Original Article

Biological Activities of Black Garlic Fermented with *Lactobacillus plantarum* PN05 and Some Kinds of Black Garlic Presenting Inside Vietnam

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ABSTRACT

Background: Black garlic has become more popular in Vietnam due to its interesting biological activities. **Purpose:** The quality of different black garlic preparations should be checked. **Methods:** 10^{10} colony forming units (CFUs) of *Lactobacillus plantarum* PN05 were mixed with 1 kg fresh garlic and then let turn into black garlic. The aqueous extract of this preparation and other black garlic products marketed in Vietnam were evaluated on antimicrobial, anticancer cell line activities and toxicity by using well agar diffusion, sulforhodamine B (SRB) assay and tissue observation respectively. **Results:** The tested black garlic extracts showed significant differences in the antimicrobial activities against *Salmonella* and *Listeria monocytogenes*. Moreover, different kinds of black garlic extracts showed different effects against human liver cancer cell line (HepG2). Surprisingly, higher amount of black garlic extracts showed the lower anti-HepG2 activity in some samples except extract of black garlic fermented with *Lactobacillus plantarum* PN05. However, mice used black garlic extracts could give the spleen to be larger significantly than control mice. **Conclusion:** The study warned human to be careful when using black garlic without any check.

Key words: Black Garlic, *Lactobacillus plantarum* Pn05, Antimicrobial Activity, Anticancer Activity, Spleen Toxicity.

INTRODUCTION

For a long time, garlic (*Allium sativum L.*) has been used as a basic ingredient in cooking as well as a medicinal substance. Garlic exhibits various pharmacological effects such as antimicrobial, antioxidant, antitumor, and immunomodulatory activities.[1-7] However, garlic consumption is still limited because of its strong flavor and odor. Moreover, fresh garlic causes some adverse effects including gastrointestinal disorder, hemolytic anemia and allergic reactions.[8-10]

Recently, a preparation of garlic called black garlic was marketed widely. It is produced by heating whole garlic bulbs at high temperature (70°C) and high humidity (90%).[11,12] Throughout this process, the white fresh garlic turns into a black color caused by Maillard and Browning reaction. Unlike fresh garlic, black garlic has a sweeter taste as a fruit so that it can be used easily. Particularly, there is a significant increase in the amounts of S-allyl cysteine (SAC), amino acids, flavonoids, and polyphenols, etc. in black garlic. SAC is one of the most bioactive compounds and plays an essential role in black garlic's pharmacological effects. Raw garlic only contains 20-30 μg/g of SAC[13] while the amount of SAC in black garlic is higher five to six times.[14,15] Compared to fresh garlic, black garlic showed stronger antioxidant and free radical scavenging ability.[16] Black garlic also had antimicrobial activity against some common pathogens such as methicillin-resistant *Staphylococcus aureus*

Submission Date: 22-01-2017;
Revision Date: 02-03-2017;
Accepted Date: 10-04-2017

DOI: 10.5530/ijper.51.4.99

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(MRSA), *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans* but this activity was lower than that of fresh garlic.[17] Besides, black garlic exhibited strong anti-tumor and anticancer action. Black garlic could inhibit the growth of many human cancer cell lines such as SCG-7901 gastric cancer cell and HT29 colon cancer cell.[18-19] Furthermore, black garlic has been reported to have a better immunomodulatory effect than raw garlic.[20]

Nowadays, foodborne infection has become the health problem that could be caused by *Salmonella* and *Listeria monocytogenes* commonly. Human often infected with these bacteria by consuming contaminated food or water. In recent, antibiotic resistance to these foodborne pathogens has been increased. It is essential to find new antimicrobial agents, especially those were isolated from natural sources.

According to WHO, liver cancer is one of the most common forms of cancer in the world, in which hepatocellular carcinoma is the most prevalent, accounts for 85-90% of primary liver cancers.[21] Until now, no study has been reported the effect of black garlic on HepG2 (human hepatocellular liver carcinoma cell line) so that this cell line was selected to perform the anticancer activity assay.

Due to black garlic is the results of oxidation of fresh garlic, probably, the quality of black garlic depends on the oxidation process. Because of *Lactobacillus plantarum* PN05 derived from *Coriandrum sativum* plant as well as giving the antioxidant activity based on exopolysaccharide of *Lactobacilli* group, *L. plantarum* was used in black garlic preparation to more understand the black garlic quality.

In Vietnam, black garlic is used extensively although the interesting biological activities are still suspected. There are various brands of black garlic appearing in the market without any quality information. The aim of this study was to examine the quality of different kinds of commercial black garlic in Vietnam and black garlic prepared with *L. plantarum* about their antimicrobial and anticancer cell line activities and toxicity.

