran (Bandung, Indonesia) and cultured in cultured medium containing RPMI-1640, 10% Fetal Bovine Serum, 1% penicillin streptomycin at 37^0C in humidified incubator containing supplemented with 5% CO_2. After confluency, MCF-7 was sub-cultured and seeded at a density of 10.000 cells/well in 96-well plates in culture medium cultured and incubated for 24h. After that, the culture medium

replaced with treated medium (culture medium containing varied concentration of BG extract) at various concentration in the 0.4 – 409.6 µg/mL range and each sample was examined in duplicate. Untreated cell (cell in culture medium without treated medium) used as negative control and Doxorubicin (0.5 μg/mL) was used as a positive control. After 24h incubation, 100 µL (5 mg/mL) MTT solution was added to each well to asses cell viability, followed by the addition of 100 µL DMSO after 4 hours incubation at 37^0C. The absorbance value (OD) was measured by microplate reader detection instrument at 570 nm and each experiment was repeated three times. All samples were duplicated into triplicate. Percentage of cells viability and cytotoxicity was determined by the standar MTT assay method and IC_{50} values was calculated.

RESULTS AND DISCUSSION

Plant Extraction

Fresh garlic (FG) exhibited a light yellowish colour; however, during the aging incubation process at 70–80 °C, the colour gradually darkened to brown and eventually turned black, resulting in black garlic (BG) (Figure 1). This pronounced colour transformation is a characteristic feature of BG production and is primarily attributed to the formation of Maillard reaction products (MRPs) during prolonged thermal treatment (Tamanna et al., 2015). The Maillard reaction is a non-enzymatic browning process that occurs between reducing sugars and amino acids under elevated temperature and controlled humidity, leading to the generation of melanoidins and other high-molecular-weight compounds responsible for the dark coloration of BG (Mandge et al., 2025). The extent of MRP formation is strongly influenced by processing parameters, particularly temperature and incubation time, which explains the progressive colour intensification observed with longer aging durations.

In addition to visual changes, thermal aging markedly affected the chemical composition of garlic, as reflected by the extraction yields obtained using solvents of different polarities (Table 1). The percent yield (w/w) of crude extracts varied according to both the duration of heating and the polarity of the extraction solvent, indicating that thermal processing altered the distribution and solubility of bioactive compounds. Prolonged heating is known to induce degradation of unstable sulfur compounds while simultaneously promoting the formation of more stable secondary metabolites, such as phenolic compounds and Maillard-derived antioxidants, which preferentially partition into semi-polar solvents such as EtOAc (Kimura et al., 2017; Ahmed & Wang, 2021).

Interestingly, the EtOAc and *n*-hexane crude extracts of FG and BG aged for 50 days showed a decrease in extraction yield, suggesting a reduction in compounds with similar polarity to these solvents. This phenomenon may be attributed to further polymerization or condensation reactions of Maillard intermediates at extended aging times, resulting in the formation of high-molecular-weight melanoidins that are less soluble in low- to medium-polarity solvents (Shin et al., 2024). Similar observations have been reported in thermally processed foods, where excessive heating leads to decreased extractable low-molecular-weight compounds despite increased overall browning intensity (Mandge et al., 2025).

Furthermore, these findings demonstrate that thermal aging not only governs the physical appearance of BG but also critically modulates its chemical profile and extractability. The observed variations in extraction yield across solvents and aging periods highlight the importance of optimizing processing conditions to preserve and concentrate bioactive constituents. These compositional changes provide a chemical basis for the enhanced antioxidant and anticancer activities observed in BG extracts and underscore the relevance of controlled aging duration in maximizing the functional properties of black garlic.

Figure 1. The changes black garlic colour during aging incubation time

Table 1. Extractive yields of various extract solvent and aging time of Indonesian Black Garlic

Days	Yields (%)			
	EtOH	EtOAc	n-BuOH	n-Hex
30	13.90	9.30	12.70	15.50
40	14.10	8.90	13.10.	14.60
50	13.90	6.70	1.90	2.10
60	54.90	8.40	13.80	14.60

Free-radical Scavenging Activity

Garlic has been long recognized for its medicinal properties and has been used for sterilization and anti-inflammation throughout history. Allicin, the main active ingredient of garlic, is currently extensively investigated for antitumor therapy. Black garlic is a type of fermented garlic. Numerous in vivo and in vitro studies demonstrated that aged black garlic possesses strong antioxidant and anticancer properties and may inhibit the proliferation of a variety of tumor cell lines by altering the cell cycle and inducing apoptosis (Mandge et al., 2025; Ryu & Kang, 2017). In this present study, free-radical scavening activity was determined based on the ability of crude extract to reduce the stable DPPH radical with following reaction in Figure 2.

