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RESEARCH ARTICLE

VEGF-A Expression in Preeclampsia Rats' Liver Treated with Andaliman Seed (*Zanthoxylum acanthopodium* DC.) Extract

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Abstract

Preeclampsia is a life-threatening pregnancy disorder diagnosed after 20 weeks of gestation, characterized by hypertension (≥140/90 mmHg) and often accompanied by proteinuria (≥300 mg.24 hours^{-1}). Preeclampsia causes an imbalance in angiogenic factors, increasing levels of soluble fms-like tyrosine kinase-1 (sFlt-1), which reduces the expression of Vascular Endothelial Growth Factor (VEGF-A) protein. This condition affects other organs, such as the liver, causing hypoxia. Andaliman (*Zanthoxylum acanthopodium* DC.), rich in bioactive compounds, is a potential alternative treatment for preeclampsia due to its hepatoprotective, anti-inflammatory, and angiogenic-modulating effects. This study aims to identify changes in VEGF-A protein expression in the liver of a preeclampsia rat model (*Rattus norvegicus*), divided into four groups, with treatment using Andaliman seed extract. Protein was extracted from the liver and measured using the Bradford method, followed by SDS-PAGE and retardation factor (RF) calculation, and then continued with Western blotting. Band intensity data were analysed quantitatively. The results showed that the preeclampsia group had a lower VEGF-A fold change (0.67 ± 0.28) than the control group (1.00). This indicates a decrease in VEGF-A expression, possibly due to an increase in the anti-angiogenic factor sFlt-1. A dose of 100 mg.kg^{-1} body weight of Andaliman extract (0.96 ± 0.08) was more effective than 200 mg.kg^{-1} body weight (0.78 ± 0.24) because it increased the protein intensity of VEGF-A based on western blotting. These findings suggest that Andaliman seed extract, particularly at a dose of 100 mg.kg^{-1} body weight, has potential as a hepatoprotective agent in preeclampsia through the restoration of VEGF-A expression.

Keywords: Andaliman seed, liver, preeclampsia, VEGF-A, Western blot.

INTRODUCTION

Preeclampsia is a life-threatening pregnancy disorder for both the mother and fetus [1]. Preeclampsia is diagnosed after 20 weeks of pregnancy and has a significant impact, causing death in 2-8% of cases [2]. This disease generally affects both mothers and fetuses, resulting in 70.000 maternal deaths and 500.000 infant deaths each year [3]. This case also occurs in Indonesia, where an estimated 24% of maternal deaths are caused by preeclampsia [1]. The diagnosis of this disease is based on symptoms, including blood pressure reaching 140/90 mmHg, and is sometimes accompanied by proteinuria of 300 mg after 24 hours of urine sampling. Other effects caused by preeclampsia include renal failure, thrombocytopenia, pulmonary edema, headaches, and liver damage [1,2,4].

Liver damage in patients with preeclampsia is characterized by elevated liver enzymes,

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particularly aspartate aminotransferase (AST) and alanine aminotransferase (ALT), which can lead to organ dysfunction [2,4]. These enzymes are produced in twofold levels due to vasoconstriction of blood vessels, leading to liver hypoxia, necrosis, and hepatocyte degeneration. Liver dysfunction in preeclampsia may result from an imbalance between proangiogenic and antiangiogenic factors. In this condition, circulating soluble fms-like tyrosine kinase-1 (sFlt-1), an antiangiogenic factor released by the syncytiotrophoblast, is increased and binds VEGF (primarily VEGF-A), thereby reducing the bioavailable fraction of VEGF [4,5].

Since VEGF is essential for maintaining endothelial integrity and nitric oxide production, its reduction promotes endothelial dysfunction, vasoconstriction, and impaired hepatic microvascular perfusion. This process may lead to hepatic ischemia, hepatocellular injury, and elevated liver enzymes [6]. This imbalance is also associated with an increased risk of developing HELLP (hemolysis, elevated liver enzymes, and low platelets) syndrome in pregnant women [4].

