ANTIHYPERTENSION STUDY OF *ANREDERA CORDIFOLIA* (TEN). V. STEENIS EXTRACT AND ITS FRACTIONS IN RATS THROUGH DEXAMETHASONE INDUCTION AND NITRIC OXIDE RELEASE

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ABSTRACT

Objective: The aims of this study were to prove the antihypertensive effect of *Anredera cordifolia* (Madeira vine) in dexamethasone-induced hypertensive rat and to determine the release of nitric oxide (NO).

Methods: The rat's blood pressure was measured by CODA® tail-cuff blood pressure system. A hypertensive rat model was developed on day 7 after administration of dexamethasone injection 0.5 mg/kg body weight (bw). NO levels were measured by spectrophotometry at a wavelength of 546 nm after reacting the serum sample with Griess reagent.

Results: Ethanol extract of Madeira vine (EEMV), ethyl acetate fraction (EF), and water fraction (WF) could reduce systolic blood pressure at day 14 with a diastolic blood pressure (DBP) reduction of 26.8, 34.1, and 40.5 mmHg, respectively. DBP began to decrease from day 8 in the EEMV group with a DBP reduction of 24.1 mmHg. In the HF, EF, and WF groups, decreasing in DBP occurred on day 14 which were 22.0, 24.5, and 35.4 mmHg, respectively. NO level in rat serum was increased significantly at 90 min after administration of EEMV 100 mg/kg bw and WF 40.73 mg/kg bw. Increasing in NO levels due to EF with a dose of 1.66 mg/kg bw was not significantly different to control group.

Conclusion: Ethanol extract of *A. cordifolia* had the antihypertensive effect in dexamethasone-induced hypertensive rats, so does its WF and EF. The mechanism of ethanol extract of Madeira vine leaves and its WF most likely due to vasodilation effect through NO-pathway, whereas EF could have other mechanism(s) of action.

Keywords: *Anredera cordifolia*, Antihypertensive, Dexamethasone, Nitric oxide.

INTRODUCTION

Hypertension is defined as blood pressure ≥140/90 mmHg. It affects millions of people, and the prevalence in adults aged 25 and over was around 32.5% in man and 29.3% in woman [1]. It is estimated that, by 2030, 40.5% of the US population is projected to have some form of cardiovascular disease (CVD), including 37.3% hypertension [2]. Hypertensive patients, which were related to CVD, need a medical attention [3]. Hypertension can be treated with pharmacological (using antihypertensive medicine) and non-pharmacological therapy (lifestyle modifications). If hypertension cannot be treated with only lifestyle modifications, antihypertensive medicine needs to be taken [4]. However, antihypertensive medicines have many side effects that can complicate the clinical problem. Diuretics may cause abnormalities of fluid and electrolyte balance. Side effects which are caused by angiotensin converting enzyme (ACE) inhibitors are cough, skin rash, hyperkalemia, kidney failure, and others. Calcium channel blockers may cause headache, flushing, dizziness, peripheral edema, constipation, and skin rash [3].

According to the World Health Organization survey, about 70–80% of the world populations rely on non-conventional medicine mainly on herbal sources in their primary health care [5]. It could be due to several advantages of herbal medicine such as: It is comparatively cheaper than modern remedies and treatments; complementary therapy is easily available; traditional medicines using herbs, vegetables, and fruits are free from any unwanted, undesired side effects; natural remedies, being general daily health supplements, not only help in curing the main disease but also soothe other body systems; and therefore, holistic remedies help in rejuvenating and revitalizing the human health [6]. However, the increasing popularity of herbal products as medications and food supplements has also raised concerns about their quality, safety, and efficacy (QSE) with uncertainty about their active compounds, their unsupervised use, as well as the legal responsibilities of practitioners. To ensure QSE of the herbal product, basic research programs should be focused on the toxicity and efficacy relationship for those potent or poisonous herbal substances [5]. One of the experiments to assess efficacy and safety of herbal is preclinical study in animal models. That study may provide an overview of efficacy, mechanism of action, effective dosage, or toxicity of herbal.

