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Research article

Methanol extract of black garlic protects against hyperuricemia by xanthine oxidase inhibitory activity *in-vitro*

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ABSTRACT

Garlic (Allium sativum L.) has been used as a medicative food for a long time. However, some individuals are averse to raw garlic owing to its pungent taste and aroma. As a result, a variety of garlic formulations have been created to minimize these characteristics without reducing their functions. Extensive in vitro and in vivo research has shown that black garlic has several benefits, including anti-oxidant, anti-inflammatory, anti-cancer, anti-obesity, anti-diabetic, anti-allergic, cardioprotective, and hepatoprotective characteristics. However, it remains unclear which compounds are active ingredients in the extract and the contributions of the main identified compounds to the extract's XO inhibitory activity are unknown. This study aims to look into the potent XO inhibitors found in black garlic methanol extract. The research methodology is experimental, involving the inhibition of xanthine oxidase activity, specifically measuring the amount of uric acid formed during the reaction catalyzed by xanthine oxidase. This is accomplished in vitro via enzymatic reactions and spectrophotometric measurements. The Maillard reaction is responsible for the production of black color in garlic, and it is influenced by heating temperature, humidity, and fermentation time. Starting on the 17th day of the fermentation process, the brownish intensity of the garlic samples heated at 70 ° C and 90% humidity changed to brown. The optimum concentration is 0.3 units/mL at an optimum temperature of 30oC and an optimum pH of 8.0 at a substratum concentration of 0.30 mM at a wavelength of 284 nm. The activity of the xanthine oxidase enzyme was inhibited by allopurinol at various concentrations, with allopurinol having the highest percentage inhibition value of 78.74 percent at a concentration of 10 ppm. The present study showed that meanwhile, the highest inhibition occurred on the 29th day of fermentation, with a percentage value of 68.01 percent, and decreased the next day.

Keywords: Black garlic, Fermentation, Hyperuricemia, Xanthine oxidase.

INTRODUCTION

Globally, the prevalence of gout has gone up, maybe as a result of dietary and lifestyle changes and longer life spans [1]. A purine metabolic condition called gout is typified by the accumulation of urate crystals around the joints as a result of chronic hyperuricemia. Hyperuricemia has been associated with myocardial infarction and cardiovascular disease [2]. The World Health Organization (WHO) estimates that between 2% and 5% of people worldwide suffer from gout, with males between the ages of 40 and 50 and women before menopause being the most common populations affected. In Indonesia, the percentage of the population with gout is 1.7 percent in rural areas and 4.8 percent in urban areas [3].

The enzyme xanthine oxidase, which is present in significant quantities in the liver and digestive system, catalyzes the purine metabolism process, which converts hypoxanthine and xanthine into more uric acid [4]. Thus, one therapy option for hyperuricemia and gout should be the use of an XO inhibitor, which

limits the body's generation of uric acid [5]. Certain synthetic XO blockers, such as febuxostat, Y-700, and allopurinol, have shown notable clinical success in the management of hyperuricemia and gout [6, 7]. Nevertheless, they do have negative effects, including an increase in the toxicity of 6-mercaptopurine, dermatitis, kidney failure, allergic and hypersensitive reactions, and digestive issues [8]. As a result, academic research is beginning to focus on screening natural compounds for possible XO blockers [9, 10]. Therefore, oxidative stress levels in vivo, especially in reperfusion is chemic cells, can be significantly influenced by XO activity [11].

Hyperuricemia, a condition linked to conditions like gout and kidney stones, can also result from high uric acid levels in vivo [12]. Additionally, hepatitis (as well as mild hepatotoxicity) elevates serum levels of XO, with the concentration of XO determining the severity of brain injury and edema [13]. The involvement of XO in "thermal stress, respiratory syndrome, viral infection, and hemorrhagic shock" has also been linked in some studies [14]. Therefore, it is possible to hypothesize that xanthine oxidase activity reduction may be advantageous to health.

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However, because of its distinct flavor and odor, unprocessed raw garlic should only be used in moderation as excessive consumption can damage stomach mucosal cells. Black garlic (BG) is simply fresh garlic (Allium sativum L.) that has been fermented for some time at high temperature and high humidity. The process darkens the garlic cloves, gives them a sweet taste, and makes them chewy and jelly-like in texture (Figure 1).

