Mechanism of action vasodilation *Annona muricata* L. leaves extract mediated vascular smooth muscles

S Ismail¹,²,*, N Hayati¹ and N Rahmawati³

¹Laboratory of Pharmacology, Faculty of Medicine, University of Mulawarman.
Jl. Kerayan Kampus Gunung Kelua, Samarinda 75119, East Kalimantan, Indonesia
²Research Center on Drugs and Public Health, University of Mulawarman.
Jl. Kerayan Kampus Gunung Kelua, Samarinda 75119, East Kalimantan, Indonesia.
³Center for Research and Development of Medicinal Plants and Traditional Medicine.
Jl. Raya Lawu, Tawangmangu, Karanganyar, Central Java 57792, Indonesia.

*E-mail address: ismail8997@yahoo.com and ismail@fk.unmul.ac.id*

**Abstract.** *Annona muricata* L. leaves (AML) is used as ethnomedicine by the Dayak Abai ethnicity in North Kalimantan for its already known use to reduce blood pressure. However, the mechanism of action in the vessel is still poorly understood. Aim study to prove the mechanism of action of AML in blood vessels. AML was extracted with a maceration technique using ethanol solvent. Mechanism of action test was performed with isolated rat aortic with endothelium (endo-intact) and without endothelium (endo-denuded). AML extract intervention on rats aorta with endo-intact and endo-denuded can induction vasodilatation activity. Increasing AML extract concentration can improve decrease vasodilatation activity on isolated rats aortic with endo-intact compared to endo-denuded, it means that endothelium can weaken vasodilatation activity of aorta mediated by vascular smooth muscle after the extract was given.

1. Introduction
Hypertension is still a major health problem in the world with increasing prevalence. Hypertension is also one of the most important risk factors of cardiovascular disease, which is the main cause of death worldwide. Incidence of hypertension worldwide in 2000 is approximately 26.4% with a total of 972 million people. It causes death in 7.1 million people. Most of the patients with hypertension are in developing countries and are predicted to increase to 60% or 1.56 billion people in 2025 [1,2].

Hypertension is generally treated in a long-term duration to keep the blood pressure well-controlled. Long-term medication not only can cause adverse events, but can also increase medication cost. New anti-hypertensive medicines are needed to be studied and discovered in order to obtain safer and more affordable anti-hypertensive medication. In addition, discovery of new anti-hypertensive medicines also had economic value because of the high number of patient, increased hypertension prevalence, and long duration of medication. These medicines can be obtained through ethnomedicine research of traditional herbs, which are used by certain ethnicities for hypertension medication. These herbs are the source of potential medicinal molecules [3].

Indonesia is rich in medicinal plants variety which use these plants as medicine. Many plants are used for anti-hypertensive medicine by several ethnicities in Indonesia. In 2015, Indonesian
exploration research of local ethnomedicine knowledge and community-based medicinal plants, known as Medicinal Plants and Herbs Research (RISTOJA) on the Dayak Abai ethnicity, showed the usage of *Annona muricata* L. leaves or soursop for reducing blood pressure [4]. Water extract of *A. muricata* leaves (AML), which is administered intravenously can cause a hypotensive effect in normotensive rats [5]. There is no study about the mechanism of action of AML on blood vessels, which has to be studied to understand whether it is mediated by endothelium and/or the smooth muscle of the blood vessel. Study of the mechanism of action in endothelium or the smooth muscle of the blood vessel on vasodilatation activity of medicinal plant is very important. A medicine where its mechanism of action is in the endothelium will provide no benefit if given to patients with endothelial dysfunction. Therefore, this study aimed to prove the mechanism of action of AML vasodilatation on blood vessels.

2. Material and Methods

2.1. Instruments and Materials

Instruments used in this study were bioassay instrument for isolated rat aortic, which consisted of Octal Bridge Amplifier and PowerLab/16SP and program chart ver. 5.0 from AD Instrument. Isometric transducer 7003 from Ugo Basile. Materials used in this study: ethanol, DMSO, HCl, NaOH, NaCl, KCl, CaCl₂, MgSO₄, KH₂PO₄, NaHCO₃ was analytical grade and glucose for biochemistry from Merck. Carbogen gas (Mix 95% O₂ + 5% CO₂) from Aneka Gas. Phenylephrine and acetylcholine from Sigma Aldrich.

2.2. Animal Subject

The animal subject used in this study was a male white Wistar rat, which was obtained from the Pharmacology Laboratory, Medicine Faculty, Mulawarman University, with 4-6 month of age, 200-300 gram of weight. The study protocol approved by Ethical Committee of Health Research, Medicine Faculty of Mulawarman University with voicher number 170/KEPK-FK/VI/2016 on 13 June 2016.

2.3. Sample Collection and Identification

AML was collected in July 2016 from the place of origin of the information regarding traditional medicine in RISTOJA 2015 that was Malinau Regency, North Kalimantan. Once the AML arrived at the Pharmacology Laboratory of Medicine Faculty, the AML was sorted, washed, and dried at 60°C for 5 days. The herbarium specimens were stored in the Pharmacology Laboratory of Medicine Faculty, Mulawarman University with voicher number ALR.2016.S001, which can be used for re-identification if needed. Identification of the plants was performed by taxonomy specialist in Mulawarman University with voicher number 56/UN17.4.3.08/LL/2016.