Cases of preeclampsia and maternal organ damage continue to rise, and research into preeclampsia treatments continues to advance. One area of ongoing research is the use of plant extracts for their bioactive compounds [1]. The Andaliman plant (*Zanthoxylum acanthopodium* DC.), often referred to as "Batak pepper", is deeply integrated into the culinary practices of the Batak ethnic group, primarily serving as a seasoning for meat and fish due to its distinct aroma and spicy flavor. This harvested fruit adds a unique flavor to traditional cuisine and is believed to enhance the shelf life of foods. Beyond its significant role in food preparation, the Batak community also utilizes Andaliman as a traditional medicine [7]. The recognized medicinal potential of this plant stems from its rich profile of secondary metabolites, including flavonoids, terpenes, pyrroloquinolines, quaternary isoquinolines, and aporphine alkaloids. Previous studies have shown that compounds from the flavonoid group, particularly flavanols, lower blood pressure, improve endothelial function, inhibit platelet aggregation, and reduce inflammatory responses [7,8].

Andaliman has been widely recognized as a medicinal plant with pharmacological activities, including antibacterial, anti-inflammatory, antifungal, anticancer, cardioprotective, nephroprotective, hepato-protective, placental trophoblast-protective, and pregnant women - anti-preeclampsia effects, which have been summarized in several reviews [7,9].

Experimental studies in animal models have shown that administration of Andaliman can reduce the risk of preeclampsia, primarily through its anti-inflammatory, antioxidant, and antimicrobial properties [7]. While previous studies have examined Andaliman's effect on placental histology [1], the present study focuses specifically on VEGF-A protein expression in maternal liver tissue, an area that remains understudied [1]. Other studies have shown that Andaliman can lower blood pressure, mean arterial pressure (MAP), and pro-inflammatory cytokine levels, namely TNF-α and IL-6, in preeclampsia rat models [10]. Therefore, Andaliman was selected in this study because its bioactive compounds exhibit antihypertensive, anti-inflammatory, antioxidant, and hepato-protective properties, making it a promising candidate for the treatment of preeclampsia and its associated maternal liver injury.

Although the effects of preeclampsia on maternal organs have been well documented, the molecular mechanisms underlying liver damage, particularly changes in VEGF-A expression, remain poorly explored [6]. The role of sFlt-1/VEGF imbalance in the pathophysiology of preeclampsia has been identified in primary isolated trophoblast and primary endothelial cells, but research specifically targeting angiogenesis dysregulation in liver tissue remains limited, and effective therapeutic strategies to restore VEGF expression in affected maternal organs have not been established [11]. Previous research showed that Andaliman promotes liver recovery and improves histological hepatic changes via FasL and cytochrome c, without mentioning the interaction between VEGF and sFLT-1 [1].

However, further research is needed to clarify the relationship between preeclampsia and liver involvement, particularly to evaluate the long-term hepatic consequences in the increasingly large population of patients affected by preeclampsia [12]. Current treatment of preeclampsia largely relies on clinical monitoring and fetal safety, with limited research on the effects on mothers affected by angiogenic dysfunction in the liver. Therefore, this study aims to address this gap by comprehensively evaluating VEGF-A protein expression in the liver of a preeclampsia animal model and investigating the hepatoprotective potential of Andaliman seed extract (*Zanthoxylum acanthopodium* DC.).

MATERIAL AND METHOD

Andaliman Seed Extraction

The plant identification was verified at the Taxonomy Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Brawijaya. The Andaliman seeds were dried, ground into powder, and macerated in absolute methanol (1:4, w/v) for 24 h under static conditions. The macerate was then concentrated using a rotary evaporator at 40°C to obtain the Andaliman seed extract [13].