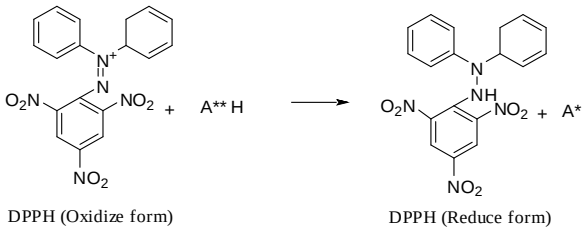


Figure 2. Free-radical scavenging reaction with antioxidant compound (Nimse et al., 2015).

Table 2. Free-radical scavening ability of each crude extract.

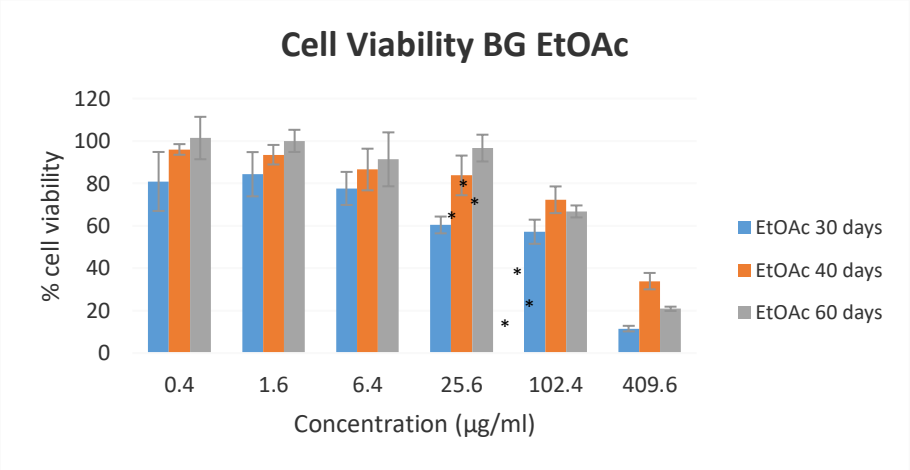
IC_{50} of Crude Extract (mg/mL)				
		FG		
Days	**EtOH**	**EtOAc**	***n*-BuOH**	***n*-Hex**
0	1287.72	487.87	1520.32	530.49
		BG		
30	130.32	12.76	146.24	9.03
40	190.51	12.36	103.86	11.42
50	825.51	633.02	1009.21	851.51
60	131.43	4.48	150.12	8.59

From table 2, it can be observed that radical scavenging activities of BG in a various days of heated show strong ability than FG as control (without any process heated). These findings are consistent with previous investigation that Japanese Garlic has IC_{50} for BG is 4.1 mg/mL and for FG is 114.9 mg/L (Ryu & Kang, 2017) and the number of total fenolik and flavonoid content of Korean garlic has increased during the aging process which improve the ability of radical savenging DPPH radical (Kim et al., 2012). DPPH radical can accept an electron or hydrogen radical and form a stable diamagnetic molecule such as flavonoid. The color of DPPH changes from purple becomes yellow signed to measure the radical scavenging activity.

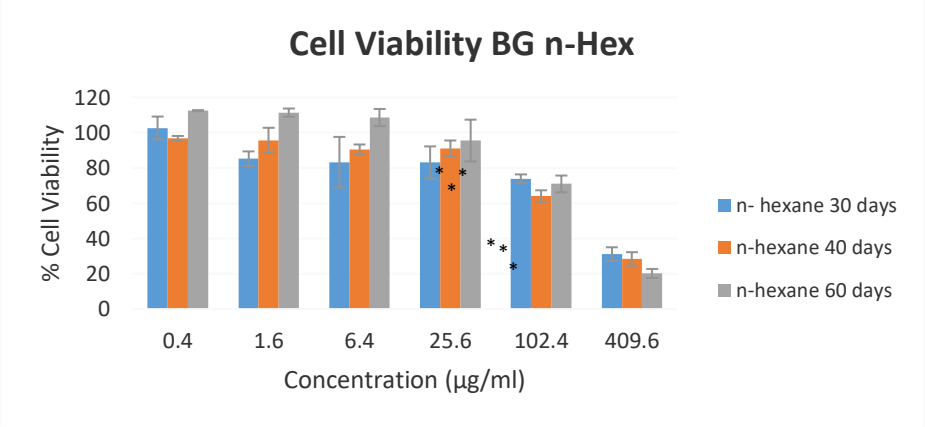
Crude Extract from BG with heated process 30; 40; 60 days showed good activity, depend on period of heated. IC_{50} for crude extract EtOH was under 200 mg/mL which represented weak activity. Meanwhile crude extract from EtOAc and *n*-Hex represent high radical scavenging activities (<50 mg/mL) which have potential as an antioxidant agent and further analysis. BG 50 days showed poor activities in every crude extract, this studies must be fully analyzed, as there not enough studies available. Components and content are vary with aging time, moreover variation of physicochemical properties and contents could result from different garlic cultivar including agronomic, genetic, and enviromental factors (Chhouk et al., 2017).