The duration of fermentation varies depending on the culture, manufacturer and intended use Fermented black garlic, a type of heat-treated garlic, has been reported to have biological effects such as anti-cancer, anti-obesity, hepato protective and anti-inflammatory [20, 21]. However, BG has long been used in South Korea, Japan and Thailand for centuries, and was introduced to Taiwan and other countries about 10 years ago. In recent years, top chefs have paid close attention to BG, using it to flavor chicken, fish, soup, and risotto [22].

Compared to fresh garlic, black garlic does not emit a strong off-flavor because the content of allicin has decreased and during aging it has been converted into antioxidants such as bioactive alkaloids and flavonoid compounds [15]. Changes in physicochemical properties are the main reasons why BG has improved bioactivity compared to fresh garlic. In addition to daily consumption, several studies have reported that BG extract has several functions such as antioxidant, anti-allergic, anti-diabetic, anti-inflammatory and anti-carcinogenic. In 1990, the Designer Foods program listed garlic as one of the best cancer fighters. Although the Designer Foods program no longer exists, scientists are still looking for so-called bioactive components in various foods.

As a continuation of our screening program for plants with XO-inhibitory effects, this work reports for the first time a systematic in vitro XO-inhibition test of black garlic. The results showed that the methanol extract of black garlic has a strong anti-XO effect. However, it is still unclear which compounds are the active components of the extract, and the contribution of the main identified compounds to the XO-inhibitory effect of the extract is unknown. This study aims to investigate the effective XO inhibitors of black garlic methanolic extract. The benefits of the study would clarify and support the use of methanol extract from fermented black garlic as an additional medicine for the prevention and treatment of hyperuricemia.

MATERIAL AND METHODS

The following instruments were used in this study: a Rotary Evaporator (Eyela), a microcentrifuge (Hettich), glassware (Phyrex), an oven (Memmert), a micropipette (Socorex), a blender, a vortex, an analytical balance (O'haus), a climatic chamber, a UV-Vis Spectrophotometer (Shimadzu UV-1900i), centrifuges, glassware (Phyrex), mortar, stamps, and filter paper. The research materials used in this study were garlic from Lumajang-East Java, fermented black garlic, aqua bidestylation, methanol (Merck), hydrochloric acid, xanthine oxidase (Sigma), xantin (Sigma).

Sample Preparation

Plant determination was performed on the single garlic bulb used in this study at the Herbarium Bogoriense, Botanical Sector Biology Research and Development Center-LIPI Cibinong, Jl. Raya Jakarta - Bogor KM. 46 Cibinong 16911. Determination is carried out to ensure that the plants used in the study correspond to the desired plant types and species. The results of the determination indicated that the single garlic obtained from Lumajang-East Java and used in this study is Allium sativum L., a member of the Amaryllidaceae tribe.

Procedure

Garlic was fermented for 35 days at a temperature of 70°C and a relative humidity (RH) of 90% [16]. On days 0, 1, 5, 9, 13, 17, 21, 25, 29, 33, and 37 of fermentation, organoleptic profiles, yield and drying losses, and ash content, as well as polar and non-polar metabolites (organosulfur, flavonoids) of onions, were analyzed. Each

sample was frozen and then mashed with a blender before being placed in a freeze-dry instrument. Methanol was used to extract the freeze-dried sample.

Anti-hyperuricemia *in vitro*

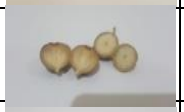
XO activity was measured using a model of Cos et al method's [25]. In summary, the assay measures the amount of uric acid that produces large amounts of XO on a xanthine substrate over a specified period. The potency of blockers can be determined by measuring changes in levels of uric acid obtained in the existence of tests performed. Each test solution contained xanthine, hydroxylamine, and EDTA in 0.2 M sodium phosphate buffer (pH 7.5). Following calculations of xanthine oxidase were kept at 5-8 °C. The reaction started when 0.2 mL XO (23.42 Milliunit/mL, 0.2 M phosphate buffer) were added and incubated for 30 minutes (37 °C). HCl was used to terminate the reaction (0.1 mL, 5 M). Uric acid levels were measured by spectrophotometer (at room temperature) at a wavelength of 290 nm. In testing the inhibition of xanthine oxidase activity, testing was carried out against allopurinol and black garlic standards.