Experimental Animal

This experiment was approved by the Animal Care and Use Committee, Universitas Brawijaya (No. 107-KEP-UB-2024). Pregnant rats were divided into four groups (n = 3 per group): normal pregnant rats as the control group (LC), preeclampsia-induced rats (LPre), preeclampsia-induced rats treated with Andaliman extract at 100 mg kg^{-1} body weight (LPre + And100), and preeclampsia-induced rats treated with Andaliman extract at 200 mg kg^{-1} body weight (LPre + And200). The doses were selected based on a previous study showing that 400 $mg.kg^{-1}$BW caused a significant decrease in body weight; therefore, only 100 and 200 $mg.kg^{-1}$BW were used [13]. Pregnancy was established by housing female and male rats in a 2:1 ratio for 4 nights and confirmed by the vaginal plug method. Preeclampsia was induced by administering 18% NaCl at 10 $mL.g^{-1}$ body weight from gestational days 14 to 21, and Andaliman extract was given during the same period. Blood pressure was measured on days 7, 14, and 21 using the tail-cuff method, and proteinuria was assessed using a dipstick [1]. Pregnant rats were considered preeclamptic when blood pressure reached ≥140/90 mmHg and proteinuria ≥300 mg.24 $hours^{-1}$ [4,5]. On day 22 of pregnancy, the rats were euthanized with ketamine and xylazine at the approved dose, and liver tissues were collected and stored in PBS at −80°C for further analysis.

Isolation of Liver Proteins

Liver proteins were isolated from 0.5 g of liver tissue, which was washed three times with phosphate-buffered saline (PBS) to remove residual blood and contaminants. The tissue was then homogenized under cold conditions using a mortar and pestle with the gradual addition of 500µL of extraction buffer containing 1 mM phenylmethylsulphonyl fluoride (PMSF), 50 mM KH_2PO_4 (pH 7.4), 0.5% Nonidet P-40, and double-distilled water (ddH_2O). The homogenate was transferred to a 1.5 mL microcentrifuge tube, mixed with an additional 500 µL of extraction buffer, and further homogenized to ensure uniform protein extraction. The sample was centrifuged at 10,000 rpm for 20 min at 4 °C, and the resulting supernatant containing soluble liver proteins was collected into a new sterile microcentrifuge tube. The isolated proteins were stored at 4 °C for immediate use and at −20 °C for further analysis [14].

Bradford Protein Assay

The Bradford protein assay was performed using Bovine Serum Albumin (BSA) as the standard protein at concentrations of 0, 125, 250, 500, 750, and 1000 $\mu g.mL^{-1}$ to generate a standard calibration curve (Fig. 1). The resulting regression equation was y = 0.0008x + 0.8393, with an R^2 value close to 1.0, indicating a strong linear relationship between absorbance and protein concentration. For protein measurement, 10 µL of each sample was mixed with 90 µL of 0.9% NaCl and 5 mL of Bradford reagent, then vortexed and incubated at room temperature for 10 min. Absorbance was measured at 595 nm using a UV-Vis spectrophotometer (Shimadzu UV-1280). The standard curve equation was then used to determine the total protein concentration in all liver samples and served as the basis for normalizing VEGF-A expression [15].

Protein Analysis of Rat Liver

Liver protein separation was performed using SDS-PAGE with a 14% separating gel and a 5% stacking gel. Protein samples from all experimental groups were diluted in Tris-HCl buffer (pH 6.8) to equalize concentration, mixed with 1× sample reduction buffer, and heated at 95 °C for 5 min for protein denaturation. A total of 20 µL of each sample was loaded into the gel, and electrophoresis was performed at 100 V for 80 min. After separation, the gel was stained with Coomassie Brilliant Blue R-250 and destained with methanol, acetic acid, and distilled water until clear protein bands were obtained. The gel was then documented for further analysis [16,17,18].

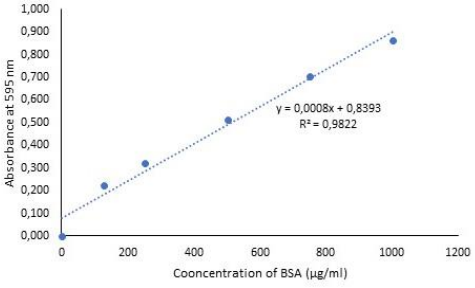

Figure 1. Standard curve of BSA

Quantification of VEGF-A Protein Expression

After SDS-PAGE, proteins were transferred onto a PVDF membrane using a transfer sandwich consisting of sponge, filter paper, gel, membrane, filter paper, and sponge. Before transfer, the gel and transfer materials were equilibrated in blotting buffer, and the PVDF membrane was activated in methanol. Electro transfer was performed at 20 V for 17 h. The membrane was then blocked with 2% Blotto in TBST for 1 h and washed three times with TBST. Primary antibodies against VEGF and β-actin were diluted 1:1000 in 2% Blotto and incubated with the membrane for 1 h, followed by incubation with goat anti-mouse IgG secondary antibody conjugated with alkaline phosphatase (1:2000) for 1 h. Protein bands were visualized using NBT/BCIP substrate and documented with a GelDoc imaging system (ImageQuant LAS 500) [17,18].