2.4. Extraction

The crushed simplicia of AML was macerated using ethanol solvent. The production of the extract followed the description by Indonesia Herbal Pharmacopoeia [6]. After the dried extract was obtained, the yield was measured using percentage of weight (w/w) between the yield with powder weight of simplicia used by weighing. The extract was also tested for its water level quality using Gravimetric method by following the procedure described in Indonesia Pharmacopoeia [7]. Dried extract stored in the freezer at -20°C before further study.

2.5. Vasodilatation Activity Test

The vasodilatation activity test was performed using the isolated organ of rat aorta with endothelium (endo-intact) and without endothelium (endo-denuded) to understand whether the mechanism of action of AML extract is affected by endothelium or only by the smooth muscle of the blood vessel. The repetition was performed five times using five rats. The rats were euthanized using high dose of ketamine and their neck was dislocated after no movement was observed. Aorta was collected and
prepared by the method described by Pieper & Dondlinger [8]. It was then placed in \textit{Kreb's-Henselheit} solution, which consisted of (mM): NaCl 118, KCl 4.7, CaCl$_2$ 1.5, MgSO$_4$ 1.1, KH$_2$PO$_4$ 1.2, NaHCO$_3$ 25, and glucose 10 with pH of 7.4.

2.6. \textit{Vasodilatation Activity Test in Rat’s Aorta with Endothelium-intact}

Aorta was prepared and placed in a 10-ml bath organ. After being equilibrated for 60 minutes, the aorta was contracted using KCl solution to observe the contraction response (the increased tonus of aorta indicates that the contraction of the smooth muscle is good). Next, the aorta was washed using \textit{Kreb's-Henselheit} solution for three times every 15 minutes and was tested using phenylephrine plus acetylcholine to test the endothelial integrity. After being equilibrated, the aorta was contracted using phenylephrine [10$^{-6}$ M] until it reached flat contraction peak and acetylcholine [10$^{-5}$ M] was given. If there is a response of relaxation, it indicates that the aorta have an endothelium-intact. Intact endothelium of the aorta, or aorta with endothelium-intact, used for this study was the one with relaxation response of more than 60% [9].

After contraction responses test and endothelial integrity test, aorta was washed three times with \textit{Kreb's-Henselheit} solution, and then washed every 15 minutes until it returned to basal tonus and ready for vasodilatation test. In vasodilatation test, aorta was contracted with phenylephrine solution [10$^{-6}$ M], after flat contraction was reached, we added extract solution cumulatively 0.03, 0.1, 0.3, and 3 (mg/ml) [10]. If a decrease in aorta tonus was observed, it mean that the extracts have vasodilatation activity. Negative value signifies vasodilatation activity and positive value signifies vasoconstriction activity. We also experiment on negative control (extract solvent or DMSO-ethanol 10%).

2.7. \textit{Vasodilatation Activity Test on Rats Aorta with Endothelium-denuded}

Aorta endothelium were removed mechanically as. Aorta was considered to not have endothelium when vasodilatation activity become $<$10% after being contracted with phenylephrine [10$^{-6}$ M] and reaching flat maximal contraction response after being added acetylcholine [10$^{-5}$ M] [11]. Aorta was washed three times with \textit{Kreb's-Henselheit} solution, and then washed again every 15 minutes until it returned to basal tonus and ready for vasodilatation test, similar in aorta with endothelium. We also experiment on negative control. Results were compared to aorta with endo-intact.

2.8. Data Analysis

Data presented as mean $\pm$ SEM and dose-response curve. Statistic test used was $t$-test for control and extract group on aorta with endo-intact and endo-denuded, results differ significantly if $p<0.05$. Data processing software and analysis used was SigmaPlot version 12.5.

3. Result and Discussion

The yield from AML extraction is 9.24% and quality test result on the extract found that it contained water as much as 3.15%. AML extract intervention on rats aorta with endo-intact and endo-denuded can induction vasodilatation activity. Increasing AML extract concentration can improve decrease vasodilatation activity on isolated rats aortic with endo-intact compared to endo-denuded. Clear results can be seen on dose-response chart in Figure 1.

Figure 1 shows vasodilatation activity test results on isolated rats aortic with endo-intact and endo-denuded in each concentration intervention. It can be seen that control intervention on rat’s aorta with endo-intact and endo-denuded can induce vasodilatation activity. Increasing control concentration can improve vasodilatation activity in isolated of rats aortic with endo-intact compared to the endo-denuded.
Figure 1. Vasodilatation activity on aorta in Control and AML intervention in rats aorta with endo-intact and endo-denuded

Note: n=5 rats; data in mean ± SEM; (E+)=endo-intact; (E-)=endo-denuded; K=Control (DMSO-Ethanol10%); AML= Extract ethanol AML; Statistical analysis with t-test. *differs significantly if compared to K(E+). †differs significantly if compared to (E-). #differs significantly if compare to K(E+) and K(E-).