MATERIALS AND METHODS

Experimental chemicals and instruments

Four types of black garlic were purchased from different manufacturers in the local market such as black garlic prepared by Vietnam Military Medical University named VMMU, Linh Dan black garlic prepared by Food and Nutrition Research Center (LD), black garlic produced by Ca Tha company (UMA), and Dong A black garlic of Dong A pharmaceutical company (DA) were used to extract for biological tests. Our LP prepared by mixed with *Lactobacillus plantarum* (*L. plantarum*) PN05 isolated from *Coriandrum sativum* was used in the study.[22] Fresh garlic was purchased by Ly Son company (Thu Duc, Ho chi minh, Vietnam).

Lactobacilli MRS broth, sodium chloride (Merck, Germany), standard ceftriazone, tetracycline, mice (Institute for Drug Quality Control, Ministry of Health, Vietnam), penicillin, streptomycin, tricloroacetic acid, tris base, dimethyl sulfoxide (Sigma, USA), yeast extract and peptone (Santa cruz, USA), HepG2 cancer cell line supplied by University of Science, Vietnam National University Ho Chi Minh City (Vietnam), minimum essential medium (MEM), fetal bovine serum (FBS), centrifugator (Kubota, Japan), optical microscope (Labomed, USA), pH meter (Schott, Germany), ten different strains of gram-negative *Salmonella* and four strains of gram-positive *Listeria monocytogenes* obtained from Center of Analysis Services and Experimentation in Ho Chi Minh City (CASE, Vietnam), oven (Memmert, Germany), CO_2 incubator (New Brunsik, USA), freeze dryer (Labcono, USA), enzyme-linked immunosorbent assay plate reader (Molecular Devices, Sunnyvale, CA, USA) were used in the study.

Preparation of black garlic in the supplement of *Lactobacillus plantarum* PN05

L. plantarum was cultured to prepare a stock containing 10^8 colony forming units (cfu)/ml. To prepare black garlic, 1 kg of fresh garlic was mixed with *L. plantarum* (10^{10} cfu). The mixture was heated at 70°C in the humidity (90%) for about 90 days. The black garlic prepared in the study was named LP.

Preparation of black garlic extracts

All black garlic samples were extracted with distilled water according to an optimal ratio (1:3) at 60°C for 72 h. The extracted solutions were centrifuged at 8,000 rpm for 15 minutes. The supernatants were filtered through filter papers and then freeze-dried. Fresh garlic extract was also prepared in the study to compare its bioactivities with black garlic extracts. The extraction yield is calculated following equation:

$$\text{Yield}\ (\%) = \frac{Wf}{Wi} \times 100,$$ where W_f is the dry weight of extract after freeze-drying and W_i is the initial weight of the raw material.

Experimental animals

All animals were housed in an air-conditioned room and given standard diet. Mice were kept for one week before starting the experiment for adaption. All the procedures were followed the rules of Hochiminh City International

university. The rules forced that mice shouldn't be pained in the study. All mice should be enthusiazed before surgery.

Antimicrobial activity assay

Salmonella strains were cultured in Luria broth (LB) medium while *Listeria* strains were cultured in *Listeria* medium to obtain bacterial suspension (10^8 cfu/ml).

The antimicrobial activities of black garlic extracts were determined by applying agar well diffusion method. Briefly, 100 µl of each bacterial suspension was spread on the surface of LB agar plate using sterile cotton swab. The wells were made in the agar using a sterile cork borer (8 mm in diameter). Then, 100 µl of black garlic extracts (300 mg/ml) was added to each well. All the plates were incubated at 37°C. The inhibition zones were measured after 16-18 h.

To evaluate the potency and efficacy of the antimicrobial activities of black garlic extracts, the references those were ceftriaxone against *Salmonella* and tetracycline against *Listeria monocytogenes* were used in the tests. Sterile distilled water was used as negative control.

Anticancer cell line activity assay

The anticancer cell line activity of black garlic was determined by using SRB assay. The cells were seeded into 96-well plates in growth medium (10^4 cells/well). After 24 h incubation, the medium was replaced with fresh growth medium containing different concentrations of the extracts. The cells were incubated for a further 48 h and then fixed with trichloroacetic (TCA) (10%) for 1 h at 4°C. The TCA treated cells were washed five times with water and dried in air. The SRB solution (50 µl) was added to each well at room temperature for 10 min. The wells were washed five times with acetic acid (1%) to remove unbound dye and dried in air. The bound dye was dissolved in 200 µl of tris base (10 mM) for 10 min on a shaker and measured at the absorbance (520 nm) using a microplate reader. The negative control used in the tests were DMSO. The cytotoxicity was evaluated based on the percentage of viable cells determined by the base on the difference of the optical density of experimental group and control group with the optical density of control group.