Data Analysis

The molecular weight of protein bands separated by SDS-PAGE was estimated using the retardation factor (Rf), calculated as the ratio of the distance traveled by the protein band to that traveled by the tracking dye. The obtained Rf values were then entered into a linear regression equation derived from the molecular weight marker to estimate the molecular weight of each visualized band. Western blot band intensity was analyzed using ImageJ software. The intensity of the target protein was normalized to the internal loading control (β-actin), and fold change was calculated as the ratio of target protein intensity to β-actin intensity. A fold change value of >1.0 indicated increased expression relative to the control, whereas a value of <1.0 indicated decreased expression. Statistical analysis was performed using one-way ANOVA followed by Dunnett's test in GraphPad Prism 9.1.2.226 [19–21].

RESULT AND DISCUSSION

Bradford Protein Assay

The Bradford protein assay is a reliable method for determining protein concentration, operating through the specific interaction between the dye Coomassie Brilliant Blue G-250 and proteins under acidic conditions. The dye specifically binds to basic amino acid residues, including arginine, histidine, phenylalanine, tryptophan, and tyrosine, reinforced by hydrophobic interactions within the protein structure.

Table 1. Absorbance and concentration of sample

Sample	Absorbance at 595 nm	Protein concentration ($\mu g.mL^{-1}$)
LC	1.667	1,034.625
LPre	1.458	773.375
LPre + and 100	1.88	1,300.875
LPre + and 200	2.074	1,543.375

This induces a crucial spectral shift in the dye's absorption maximum, moving from 465 nm to the measurable wavelength of 595 nm, thus generating the characteristic blue coloration. This spectrophotometric reading at 595 nm is then used to calculate protein content, assuming a direct proportional relationship between the resulting absorbance and the protein quantity, as confirmed by a linear standard curve [15].

The Bradford method was employed to quantify total protein concentration in liver tissue samples, which was important for normalizing VEGF-A expression levels. The results obtained from this assay ensured the accuracy and comparability of protein expression measurements across experimental groups. The absorbance of each sample was measured at the same wavelength to determine its protein concentration. The absorbance value acts as the y-value of the equation, and the x-value is the protein concentration. The result showed protein concentration in the liver of control (LC) and preeclampsia (LPre) 1034.625 $\mu g.mL^{-1}$ and 773.375 $\mu g.mL^{-1}$. Meanwhile, preeclampsia-induced rat liver treated with Andaliman at 100 and 200 $mg.kg^{-1}$ showed increasing protein concentration of 1,300.875 $\mu g.mL^{-1}$ and 1,543.375 $\mu g.mL^{-1}$ (Table 1).

Protein concentrations in liver samples from rats with preeclampsia were lower than those in other samples. The increase in protein concentration in the Andaliman-treated groups (1,300.875 and 1,543.375 $\mu g.mL^{-1}$) compared with the preeclampsia group (773.375 $\mu g.mL^{-1}$) suggests that Andaliman may contribute to restoring hepatic protein synthesis, which is impaired in preeclampsia. This finding is supported by earlier research showing that total protein levels in the preeclampsia patient group were lower than in patients with normal pregnancies, indicating liver dysfunction [22].

SDS-PAGE

A 15 µg of the prepared protein sample was loaded into the SDS-PAGE well to achieve optimal

separation based primarily on molecular weight (Fig. 2). Analysis of the Rf and log MW calculations obtained from the molecular weight marker was essential to generate a calibration curve. This calibration yielded a strong linear regression equation of y = -1.7384x + 2.6953 with an excellent coefficient of determination, R^2=0.9808 (Figure 3). This equation is then used to accurately estimate the molecular weight of every protein band visualized in the sample.