Madeira vine (*Anredera cordifolia*) traditionally used to reduce blood pressure. Preliminary studies of blood pressure lowering effect of Madeira vine (*A. cordifolia*) had been done [7]. That examination showed that the ethanol Madeira vine leaves extract had a potential antihypertensive effect. It could prevent heart rate increment which was induced by adrenaline. Sukandar *et al.* [8] showed vasodilation effect of *A. cordifolia* on rabbit aorta. Toxicity studies of *A. cordifolia* had been done and published in several journals [9-11], which showed that *A. cordifolia* quite safe to be used as herbal medicine even in pregnant women. Nevertheless, the antihypertensive effect of *Anredera cordifolia* have not been studied in chronic hypertensive animal model. One example of such animal model is dexamethasone-induced hypertensive rat, which had elevated blood pressure due to increased peripheral resistance [12,13]. On that model, elevated blood pressure can be antagonized by vasodilation due to increase of nitric oxide (NO) bioavailability, inhibition of α1 receptors, inhibition of ACE or AT1 receptor, or inhibition of calcium channels. This experiment intended to prove the antihypertensive effect of *A. cordifolia* in dexamethasone-induced hypertensive rat and elucidate the mechanism of its action.

METHODS

Preparation of ethanol extract and its fractions

The leaves of *A. cordifolia* were collected from Lembang-Bandung and identified in Herbarium Bandungense, School of Life Science and Technology, Bandung Institute of Technology. Ethanol extract was prepared as described in the study of Garmana *et al*. Briefly, the Madeira vine leaves crude drug was extracted using 96% ethanol for 3 h at 80°C by reflux and repeated in 3 times. The ethanol extract was concentrated using a rotary evaporator (namely, ethanol extract of Madeira vine [EEMV]). The fractionation was carried out by liquid-liquid extraction method with n-hexane and ethyl acetate as solvents. A total of 100 g of extract was put in 500 ml of boiling aquadest, then stirred and filtered while still hot. The filtrate was cooled, then put into separating funnel, and n-hexane was added with a volume ratio of 1:1. The mixture was shaken gently. The n-hexane fraction was separated, whereas the water fraction (WF) was reextracted using n-hexane up to 3 times. The water layer was then further extracted by adding ethyl acetate. The n-hexane (HF) and ethyl acetate fractions (EF) were collected and concentrated using a rotary evaporator. The WF was dried with freeze-dryer.

Phytochemical screening and determination of marker in fractions

Phytochemical screening was conducted to determine the presence of saponin, quinone, flavonoid, tannin, alkaloid, and steroid/triterpenoid in extract and fractions. Thin layer chromatography (TLC) profile was determined using silica gel gel GF254 plate with toluene-ethyl acetate-formic acid (35:15:1) as mobile phase for n-hexane fraction; ethyl acetate-methanol-water (23:2:1) for ethyl acetate fraction.

Experimental animal

Male Wistar rats (200–300 g) were provided by School of Pharmacy, ITB. They were housed in clean and transparent polypropylene cages and maintained at 25–27°C and at a relative humidity of 55–75%, under a 12-h light-dark cycle, and free access to food and water at all times. This study was approved by the Animal Research Ethics Committee - Bandung Institute of Technology (ethical approval No. 01/KEPHP-ITB/09-2016).

Evaluation of antihypertensive effect

In the evaluation of chronic antihypertensive effect of Madeira vine leaves extract, seven groups (n=5) were used: Negative and positive control groups were given sodium carboxymethyl cellulose (Na CMC), EEMV group was given 100 mg/kg bw EEMV leaves, HF group was given 0.02 mg/kg body weight (bw) of n-hexane fraction, EF group was given 1.66 mg/kg bw of EF, WF group was given 40.73 mg/kg bw of WF, and standard group was given 0.45 mg/kg bw of amlodipine. Dexamethasone 0.5 mg/kg bw was given subcutaneously for 14 days. Extract, fractions, and amlodipine were administered on day 8–14. Blood pressure was measured by CODA® tail-cuff blood pressure system on day 0 (before induction), day 7 (after induction), and days 8, 10, and 14 (1, 3, and 7 days after therapy) [14-16]. The obtained data were analyzed statistically using SPSS version 24.

