



Research Article

Study of Combination of Nanoherbal Andaliman (*Zanthoxylum acanthopodium*) and Extra Virgin Olive Oil (EVOO) Effects in the Expression of Malondialdehyde (MDA), Heat Shock Protein-70 (HSP70) and Placental Histology of Preeclamptic Rats

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ABSTRACT

Background: Pre-eclampsia (PE) contributes to the second cause of maternal death in Indonesia. Andaliman is a typical spice of the Batak ethnic in Northern Sumatera Province, Indonesia. This study aimed to explore the potential of novel herbal medicine compound of nanoherbal andaliman and *extra virgin olive oil* (EVOO) as PE treatment.

Methods: Nanoherbal andaliman was generated using *High-energy Milling* (HEM). The treatments were divided into the following five groups: K⁻ (control): pregnant rats; K⁺: PE model rats; P1: PE model rats + 0.45 g of EVOO/200 g BW on the 13th–19th day of pregnancy; P2: PE model rats + nanoherbal andaliman 100 mg/200 g BW on the 13th–19th day of pregnancy; and P3: PE model rats + combination of 0.45 EVOO/200 g BW and nanoherbal andaliman 100 mg/200 g BW on the 13th–19th day of pregnancy. Rats were dissected on the 20th day of pregnancy. The observed parameters were blood pressure, proteinuria, malondialdehyde (MDA), Heat Shock Protein-70 HSP-70 and histology of placenta.

Results: A significant difference was noticed ($p < 0.05$) in blood pressure, proteinuria, foetal weight, haematocrit, erythrocytes and trophoblastic cells after the administration of combined nanoherbal andaliman and EVOO. No significant differences in placental weight, foetal number, leukocytes, MDA and HSP-70 were found ($p > 0.05$).

Conclusion: The combination of nanoherbal andaliman and EVOO decreased systolic blood pressure and induced the expression of MDA and HSP-70, as well as placental histology of pre-eclamptic rats.

Introduction

Pre-eclampsia (PE) is one of the major causes of morbidity and maternal and perinatal mortality, with characteristics such as hypertension and proteinuria in pregnancies over 20 weeks and complex disorders involving the maternal and placental components.^{1,2} According to the Indonesian Demographic and Health Survey, PE contributed to 24% of pregnant women and is the second cause of maternal death in Indonesia.³ This disease is one of the most complicated hypertensive disorders during pregnancy.⁴ Hypertension cases in pregnancy including PE have notably increased in number and caused frequent medical complications in pregnancy. Around 70% of women diagnosed with hypertension in pregnancy are cases of PE.⁵ Annually, roughly 50 000 women died due to PE.⁶ Incomplete placentation occurs, including the arterial spiral remodelling and trophoblastic invasion in the decidua, causing the reduction of blood perfusion, hypoxia/ischemia and oxidative stress in PE.⁷ Oxidative stress in PE can occur both in the maternal

circulation and in the placenta.¹ The pathophysiology of PE is still unclear but ROS imbalance and presence of antioxidants are two of the factors that can affect this disease. ROS strongly contributes to endothelial dysfunction in PE.

Nanotechnology can produce a herbal drug preparation on the atomic and molecular scales of chemistry, biology, and catalytic activity.⁸ Nanotechnology has several advantages such as ability to modify surface characteristics, small sizes and high loading capacity for high-concentration application.⁹ Nanomaterials are used in medicine especially in tumour therapy and researchers have developed various nanomaterials such as nano-oxide, carbon nanomaterials, quantum dots, and metal nanomaterials to deliver drugs to the placenta.¹⁰

Andaliman fruit (*Zanthoxylum acanthopodium* DC.) is one of the special ingredient spices of the Batak ethnic (Karo, Simalungun, South Tapanuli) in Northern Sumatera, Indonesia and it has antioxidant activity. *Zanthoxylum* genus has been widely studied for several