Toxicity test

Mice were administered with the extracts at a dosage of 10 mg/0.1ml/day until they were administered to 20 mg/ml, 75 mg/ml and 100 mg/ml. One mice group received distilled water were used as control. After 7 days and 14 days of the treatment, liver and spleen tissues were collected, weighted and observed.

Statistical analysis

All experiments were carried out in triplicate. The results were expressed as mean ± standard deviation (SD). Statistical analyses were performed using SPSS 16.0 software.

RESULTS AND DISCUSSION

Determination of extraction yield

The extraction yields (%) of different extracts were presented in Figure 1. Two extracts of LD and UMA showed highest extraction yield and equally around 61%. The lowest yield in black garlic extraction was observed in DA, which was 42.00±1.50%. The yield of LP (56.00 ± 0.87 %) was higher than DA, however, it was still lower when compared to other kinds of black garlic extracts. Yield of fresh garlic extract was only 26.50 ± 0.87%, lower than that of black garlic extracts. Probably, there were many compounds in fresh garlic converting into water soluble compounds in black garlic that were not focused before.

Antimicrobial activities

The results of antimicrobial activities of different extracts were described in Table 1 and Table 2. As be presented, all tested samples were effective against both gram-negative *Salmonella* (Table 1) and gram-positive *Listeria monocytogenes* (Table 2). However, their activities were lower than fresh garlic extract (Table 1, Table 2). LP and DA gave the higher antimicrobial activities than VMMU, LD and UMA. UMA had the lowest antimicrobial effects against these bacteria. It was meant that it should be studied well in fermentation of fresh garlic so that black garlic will give the higher antimicrobial activities. Probably, the antimicrobial agents in fresh garlic were spoiled in fermentation. Otherwise, the antimicrobial agents in fresh garlic can be converted into the compounds without activities. In the study, it took three months to let LP turn black completely, suggesting that the slower

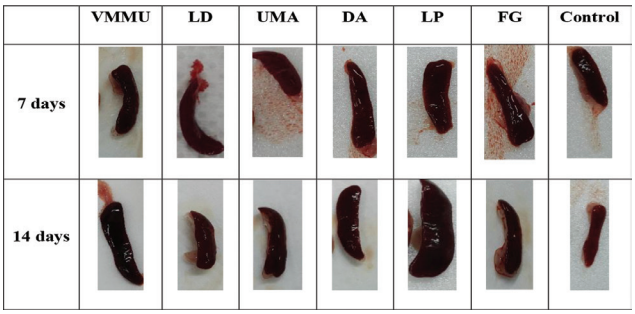

Figure 1: Spleen tissues of mice which administrated different extracts.

Table 1: Potency of antimicrobial activity of different extracts to the activity of ceftriaxone against *Salmonella*

Pathogens	Equivalent concentration of ceftriaxone (µg/ml) to concentration of the extracts (300mg/ml)					
	VMMU	LD	UMA	DA	LP	Fresh garlic
Sal1	0.39±0.02	0.52±0.04	0.36±0.03	0.71±0.06	0.93±0.12	0.75±0.03
Sal2	0.40±0.03	0.62±0.04	0.35±0.02	0.64±0.02	0.74±0.07	0.63±0.04
Sal3	0.61±0.08	0.55±0.05	0.39±0.04	0.65±0.05	0.77±0.01	0.88±0.09
Sal4	0.41±0.03	0.45±0.05	0.29±0.02	0.57±0.06	0.54±0.04	0.49±0.02
Sal5	0.58±0.03	0.82±0.04	0.53±0.03	1.13±0.11	1.25±0.06	1.36±0.08
Sal6	0.59±0.09	0.65±0.03	0.43±0.04	0.76±0.09	0.93±0.03	0.71±0.06
Sal7	0.43±0.03	0.60±0.06	0.30±0.04	0.48±0.02	0.71±0.05	0.63±0.05
Sal8	0.44±0.02	0.66±0.07	0.39±0.06	0.70±0.03	0.86±0.08	0.98±0.09
Sal9	0.31±0.01	0.39±0.03	0.30±0.02	0.46±0.02	0.58±0.04	0.44±0.03
Sal10	0.39±0.03	0.49±0.05	0.36±0.03	0.63±0.03	0.79±0.06	0.91±0.07

Values were expressed as mean ± standard deviation.

