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# *IN-VITRO* EVALUATION OF CYTOTOXICITY, ANTIMICROBIAL, AND ENZYME INHIBITION ACTIVITY OF BLACK GARLIC AND ITS NANOPARTICLES

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## ABSTRACT

Black garlic (aged garlic) supplementation in diet has been shown to be beneficial to patients. Recently, its nano-size and pharmacological role in the prevention and treatment of cancer has received increasing attention. The present study was designed to use cancer cells, harmful microbes, and enzymes cause diabetes and Alzheimer's disease as test models to evaluate their effects. The obtained data from the assay indicated that black garlic nanoparticles system are positive for oral cancer cells, liver cancer cells, lung cancer cells, monkey kidney cancer cells, skin cancer cells and negative for breast cancer cells. Black garlic did not show activity against *Staphylococcus aureus*, *Enterococus faecium*, *E. coli* and *Candida albicans* at concentrations less than 256 µg/mL while black garlic nano-particles system showed inhibited activity on seven microorganism lines in this study. Black garlic nanoparticles might inhibit α-glucosidase enzyme which is involved diabetic but not AChE that is involved Alzheimer's.

**KEYWORDS**: Antimicrobial, black garlic, cancer, cytotoxicity, inhibition, nanoparticles.

## 1. INTRODUCTION

Garlic is known for many health benefits because it contains disease-causing antibacterial, antibiotics, and free radical fighting; anti-atherosclerosis; anti-cancer and anti-highcholesterol compounds (Kim *et al.*, 2012). However, the consumption of unprocessed fresh garlic bulbs was limited due to its characteristics pungent odor and tendency to upset the human's stomach. Black garlics were made from fresh garlic by aging at suitable temperature and humidity conditions for a certain period of time. Fresh garlic's unpleasant odor compounds were lost after processing, the black color of garlic was formed due to biochemical and chemical reactions (primarily the Maillard reaction). In addition, nanomaterials loaded drugs could show many outstanding biological activities. Besides that, the activity of black garlic nanoparticles has not been published, but it could be confirmed that when nanoizing the bioactive compounds in black garlic, their bioactivity will increase many times (Shaikh *et al.*, 2009). Therefore, the aim of this study be to evaluate the activity of black garlic and its nanosize through assay models on cancer cell lines, pathogenic microorganisms and enzymes that cause diabetes and Alzheimer's disease.

## 2. MATERIALS AND METHODS

### 2.1. Materials

Fresh softneck garlic materials (local varieties) were harvested and selected at the age of 130-135 days (after planting) in Van Hai ward, Phan Rang - Thap Cham city , Ninh Thuan province.

### 2.2. Methods

Black garlic was processed through the aging process of ten kg of freshly garlic bulbs and put into an aging chamber. Setting the temperature at 70°C until getting the black garlic products. Black garlic was extracted with a ratio of black garlic/50% ethanol solution is 1/10 at 60°C for 90 minutes (Nguyen Ai Thach and Nguyen Minh

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Thuy, 2017). Black garlic nanoparticles were made by alginate with a ratio of black garlic extract/alginate solution (1 mg/mL) was 2/1 (Nguyen Ai Thach and Nguyen Minh Thuy, 2019).

***2.2.1. Evaluation of the cytotoxic effects***

Cancer cell lines derived from American Type Culture Collection (ATCC), including: human mouth epidermal carcinoma KB (CCL-17$^{TM}$), liver cancer Hep G2 (HB - 8065$^{TM}$), lung cancer LU-1 (HTB- 57$^{TM}$), breast cancer MCF-7 (HTB - 22$^{TM}$), Vero monkey kidney carcinoma and skin cancer SK-Mel 2 (HTB - 68$^{TM}$). The cytotoxic activity was performed based on the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) method which was described and modified by Scudiero *et al*. (2006). Experimental results were determined by the absorbance values/optical density (OD) that were measured at 540 nm on the Genios Tecan spectrophotometer. The experiment was repeated three times. The value of $IC_{50}$ was determined by the value of inhibition of cell growth and the Rawdata software.

$$\% \text{ inhibition} = (OD_{control\ (+)} - OD_{sample})/(OD_{control\ (+)} - OD_{control\ (-)}) \times 100$$

$$IC_{50} = High_{conc} - \frac{(High_{Inh\%} - 50)x(High_{conc} - Low_{conc})}{High_{Inh\%} - Low_{Inh\%}}$$

(In there, $High_{Conc}$/$Low_{Conc}$: test substance at high concentration/test substance at low concentration; $High_{Inh\%}$/$Low_{Inh\%}$: percent of inhibition at high concentration/percent of inhibition at low concentration).

