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In vitro evaluation of the vasodilatory activity of ethanol extracts of *Eleutherine bulbosa* bulbs and leaves

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ABSTRACT

The aim of this study was to examine and compare the vasodilatory activities of ethanol extracts of *Eleutherine bulbosa* bulbs (EBBs) and leaves (EBLs) on isolated aortas from Wistar rats, with and without endothelium. EBBs and leaves EBLs were washed with clean water and chopped and dried in a 60°C drying cabinet. Extraction was carried out by maceration using an ethanol solvent. The collected filtrate was concentrated using a vacuum evaporator, and the concentrated liquid was dried further in a 60°C oven. By carrying out relevant bioassays, the ethanol extracts of EBBs and leaves EBLs were evaluated using Wistar rat aortic rings, with and without endothelium in Krebs–Henseleit solution. Aortic rings were precontracted with phenylephrine solution, and after reaching the plateaued peak contraction, a single dose of the extract solution was administered, and the contractility was measured. The percentage of aortic dilation was presented as mean ± SD and statistically analyzed using the *t*-test, deemed significantly different if the *p*-value was < 0.05. The results showed that E. bulbosa leaf extracts induced vasodilation of aortic rings without endothelium, which was stronger than *E. bulbosa* bulb EBB extracts, indicating the vasodilatory activity through the endothelium-independent vascular smooth muscle.

INTRODUCTION

Eleutherine bulbosa (Mill.) Urb. (*E. bulbosa*), also termed Eleutherine americana Merr (Couto *et al*., 2016), belongs to the Iridaceae family and is widely available in Kalimantan under the local name *Bawang Tiwai*. It is usually used as food and has not been explored as a raw material for medicines or herbs with economic potential. An ethnobotanical search has revealed that the Kalimantan Dayak ethnic group has utilized this herb to reduce blood pressure (Mayasari *et al*., 2018; Syamsul *et al*., 2015). The E. bulbosa bulb (EBB) extract reportedly decreases blood pressure in hypertension-induced rats, causing vasodilation in the rat's aorta with endothelium *in vitro* (Hasimun *et al*., 2017; Yuliandra *et al*., 2018).

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It has been known that vasodilation and vasoconstriction activities in blood vessels can be mediated by endothelium-dependent vascular smooth muscle cell (VSMC), by inducing endothelium to release endothelium-derived relaxing factor (EDRF) such as nitric oxide or prostacyclin or other EDRF, which is a vasodilator (Ismail *et al*., 2017), and also via endothelial-independent VSMC, by inhibiting the calcium channel which causes a reduction in Ca^{2+} influx (Luna-Vázquez *et al*., 2013). To date, there are no data showing whether the vasodilatory activity of EBB extract depends on the endothelium or not. Therefore, this study examined the vasodilatory activity of EBB extract to see whether it is endothelium-dependent. If proven otherwise, this herb has the potential to be developed as an antihypertensive drug in hypertensive patients with endothelial dysfunction as blood vessels with endothelial dysfunction will fail to show a vasodilatory effect if the drug action only occurs in the endothelium-dependent VSMC (Sandoo *et al*., 2010).

Currently, *E. bulbosa* leaves (EBLs) are disposed of as waste with no economic potential. A previous study has reported that EBLs possess antioxidant activity, as well as total phenolic content and secondary metabolites almost comparable with those present in bulbs (Pratiwi *et al*., 2013; Shi *et al*., 2018). (EBBs)

demonstrate a strong vasodilatory potential as they grow from the bulging base of the leaf, warranting further investigation owing to its economic potential.

EBBss and EBLs are extensively available in East Kalimantan and possess economic potential. Hence, further research needs to be undertaken to discover herbal/medicinal raw materials with antihypertensive properties. Hypertension requires long-term therapy to control blood pressure and reduce the risk of stroke and coronary heart disease. Therefore, this plant presents immense opportunities as the number of individuals with hypertension in Indonesia, as well worldwide, continues growing and is estimated to reach more than 1.5 billion in 2025 (Kearney *et al*., 2005). Furthermore, this research is in accordance with the strategic plan of Mulawarman University in the Health Sector, on the prioritized topic of utilizing the East Kalimantan biodiversity-based plants for medicine and cosmetics, and further is in accordance with the vision and mission of the Medical Faculty of Mulawarman University.