The similarity of the protein bands obtained from the SDS-PAGE separation showed similarities between the control liver, preeclampsia, and those treated with Andaliman (Suppl. 1). Differences were seen in the loss of proteins with lengths of 256 and 39 kDa in pre-eclampsia rat liver and 256 kDa in pre-eclampsia rats treated with 100 mg kg^{-1} BW Andaliman. The group of preeclampsia rats treated with mg kg^{-1} BW Andaliman showed clearer bands.

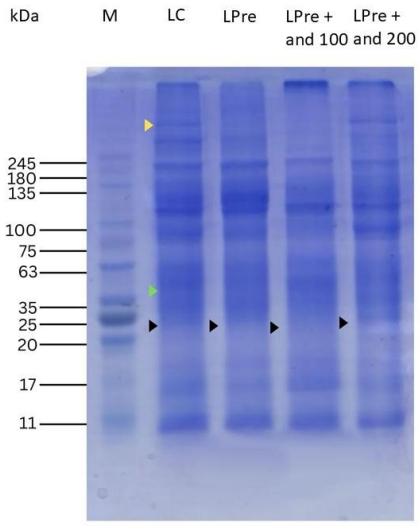

Figure 2. Protein profile of rat liver using SDS PAGE. (M) marker, (C) control, (L) liver, (Pre) preeclampsia, (and) Andaliman. Yellow arrowhead indicates 256 kDa protein; green arrowhead indicates 39 kDa protein; black arrowhead shows 21 kDa protein.

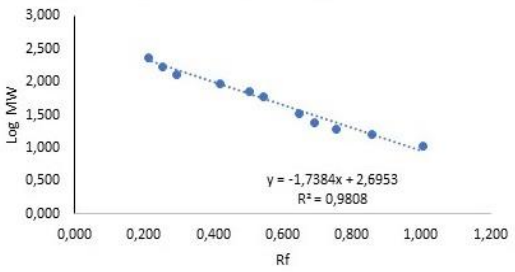

Figure 3. Log molecular weight of protein markers compared to Rf values

Protein bands with molecular weights of 256 kDa and 39 kDa appear thinner in the preeclampsia group, indicating impaired expression or degradation of specific proteins due to oxidative stress and inflammation associated with preeclampsia. Preeclampsia is known to cause systemic endothelial dysfunction triggered by the release of antiangiogenic factors from the placenta undergoing hypoxia and ischemia, which also affects other maternal organs [2]. The SDS-PAGE results showed that the protein, estimated at 21 kDa, was present in all samples. The estimated protein with a molecular weight of 21 kDa was VEGF, which was further analyzed by Western blotting [5,12].

VEGF-A Protein Expression in Liver by Western Blotting

The proteins were examined further using Western blotting. Western blotting was performed to compare the intensities of the target protein (VEGF) and the internal loading control (β-actin) (Fig. 4). Subsequently, band thickness analysis of the resulting protein bands was performed in ImageJ to determine normalized density and fold-change values.

Western blotting analysis revealed that the control group (LC) showed the optimal level of VEGF expression, with a normalized fold change of 1.00. This was significantly higher than in the treated and untreated pre-eclampsia groups (Fig. 5). The untreated pre-eclampsia group (LPre) showed a significant 37% reduction in fold change (0.67 ± 0.28). Andaliman treatment effectively restored VEGF expression in both dose groups. The 100 mg.kg^{-1} BW dose was more effective, achieving 96% recovery towards normal levels (0.96 ± 0.08), compared to 78% recovery with the 200 mg.kg^{-1} BW dose (0.78 ± 0.24; P = 0.1757).

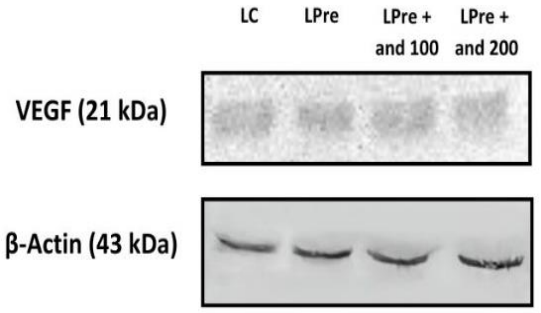

Figure 4. Protein expression of VEGF in rat liver using Western blotting; legend: (M) marker, (C) control, (L) liver, (Pre) preeclampsia, (and) Andaliman.