***2.2.2. Evaluation of antimicrobial activity***

Representative strains of bacteria and mold that cause disease in human of the Institute of Chemistry - Vietnam Academy of Science and Technology, including gram-negative bacteria: *Pseudomonas aeruginosa* (Pa) ATCC 15442, *E. Coli* (Ec) ATCC 25922; gram-positive bacteria: *Staphylococcus aureus* (Sa) ATCC 13709, *Bacillus subtilis* (Bs) ATCC 6633, *Lactobacillus fermentum* N4, *Enterococus faecium* B650 and mold: *Candida albicans* (Ca) ATCC 10231. Assays of antibiotic activity according to the method which was modified by Paul *et al*. (2005).

***2.2.3. Evaluation of inhibitory activity of enzymes which causing diabetes (α-glucosidase) and Alzheimer's disease (acetylcholinesterase)***

*a. Enzyme α-glucosidase*: The inhibitory activity of enzyme α-glucosidase was conducted by two methods:

First method (1) (Yamaki and Mori, 2006): Each well consists: 20 µL of sample (control well replaced with 20 µL of 0.5 M potasium phosphate buffer, pH 6.7), 50 µL of 25 mg/mL emzyme α-glucosidase concentration, 50 µL of 3 mM p-NPG substrate ( ρ-nitrophenyl α-D-glucopyranoside) and 120 µL of 0.5 M potasium phosphate buffer, pH 6.7. The mixture was incubated at 37°C for 45 minutes, then added 50 µL of 0.67 M $Na_2CO_3$ to each well to stop the reaction. Reaction mixture was measured at 415 nm on Elisa reader (Biotek, ELx800, USA). The degree of α-glucosidase inhibition was calculated by the formulation: % Inhibition = $[(A_{415control} - A_{415sample})/A_{415control}]$x 100. In there: $A_{415control}$ and $A_{415sample}$ are OD values of control samples and testing samples which measured at 415 nm .

Second method (2) modified by Hakamata *et al*. (2009): The absorbance of the reaction was determined by Tecan GENios equipment with a wavelength of 405 nm (A). The inhibition ability of α-glucosidase of samples was determined by formulation: Inhibition (%) = $[A_{(negative\ control)} - A_{(test\ sample)}]/A_{(negative\ control)}$ x 100% and $IC_{50}$ values were calculated by Tablecurve software.

*b. Enzyme acetylcholinesterase (AChE)*: The inhibitory activity of AChE that causes Alzheimer's disease was tested by using a test method that modified by Karthikeyan *et al*. (2015). The reaction well includes: 10 µL of test compound diluted at concentrations; 15 µL Phosphate buffered saline (PBS buffer); 25 µL in positive control wells (enzyme with 100% activity) and 50 µL in blank wells (without enzyme); 25 µL enzyme AChE (Electriceel) was provided by Sigma company in PBS buffer with concentration of 0.22 U/mL; 25 µL of 15 mM acetylthiocholine chloride; 125 µL of 3 mM Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid) or DTNB). Reaction for 30 minutes at 37°C. Read the absorbance of the reaction at 410 nm by using a Microplate reader (Biotek, USA). The percentage of enzyme inhibition activity was calculated by the formulation: Inhibition (%) = $[(Od_{sample\ well} - OD_{blank\ well})/(OD_{positive\ control\ well} - OD_{blank\ well})]$x100. The value of $IC_{50}$ was calculated by the percent value of enzyme active level that correlates with different concentrations of the test substance.

**2.3. Data analysis**

The data were presented as mean values ± standard deviation (STD).

**2.4. Time and place of study**

This research was conducted from August 2017 to March 2018 at the Institute of Materials Sciences, Institute of Chemistry, Institute of Biotechnology - Vietnam Academy of Science and Technology.

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## 3. RESULTS AND DISCUSSION

### 3.1. Cytotoxic effect

Therapy using nanoparticle is an emerging method of treating cancer and other inflammatory disorders. The National Cancer Institute in the USA has recognized nanotechnology as a potential field in the modern medical revolution to detect, treat and prevent cancer (Nair *et al*., 2010). The results in Table 1 shown that both black garlic and black garlic nanoparticles were positive for tested cancer cell lines, except breast cancer cells. This showed that the positive effects of black garlic and its nanosized system in the treatment of cancer in-vitro. Besides that, when nanotechnology was applied, drug particles are easy to contact and destroy cancer cells. The rate of inhibition of mouth epidermal carcinoma cancer cells, liver cancer, lung cancer, monkey kidney carcinoma cancer and skin cancer which increased significantly were 43-42.4%, 16.7-85.6%, 8.50-47.8%, 10.6-36.7% and 2.98-54.3%, respectively, when tested by black garlic nanoparticles. In particular, the black garlic nanoparticles system has a very good effect in inhibiting liver cancer cells up to 85.6%.