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types of biological activities such as larvacide (insecticide that is specifically targeted against the larval), anti-inflammatory, analgesic, antinociceptive, antioxidant, antibiotic, hepatoprotective, antiplasmodial, cytotoxic, antiproliferative, anthelmintic, antiviral, anticonvulsant and antifungal activities.¹¹⁻¹⁶ Antioxidants are components that can inhibit free radicals and nanoherbal andaliman can reduce free radicals in PE placentas. People use olive oil not only as food and for cooking but also as the most common ingredients of perfumes and soaps. Olive oil in *Extra Virgin Olive Oil* (EVOO) at a low dose could control serum Heat Shock Protein-70 (HSP-70) levels; thus, apoptosis did not occur excessively especially in PE.¹⁷ Previous studies upon the antioxidant activity of this extract showed that this extract inhibited the formation of oxygen free radicals, and it served as a xanthine oxidase inhibitor and possessed cytotoxic activity.¹⁸ It has been demonstrated that phenolic compounds in EVOO exert beneficial effects on lipid oxidation, DNA oxidative damage and in general, oxidative stress in vitro and in vivo.¹⁹ Thus, the combination of these features can possibly reduce PE because of the antioxidant activity in andaliman and phenolic compounds in EVOO.

Materials and Methods

Nanoherbal Andaliman

Andaliman fruits (*Z. acanthopodium*) were obtained from Dairi, Northern Sumatera Province, Indonesia. Nanoherbal andaliman samples were generated using *High-energy Milling* (HEM) methods with HCl 2M activator solution (Tokyo, Japan) in an Indonesian research institute (LIPI, Jakarta). They were washed and dried in accordance to the requirements of water content using HEM. The destructive medium that is *Simplicia* was inserted into a jar. Larger-diameter balls, small balls and the last sample were then inserted into the jar. The total volume of the balls and samples did not exceed 2/3 of the volume of the jar. It was filled with the ball and samples, and it was closed tightly. Then HEM was turned on for 2 h. The morphology and structure of nanoherbal andaliman were characterised using an electron scanning microscope (SEM) with 10000× magnification (JSM-6390A, Tokyo, Japan). The SEM showed that nanoherbal andaliman distributed the colts. The size of nanoherbal andaliman was determined using the *Particle Size Analyser* (PSA) with ethyl alcohol as the diluent. An oral dose of nanoherbal andaliman was tested for its toxicity by using the *Thompson–Weil's formula*.²⁰

Characteristics of Nanoherbal Andaliman

Ash content (AC)

In this test, 2 g of nanoherbal andaliman was placed into a porcelain crucible for incandescence and then matched until the weight remained.²¹ The value of ash weight (AW) was 0.1269 g.

Insoluble ash in acids (IAIA)

The ash obtained from the previous examination was

heated with 25 ml of diluted HCl for 5 min until it boiled. The insoluble ash in the acid was filtered and washed with hot water.^{21,22} The value of the AW was 0.0245 g.

The following formula was used for AC and IAIA:

$$\% \text{ AC / IAIA} = \frac{\text{AW}}{\text{SW}} \times 100 \quad \text{Eq. (1)}$$

Substances soluble in water (SSIW)

SSIWs were obtained from 5 g of nanoherbal andaliman and macerated with 2.5 ml of chloroform in water to 100 ml for 24 h in a clogged flask. The mixture was then shaken for the first 6 h, then filtered after leaving it for 18 h. Subsequently 20 ml of the filtrate was air-dried and then matched in a porcelain dish. Lastly, the remainder was heated at 105 °C until the weight remained.^{21,22} The substance weight (Su W) value was 0.1430 g.

Substances Soluble in Ethanol (SSIE)

SSIEs were obtained from 5 g of nanoherbal andaliman and macerated in 100 ml of 96% ethanol for 24 h in a clogged flask. The mixture was shaken in the first 6 h and left as it was for 18 h. Then, 20 ml of the filtrate was air-dried and then matched in a porcelain dish. The remainder was heated at 105°C until the weight remained.^{21,22} The Su W value was 0.1767 g.

