ABSTRACT

Antioxidant and Anti-inflammatory Effect of *Black Garlic* on LPS induced Vero cell

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Inflammation is the process that occurs in the body as an immune response due to risk stimulate. Serious damage can occur if the inflammatory response exceeds the protective anti-inflammatory effects. Black garlic (BG) is one of the natural resources that has been reported can be used as an anti-inflammatory agent. This research aimed to study the effect of aqueous extract of Black garlic as an anti-inflammatory. The methods of this research were used the extraction of BG by maceration method using aqueous as a solvent, S-allylcysteine as a potential compound was tested using HPLC technique. Antioxidant properties of BG was tested by using DPPH and H_2O_2 scavenging assays. Cytotoxicity test was done with WST-1 assay in 96-well plate. The anti-inflammatory effects of BG were evaluated in the lipopolysaccharide (LPS)- induced Vero cell. Nitric Oxide (NO) production was determined by the Griess assay, while expression of TNF- α, iNOS and COX-2 in mRNA and protein levels were measured using RT-PCR and Western blot analysis. The result showed that BG extract contains S-allylcysteine and has an ability to scavenging free radical activities. The BG aqueous extract did not show cytotoxicity on Vero cells up to 750µg/ml. LPS-induced nitric oxide production in Vero cell was reduced after BG treatment, as well as the results of RT-PCR showed that BG markedly inhibited LPS-stimulated iNOS and COX-2 gene and protein expression, as well as TNF- α. This study concluded that the administration of aqueous extract of Black Garlic has the potential as an antioxidant and anti-inflammatory by inhibiting the expression of NO, iNOS and COX-2 mRNAs.

Keywords: Black garlic, Vero cell, anti-inflammatory, TNF α, iNOS, COX-2.

CHAPTER I

INTRODUCTION

A. Background

Inflammation is a defence response to harmful stimuli. The inflammatory response must be approximately terminated to prevent tissue damage, and failure to terminate inflammation results in chronic inflammation and cellular destruction. Chronic inflammation can lead to a variety of diseases, such as atherosclerosis, rheumatoid arthritis, cancer, and allergies. During the inflammatory process, many kinds of cells are activated, and these cells secrete various pro-inflammatory molecules, including cytokines and nitric oxide (NO) (Nguyen *et al*., 2012).

Inflammation is the intricate physiological process underlying the body's defence mechanism against foreign substances and infectious agents, and the repair of consequential injury. Inflammation increases the expression of anti-inflammatory proteins during the initial immune stages and is suppressed in subjects with decreased expression of inflammatory cytokine mediators (O'Shea & Murray, 2008). If the inflammatory response exceeds the protective anti-inflammatory effects, serious damage can occur. The accumulation of this damage throughout life has been associated with aging and age-dependent diseases such as cardiovascular disease, cancer, neurodegenerative disorders, and other chronic conditions (Wang *et al*., 2010).

On the other hand, Oxidative stress can also activate a variety of inflammatory mediators involved in several chronic diseases. Clinical evidence suggests that oxidative stress and inflammation linked to overproduction of ROS are likely to represent an important component for the development of several diseases including inflammation-associated chronic diseases (T. Hussain *et al*., 2016). These ROS, especially those derived from mitochondria, stimulate the activation of mediator signaling molecules as the transcription factor nuclear factor kappa-B (NF-κB) (Buelna-Chontal & Zazueta, 2013), that up-regulates the production of inflammatory cytokines, such as interleukin-1β (IL-1β) or tumor necrosis factor-α (TNF-α) (Naik & Dixit) and others mediators, as iNOS or cyclooxygenase-2 (COX-2) (K.-M. Lee *et al*., 2004).

Moreover, ROS can damage cellular lipids, lipid peroxidation products, and lipid-derived aldehydes as malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE), and acrolein, which are implicated in numerous oxidative stress-induced inflammatory diseases with harmful effects (Yadav & Ramana, 2013). Proteins and DNA may also be damaged by ROS; the DNA damage can cause mutations and is implicated in the initiation and/or promotion of inflammation-mediated carcinogenesis (Ohnishi *et al*., 2013). NF-κB is activated by an important type of membrane receptor that is related to oxidative stress and inflammation, and whose regulation is the mechanism of action for certain antioxidant molecules to inhibit the inflammatory process. These membrane receptors are the toll-like receptor 4 (TLR-4) (Medzhitov, 2001).