Figure 1 also shows vasodilatation activity test result on isolated rats aortic with endo-intact in each concentration intervention of AML extract. It can be seen that AML extract intervention on rats aorta with endo-intact and endo-denuded can induction vasodilatation activity. Increasing AML extract concentration can improve decrease vasodilatation activity on isolated rats aortic with endo-intact compared to endo-denuded, it means that endothelium can weaken vasodilatation activity of aorta mediated by vascular smooth muscle after the extract was given. Clear results can be seen on extract intervention at 3 mg/ml concentration.

In intervention with 3 mg/ml concentration, the extracted AML effect on aorta with endo-intact was a vascular vasodilatation activity by as much as -39.73% and -66.44% in aorta endo-denuded. This showed that vascular endothelium plays a role in weakening vasodilatation activity in aorta with endo-intact by 18.33%, meaning that intervention of AML extract at vascular endothelium can reduce vasodilatation, mediated by vascular smooth muscle. In intervention with 3 mg/ml concentration on control group, greater vasodilatation activity can be observed on aorta with endo-intact (-4.37%), compared to the one with endo-denuded (-3.20%).

Vascular tone is mediated by endothelium and smooth muscle of the vessel itself. Vascular endothelium is responsible in vasoconstriction and vasodilatation. Endothelium role in vasodilatation is mediated by muscarinic-M, histaminergic-H2, vasopressin, adrenergic-α2, serotoninergic, kinin, thrombin, purinergic, ET_B1, and AT2 receptors which activate endothelial nitric oxide (NO), resulting...
in a decrease of calcium ion inside cytosol of the vascular smooth muscle, eventually causing vasodilatation. Endothelium is also responsible for vasoconstriction. Some study proved an existence of endothelium-dependent contractions factors (EDCF) that caused vascular vasoconstriction that is affected by the mediator released from endothelium [12], but what mediator was released remained a question. EDCF role in this study may be influenced by the AML extract intervention that can reduce vasodilatation activity mediated by vascular smooth muscle, but further research is needed.

Vasodilatation mechanism on blood vessel mediated by endothelium, can occur immediately on vascular smooth muscle through the effect of (1) calcium antagonist channel; (2) AT1 antagonist receptor; (3) ET antagonist receptor; (4) α1-receptor antagonist; (5) opening of potassium ion channel resulted in hyperpolarization; or in presence of (6) NO donor that caused sGC activation. All these processes cause a decrease of calcium ion in the cytosol of vascular smooth muscle, making the calcium calmodulin-ion complex to not be formed, causing the MLCK that activates myosin ATPase to not happen, therefore the myosin head will be detached from actin, causing vascular relaxation [12].

Study on rats proved that AML can reduce blood pressure, and its mechanism of action is not influenced by muscarinic, histaminergic, adrenergic or endothelium-dependent pathways. The results were not clearly explained. This confirmed our study that AML extract on aorta with endo-intact can weaken vasodilatation effect mediated by vascular smooth muscle, but mechanism of how the endothelium contributed in reducing vasodilatation effect will need further research. Hypotension effect from AML was related with alkaloids and essential oil contained in it. Alkaloids isoquinoline, coreximine, and anomurine are suspected to have weak lowering effect on blood pressure, and essential oil beta-cryophyllene shows hypotension and vasodilatation effect. Furthermore, reticuline as another alkaloid from AML was suspected to cause hypotension through voltage-dependent Ca$^{2+}$ channel blocker and/or inhibiting the release of calcium ion from intracellular reserve, but further research needed to prove this [5]. AML also contain quercetin, it can dilate the vessels through sGC activation and cyclooxygenase-dependent pathway [13]. This might contribute to vasodilatation that happened in rat aorta with endo-denuded, hence further research on quercetin needed to be considered.

AML is already used by Dayak ethnicity at Abai in North Borneo from generation to generation as a treatment for hypertension. This herbal potion has been used in other countries, the results of the ethnobotany research has been widely applied in countries such as Bolivia [14], Nigeria [15], and Mauritius [16]. This research also confirms that the role of ethnobotany exploration is very useful in finding medicinal plants efficacy that are needed to be proved scientifically in pre-clinical and clinical studies. Our research proved the efficacy of medicinal plant of AML that has been used by Dayak Abai ethnicity to treat hypertension from generation to generation that was explored through Local Scientific Research of Community-based Ethnomedicine and Medicinal Plants in Indonesia at Abai ethnicity. Data from Local Scientific Research of Community-based Ethnomedicine and Medicinal Plants in Indonesia should have been explored by government and conserved so not to become lost as time goes on, and this intellectual property of local societies need to be thought out and protected. This abundant data is coming from Indonesia itself that is rich in medicinal plants and have more than 200 ethnics utilizing it as treatment for many kinds of diseases. If this database was available, it is the focus of the next generation researchers to scientifically prove the benefits of those medicinal plants so that they can be used in many health services and for the sake of Indonesia’s prosperity.

4. Conclusion
Vasodilatation mechanism of action from ethanol extract of *A. muricata* was mediated by smooth muscle of blood vessel and endothelium that contributes to the weakening of vasodilatation activity.

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