The aim of this study was to examine if the vasodilatory activity is endothelial-dependent and to compare the vasodilatory activity of the extracts of EBBs and EBLs, bioassayed using isolated rat aorta.

MATERIALS AND METHODS

Materials

The bulbs and leaves of *E. bulbosa* were obtained from farmers in Samarinda City, East Kalimantan province, Indonesia. Bulbs demonstrating no rot or fungal growth were selected. The criteria for the leaves used were as follows: healthy, green, exposed to direct sunlight, and with no fungal growth or rot. Ethanol for extraction was purchased from PT Sumber Bahagia Surabaya. Ketamine for rat anesthesia was obtained from the Department of Pharmacy at AW Sjahranie regional general hospital Samarinda. Chemicals for making Krebs–-Henseleit solutions were purchased from Sigma- Aldrich distributor in Surabaya. Carbogen gas (containing 95% O_2, 5% CO_2) was purchased from PT Murni Gas Raya, Samarinda.

Extracts preparation

EBBs and EBLs, weighing 1 kg each, were selected according to the above-mentioned criteria and were washed and dried in a drying cabinet maintained at 60°C. After drying, the bulbs and leaves were cut into small pieces and macerated for 3 days using an ethanol solvent. The collected filtrate was concentrated using a rotary evaporator at 60°C, dried in a desiccator for 1 week, and placed in a 60°C oven. Finally, ethanol extracts of EBBs and EBLs were obtained for testing.

Rat aortic ring preparation

The bioassays were carried out using aortic rings isolated from male Wistar rats (*Rattus norvegicus* strain Wistar) aged 3–4 months and weighing 200–250 g, obtained from Pharmacology Laboratory, Faculty of Medicine of Mulawarman University. First, rats appearing healthy were randomly selected and anesthetized, followed by abdominal surgery. Next, the rat's aorta, separated from the surrounding tissue, was cut into a ring of 3 mm length, with its endothelial layer and the aortic smooth muscles intact, and put into an organ bath containing 10 ml of Krebs–Henseleit solution, consisting of (mM) NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $KH2_2PO_4$ 1.2, $NaHCO_3$ 24, and glucose 11, maintained at temperatures 37°C, pH 7.4, and diffused with carbogen gas. The study protocol was approved by the Health Research Ethics Committee (KEPK) of Mulawarman University (No. 159/KEPK-FK/IX/2019).

Vasodilatory evaluation of EBB and EBL

For EBB and EBL, vasodilation was bioassayed as described by Ismail and Yuniati (2016). The aortic integrity was evaluated by administering a methacholine solution, which induces vasodilation in the aortic ring. The aortic endothelium was mechanically removed as described by Ismail and Yuniati (2016), with the aorta pronounced without endothelium if < 10% vasodilation occurred after administration of the methacholine solution. Aortic precontraction was carried out using phenylephrine (PE) solution. After the plateaued peak of contraction was reached, 200 µl of a 10% concentration of EBB, EBL, and extract solvent was administered, and the contractile response was observed. The evaluation was carried out on aorta with an intact endothelium, as well as aorta with the endothelium removed, repeated six times using six rats.

Following extract administration, changes in aortic tone were calculated as Δg, defined as the difference between the final aortic tone after treatment and the initial tone at submaximal contraction (in grams). The percentage of aortic vasodilatory response was calculated using the following formula:

$$\% \text{ aortic vasodilation} = \frac{\Delta g\ (g)}{\textit{Initial tone at submaximal contraction (g)}} \times 100$$

Data regarding the percentage of aortic vasodilatation were statistically evaluated and are presented as mean ± SD. Statistical analysis was carried out using a *t*-test and results were deemed as significantly different if the *p*-value was < 0.05.