RESULTS AND DISCUSSION

Single garlic fermentation process

The single garlic is fermented in the Center for Biotechnology Assessment and Application of Technology (BPPT-Serpong) with a temperature of 70ºC and 90% humidity in the Climatic Chamber for a period of 1 to 37 days. The samples were black garlic days 0, 1, 5, 9, 13, 17, 21, 25, 29, 33, and 37, with organoleptic differences in each of these samples (Table 1). The single white garlic becomes black during the fermentation process. In general, the Maillard reaction is responsible for the production of black color in garlic, it is influenced by heating temperature, humidity, and fermentation time. The degree of browning in the heated garlic samples increased with the duration of fermentation; the longer it was brown, the more intense the browning. On the 17th day of the fermentation process, the brownish intensity of the garlic samples heated at 70°C and 90% humidity changed to brown (Table 1).

Table 1: Results of single garlic fermentation

Fermentation time (day)	Figure	Texture	Scent	Ash content (%)	Drying shrinkage (%)
Day 0		Hard	Typical garlic	2.64	7.50
Day 1		Hard	Typical garlic	2.10	8.40
Day 5		Hard	Typical garlic	2.29	7.80
Day 5		Little hard	Typical garlic	2.76	6.50
Day 13		Little hard	Typical garlic	2.59	8.00
Day 17		Soft	Sweet	2.67	7.00
Day 21		Soft	Sweet	2.24	8.00
Day 25		Soft	Sweet	2.23	6.,90
Day 29		Soft	Sweet	**2.91**	**5.90**
Day 33		Soft a little hard	Sweet	2.43	8.10
Day 37		Soft a little hard	Sweet	1.63	6.00

The fermentation process produced a change in taste on the 17th day. This is due to the Maillard reaction, which is a non-enzymatic browning reaction that occurs when reducing sugars and primary amino acids are heated to create a dark brown color

(melanoid) substance with no pungent scent and a sweet taste [18]. As in the 17th-day sample, the distinctive garlic aroma will gradually fade and be replaced by a caramel scent.

The ash content of the sample is 1.63-2.91 percent (Table 1), indicating that it meets the standard requirements for garlic ash content, which is less than 3.0 percent. The decision was made to provide an overview of the internal and external mineral content from the beginning of the process to the formation of fermented garlic. The ash content is critical since it may indicate whether a sample is suitable for further processing. The higher the purity of a raw material and the lower the ash content of a sample, the greater the efficiency and quality.

The drying shrinkage of the sample varies from 5.90 to 8.40 percent (Table 1), meaning that it meets the garlic drying shrinkage minimum of not more than 10.0 percent. The drying shrinkage was calculated to provide a maximum limit on how much compound was lost during the drying process. Methanol 80 percent solvent was used for sample extraction. The resulting sample was refined with a blender to obtain smaller particles and extend the surface of the substance for more efficient extraction, thus promoting bioactive component solubility and increasing extraction yield. Each sample was weighed up to 200 grams, and 400 ml of 80 percent methanol was added. Sonication was then performed on the sonicator at 3000rpm centrifuge, for 25 minutes and filtered into a 500 ml bottle to obtain filtrate, for 20 minutes. The filtrate obtained during the extraction process is placed in a rotary evaporator and heated to 50ºC until thick to separate the extract from the solvent. The extract was vacuum freeze-dried after being frozen at -80ºC in the fridge (VFD).

Xanthine Oxidase Inhibition Activity Determination

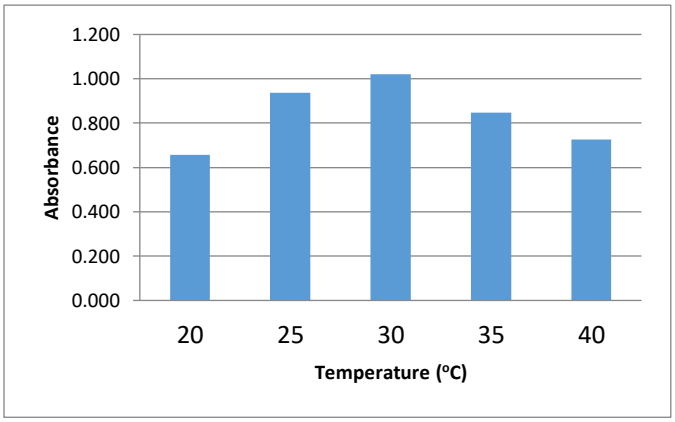
The incubation process is divided into two phases. The first stage consisted of a 10-minute pre-incubation to adjust the test solution's conditions to the optimal environmental conditions, followed by a 30-minute incubation for enzymatic reactions. This test uses the enzymes 0.1, 0.2, and 0.3 units per milliliter. The higher the absorption, the more products are made, and the enzyme activity rises even higher.