The following formula was used for SSIW and SSIE:

$$\% \text{ SSIW/SSIE} = \frac{\text{Su W}}{\text{SW}} \cdot \frac{100}{20} \times 100 \quad \text{Eq. (2)}$$

Water content (WC)

In this test, 200 ml of toluene and 2 ml of distilled water were added into the flask and then distilled for 2 h until the last drop. Then, 5 g of nanoherbal andaliman was heated for 15 min. After the toluene started to boil with the regulated speed of 2 drops per second, the temperature was raised so that most of the penetrating speed was accelerated to 4 drops per second. When the volume of water increased, distillation was continued for 5 min. The receiving tube was then allowed to cool at room temperature.^{21,22} The volume of the water (V) was 0.5 ml. The following formula was used:

$$\% \text{ WC} = \frac{V}{\text{SW}} \times 100 \quad \text{Eq. (3)}$$

where AW is the ash weight, SW is the sample weight, Su W is the substance weight, and V is the water volume.

Extra Virgin Olive oil (EVOO)

EVOO was obtained from the city supermarket of Medan, Indonesia (Bertolli, Italy; Certificate: IFS–BRC). Bertolli EVOO was produced by squeezing olives directly with cold techniques right after the olives were harvested. EVOO dose calculations were based on previous research.²³

Animal

A total of 25 pairs of *Rattus norvegicus* at around 180–200 g in weight were used in this research. Rats were mated at the Biology Laboratory Animal House in Universitas Sumatera Utara. The handling of the experimental animal or ethical issue was demonstrated

ethically (*Ethical Clearance*: 0492/KEPH-FMIPA/2018). The control groups were divided into the following two groups. K⁻ (negative control): pregnant rats without treatment; and K⁺ (positive control): pregnant rats given with 6% NaCl at 3 ml/day/200 g BW subcutaneously on the 6th–12th day of pregnancy. The treatments were divided into the following three groups. P1: pregnant rats given with 6% NaCl at 3 ml/day/200 g BW subcutaneously on the 6th–12th day of pregnancy + 0.45 g of EVOO/day/200 g BW orally on the 13th–19th day of pregnancy; P2: pregnant rats given with 6% NaCl at 3 ml/day/200 g BW subcutaneously on the 6th–12th day of pregnancy + nanoherbal andaliman 100 mg/day/200 g BW orally on the 13th–19th day of pregnancy; P3: pregnant rats given with 6% NaCl at 3 ml/day/200 g BW subcutaneously on the 6th–12th day of pregnancy + combination of 0.45 g of EVOO/day/200 g BW and nanoherbal andaliman 100 mg/day/200 g BW orally on the 13th–19th day of pregnancy. Pregnant rats were dissected on the 20th day of pregnancy, and paraffin blocks were created and further stained with *Haematoxylin and Eosin* (HE). Trophoblastic cells in the placenta were counted with five fields of view in every treatment.

Research Parameters

Blood pressure

Blood pressure levels were measured using an electronic blood pressure device (noninvasive sphygmomanometer) manufactured in Jakarta, Indonesia, in 2016. These levels were measured at the base of the rat's tail on the 5th, 13th and 20th day of pregnancy.

Proteinuria

Proteinuria levels were measured using urine dipstick (Verify urinalysis reagent Strips 3 parameters) manufactured in Jakarta, Indonesia (2017). Urine specimens were collected and measured for proteinuria at 7 am until 10 am on the 5th, 13th and 20th days of pregnancy.

Creatinine

The sample for the creatinine test was blood in the form of serum or plasma heparin. The tool used was UV2400 spectrophotometer obtained from Zhejiang, China, in 2014. Furthermore, 3 ml of the plain tube sample was then centrifuged to separate the serum from plasma. Standard blank and creatinine solutions were prepared first, and approximately 50 µL of it was added to the cuvette, followed by 1000 µL of reagent 1 (NaOH). The mixture was incubated for 5 min. Thereafter 250 µL of Pikrat acid was added, and then, the solution was incubated for 1 min. It was then measured on a spectrophotometer with a wavelength of 546 nm. The standard absorbance with the value of 1 was obtained. Approximately 50 µL of serum was added to the cuvette, then added with 1000 µL of reagent 1 (NaOH). Subsequently, the mixture was incubated for 5 min, and 250 µL of Pikrat acid reagent

was added using a spectrophotometer with a wavelength of 546 nm.