Lipopolysaccharide (LPS / endotoxin), the main component of the outer membrane of Gram-negative bacteria, can lead to a variety of inflammatory reactions, including the release of a variety of inflammatory cytokines, such as tumor necrosis factor (TNF) -α and interleukins (ILs) and pro-inflammatory mediators such as nitric oxide (NO) and prostaglandin E2 (PGE2), which are produced by induced nitric oxide synthetase (iNOS) and cycloxygenase (COX-2) respectively; these factors participate in the main cytotoxic and pro-apoptotic mechanisms involved in the innate response in macrophages (Chun *et al*., 2012).

Nitric oxide is known as neurotransmitter in central nervous system and a potent vasorelaxant regulating the blood pressure in vascular system, and antimicrobial reagent against bacterial pathogen. Moreover, excess amount of NO produced by inducible nitric oxide synthase (iNOS) and its derivatives, such as nitrogen dioxide and peroxynitrite, is implicated in pathogenesis of septic shock, inflammation and carcinogenesis (Nicholas *et al*., 2007). iNOS is widely expressed in various cells, including vascular smooth muscle cells, hepatocytes and kupffer cells, and is highly expressed in LPS-activated macrophages (Nathan and Xie 1994). Prostaglandin (PG) produced at inflammatory sites by inducible cyclooxygenase-2 (COX-2), is a precursor of a wide group of biological active mediators such as prostaglandin E2 (PGE2), prostacyclin, and thromboxane A2. Over-expression of COX-2 occurs in certain epithelial cancer tissue. COX-2 is also markedly expressed in inflammatory cells stimulated by LPS, pro-inflammatory cytokines and tumor promoters (Ohshima & Bartsch, 1994).

Vero cells are one of the most common mammalian continuous cell lines used in microbiology, and molecular and cell biology research. Derived from the kidney of an African green monkey (Cercopithecus aethiops) in the 1960s, This anchorage-dependent cell line has been used extensively in virology studies, but has also been used in many other applications, including the propagation and study of intracellular bacteria (e.g., Rickettsia spp.; UNIT 3A.4) and parasites (e.g., Neospora), and assessment of the effects of chemicals, toxins and other substances on mammalian cells at the molecular level. In addition, Vero cells have been licensed in the United States for production of both live (rotavirus, smallpox) and inactivated (poliovirus) viral vaccines, and throughout the world Vero cells have been used for the production of a number of other viruses, including Rabies virus, Reovirus and Japanese encephalitis virus (Ammerman *et al*., 2008)

The use of herbal medicines is becoming an increasingly attractive approach for treating various inflammatory disorders. Indonesia is abundant with natural resources, including the diversity of plants. More than 30,000 plant species are found there in where 9,600 species are medicinal plants (SUHARNO, 2016). Over than 1,800 plant species known exist and were planted in several forest formations, with 940 plant species used by local people for traditional herbal medicine only 300 species by drug industries (PUTRI *et al*., 2016),

Plants occupy a unique position on this planet because they are the foundation of life on Earth. They are the main producers of all food chains. Plants directly supply 90% of human calories and 80% of the amount of proteins. Plants are used as a potential source of the drug for immemorial time, the current millennium sets the goal of free treatment for side effects with antibiotics. Global interest in traditional medicine has gained importance as several plants have been successfully cited in treating diseases. (Farnsworth *et al*., 1985)

Plants of the Genus Allium are known for their production of organosulfur compounds, which process interesting biological and pharmacological properties.

Among these, garlic (Allium sativum) is one of the most widely used ones. When extracted and isolated, these compounds exhibit a broad spectrum of beneficial effects against microbial infections as well as cardioprotective, anticancerogenic, and anti-inflammatory activity(Salman *et al*., 1999). The benefits of garlic to health have been proclaimed for centuries; however, only recently have Allium sativum and its derivatives been proposed as promising candidates for maintaining the homeostasis of the immune system. The complex biochemistry of garlic makes it possible for variations in processing to yield different preparations with differences in final composition and compound proportion (Arreola *et al*., 2015).