RESULTS AND DISCUSSION

The extraction of EBBs and EBLs with ethanol resulted in yields of 8.94% and 8.84% with water contents of 8.89 ± 0.07 and 9.35 ± 0.04, respectively. This water content was within the tolerated limits established in the Indonesian Herbal Pharmacopoeia.

In this study, our results demonstrated that dimethyl sulfoxide) ethanol as an extract solvent could induce mild vasodilation in the aortic blood vessels with intact endothelium. Following the administration of extract solvents, increased contractions were observed in the endothelium-denuded aorta (Fig. 1A), with the *t*-test analysis revealing statistically significant differences ($p < 0.05$). This suggested that the extracting solvent could induce aortic vasodilation in blood vessels with intact endothelium.

In the EBB intervention study, EBB induced vasodilation in endothelium-denuded aortic blood vessels, as well as vasoconstriction in the aorta with intact endothelium (Fig. 1B), presenting statistically significant differences ($p < 0.05$) following the *t*-test. This indicated that EBB could induce the endothelium

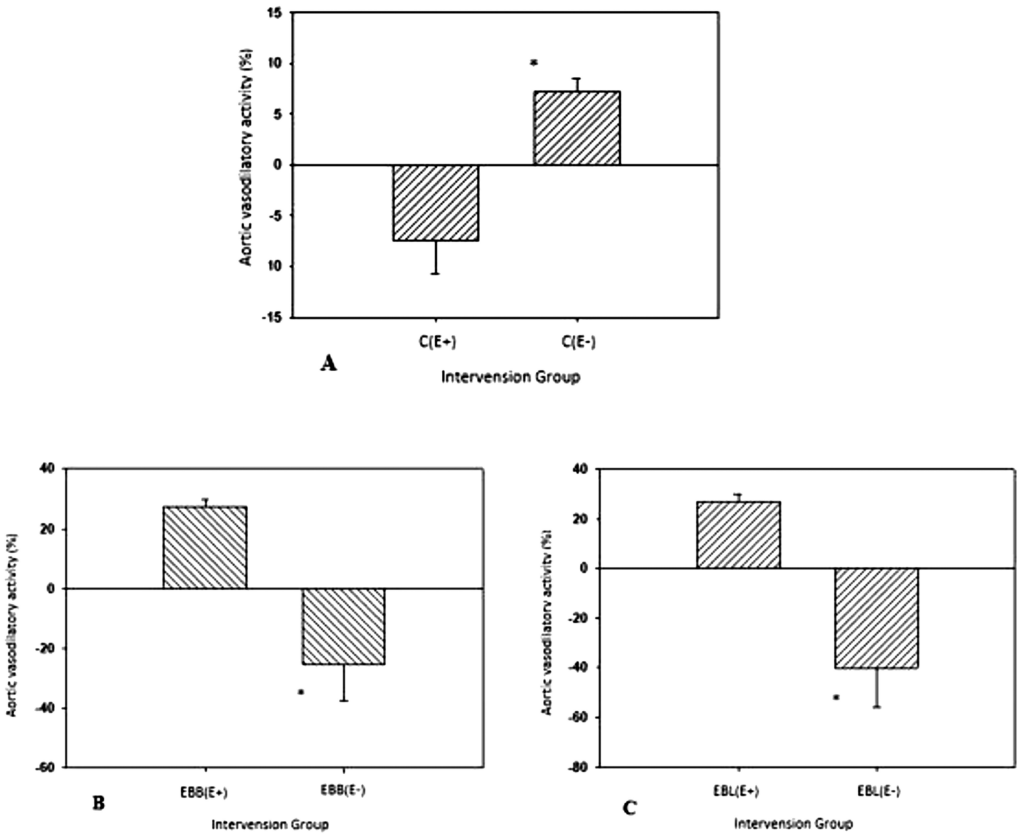

Figure 1. Vasodilatory activities of rat-isolated aorta rings with endothelium- and endothelium-denuded aorta following the intervention of the extract solvent (A); EBB (B); and EBL (C). n = 6 rats; data are presented as mean ± SD; C = control group = extract solvent; EBB = *E. bulbosa* bulb extract; EBL = *E. bulbosa* leaf extract; (E+) = aorta with endothelium; (E−) = endothelium-denuded aorta; **t*-test analysis revealed statistically significant differences ($p < 0.05$).