Determination of serum NO levels

The NO-involved vasodilation effect was studied *in vivo* by measuring the nitrite level in serum with Griess reagent. The rat blood was taken before and after administration of extract, fractions, or isosorbide dinitrate (ISDN)1.8 mg/kg bw on min 0 (before drug administration), 30, 60, and 90. Blood samples were centrifuged at 10,000 rpm for 10 min. A total of 100 μl serum was deproteined with the addition of 20 μl $ZnSO_4$ 6%, then centrifuged again. The supernatant was reacted with cadmium for 15 min. A total of 50 μl aliquots was put in a microwell-plate and 50 μl of Griess reagents were added, and then incubated for 30 min. The absorbance was measured by Microwell-plate reader at a wavelength of 546 nm [17-19]. It was proportional to NO level in the serum samples. The obtained data were analyzed statistically using SPSS version 24.

RESULTS

Phytochemical screening of the EEMV leaves showed that it contained flavonoid, phenol, and steroid/triterpenoid. The fractionation yields

0.02%, 1.66%, and 40.73%, respectively, of n-hexane, ethyl acetate, and WF. Phytochemical screening of the fractions was presented on Table 1. The TLC profile showed that ursolic acid could be a marker compound in HF, apigetrin (apigenin-7-O-glycoside) could be a compound marker in EF, while in WF marker compound was a flavonoid compound.

Administration of extract, fractions, and amlodipine showed an effect on rat blood pressure. In general, it can be seen that the extract and all of fractions can lower blood pressure in hypertensive animal models (Fig. 1). Ethanol extract, WF, and EF could decrease systolic blood pressure (SBP) significantly on day 14 (day 7 after therapy). EEMV, EF, and WF reduced SBP by 26.8, 34.1, and 40.5 mmHg, respectively. Amlodipine decreases SBP gradually. It reduced SBP significantly from day 10 (day 3 after amlodipine administration). After 7 days administration of amlodipine, SBP reduced by 34 mmHg.

Similar results were observed in diastolic blood pressure (DBP) (Fig. 2). The extract, fractions, and amlodipine expressed significantly decrease the DBP on day 14 (day 7 after therapy). In the extract group, the DBP decreased significantly from the first day of extract administration (day 8). After 7 days of EEMV administration, DBP decreased by 24.1 mmHg. HF, EF, and WF reduced DBP by 22.0, 24.5, and 35.4 mmHg, respectively. Amlodipine reduced DBP by 29.1 mmHg.

Fig. 3 denoted experimental results of the determination of NO levels in rat serum. In general, it could be seen that the administration of ISDN, EEMV, ethyl acetate, and WF could increase NO levels in rat serum. Administration of ISDN caused a gradual increase of NO levels from the min 30. A significant increase occurred on min 60 and then slightly decreased on min 90. In the extract and fractions groups, increasing in NO levels started on min 30. A significant increase in the extract group occurs on min 60 and 90, while in the WF group, NO levels increased significantly on min 90. In the EF group, the highest increase of NO levels occurred on min 90, but it was not significant compared to the negative control group which also gave a slight increase of NO levels.

DISCUSSION

Arterial blood pressure is strongly influenced by blood flow (cardiac output) and resistance to blood flow (peripheral resistance). Cardiac output is a function of stroke volume, heart rate, and venous capacitance, whereas total peripheral resistance reflects vascular resistance, blood viscosity, and turbulence [4,20]. The effects of ethanol extract of Madeira vine leaves extract on heart rate and cardiac output have been investigated. Madeira vine leaves extract could prevent heart rate increment induced by adrenaline. It also showed diuretic and natriuretic activity [7]. In this study, its effect on vascular resistance was studied.

