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by Lia Agustina, Et Al.

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RESEARCH ARTICLE

In vitro Antiplatelet Activities of Aqueous Extract of Garlic (*Allium sativum*) and black Garlic in Human Blood

Lia Agustina[1], Emilia Gan[2], Ninis Yuliati[3], Giftania W. Sudjarwo[4]
[1,2,3]Department of Pharmacy, Faculty of Pharmacy, Institut Ilmu Kesehatan Bhakti Wiyata, Kediri, Indonesia.
[4]Department of Pharmacy Biology, Facultthy of Pharmacy, Hang Tuah University, Surabaya, Indonesia.
*Corresponding Author E-mail: **lia.agustina@iik.ac.id**

ABSTRACT:
Coronary heart disease is one of the deadliest cardiovascular disease in the world. Recent studies have shown an increase in aspirin resistance as a gold standard therapy causing recurrent heart attacks. Antiplatelet of natural resources thus need to be explored. Garlic (*Allium sativum*) is known to be effective as an antiplatelet, but the problem arose after consuming garlic prolonged is the presence of unpleasant breath and body odor which reduces patient compliance in therapy. Black Garlic is a product made of garlic which tastes delicious and does not smell pungent like raw garlic. There has been no study of Black Garlic as an antiplatelet before. The aim of this research was to determine the antiplatelet activities of aqueous extract of garlic (AEG) and Black Garlic (AEBG) and to evaluate their differences in human blood *in vitro*. This research was designed to be experimental posttest only, which was performed in 10 groups, namely the vehicle controls, positive controls, as well as the concentration groups of AEG and AEBG (each 1-4µg/µL). The antiplatelet activity was tested using the Born method, which measured the plasma uptake before and after induced with Papain. Evaluation was done by calculating the percent inhibition of platelet aggregation of the concentration groups relative to vehicle controls. The results showed that both AEG and AEBG have antiplatelet activities ([H=16.664; 5 df; p=0.005] and [H=16.225; 5 df; p=0.006] respectively). The inhibition of platelet aggregation is also shown to be significantly different in both samples [H=21.693; 7 d.f; p=0.003].

KEYWORDS: *Allium sativum*, Antiplatelet, Black Garlic, Garlic, Papain.

INTRODUCTION:

Coronary hearth disease (CHD) is leading cause of death in the world. CHD is accrual plaque which narrow's the heart's arteries and reduced blood flow. Its lead to the lack of oxygen-rich blood suply to organ caused ischemia of myocardial tissue and alteration of heart function. Hypertension, hypercholestherolemia, diabetes and smoking are several risk factor to CHD[1,2,3,4]. These risk factor are varies though individual and population change through monitoring of cholesterol, blood glucose and others risk factor[5]. To reduce mortality and prevent heart attack, antitrombotic can be given to CHD patients[6], which include antiplatelet, anticoagulant and fibrinolytics[7].

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Antiplatelet works by inhibiting trombus formation in the arterial circulation, thus reducing platelet aggregation. Antiplatelet drugs are highly prescribed to patients with ischemic heart disease[8]. Anticoagulants or blood thinner prevent coagulation (conversion of liquid to gel form) of blood[9]. Fibrinolytic works by activating plasminogen to form plasmies, thus degrades the fibrillus until the thrombus ruptures. This drug is given to the patients who experience angina attacks due to ischemia in the golden time range (12-24 hours after the attact)[10].

A review literature from previous study showed that garlic has multiple beneficial effect including hypolipidemic, antimicrobial, hypoglicemics, antithrombotic, antioxidant, etc[11,12,13]. Garlic also shown the ability to reduce BUN (blood urea nitrogen), glucose, triglyceride, cholesterol[14]. Furthermore, garlic also shown antibacterial effect against resistant *Staphylococcus aureus*[15]. The garlic effect could be

different in different varieties[16].

Garlic (*Allium sativum* Linn.) is a member of Liliaceae family. The main component of garlic are organosulfur alliin and γ-glutamilcystein. γ-glutamilcystein reacts with γ-glutamyl-transpeptidase and γ-glutamyl-peptidase peroxidase forms alliin. When garlic sliced or crushed, alliin will reacts with aliinase produce unstable allicin (marked by stinging garlic odor). Allisin will then formed allyl sulfide, ajoene and dithiin[17].