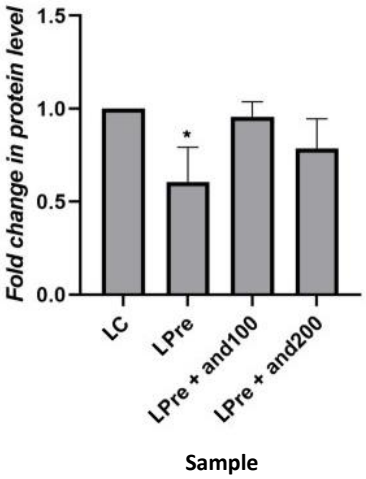

Figure 5. Comparison of VEGF protein fold change values for each sample; legend: (M) marker, (C) control, (L) liver, (Pre) preeclampsia, (and) Andaliman (*) indicates statistically significant difference compared to the control group ($p < 0.05$)

The results showed that VEGF expression in the liver of the preeclampsia group decreased significantly by 37% compared to the normal control. A dose of 100 mg.kg^{-1} BW showed the best recovery of VEGF expression, which was not significantly different from the control, indicating a recovery of 96% from normal levels. Meanwhile, the 200 mg.kg^{-1} BW dose showed recovery reaching 78% of the control level. Previously mentioned, Andaliman contains many compounds that are believed to have anti-preeclampsia potential [7]. Compounds from the flavonoid group, especially flavonols, can lower blood pressure, improve endothelial function, inhibit platelet aggregation, and reduce the inflammatory response, which has a positive effect on improving preeclampsia [8].

As mentioned previously, the decrease in VEGF protein levels may be due to lower total protein levels in the preeclampsia group compared with patients with normal pregnancies [22]. A more detailed possibility is a compound that has been proven to be an anti-pre-eclampsia: resveratrol. Resveratrol possibly reduces the secretion of the anti-angiogenic factor sFlt-1 from placental cells by directly suppressing the gene expression of its primary splice variants, sFlt-1 e15a and sFlt-1 i13. By lowering sFlt-1 levels, resveratrol may increase the bioavailability of VEGF, as less VEGF is bound and neutralized [11]. This restoration of pro-angiogenic VEGF signaling is crucial for mitigating the endothelial dysfunction characteristic of preeclampsia [23].

This result, in accordance with existing theory, is consistent with VEGF-A normally binding to and activating VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1) [24]. However, in patients with preeclampsia, there was possibly an increase in angiogenic factors such as soluble fms-like tyrosine kinase-1 (sFlt-1) and soluble endoglin (sEng), which causes endothelial dysfunction [25]. sFLT-1 is a soluble receptor of VEGF produced by the syncytiotrophoblast layer, so that when sFlt-1 increases, it causes a decrease in VEGF that mediates endothelial cell function [3,4]. sFlt1 has a very strong binding activity to VEGF-A. An increase in soluble Flt1 will reduce the availability of local VEGF, thereby inhibiting VEGF responsiveness in endothelial stalk cells and preventing the formation of new blood vessels [24,25].

These findings indicate that the 100 and 200 mg.kg^{-1} BW doses were effective in increasing VEGF-A expression to near-normal levels, suggesting that the bioactive components of Andaliman, particularly resveratrol, act through a mechanism that reduces sFlt-1 secretion [11,26]. Resveratrol inhibits activation of the Flt-1 gene promoter (the gene that encodes sFlt-1) and prevents increases in the Egr-1 transcription factor, a regulatory protein that controls flt-1 gene expression. Research on resveratrol reducing the secretion of the anti-angiogenic factor sFlt-1 from placental cells by directly suppressing the expression of its main splicing variants, sFlt-1 e15a and sFlt-1 i13, was reported. By lowering sFlt-1 levels, resveratrol likely increases the availability of VEGF-A, as less VEGF is bound and neutralized. The restoration of this pro-angiogenic VEGF signal is crucial for reducing the endothelial dysfunction characteristic of preeclampsia [11]. Decreased sFlt-1 increases VEGF bioavailability in maternal organs [4].