Erythrocytes and Leukocytes

The number of erythrocytes was obtained by haemocytometer obtained from Bandung, Indonesia (2014). The diluent pipette was used in a scale of 101 with a red glass core. Blood was inserted with a diluent pipette to a scale of 0.5 then the Hayem's fluid (used for checking the amount of erythrocytes in the blood) solution was sucked on a scale of 101. After becoming homogeneous, the samples were dropped and the suspension was allowed to flow around the counting chambers. The number of cells was observed using a microscope and a calculated Improved *Neubauer Chamber*. A number of leukocytes were collected by inserting 500 µl of TURK solution. Subsequently, 20 µl of blood was added to the tube containing the TURK solution. Then it was mixed until homogeneous and allowed to stand for 3 min. Blood diluted with TURK solution was inserted into an improved Neubauer. Leukocyte cells were counted under a microscope with a weaker magnification (10×).

Haematocrit

Microcapillary tubes were filled with blood. One of the ends of each tube was then closed by burning (by flame) or using a special covering material. These tubes were then centrifuged 2014 TGM12 centrifuge (Zhejiang, China) at a speed of more than 16000 rpm. Then the haematocrit value was calculated using the graph contained in the haematocrit centrifuge.

Placental and Foetus

Placental and foetal weights were measured using organ scales and by macroscopic observations.

Malondialdehyde (MDA)

MDA level was measured using blood specimen and this measurement was conducted in the Immunology Laboratory Faculty Healthy, Universitas Sumatera Utara. Then 0.5 ml of blank was added with 0.5 ml of standard and 0.5 ml of serum by using vortex mixer and then centrifuged for 10 min at a speed of 2000 rpm. Each of the filtrate was pipetted at 1 ml and placed in a boiling water bath for 30 min. Then, the absorbent was cooled down and read with a spectrophotometer (UV2400 Spectrophotometer: Zhejiang, China) with a wavelength of 530 nm.

HSP-70

HSP-70 was examined using *Well Reader-Elisa Reader R-Biopharm* (Germany). Blood samples were centrifuged at 3000 rotations per min for 15 min using the DT5-6A (2) low-speed centrifuge (Tianjin, China). Furthermore, 100 ml pipette from buffer solution was placed in a container. The petri dishes were covered, incubated for the next 2 h and then agitated at room temperature. The contents were then emptied from the container. The container was washed by adding 400 ml of buffer solution

to each container. This procedure was repeated 3-4 times. After the last washing, the container was emptied, and the petri dish was suctioned carefully to remove the remnants from the buffer solution. Then, 100 ml of HSP-70 antibody solution was pipetted into each container except for the empty container. The dish was covered, incubation and stirred for 1 h at room temperature. The same previous washing method was then performed. Next 100 ml of conjugated blue solution was added in each container except the empty container. The dish was then covered, incubated and agitated for 1 h at room temperature. All containers were pipetted by 100 ml of solution substrate, and they were incubated and stirred for 30 min at room temperature. The pipetting of 100 ml of solvent was stopped into each solution. The results were read with a wavelength of 450 nm.

Trophoblastic cells

Pregnant rats were dissected on the 20th day of pregnancy. Then, paraffin blocks were created and stained by HE. Trophoblastic cells in placenta were counted with five fields of view in each treatment and observed using the Axiocamp ERc 5s microscope (Germany, 2015) with 40× magnification.

Statistical analysis

Data of blood pressure level, proteinuria, foetal number, foetal and placental weights, number of leukocytes and erythrocytes, and haematocrit, HSP-70 and MDA levels were processed with *Kruskal–Wallis test* in SPSS 22 program.