The most recent experimental results, which indicate that garlic appears to enhance the functioning of the immune system by stimulating certain cell types, such as macrophages, lymphocytes, natural killer (NK) cells, dendritic cells, and eosinophils, by mechanisms including modulation of cytokine secretion, immunoglobulin production, phagocytosis, and macrophage activation. Finally, because immune dysfunction plays an important role in the development and progress of several diseases, we critically examined immunoregulation by garlic extracts and compounds isolated, which can contribute to the treatment and prevention of pathologies such as obesity, metabolic syndrome, cardiovascular disorders, gastric ulcer, and even cancer (Jung *et al*., 2013).

Although garlic (Allium sativum L.) has been used for remedies and food for more than a thousand years, most people used garlic based on their experiences without any knowledge about relationships between biological activities and constituents of garlic or its transformation products (Block *et al*., 1992). The discovery of allicin in 1944 ignited intense investigation of the sulfur containing compounds in garlic and its unique sulfur chemistry (Cavallito *et al*., 1944). Numerous sulfur-containing compounds derived from garlic have since been identified, and their biological activities have been investigated (Koch et al., 1996).

Besides garlic, there is also black garlic which is obtained from fresh garlic (Allium sativum L.) that has been fermented for a period of time at a controlled high temperature (60-90C) under controlled high humidity (80-90%). When compared with fresh garlic, black garlic does not release a strong offensive flavor owing to the reduced content of allicin. Enhanced bioactivity of black garlic compared with that of fresh garlic is attributed to its changes in physicochemical properties (yuan *et al*., 201).

To find out the potential of black garlic as an anti-inflammatory, there will be a study of the potential of black garlic as anti-inflammatory in Vero cell.

B. Research Problems

1. Is the aqueous extract of black garlic contain S-ally cystine compound?
2. Is the aqueous extract of black garlic able to scavenge free radical of DPPH and hydrogen peroxide (H2O2)?
3. Is the aqueous extract of black garlic toxic for the Vero cells?
4. Is the aqueous extract of black garlic able to inhibit nitric oxide (NO) production in LPS-induced Vero Cells?
5. Is the aqueous extract from Black Garlic inhibit iNOS, COX-2 and TNF-α in LPS induced Vero cells?

C. Research Objectives

1. To know if the aqueous extract of black garlic contains S-ally cystine compound.
2. To know if aqueous extract of black garlic able to scavenge free radical of DPPH and hydrogen peroxide (H2O2).
3. To know if the aqueous extract of black garlic toxic for the Vero cells.
4. To know if the aqueous extract of black garlic able to inhibit nitric oxide (NO) production in LPS-induced Vero Cells.
5. To know if the aqueous extracts from Black Garlic inhibit iNOS, COX-2 and TNF-α in LPS induced Vero cells.

D. Research Benefits

The benefit of this research provides information to be able to develop knowledge in particular, can be used as a source of additional information on knowledge about the usage of natural product as traditional medicine, especially for Black garlic single clove.

E. Research Scopes

Due to the factors that may encounters that inadvertently narrow the scope of the study. and to avoid the limitations that may affect the results of the research the boundary of this research was established as follow:

1. Extraction of Black Garlick was done by maceration method using aqueous.
2. Vero cell line were used as cell model and cultured at the FALITMA.
3. Induction of inflammation was done by Lipopolysaccharide LPS.
4. HPLC was done to determine SAC at Muhammadiyah Universitas Malang.
5. Cytotoxicity assay was carried out by using WST-1.

6. Antioxidant properties was done by using DPPH and H2O2.
7. NO measurement was carried out by using Griess reagent.
8. Effects of Black garlic on LPS-induced iNOS and COX-2 expression were carried out by RT-PCR and western blotting.

CHAPTER II

LETRATURE REVIEW

A. Black Garlic

Garlic (Allium sativum L.), belonging to the Alliaceae family, is a frequently used ingredient in gastronomy. Garlic has additionally been used as a conventional medication for a range of biological effects, like increasing stamina, aiding digestion to prevent diarrhea and worm infestation, and treating heart disease, arthritis, and fatigue (Yuan *et al*., 2016).

The classification of garlic Allium sativum L.