to release endothelium-derived contracting factors, which release substances provoking blood vessel contraction and EDRF, which cause vasodilation in blood vessels. EDRF activity can be used as an indicator of blood vessel dysfunction. Furthermore, EBL showed vasodilatory activity in endothelium-denuded rat aorta and vasoconstriction in aortas with intact endothelium (Fig. 1C), revealing statistically significant differences ($p < 0.05$) using the *t*-test. These results show that the vasodilatory effects of EBB and EBL were not affected by solvent extracts that induced contraction in the endothelium-denuded aorta.

Figure 2A shows the vasoconstriction induced by EBL and EBB in aorta with endothelium, which failed to demonstrate statistical significance ($p > 0.05$). As shown in Figure 2B, the vasodilatory activity of EBL in the endothelium-denuded aorta was stronger than that induced by EBB, with the *t*-test presenting statistically significant differences ($p < 0.05$).

The aorta is the largest artery among all blood vessels, demonstrating a structure similar to arteries and arterioles, as well as almost evenly distributed cholinergic and adrenergic receptors (Rameshrad *et al*., 2016). Therefore, the aorta is used to evaluate blood vessel dilatation *in vitro*. Moreover, the aorta can be easily prepared and installed in an organ bath when compared with other smaller arteries.

Before examining the aortic dilatation activity, each treatment was carried out with a precontraction, by administering a 10^{-3} M submaximal concentration of PE to the prepared aorta (Ismail and Yuniati, 2016; Ismail *et al*., 2013). PE is a selective α_1 receptor agonist, activating α-adrenergic receptors in blood vessels. Activation of α-adrenergic receptors results in contractions that increase peripheral vascular resistance and blood pressure (Brunton *et al*., 2011). In the vascular smooth muscle, there are two types of Ca^{2+} transmembrane channels: receptor-operated calcium channels (ROCC) and voltage-dependent calcium channels (VDCC). PE activates ROCC and regulates extracellular Ca^{2+} influx, causing the aorta to contract topically. In addition, PE activates specific inositol-1,4,5-triphosphate (IP_3) receptor channels in the sarcoplasmic reticulum membrane and induces the release of Ca^{2+} from sarcoplasmic reticulum, thereby causing vascular smooth muscle contraction (Gan *et al*., 2016).

In this study, vasodilatory activities of EBB and EBL were observed in the aorta with and without endothelium. EBB and EBL demonstrated contraction in the rat aortic ring with endothelium, indicating that EBB and EBL did not induce endothelium to release nitric oxide or prostacyclin, or other EDRF, which is a vasodilator. On the other hand, EBB and EBL relaxed the PE-contracted aortic ring with endothelium-denuded, which showed that EBB and EBL were active in the vascular smooth muscle to inhibit calcium channels or to induce other mechanisms that reduce Ca influx, causing vasodilation. Further studies are required to find out the exact mechanism.

The vasodilatory activity is presumably owing to the phytochemicals in EBB and EBL. Previous investigations on