Results

Nanoherbal andaliman

When viewed by electron microscopy, nanoherbal andaliman had extremely small sized clots at 0.440 μm (Figure 1a), as well as large clots at 44 × 103 nm, thereby more effective in penetrating cells. Changing drug molecules into a nanometre scale can improve the efficacy of the drug molecule. Based on PSA, nanoherbal andaliman has an average diameter of distribution of

783.9 nm ± 173.4 (Figure 1b), in which the cumulant result was 3783.1 nm and the polydispersity index value was 1.223, with measurement condition at 25°C. Furthermore, the refractive index was 1.3611, the cP viscosity was 1.1015, and the CPS scattering intensity was 9963. The emulsion characterisation of nanoherbal andaliman based on the characteristic test is shown in Table 1. Nanoherbal andaliman has minimal water and ash contents. Thus it can be potentially developed into medicine.

Table 1. Characteristic test of nanoherbal andaliman.

Parameters	Results
Water Content (WC)	9.99%
Substances soluble in water (SSIW)	14.30%
Substances soluble in ethanol (SSIE)	17.67%
Ash content (AC)	6.34%
Insoluble ash in acids (IAIA)	1.22%

Blood pressure

Based on the statistical data of blood pressure before injecting of 6% NaCl, no significant difference was observed in the systole ($p=0.284$) and diastole ($p=0.529$). The pregnant rats were given 6% NaCl at 3 ml/day/200 g BW subcutaneously on the 6th–12th day of pregnancy. Diastole was observed and showed no significant difference ($p=0.056$). Conversely, a significant difference was observed in systole between the groups ($p=0.006$) on the 13th day of pregnancy. Based on the data 6% NaCl can increase systolic blood pressure in pregnant rats. Thus, pregnant rats experienced PE, because their blood pressure levels exceeded 120/80 mmHg. Pregnant rats were given with nanoherbal andaliman and EVOO on the 13th–19th day of pregnancy. Before dissecting on the 20th day of pregnancy, a significant difference in systolic blood pressure ($p<0.01$) was found between the groups of K⁺;P1;P2 and P3 but no significant difference ($p>0.05$) was observed in the diastolic blood pressure (Figure 2). Based on the graph nanoherbal andaliman and EVOO can reduce systolic blood pressure in pregnant rats.

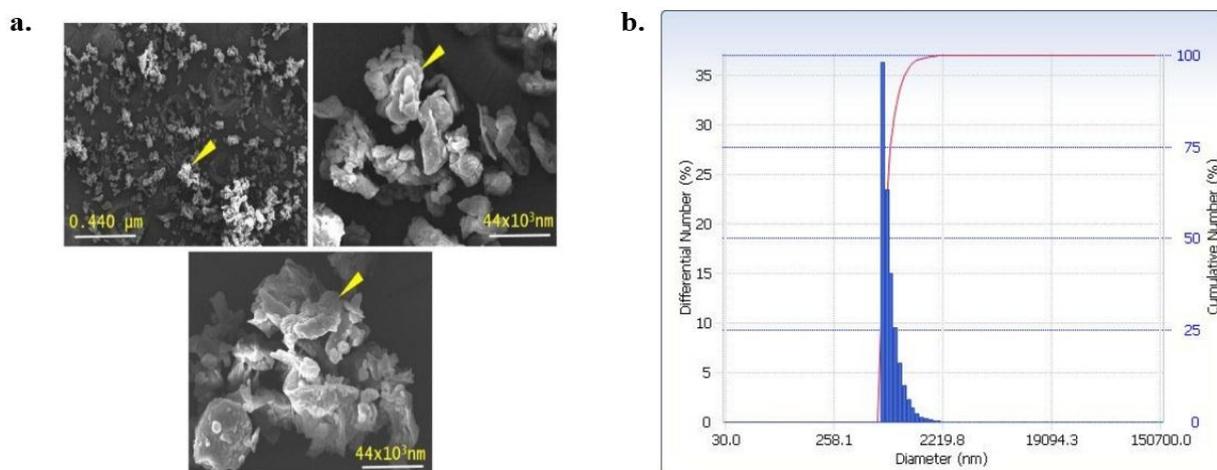


Figure 1. a. Nanoherbal andaliman (*Z. acanthopodium*) in Scanning Electron Microscope (10000x). b. Range value of diameter of distribution nanoherbal andaliman with particle size analyzing.