Kingdom	:	Plantae
Clade	:	Angiosperms
Clade	:	Monocots
Order	:	Asparagales
Family	:	Amaryllidaceaega
Subfamily	:	Allioideae
Genus	:	Allium
Species	:	Allium sativum L.(anonymous, 2017)

Single Clove Garlic, also known as Solo garlic, monobulb garlic or pearl garlic, is a type of Allium sativum (garlic). The size of the single clove differs from approximately 25 to 50 mm in diameter. It has the flavour of the garlic clove but is somewhat milder and slightly perfumed. The appearance is somewhat akin to that of a pickling onion, with white skin and, often purple stripes (Satti *et al*., 2018).

Single clove garlic has been grown at the foothills of the Himalayan Mountains for about 7,000 years. Normal garlic is planted as a single seed clove which is then triggered into developing multiple cloves by cold weather. But in the temperate climes of the Yunnan province the garlic seeds stay as a single clove, growing to around two inches in diameter. The seeds are planted roughly 2,000 metres above sea level in warm temperatures and with a large amount of sunlight. Solely fifty % of the garlic seeds that are planted in these specific conditions will develop into a single clove garlic. This is not a species - rather it is the conditions underneath that the seeds are cultivated that lead to one clove garlic (Anonymous, 2013).

B. Comparison between fresh garlic and Black garlic.

When compared with fresh garlic, black garlic does not release a strong offensive flavor owing to the reduced content of allicin (Yuan *et al*., 2016). During the heat treatment process, the raw garlic (Figure.1. A) turns into black colour (Figure.1. B), and the texture of the final product is sticky and jelly-like, with a sweet and sour flavour. Black garlic has been readily adopted as an ingredient in processed food materials, such as beverages, candy and ice cream, due to its sweet and syrupy taste(Jeong *et al*., 2016). Enhanced bioactivity of black garlic compared with that of fresh garlic is attributed to its changes in physicochemical properties.

(a) (b)

Figure 1. Garlic. Fresh Garlic (a); Black Garlic (b) (Tamara, 2013)

Fresh garlic contains approximately 63% of water, 28% of carbohydrate (fructans), 2.3% of organosulfur compounds, 2% of proteins (alliinase), 1.2% of free amino acids (arginine), and 1.5% of fiber (Santhosha *et al*., 2013), Nontreated fresh garlic also contains a high amount of g-glutamylcysteines (Amagase *et al*., 2001). These compounds can be hydrolysed and oxidized to form alliin, which accumulates naturally during the storage of garlic at a cool temperature, allicin contributes to the characteristic flavor and taste of garlic. Allicin and other thiosulfinates are promptly disintegrated to other compounds such as diallyl sulfide, diallyl disulfide, and diallyl trisulfide, dithiins, and ajoene(Corzo-Martínez *et al*., 2007).

At the same time, glutamylcysteines are converted to S-allylcysteine (SAC) through its catabolism pathway other than the alliineallicin pathway (Choi *et al*., 2014). The concentration of S-allyl-cysteine, one of the most important organosulfur bioactive compounds of garlic, also increases in black garlic from 4.3- to 6.3-folds depending of heating treatment (Bae *et al*., 2012). S-allylcysteine contributes to health benefits of garlic, such as its antidiabetic, antioxidant, and anti-inflammatory activities (Colín-González *et al*., 2012).

As for Black garlic (BG), during the thermal process, some chemical compounds from fresh garlic are converted into Amadori/ Heyns compounds, which are key intermediate compounds of Maillard reaction (Yuan *et al*., 2016).The chemical compounds of Black garlic are complicated, and the quality of its products depends on the manufacturing process. Nevertheless, BG contains much more functional compounds such as SAC than fresh garlic. (Choi *et al*., 2014) has declared that many valuable components within Black garlic against diseases increased during the aging process, especially polyphenol, flavonoids, and some intermediates of Maillard reaction have been known as antioxidant agents. Furthermore, the antioxidant activity of garlic varies across regions; nevertheless, BG demonstrates significantly much higher biological activity, such as antioxidant properties, than fresh garlic (Sato *et al*., 2006).

Three of Amadori and three of Heyns compounds in BG increased significantly up to 40-100-fold higher than those in fresh garlic. In contrast, through the aging process for converting fresh garlic to BG, the amount of fructans decreased simultaneously, owing to the fact that fructose and glucose with some of amino acids play important roles in Maillard reaction in garlic processing.