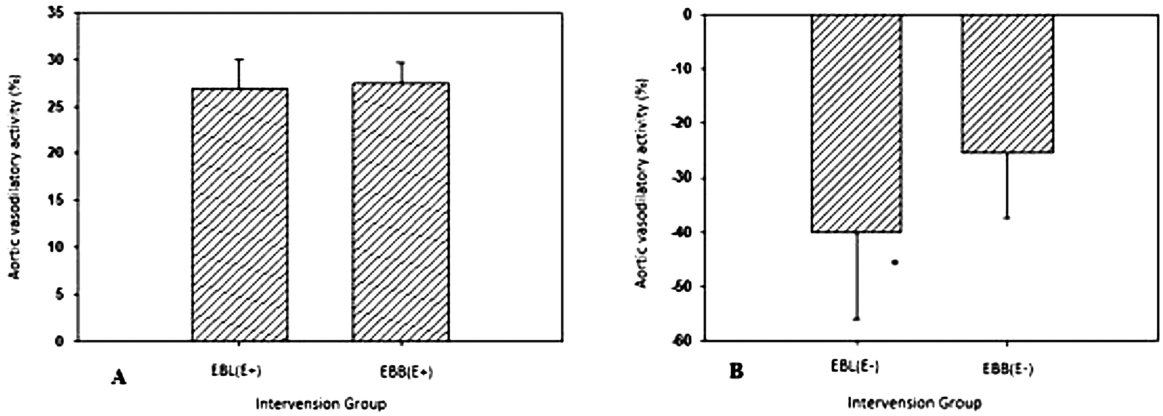

Figure 2. Comparison of vasodilatory activity of EBLs and EBBs in the isolated aorta with endothelium- (A) and endothelium-denuded (B) aorta. n = 6 rats; data are presented in mean ± SD; EBL = *E. bulbosa* leaf extract; EBB = *E. bulbosa* bulb extract; (E+) = aorta with endothelium; (E−) = endothelium-denuded aorta; **t*-test analysis revealed statistically significant differences ($p < 0.05$).

the bulbs of *E. americana* Merr. have confirmed that the bulbs contain alkaloids, flavonoids, glycosides, tannins, saponins, and triterpenoids/steroids compounds (Kamillah, 2014), with the leaves also revealing a similar composition of phytochemicals. Notably, the detected flavonoids, alkaloids, tannins, saponins, and triterpenoids are strongly suspected to play a role in blood vessel dilatation.

Reportedly, several studies have revealed that some flavonoid compounds can demonstrate a direct effect on the vascular smooth muscle. Flavonoids inhibit Ca^{2+} influx through inhibition of L-type Ca^{2+} voltage channels, with a reduced Ca^{2+} influx in smooth muscle cells resulting in vascular smooth muscle relaxation (Liu *et al*., 2014). For example, epigallocatechin-3-gallate, a flavonoid found in green tea, as well as in plants of the Malvaceae family, significantly reduces systolic blood pressure in hypertensive rats (Rezende *et al*., 2016); hesperetin, a flavonoid present in *citrus*, has a vasospasmolytic effect in rat coronary artery (Liu *et al*., 2014); and flavonoids isolated from *Nolana ramosissima* I.M. Johnst relax rat aorta through an endothelium-independent mechanism (Cifuentes *et al*, 2020) and can inhibit Ca^{2+} influx through Ca^{2+} L-type channels in VSMCs. Furthermore, flavonoids are known to inhibit the enzyme phosphodiesterase (PDE) in smooth muscle cells. In cells, PDE breaks down cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP). In smooth muscle cells, high concentrations of cAMP and cGMP cause blood vessel dilation, as these molecules inhibit myosin light chain kinase. Additionally, PDE inhibition is directly related to the ability of flavonoids to block the calcium–calmodulin complex activity, suppressing the contraction of smooth muscle cells (Elvebak *et al*., 2010; Rezende *et al*., 2016). For example, quercetin, which is a flavonol triglycoside, a subclass of flavonoids, can increase cGMP concentrations through its role as an antioxidant in VSMCs, demonstrating a vasorelaxant effect in isolated rat aorta and porcine coronary arteries (Suri *et al*., 2010). Reportedly, quercetin levels are considerably high in the bulbs of *E. americana* Merr., making this plant a potential therapeutic agent (Rosa, 2013). Additionally, pure quercetin has been known to demonstrate a role in lowering blood pressure by inducing dilatation in blood vessels (Perez-Vizcaino *et al*., 2002). Quercetin can also inhibit vascular smooth muscle contraction by inhibiting the formation of angiotensin II, protein kinase activity, and blood vessel superoxide production (Ozarowski *et al*., 2018).