The maximum wavelength was found to be cf284 nm with an absorbance value of 1.21 when the maximum wavelength was tested with the xanthine oxidase enzyme 0.3 U / mL (Appendix 3). Although the wavelengths in each of the previous works of literature vary, the wavelengths of 268-295 nm are also used in previous studies. This is due to a variation in detection on the measuring instrument, as well as varying conditions at the time of the test, which results in different absorption.

Analysis of xanthine oxidase inhibition by UV-V is spectrophotometry, the measured absorbance shows how much uric acid production can be identified as the compound has a strong chromophore group. Chromophore groups are molecules or molecular components that absorb a large amount of light in the ultraviolet-visible region [17].

Temperatures of 20, 25, 30, 35, and 40 C were used to test the optimal temperature conditions. The optimal temperature was obtained and used for pre-incubation and incubation during sample testing. Following the measurement, the optimum condition was determined to be 30ºC with an absorbance value of 1.02, which decreased as temperature increased (Figure 1). The resultant product is defined by the absorption value. If more products are produced, the activity will be at its peak. The decrease in absorption is caused by the decomposition and denaturation of the polypeptide chains in the enzyme, which reduces the enzyme's kinetics efficiency.

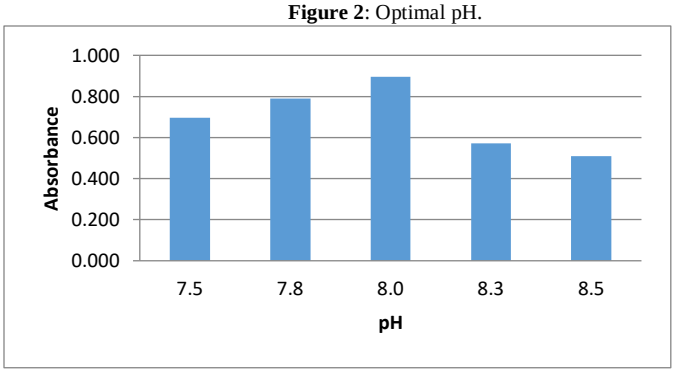
Figure 1: Optimal temperature condition.



The reaction's maximum temperature is reached the quickest. In general, the higher the temperature, the faster the reaction, both non-catalyzed and catalyzed by the enzyme [19]. The optimum temperature for achieving the highest absorbance of 1.02055 is 30°C. This indicates that the formation or reaction that occurs between the xanthine enzyme and its substrate is maximal at this temperature and that the subsequent temperature increase results in a decrease in temperature.

The test for optimizing the pH was performed with a pH difference of 7.5; 7.8; 8; 8.3 and 8.5. The optimal condition is pH 8.5, which has higher absorption than pH 7.5, 7, 8, 8, and 8.3. The pH value is used to test the inhibitory activity. When enzyme activity is quantified at various pH values, the majority of enzymes in the body perform optimally between pH 5.0 and 9.0. [19]. The optimal pH values are then calculated to determine the optimal pH range for enzyme activity. The optimum pH is 8.0, which has a maximum absorbance of 0.89504 (Figure 2).

Figure 2: Optimal pH.



The xanthine substrate concentration optimization measure, which aims to find the optimum concentration in accordance with the enzyme used, is 0.3 units/mL. The tests were conducted at a temperature of 30°C and a pH of 8.0, with substrate concentrations of 0.05, 0.1, 0.15, 0.2, 0.25, and 0.30 mM, respectively (Table 2). The highest enzyme activity was observed at a concentration of 0.30 mM and an absorbance of 1.20870. An increase in the concentration of the substrate will increase the reaction rate. If the substrate concentration (S) rises while the other conditions remain constant, the reaction rate rises to a maximum. The enzyme is saturated with the substrate at this stage. Since not all enzymes react with the substrate at concentrations below 0.30 mM, adding the substrate causes the number of enzymes to increase and the reaction speed to increase, due to the addition of the substrate until a concentration of 0.30 mM is reached at which all enzymes have reacted with the substrate