Cytotoxicity Assay

Cytotoxicity assay was used to evaluate in vitro human cancer cell line MCF-7 activity using MTT in vitro proliferation assay (Riyadi et al., 2022). According to the percentage of cell viability (Figure 3), BG crude extract of *n*-hexane and EtOAc solvent at six extract concentrations (0.4; 1.6; 6.4; 25.6; 102.4; 409.6 µg/mL) showed significant differences ($p<0.05$) cytotoxicity from 25.6 to 409.6 μg/mL compared to the negative control group (MCF-7 without BG treatment), but the cytotoxicity was not significant ($p<0.05$) between days of each group. The highest concentration showed that BG crude extract inhibitted 30% cell viability compared to the negative control group, although it has not been able to inhibit cell viability until 2% like positive control (doxorubicin).



(A)



(B)

Figure 3. Percentage of viability cells of MCF-7 human cell line breast cancer after treated with Black Garlic (BG) extract with EtOAc solvent (A) and *n*-Hexane solvent (B) of various concentration and time of processing. MCF-7cells without treatment were used as a negative control. The (*) indicated significant difference ($p<0.05$). Result represents the mean ±SD (n=3)

Table 3. IC_{50} values of MCF-7 cell proliferation of Indonesian Black Garlic Extract (μg/mL)

Cells	BG *n*-Hexane			BG EtOAc		
	30	40	60	30	40	60
MCF - 7	343.06	280.36	250.85	47.27	450.46	228.063

The IC_{50} (50% Inhibitory concentration), evaluate the potentially toxic concentration that affect in vitro cytotoxicity cell after treatment (Table 3). The IC_{50} showed that BG crude extract of EtOAc solvent in 30 days incubation time had smallest IC_{50} value at 47.27 μg/mL, while BG crude extract of EtOAc solvent 40 days incubation time had lowest IC_{50} value at 450.46 μg/ml. The smaller IC_{50} value shows the higher extract activity to inhibit cancer cells proliferation (Deepika et al., 2024; Riyadi et al., 2024a). According to The American National Cancer Institute, a significant effect of cytotoxic agent for further purification $IC_{50} < 30$ μg/mL (Deniz et al., 2017; Riyadi et al., 2024b; Hidayat et al., 2022). Although, the cytotoxic activities of BG crude extract of EtOAc solvent in 30 days incubation in this research $IC_{50} > 30$ μg/ml, but we suggest an extract can be potential as an anticancer and has effect to inhibit on MCF-7 cell lines. Optimization BG crude extract method of EtOAc solvent was needed.

Based on table 3, it can be observed the IC_{50} values of various black garlic extracts against the death of MCF-7 breast cancer cell lines by the MTT method. Other studies have shown the potential of black garlic as an anticancer in human lung carcinoma A549, breast adenocarcinoma cells MCF-7, stomach adenocarcinoma cells, hepatocarcinoma cells HePG2, U937 leukemia cells (Park et al., 2014) SGC-7901 human gastric cancer cells and HT29 colon cancer cells (Ryu et al., 2017). BG extract can inhibit cell growth by inducing the apoptosis intrinstic pathway of cancer cells via upregulation of death receptor 4 and fas ligand as well as increasing the expression ratio of Bax / Bcl2 protein (Park et al., 2014; Purev et al., 2012). This research is a preliminary study to evaluate the crude extract that have highly cytotoxic activities based on isolation method and shows first evidence BG crude extract has an effective anticancer in MCF-7 cell lines. Further investigation is needed to evaluate the isolation BG crude extract of EtOAc solvent, and characterization of cytotoxic compound of black garlic.

CONCLUSION

Black garlic (BG) exhibited strong DPPH radical scavenging activity across different thermal aging durations. The ethyl acetate (EtOAc) and *n*-hexane extracts of BG aged for 30, 40, and 60 days showed high antioxidant activity, with IC_{50} values below 50 μg/mL. Importantly, this study demonstrates for the first time that BG aged for 30 days and extracted with EtOAc yields the most potent antioxidant fraction and the lowest IC_{50} value (47.27 µg/mL) against MCF-7 breast cancer cells, identifying thermal aging duration at 30 days as a critical determinant of anticancer potential. These findings provide a scientific basis for optimizing and standardizing black garlic processing to enhance its functional and therapeutic value. Further studies are required to isolate and characterize the bioactive compounds responsible for the observed anticancer activity.

RECOMMENDATIONS

Further studies are recommended to isolate and identify the bioactive compounds responsible for the antioxidant activity of BG, particularly from the ethyl acetate extract heated for 30 days. Advanced analytical techniques should be applied to characterize the active constituents. In addition, *in vitro* and *in vivo* anticancer evaluations are necessary to confirm the anticancer potential and elucidate the underlying mechanisms. These studies will support the development of BG as a potential natural antioxidant and anticancer source.

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