***Table 1.*** *Bioactivity of reagents on cancer cell lines*

| Reagents | Inhibited value (%) on cancer cells at a concentration of 20 times diluted | | | | | |
|---|---|---|---|---|---|---|
| | Mouth epidermal carcinoma KB | Liver Hep G2 | Lung LU-1 | Breast MCF-7 | Vero monkey kidney carcinoma | Skin SK-Mel 2 |
| Black garlic nanoparticles | 42.4±3.1 | 85.6±4.5 | 47.8±2.7 | 0 | 36.7±5.7 | 54.3±8.4 |
| Black garlic | 5.43±0.34 | 16.7±1.8 | 8.50±0.67 | 0 | 10.6±0.9 | 2.98±0.21 |
| Ellipticine | 98.3 | 95.6 | 93.2 | 90.2 | 94.7 | 94.6 |

### 3.2. Antibiotic activity

The results of testing antibiotic activity of black garlic and its nanoparticles system were shown in Table 2. Black garlic samples did not present bioactivity against *Staphylococcus aureus*, *Enterococus faecium*, *E. Coli* and *Candida albicans* at ≤256 µg/mL. While the black garlic nanoparticles system showed bioactivity on seven tested microbial strains. At a concentration of 256 µg/mL, the black garlic nanosize system was inhibited *Staphylococcus aureus* and *Enterococus faecium*. The next, concentrations of 64 µg/mL and 16 µg/mL showed inhibition with *Lactobacillus fermentum*, *Pseudomonas aeruginosa* and *Bacillus subtilis*, *E. Coli*, *Candida albicans*, respectively.

***Table 2.*** *Antimicrobial activity of black garlic and black garlic nanoparticles*

| | | | Reagents | |
|---|---|---|---|---|
| | | | Black garlic nanoparticles | Black garlic |
| $IC_{50}$ value (%) | Gram-positive bacteria | *Staphylococcus aureus* | >64 | >256 |
| | | *Bacillus subtilis* | >4.0 | >64 |
| | | *Lactobacillus fermentum* | >16 | >64 |
| | | *Enterococus faecium* | >64 | >256 |
| | Gram-negative bacteria | *E. Coli* | >4.0 | >256 |
| | | *Pseudomonas aeruginosa* | >16 | >64 |
| | Mold | *Candida albicans* | >4.0 | >256 |

### 3.3. Inhibiting activity of enzyme α-glucosidase and acetylcholinesterase

#### *3.3.1 Enzyme α-glucosidase*

Diabetes is a metabolic syndrome that continuously affects the physiological system of the human body related to enzyme α-glucosidase. It is also one of the leading causes of death worldwide, and if left unchecked, it can threaten the multiple organ system (Zakir *et al*., 2008).

***Table 3.*** *Inhibiting activity enzyme α-glucosidase of black garlic and its nanoparticles system*

| Reagents | Method (1) | Method (2) |
|---|---|---|
| | Inhibition (%) | $IC_{50}$ (µg/mL) value |
| Black garlic nanoparticles | 33.2 | >16 |
| Black garlic | 13.4 | >256 |
| Acabose | 97.3 | 180 |

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Uncontrolled blood glucose is known to be the key features in the onset of type 1 and type 2 diabetes difficulties. Table 3 showed that the ability to inhibit α-glucosidase in both test methods (1) and (2). For method (1), black garlic nanoparticles system inhibited 33.2% higher than black garlic 13.4%. Besides that, with method (2), black garlic did not inhibit α-glucosidase at ≤ 256 μg/mL. At the same time, the black garlic nanoparticles system inhibited it at concentrations ≥ 64 μg/mL.

***3.3.2. Enzyme AChE***

Cognitive decline, dementia are the onset of dementia and the most common form of Alzheimer's disease associated with AChE, is devastating forms of damage that can occur during aging. This disease accounts for more than 70% of all causes of dementia (Seshadri *et al*., 2002). In the past decade, a growing number of data suggest that vascular factors play an important role in Alzheimer's disease and those with risk factors for cardiovascular disease and a history of stroke elevation in both vascular dementia and Alzheimer's disease (Kivipelto *et al*., 2001). The results in Table 4 showed that both black garlic and black garlic nanosystems were not able to inhibit AChE at concentration ≤ 128 μg/mL.

***Table 4.*** *Inhibitory activity enzyme AChE of black garlic and its nanoparticles system*

| Reagents | $IC_{50}$ (μg/mL) value |
|---|---|
| Black garlic nanoparticles | >128 |
| Black garlic | >128 |
| Doneperil | 0.038 |

## 4. CONCLUSION

Black garlic nanoparticles system is positive for cancer cell lines (except breast cancer cells). In particular, the ability to cause hepatocellular toxicity of black garlic nano system is more than 80%. Black garlic did not show that antibacterial activity against *Staphylococcus aureus*, *Candida albicans*, *E. Coli* and *Enterococus faecium* at 256 μg/mL concentration. However, the black garlic's nano-size expressed antimicrobial activity on all tested microbial strains. It could inhibit the enzyme α-glucosidase that causes diabetes which had not effect on AchE - causes Alzheimer's disease. Black garlic nano-materials have demonstrated that nanotechnology will be a promising technology for wider applications. Therefore, complementary and alternative medicine practices with black garlic extracts or its nano-size as a means of decreasing the burden of drug resistance and reducing the cost of the management of disease, could be of clinical and public health importance.

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