ABG has been developed as a functional food with antioxidant and anti-inflammatory activities. BG is produced by the Maillard reaction, which caramelizes sugars. Therefore, BG has a sweet and caramelized taste. Black garlic features a low concentration of allicin but a high concentration of phytochemicals with antioxidant activity compared with FRG. The compound allicin, which produces FRG's distinctive odor, is converted into SAC, an antioxidant compound, during the long heating procedure. S-allylcysteine and its metabolites, but not allicin, are detected in the plasma, liver, and kidney after oral intake (Steine *et al*., 2001).

In addition, Black garlic does not damage gastrointestinal mucosa, which contrasts with FRG (Hoshino *et al*., 2001). BG has many health benefits, but it is difficult to conclude that ABG is better for health than is FRG. Heating procedures reduce the anti-inflammatory effects of FRG (Shin *et al*., 2013). If ABG had a strong anti-inflammatory and antioxidant effect, ABG would be the best candidate for treating inflammatory diseases induced by oxidative stress. However, the antioxidant activity of ABGE is not directly proportional to its anti-inflammatory activity. FRG and ABG should be considered carefully as functional foods because they produce different effects (Jeong *et al.*, 2016).

Moreover, black garlic possesses an abundant amount of antioxidant compounds such as polyphenols, flavonoids, tetrahydro-β-carboline derivatives, and organosulfur compounds, including S-allyl-cysteine and S-allyl-mercaptocysteine, as compared with fresh garlic. Kim and collaborators suggested that the total polyphenol and flavonoid of black garlic increase 9.3- and 1.5-folds, respectively, after a program heat schedule as compared with fresh garlic (M. J. Kim *et al.*, 2014).

Black garlic is produced by aging fresh garlic at high temperature and high humidity (Lee *et al*., 2009). Throughout the aging process, polyphenol contents are increased, and unstable compounds of raw garlic are converted into stable compounds such as S-allylcysteine (SAC) and S-allylmercaptocysteine (SAMC). These organosulfur compounds are also considered as synthetic H2S donors as well as antioxidant. These compounds have been shown to be able to reduce the accumulation of ROS and appear to selectively induce nuclear factor erythroid 2-like factor (Nrf2) involved in oxidative stress defense (Shi *et al*., 2015).

C. Compounds of black garlic

When garlic undergoes treatment to turn into black garlic, allicin, the component that gives fresh garlic its notorious odor, is converted into a variety of antioxidant compounds (Ryu & Kang, 2017).

1. Amadori/Heyns compounds: These are shaped during amid the Maillard reaction. Amadori/Heyns compounds are strong antioxidants, and

Contrasted with fresh garlic, black garlic has up to 40 to 100 times more of these compounds.

2. Organosulfur compounds: Diallyl sulfide, diallyl disulfide, diallyl trisulfide, and diallyl tetrasulfide.
3. Pyruvate: is the main antioxidant and anti-inflammatory molecule which abundant black garlic. It reduces nitric oxide and prostaglandin E2, both of which prolong and intensify inflammation. The concentration of pyruvate in ABG is higher than that in Fresh garlic.
4. 5-hydroxymethylfurfural: One of the main antioxidant compounds in Black garlic , and it is also an important intermediate product in Maillard reaction, Compared to fresh garlic, black garlic has a higher amount of this useful component, as 5-HMF is created under very high heat (Zhang et al., 2016). Moreover, 5-HMF was found in chloroform extract of ABG and treated in TNF-α-stimulated HUVECs. It suppressed total protein and mRNA expression of VCAM-1 and intercellular cell adhesion molecule-1 (ICAM-1) in TNF-α-induced cell surface. It also inhibited reactive oxygen species formation, THP-1 monocyte adhesion, and activation of NF-κB transcriptional factor in TNF-α-stimulated HUVECs (M. J. Kim et al., 2014).
5. S-allyl cysteine (SAC): S-allyl cysteine, one of the major organic garlic compounds that had been known to possess a powerful anti-oxidant property, can be candidate for potential formula compared with other organosulfur compounds (J.-M. Park et al., 2014). S-Allylcysteine is formed by the catabolism of γ-glutamylcysteine and it inhibits oxidative