Garlic is known to have effectiveness in the maintenance of the cardiovascular system, especially as antithrombotic[18]. The active compund of garlic tubers is known able to reduce platelet aggregation and anticoagulant. It is also fibrinolytic effect[17,19,20]. The unwanted effects of garlic, smelly breath and body odor, arises after consuming high dose and potentially reduce patient compliance. The development of garlic into solid dosage forms has been previously studied and showed acceptable form[21,22].

The garlic processing nowadays has been developed using heating at controlled temperature and humid atmosphere for several days[23,24]. The processed garlic well known as black garlic (BG). It has sweet taste, jelly-like texture and odorless since alliin decomposes during the manufacturing process. The process increases total polyphenols and total flavonoids as antioxidants significantly[23,24] concluded from previous study that BG has antioxidant activity, anticancer, antiobesity, hepatoprotective, anti-inflammatory, anti-allergic and able to relieve dislipidemia. Our study focusing on the biological activity of BG as antithrombotic in vitro using human blood.

MATERIALS METHODS:

Materials:

Papaya enzyme (Vegavero, Vanatari International GmbH, Berlin, Germany), Acetosal (Aspilets, PT. Darya Varia, Indonesia)

Methods:

Plant selection. Garlic (*Allium sativum* Linn.) were obtained from the local market of Pasar Legi, Blitar, Indonesia. A fresh bulb with the characteristics of dark-yellow tuber and slightly reddish shell were chosen.

Black Garlic processing. A fresh garlic cleaned and stripped from the outher shell. Garlic were heated at 70-80°C for 10 days. Garlic then aerated at room temperature and protected from direct sunlight for two days.

Water extract of garlic. 5gram of fresh garlic (FG) and black garlic (BG) was crushed, then heated in 50mL aquadest at 80°C for 60 minutes. After cilled off, the solution were centrifuged at speed of 4000rpm for 15 minutes three times. Supernatant was taken and diluted at concentration 1, 2, 3 and 4μg/μL.

Blood sampling. An adult men, healthy, didnot receive any medication and treatment for the last two weeks, didnot smoke, didnot cunsume alcohol and willing to follow the procedure were selected. Blood sampling was carried out by venipuncture method vaccuum. The tube used to collect blood is containing 3.8% sodium citrate (blue lid), EDTA (purple lid) and withour additives (red lid).

Platelet-rich plasma (PRP). Fresh blood taken was placed into tube containing sodium citrate. Then the blood is anticoagulated with ACD solution (dextrose anticoagulant citrate, dextrose acid citrate and dextrose citric acid) with ratio 1:9, then centrifuged at 1000rpm for 15 minutes[25]. The top layer of tube then remove.

Platelet poor plasma (PPP) fresh blood was centrifuged at 3000rpm for 15 minutes. The supernatant (PPP) used as blank.

Antiplatelet activity assay. Carried out by method developed by Born[26,27]. 100μL sample and acetosal or water extract of FG and BG or aquadest were homogenized into centrifuged tube. The absorption was measured using spektro-Vis at 600nm wavelength (a). PPP used as blank. Into each test tube, 50mg of papain was added, then incubated at 37°C for 20 minutes. Sample were then measured again with spectro-Vis at 600 nm wavelength (b). The percentage of platelet inhibition was calculated according toVogel[26] formula:

$$\text{Platelet aggregatopm} = \frac{a - b}{a} \times 100\ \%$$

The platelet aggregation of inhition is calculated by following formula

$$\text{Platelet aggregatopm inhibition} = \frac{c - d}{c} \times 100\%$$

Where c is average percentage of negative control of platelet aggregation and d is positive or sample platelet aggregation percentage.

RESULT AND DISCUSSION:

The samples used in this study is fresh garlic (FG) and black garlic (BG). BG obtained by processing FG at controlled temperature (60–90°C) at high humidity temperature (80-90%) for 14 days. According to previous result this process produced sweet and jelly-like texture garlic.

Organoleptic examination on day seventh showed that the color was not homogenous with black skin and brown bulb, spicy and bitter taste, soft and wet texture. On the day eighth, the color, smell and taste similar to the previous day. On the ninth day, the taste is spicy and sweet with soft texture. The optimum taste and texture was obtained from tenth day. On the next day, the taste was bitter, eventhough the color, smell and texture were good. On the twelft day, the BG texture hardens and the taste was bitter.