Table 2: Potency of antimicrobial activity of different extracts to the activity of tetracycline against *Listeria monocytogenes*

Pathogens	Equivalent concentration of tetracycline (µg/ml) to concentration of the extracts (300mg/ml)					
	VMMU	LD	UMA	DA	LP	Fresh garlic
L17	2.18±0.28	4.23±0.49	1.32±0.15	2.18±0.28	5.32±0.61	6.14±1.28
L19	2.01±0.25	3.01±0.46	0.89±0.25	3.09±0.43	9.59±0.13	5.56±0.79
L20	1.78±1.03	3.40±0.24	0.94±0.07	2.95±0.23	7.14±1.85	5.92±1.02
L21	1.61±0.53	3.45±0.75	1.29±0.35	1.57±0.64	7.54±0.67	6.49±0.57

Values were expressed as mean ± standard deviation.

fermentation will make the antimicrobial agents in garlic more stable. The reason why LP was prepared for three months that *L. plantarum* PN05 might produce compounds to slow fermentation.

All the extracts showed similar antimicrobial properties against these strains of *Salmonella*, except for Sal 8 (Table 1). It was less sensitive to all black garlic extracts as well as fresh garlic extract. According to Truong *et al*. Sal 8 had its highest resistance to most antibiotics.[23] Therefore, the study opened a potential orientation for resistant mechanism in *Salmonella* in the future.

In order to study the potency of antimicrobial activities of black garlic extracts, antibiotics including ceftriaxone and tetracycline were used as the references. Because all *Salmonella* strains were still sensitive to ceftriaxone, ceftriaxone was used to study on the potency of black garlic extracts on *Salmonella*. Otherwise, *Listeria* strains were resistant to ceftriaxone, but they were sensitive to tetracycline. Therefore, tetracycline was used to study on the potency of black garlic extracts on *Listeria monocytogenes*. The standard curves of antimicrobial activities of antibiotics were established according to the diameter of inhibition zones and antibiotic concentrations. The equivalent concentrations of antibiotics to the antimicrobial activity of different extracts were calculated from standard curves.

Anticancer cell line activities

The cytotoxicity percentage of different extracts at concentration of 100 mg/ml against HepG2 cell line were presented in Table 3. The results showed that with the dose (100 mg/ml), VMMU exhibited the highest action against HepG2, which was 91.28 ± 5.88 % while LP gave the lowest activity (16.54 ± 7.84 %). LD, UMA and DA showed the cytotoxicity effects more highly than LP at this dose. However, the cytotoxicity actions in LD, UMA, DA and LP increased when the dose reduced (Table 3). Especially, LP could show the strongest cytotoxicity (92.28 ± 1.56%) at the lowest dose (25 mg/ml). Perhaps, some compounds in black garlic extracts showed antagonistic action on the cytotoxicity. Therefore, it was important to optimize the best conditions for garlic fermentation. Fresh garlic showed the lowest activity (9.16 ± 4.44 %), pointing that there were many compounds in fresh garlic converted to

Table 3: The cytotoxicity on HepG2 of black garlic extracts			
	Cytotoxicity percentage (%)		
	Extract concentration (100 mg/ml)	Extract concentration (75 mg/ml)	Extract concentration (25 mg/ml)
VMMU	91.28±5.88	Unidentified	Unidentified
LD	67.44±6.08	69.67±2.45	77.96±2.57
UMA	74.83±4.32	75.85±3.07	86.00±1.78
DA	68.22±2.19	70.01±3.17	79.24±6.47
LP	16.54±7.84	58.64±6.40	92.28±1.56

Values were expressed as mean ± standard deviation.

Table 4: Weight of mice spleen administrated different concentration of VMMU extract				
	Weight of spleen (g)			
	VMMU extract concentration (mg/ml)			Control
	100mg/ml	75mg/ml	25mg/ml	
7 days	0.30±0.05	0.40±0.13	0.28±0.09	0.24±0.04
14 days	0.29±0.06	0.28±0.12	0.27±0.05	0.23±0.08

Values were expressed as mean ± standard deviation.

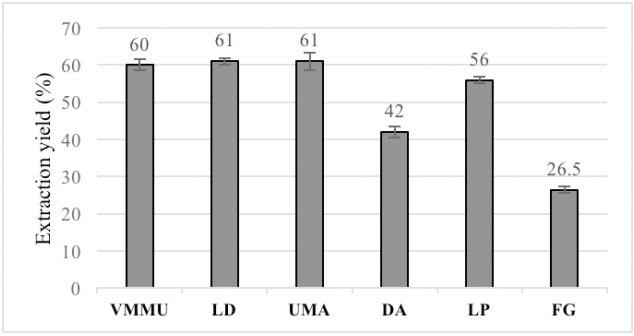

Figure 2: Extraction yield of black garlic and fresh garlic extracts.

their derivatives showing cytotoxicity in black garlic, like cysteine changing to S-allyl-L-cysteine. However, there might be have some cytotoxic agents those were antagonistic together. The reasons why VMMU showed higher activity when the dose (100 mg/ml) was higher than the other black garlic samples were still unclear. However, by using *L. plantarum* in fermentation, the cytotoxicity action could obtain to 92.28 ± 1.56 % at the low dose (25 mg/ml) that could be safer for use.