Doses were based on Simanullang's study using Andaliman at 100, 200, and 400 mg.kg^{-1} BW, with 400 mg.kg^{-1} BW causing a significant decrease in body weight [13]. Therefore, only 100 and 200 mg.kg^{-1} BW were used in this study. A dose of 100 mg.kg^{-1} of Andaliman was more effective in increasing VEGF protein levels than a dose of 200 mgkg^{-1}. Whole-plant extracts are used because the complex interactions among phytochemicals can increase bioavailability and produce synergistic therapeutic effects. According to the hormesis theory, the optimal effective dose of each herbal preparation is different. Therefore, a higher dose does not necessarily provide greater therapeutic benefits than a lower dose when used for treatment [27].

CONCLUSION

Based on the findings of this study, preeclampsia significantly downregulates VEGF-A expression in the maternal liver of rat models. Treatment with Andaliman seed (*Zanthoxylum acanthopodium* DC.) extract effectively attenuates this suppression, as evidenced by the restoration of VEGF-A levels. Notably, the 100 $mg.kg^{-1}$ BW dose demonstrated greater efficacy than the 200 $mg.kg^{-1}$ BW dose. These findings suggest that Andaliman extract may contribute to the restoration of hepatic protein synthesis. Possibly through bioactive compounds such as resveratrol, which modulate antiangiogenic pathways, this may contribute to improved preeclampsia-associated liver dysfunction by restoring angiogenic balance.

Author Contribution: Conceptualization, RK, MSS, AN, F; methodology, RK, MSS, RPV; validation, Azmi Noer; writing—original draft preparation, RK; writing—review and editing, RPV, F; supervision, AN, F; funding acquisition, F. All authors have read and agreed to the published version of the manuscript.

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Ethic Statement: This study was approved by the Animal Care and Use Committee, Universitas Brawijaya (No. 107-KEP-UB-2024).

Conflict of Interest: The author declares that there is no conflict of interest regarding the publication of this article.

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Supplementary 1. Molecular weight of protein sample based on Rf value calculations

MW (kDa)	Liver control			MW (kDa)	Liver Preeclamsia			MW (kDa)	Liver Preeclamsia + extract Andaliman 100 mg.kg^{-1} bw			MW (kDa)	Liver Preeclamsia + extract Andaliman 200 mg.kg^{-1} bw		
	Protein movement (cm)	Rf	Log MW		Protein movement (cm)	Rf	Log MW		Protein movement (cm)	Rf	Log MW		Protein movement (cm)	Rf	Log MW
256	0.5	0.104	2.409	202	0.8	0.167	2.306	202	0.8	0.167	2.306	256	0.5	0.104	2.409
202	0.8	0.167	2.306	160	1.1	0.229	2.203	160	1.1	0.229	2.203	202	0.8	0.167	2.306
160	1.1	0.229	2.203	100	1.7	0.354	1.998	100	1.7	0.354	1.998	160	1.1	0.229	2.203
100	1.7	0.354	1.998	92	1.8	0.375	1.964	92	1.8	0.375	1.964	100	1.7	0.354	1.998
92	1.8	0.375	1.964	73	2.1	0.438	1.861	73	2.1	0.438	1.861	92	1.8	0.375	1.964
73	2.1	0.438	1.861	49	2.6	0.542	1.690	49	2.6	0.542	1.690	73	2.1	0.438	1.861
49	2.6	0.542	1.690	33	3.1	0.646	1.519	39	2.9	0.604	1.587	49	2.6	0.542	1.690
39	2.9	0.604	1.587	21	3.7	0.771	1.313	33	3.1	0.646	1.519	39	2.9	0.604	1.587
33	3.1	0.646	1.519	16	4.0	0.833	1.210	21	3.7	0.771	1.313	33	3.1	0.646	1.519
21	3.7	0.771	1.313	14	4.2	0.875	1.142	16	4.0	0.833	1.210	21	3.7	0.771	1.313
16	4.0	0.833	1.210	9	4.8	1.000	0.937	14	4.2	0.875	1.142	16	4.0	0.833	1.210
14	4.2	0.875	1.142					9	4.8	1.000	0.937	14	4.2	0.875	1.142
9	4.8	1.000	0.937									9	4.8	1.000	0.937