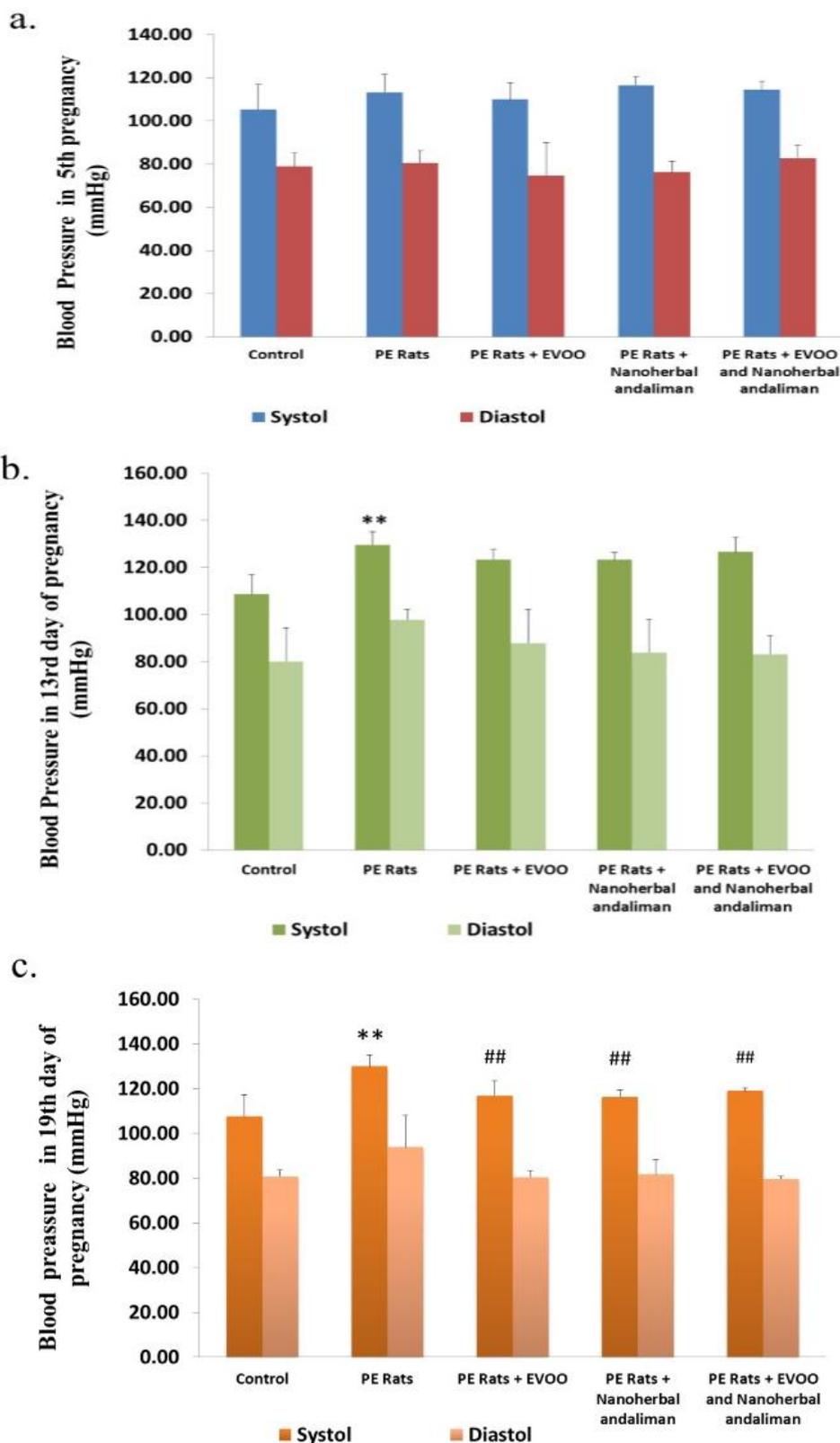


Figure 2. Systolic and diastolic blood pressures mean values in 5th, 13rd and 19th days of pregnancy. Systolic blood pressure: 5th days of pregnancy (Figure 2a) where *Kruskal Wallis* test was applied but non-significant difference was found in the mean systolic blood pressure ($p = 0.284$), 13rd days of pregnancy (Figure 2b), Mann Whitney test was applied to compare mean systolic value between all groups. ** $p < 0.01$ compared to control but non-significant difference in the other groups. 20th days of pregnancy, Mann Whitney test was applied to compare mean systol value. ** $p < 0.01$ compared to control and ## $p < 0.01$ compared to K⁺ (Figure 2c). Diastolic blood pressure: Comparison of diastolic blood pressures mean in 5th days of pregnancy (Figure 2a), after injecting NaCl 6% or 13rd days of pregnancy (Figure 2b) and 20th days of pregnancy (Figure 2c). Non-significant difference was found between the groups ($p = 0.529$; $p = 0.056$; $p = 0.071$)