Dexamethasone administration may cause functional impairment of vascular smooth muscle cells, thus increasing peripheral resistance of the blood vessels. In general, it can occur as a result of two reasons. The first reason: Increasing in sensitivity and reactivity of blood vessels to vasoconstrictors [12]. It is probably due to increased expression of angiotensinogen [21], increased expression of angiotensin II receptor

Table 1: Phytochemical screening of n-hexane, ethyl acetate, and water fraction

Group	HF	EF	water fraction
Alkaloid	+	+	-
Flavonoid	+	+	+
Saponin	-	-	-
Quinone	-	+	-
Tannin	-	-	-
Phenol	+	+	+
Steroid/triterpenoid	+	+	-

+: Detected, -: Not detected, HF: n-hexane fraction

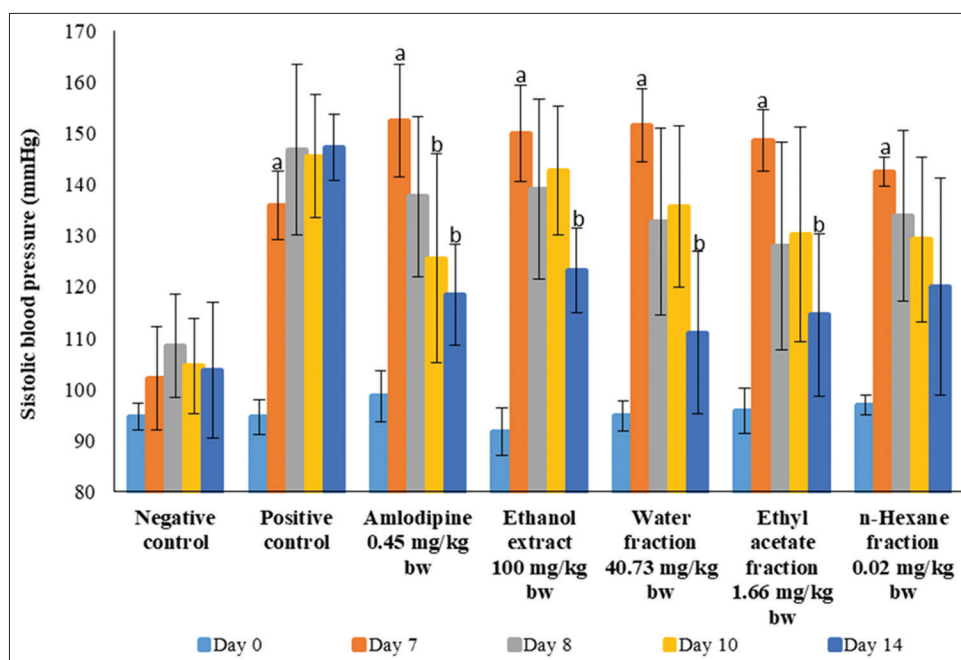**Fig. 1: Systolic blood pressure before and after administration of Madeira vine leaves extract and its fractions. Data are expressed as mean±standard deviation (n=5). [a]Significantly different compared to blood pressure day 0 (p<0.05). [b]Significantly different compared to blood pressure day 7 (p<0.05)**
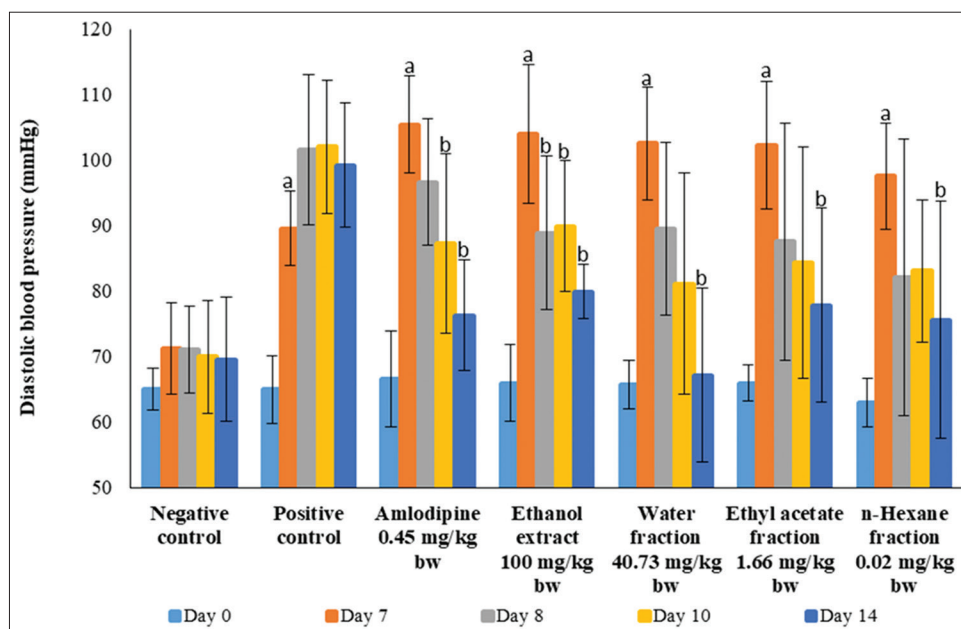