Tissue observation

To know which dose was safer for use, mice spleen and liver observation were checked. There was no change in liver. However, the weight of mice spleen administrated different concentration of VMMU became larger than mice spleen without using black garlic extracts (Figure 2, Table 4). In the study, fermentation of black garlic supplemented with *L. plantarum* PN05 could give cytotoxicity against HepG2 highly when using low dose that is safer in use.

CONCLUSION

The study showed the different antimicrobial levels of different black garlic extracts when comparing to fresh garlic extract. Also, the study showed that the cytotoxicity actions on HepG2 depending on the black garlic preparation. Moreover, mice spleens became larger than normal ones, leading to problem in immunological response. However, black garlic prepared by mixing with *L. plantarum* PN05 gave higher activities against *Salmonella* and *Listeria* strains that could be used in replacement therapy for these strains in cases of resistance to many antibiotics. Moreover, LP showed the higher cytotoxicity at the low dose (25 mg/ml) than the higher dose (75 mg/ml and 100 mg/ml), leading to the safer use. We should take consideration on conditions for producing black garlic to get good biological activities with the toxicity reduce.

ACKNOWLEDGEMENT

Thanks for the fund supported by Hochiminh City International University (HCM IU) for the research (SV2015-BT-16) signed in the contract (09-BT-2015/HDSV-QLKH).

CONFLICT OF INTEREST

There is no conflict in all authors.

ABBREVIATION USED

DA: Dong A; **LD:** Linh Dan; **VMMU:** Vietnam Military Medical University; **LP:** *Lactobacillus plantarum* product; **L. plantarum:** *Lactobacillus plantarum*; **WHO:** World Health Organization.

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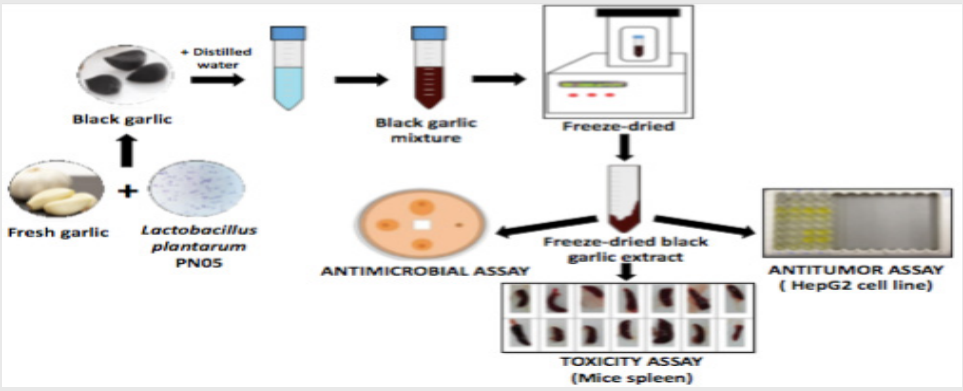
PICTORIAL ABSTRACT



SUMMARY

- *L. plantarum* (10^{10} cfu) was mixed with 1 kg of fresh garlic and heated at 70^{0}C in the humidity (90%) for about 90 days to produce the black garlic named LP.
- The LP aqueous extract and other aqueous extracts from DA, LD and VMMU were freeze-dried.
- The freeze-dried extracts were used to test the antimicrobial activities, the cytotoxicity on HepG2 and the mice liver and spleen.
- LP gave the higher activities against *Salmonella* and *Listeria* strains.
- LP showed the higher cytotoxicity at the low dose (25 mg/ml) than the higher dose (75 mg/ml and 100 mg/ml), leading to the safer use.
- The aqueous extracts didn't show effect on liver, but enlarged the mice spleens.
- We should take consideration on conditions for producing black garlic to get good biological activities with the toxicity reduce.

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Cite this article: Nguyen N, Giang M, Nguyen Tu. Biological Activities of Black Garlic Fermented with Lactobacillus plantarum PN05 and Some Kinds of Black Garlic Presenting Inside Vietnam. Indian J of Pharmaceutical Education and Research. 2017;51(4):672-8.