The assay condition was optimized through centrifugation time and volume. The optimum centrifugation time was ten minutes, while optimum volume for centrifugation was 500μL.

Papain is natural enzyme derived from papaya. In this assay, papain is used to induce platelet aggregation. The ability of papain to induce platelet aggregation was observed using light microscope. The optimum dose for papain induction was 50mg/500μL.

The antiplatelet activity test is an assay to quantify the ability of substance to inhibit platelet aggregation which could cause thrombosis or occlusion of blood vessels. We used the method developed by Born (Platelet Aggregation and Deaggregation in Platelet-Rich Plasma)[27]. The assay was conducted with positive control (Aspirin), water extract of fresh garlic, and water extract of black garlic. The platelet aggregation was observed in sample aspirin, water extract of fresh garlic and black garlic.

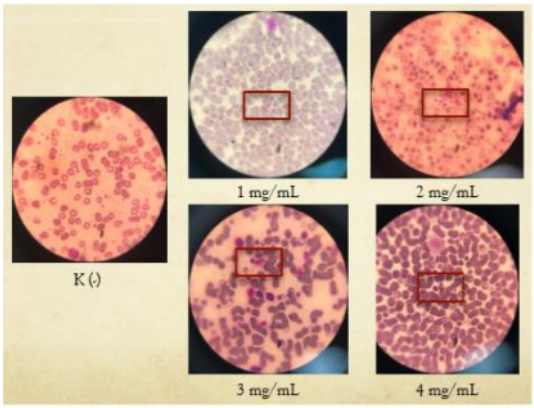

Fig. 1: Microscopic observation of platelet aggregation

Table 1: Platelet aggregation in PRP after papain induction

papain dose (mg)	platelet aggregation in PRP (min)				
	1	5	10	15	20
40	+++	+++	+++	+++	++
50	+++	+++	+++	+++	+++
75	+++	+++	+++	++	+
100	+++	+++	++	+	-

Table 2: Percent of platelet inhibitition

treatment	persent inhibitition (%)
control positive	19.370
FG extract 1 μg/μL	10.371
FG extract 2 μg/μL	16.932
FG extract 3 μg/μL	24.093
FG extract 4 μg/μL	24.993
BG extract 1 μg/μL	16.918
BG extract 2 μg/μL	17.325
BG extract 3 μg/μL	30.547
BG extract 4 μg/μL	42.692

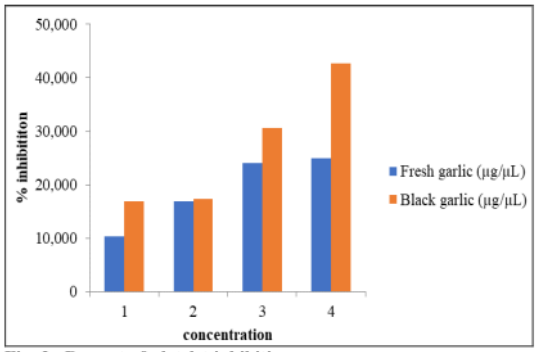

Fig. 2 : Percent of platelet inhibition

Our result here showed that the inhibition of platelet aggregation was dose dependent manner in both fresh and black garlic. We also observed that the inhibition of platelet aggregation was higher in back garlic sample. The similar result was also observed by Sukandar[28] in which water extract of fresh garlic could induce platelet inhibition.

The decrease in platelet aggregation is depends on the type of platelet aggregation inducer because it is associated with the specific damage to platelet function[29]. The platelet aggregation inducer is classified into two type, weak inducer (ADP and epinephrine) and strong (arachidonic acid, collagen and trombine). The mechanism of platelet inhibition could also predict from the type of inducer used in the assay. ADP stimulates aggregation through the purigenic specific receptor pathway (i.e P2Y12). Collagen stimulates aggregation through GPVI receptor pathway which induce the release of Ca^{2+} and increase plateet aggregation[30]. In Our study, we used papain as platelet aggregation inducer. We observed in our study that papain could induce platelet aggregation at concentration 100 mg/ mL. Since black garlic contain higher flavonoid, we assumed that black garlic inhibit arachidonat synthesize which in turn inhibit the formation of TXA2[31].

CONCLUSION:

Garlic and black garlic could inhibit the platelet aggregation. The effect of platelet aggregation is dose dependent manner. Black garlic has higher antiplatelet activity than garlic.

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