Fig. 2: Diastolic blood pressure before and after administration of Madeira vine leaves extract and its fractions. Data are expressed as mean ± standard deviation (n=5). [a]Significantly different compared to blood pressure day 0 (p<0.05). [b]Significantly different compared to blood pressure day 7 (p<0.05)

gene [22], or increased expression of α1 receptor genes in blood vessels [23,24]. The second: Decreasing in bioavailability of vasodilators such as prostaglandins and NO. It might be caused by decreasing synthesis [12,22] or increasing inactivation of prostaglandins and NO [12,13] result in oxidative stress in the blood vessels. Oxidative stress and NO deficiency will lead to vasoconstriction, increased peripheral resistance, and increased blood pressure both SBP (Fig. 1) and DBP (Fig. 2).

The increasing in SBP and DBP after giving dexamethasone 0.5 mg/kg bw for 7 days showed that induction was successful and hypertensive animal model had been established. Amlodipine can be

used as standard as it has antihypertensive and antioxidant effects that can inhibit many of the oxidative stress-dependent mechanisms involved in Ang II-mediated cardiovascular injury [25].

From the experimental results, it can be assumed that the Madeira vine leaves extract and its fractions caused vasodilation and could decrease peripheral resistance, and hence, they can antagonize the effects of dexamethasone. Vasodilation effects can be achieved through several ways, including: (a) Increased levels of NO, (b) inhibition of α1 receptors in blood vessels, (c) inhibition of ACE or AT1 receptor in blood vessels, and (d) inhibition of calcium channels.
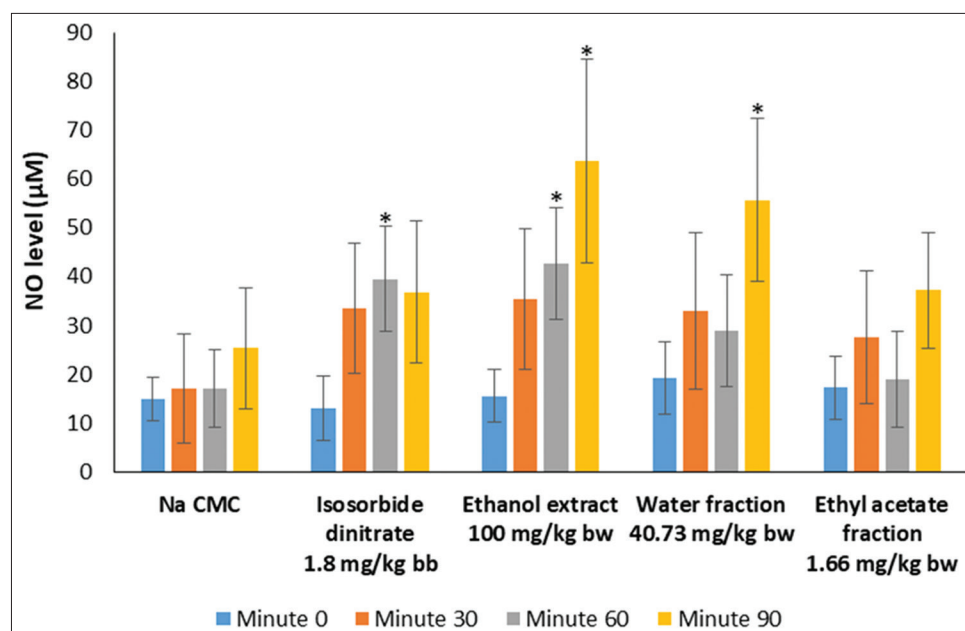
Fig. 3: Nitric oxide levels before and after administration of Madeira vine leaves extract and its fractions. Data are expressed as mean ± standard deviation (n=5). *Significantly different compared to Na CMC group (p<0.05)

Decreasing in peripheral resistance can also be attributed to the antioxidant effect of EEMV. The antioxidant properties of the extract could reduce superoxide formed when oxidative stress occurs. The antihypertensive actions may also caused by the complex pharmacological mechanism from various kinds of constituents [26]. The result of experiment on dexamethasone-induced hypertensive rats showed that the most effective were ethyl acetate and WF. Therefore, the experiment continued on both fractions by measuring the NO levels in the rat serum.