Notably, alkaloids demonstrate hypotensive and antihypertensive activities through vasodilator activity. For example, aristotelin, an indole alkaloid, derived from *Aristotelia chilensis* (Molina) Stuntz leaves, induces relaxation in endothelium-denuded rat aorta by activating potassium channels and blocking calcium channels (Romero *et al*., 2019). Furthermore, alkaloids (tropane alkaloids), found in the roots of *Erythroxylum lugens*, can inhibit Ca^{2+} voltage channels and IP3 receptors in VSMCs. If the IP3 receptor on the surface of the sarcoplasmic reticulum of smooth muscle cells is activated, the sarcoplasmic reticulum will release Ca^{2+} intracellularly. The inhibition of these receptors results in decreased intracellular Ca^{2+} levels, preventing the formation of Ca^{2+}–calmodulin complexes and resulting in the inhibition of actin and myosin cross-bridges (Oliviera *et al*., 2012).

Tannin is one of the phytochemicals present in the bulbs of *E. americana* Merr. (Noorcahyati, 2012). Tannins are divided into two groups, namely, hydrolyzed tannins and condensed tannins. Hydrolyzed tannins from the *Geum japonicum* L. plant affect NO and cGMP activities in rat aortic rings precontracted with PE. Tannins can induce the relaxation of blood vessels. Reportedly, the *Moringa stenopetala* leaf extract contains tannins and causes relaxation in spiral slices of the pig aortic, by blocking Ca^{2+} L-type channels in VSMCs (Geleta *et al*., 2016).

Saponins have also been reported to induce vasorelaxation in the rat aortic rings without endothelium, for example, ginsenoside Rg_3, a ginseng saponin, which induces endothelium-independent relaxation in rat aortic rings by inhibiting influx of Ca^{2+} and stimulating K^+ efflux (Gan *et al*., 2016). Astragaloside IV, a saponin active ingredient extracted from *Astragalus membranaceus*, relaxes the endothelium-denuded rat aortic ring, through NO that comes from the vascular smooth muscle system (Hu *et al*., 2016).

Reportedly, terpenoids relax the endothelium-denude rat aortic rings. For example, the ethanol extract of *Valeriana fauriei* root and rhizome, which contains lots of sesquiterpenoids and iridoid glycosides, has vasodilatory activity in the endothelium-denuded aortic ring, by blocking extracellular Ca2+ entry through ROCCs and VDCCs (Kim *et al*., 2019). Piperitenone

oxide, a monoterpenoid ketone, as a major chemical constituent of the essential oil from *Ziziphora clinopodioides* Lam. (Uyghur traditional herb) and *Mentha x villosa*, can relax the contraction induced by PE in both rat aortic rings with and without functional endothelium (Lan *et al*., 2017).

Based on the research reports above, flavonoids, alkaloids, tannins, saponins, and terpenoids have direct vasodilatory activity in vascular smooth muscle, not related to the endothelium. These chemical constituents can act synergistically with different strengths and amounts in each of the plant parts such as the bulbs and the leaves. In this study, showing that EBL induces a stronger vasodilatory activity compared to EBB, it is suspected that the number of phytochemicals (flavonoids, alkaloids, tannins, saponins, and triterpenoids) which have vasodilatory activity in EBL is far more than those contained in EBB. Analogous to the studies reported above, the phytochemicals contained in EBL and EBB act on endothelial-independent VSMC. To prove the correct mechanism, further research is needed.

However, bioassay-guided isolation needs to be carried out to obtain active compounds possessing vasodilatory activity in the smooth muscle of blood vessels, as well as the continued elucidation of structures. Therefore, the bulbs and leaves of *E. bulbosa* are expected to be developed as an herbal antihypertensive agent acting on the smooth muscles of blood vessels.

CONCLUSION

The ethanol extract of bulbs and leaves of *E. bulbosa* possesses a vasodilatory effect *in vitro* by acting on the endothelium-independent vascular smooth muscle. This vasodilatory activity of EBL was stronger than that induced by EBB.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

The study protocol was approved by the Health Research Ethics Committee (KEPK) of Mulawarman University (No. 159/KEPK-FK/IX/2019).

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