Table 2: Optimum temperature, pH, and substrate indicators

Optimum Temperature		Optimum pH		Optimum Substrate	
T (^{0}C)	**Abs.**	**pH**	**Abs.**	**Substrate concentration (mM)**	**Abs.**
20	0.657	7.5	0.695	0.05	0.23124
25	0.937	7.8	0.790	0.10	0.44707
30	1.021	8.0	0.895	0.15	0.61831
35	0.847	8.3	0.572	0.20	0.81161
40	0.726	8.5	0.508	0.25	0.99916
				0.30	**1.20870**

Note: Abs: Absorbance; Bold: optimum results; T: Temperature

In-Vitro Black Garlic

The measurement conditions were obtained at wavelengths of 284 nm, enzymes 0.3 oxidase/mL, incubating temperatures of 30^{o}C, pH 8.0, and substrate concentration of 0.30 mM based on initial test results from inhibition of xanthin oxidase activity. The tests were conducted on a blank solution, a blank control, an allopurinol comparison solution, an allopurinol control solution, a sample of black garlic, and a sample control solution.

The sample solution and allopurinol comparison were tested to determine the inhibitory ability of the enzyme activity provided by the allopurinol extract and comparison, while the sample control test and allopurinol comparison control were performed as a correction factor for the allopurinol extract and comparison. This test was conducted twice with the assistance of a UV-Vis spectrophotometer.

Allopurinol inhibited xanthine oxidase enzyme activity with many doses of medication, with allopurinol having the highest percent inhibition value of 78.74% at a concentration of 10 ppm. This is since allopurinol functions as a competitive inhibitor with a substrate-like structure. The competitive inhibitor will compete with the xanthine substrate for the active side of the enzyme, causing the enzyme activity to decrease or stop, resulting in the formation of uric acid as the product. Since allopurinol is a xanthine oxidase inhibitor, it lowers uric acid levels. There is no structural similarity between the substrate and the inhibitor in noncompetitive inhibitors, but the inhibitory effect occurs because the inhibitor binds to the allosteric site of the enzyme, thereby changing the shape of the enzyme's active site.

The inhibition of xanthine oxidase enzyme activity by black garlic at a concentration of 10 ppm with a difference in fermentation time showed a percentage of inhibition ranging from 50.09 to 68.01, indicating that all samples can be used as a support in the treatment of hyperuricemia. The highest inhibition occurred on the 29th day of fermentation, with a percentage value of 68.01, and decreased the next day. The fermentation time is the same as in previous research, which found that the rise in SAC content of 194.3 g/g was 6 times greater than that of garlic. The chemical composition of black garlic is dependent on the fermentation conditions; however, some researchers report that many valuable components of black garlic are increased during the process, particularly polyphenols, flavonoids, and certain intermediates in the Maillard reaction, all of which are known as antioxidant agents. The ability of black garlic to inhibit the activity of the xanthine oxidase enzyme, which has antioxidant activity, plays a role in preventing oxidation reactions. Oxygen serves as an electron acceptor in oxidation reactions. Antioxidants are electron donors that pass electrons to oxygen, inhibiting the xanthine oxidation reaction by preventing the substrate from binding to oxygen, resulting in the formation of uric acid as a component.

CONCLUSION

Fermented black garlic at 70^{o}C and 90% relative humidity for 29 days resulted in a 68.01% inhibition at a concentration of 10 ppm. In comparison, 10 ppm allopurinol resulted in 78.74 percent inhibition. However, the precise concentrations of XO inhibitors required to generate a positive effect in vivo have yet to be determined.

Effective dietary XO inhibitors have the ability to reduce XO inhibitory activity, potentially lowering hyperuricemia.

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Authors' Contribution

AAS (Conceptualization, extraction process, methodology, data validation, and writing of original draft).

SK (Conceptualization, check for final manuscript)

DR (Conceptualization, Investigation, data collecting from *in vitro,* check for final manuscript)

NDY (Conceptualization, Data collecting from In vitro, check for final manuscript)

AY (Check for final manuscript) Final manuscript)

Conflict of Interest : No conflict of interest

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