NO is released from the endothelial. Before the structure is elucidated, it is known as endothelium-derived relaxing factor. In the blood vessel wall, NO is produced mainly from L-arginine by the enzyme endothelial NO synthase (eNOS), but it can also be released non-enzymatically from S-nitrosothiols or nitrate/nitrite. NO diffuses into the smooth muscle, then stimulates guanylyl cyclase, and induces cGMP formation. Furthermore, cGMP will activate the protein kinase G which promotes calcium absorption into the sarcoplasmic reticulum until the calcium content in the cytosol decreases and evoke relaxation of smooth muscle of blood vessels [27].

Determination of NO levels can be associated with the vasodilatory effect of a substance. The higher levels of NO in the blood denoted greater vasodilation effect. NO has a short half-life and undergoes metabolism in the body to nitrate and nitrite [28,29]. Therefore, the determination of NO levels can be done indirectly by measuring the levels of metabolites. Nitrite concentration can be determined colorimetrically using the Griess reaction. The nitrate component present in the biological sample should be converted first into nitrite by adding a reductor, for example, cadmium. The nitrite component in the sample will react with sulfanilamide in an acidic condition forming diazonium ion which is then coupled with N-(1-napthyl) ethylenediamine to form a chromophoric azo product that absorbs strongly at 540 nm [19].

NO level determination showed that the NO level profile of extract and WF was different from ISDN (Fig. 3). The WF has a slower onset than ISDN. However, EEMV and WF may have a longer duration. It can be an advantage over its use since it reduces the frequency of drug administration. The EF showed a slight increase in NO levels. However, this fraction showed a hypotensive effect on dexamethasone-induced hypertensive animals. Based on the result, it can be suggested that there are other mechanisms in the antihypertensive effect of EF in addition to the effect of NO levels. Other possible mechanisms include calcium channel blocker, alpha receptor blocker, or inhibition of ACE.

Phytochemical screening showed that extract and all of fractions contained flavonoid and phenolic compound. Phenolic compounds widely distributed and the most secondary metabolite found in plants. It can act as an antioxidant since it can scavenge free radicals [30]. Phenolic compounds included phenolic acid, anthocyanin, and flavonoid [31]. Flavonoid is the biggest group of phenols found in higher plants and is effective antioxidant in plants and animals [32,33]. Flavonoid as a food supplement could decrease the risk of cancer, cardiovascular, and neurodegenerative disease [34]. Based on the chromatogram profile of Madeira vine leaves fraction, marker compound of the EF was apigenin-7-O-glycoside, whereas marker compound of WF was flavonoid compound. Flavonoids were known to have various pharmacological activities including antihypertensive effects. Its effect can be achieved through vasodilation or antioxidant properties of flavonoids [35]. Flavonoids scavenge the free radicals, so it can reduce superoxide (O_2^-) concentration and prevent the formation of peroxynitrite ($ONOO^-$). In addition, flavonoids also modulate the bioavailability of NO at the cellular level by increasing the expression of the enzyme NO synthase (NOS) [36,37]. Flavonoids could be a compound that plays a role in their antihypertensive effect.

CONCLUSION

An EEMV (*A. cordifolia*) had antihypertensive effects in dexamethasone-induced animal model so does its WF and EF. The mechanism of Madeira vine leaves extract and its WF most likely due to vasodilation effect through NO-pathway, whereas EF could have another mechanism (s) of action. The antihypertensive effect of the extract and fractions could be due to its flavonoids content. Madeira vine leaves can be exploited as a source of natural antihypertensive agents.

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