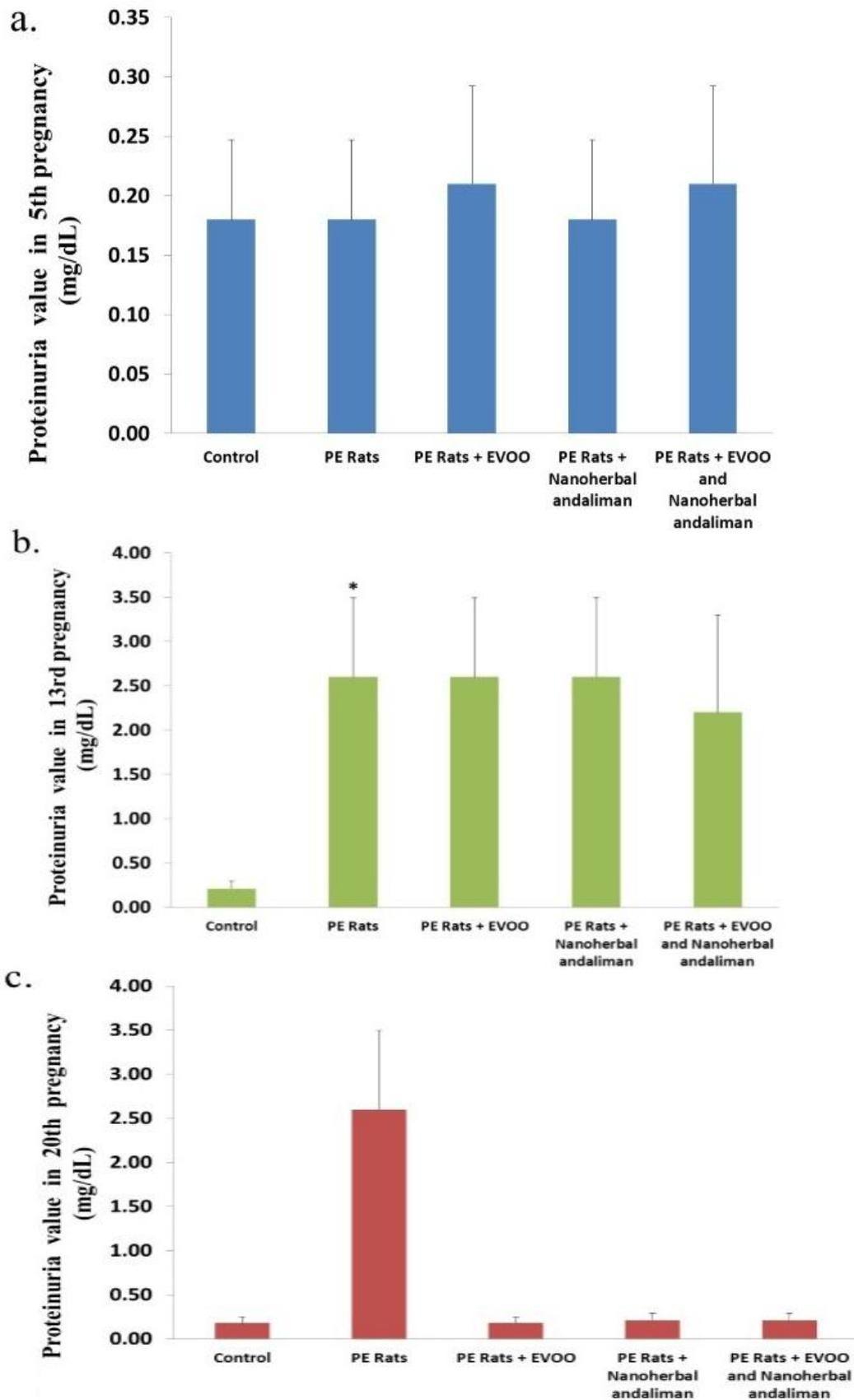


Figure 3. Comparison of proteinuria mean values in 5th days of pregnancy (Figure 3a), after injection NaCl 6% or 13rd days of pregnancy (Figure 3b) and before dissecting or 20th days of pregnancy (Figure 3c). Non-significant difference was found in 5th days of pregnancy ($p=0.404$) and 20th days of pregnancy value ($p = 0.403$). Mann Whitney test was applied to compare mean 13rd days of pregnancy value between all groups. * $p<0.05$ compared to control.

Proteinuria

No significant difference was observed in the proteinuria value $p > 0.05$ ($p = 0.404$) before the injection of 6% NaCl. After 6% NaCl (13th day of pregnancy) was injected, a significant difference was observed in the proteinuria value between the groups in K^- with K^+ (Figure 3), $p < 0.05$ ($p = 0.024$).

Furthermore, 6% NaCl can increase proteinuria values in pregnant rats, given that the proteinuria value in dipstick urine was more than 0.3 g/L. No significant difference was also observed, with $p > 0.05$ ($p = 0.403$), on the 20th day of pregnancy. However, based on the average in the graph, an increased proteinuria was observed on the 13th day of pregnancy, but after 6% NaCl injection, it decreased in each treatment. Proteinuria value decreased in the combination of nanoherbal andaliman and EVOO, but it was insignificant when EVOO or nanoherbal andaliman was administered alone.

Creatinine

No significant difference was observed, with $p = 0.254$ ($p > 0.05$) in the creatinine value in each treatment. P2 group had the highest average value, whereas the K^- group had the lowest (Figure 4). One of the complications of PE is acute kidney failure, with creatinine level as its marker. As depicted in Figure 4, no creatinine level value exceeded the normal level at each treatment. Hence, administering EVOO and nanoherbal andaliman did not lead to kidney problems.

Erythrocytes and Leukocytes

A significant difference was shown in between the groups with $p < 0.01$ erythrocytes value ($p = 0.007$) in K^- with K^+ and P3 (Figure 5). The highest erythrocyte level was in the K^- group where as the lowest was in the K^+ group. PE disease can reduce the number of erythrocytes during pregnancy. Pregnant women need blood for foetal safety. EVOO, nanoherbal andaliman and their combination can increase the number of normal erythrocytes in pregnant women. Thus, this combination is safe. However, the number of leukocytes in pregnant rats showed no

significant difference with $p > 0.05$ ($p = 0.064$) in each treatment. However, the highest number of leukocytes was in the PE group, and the lowest was in the control group. Leukocytes cell activity can cause an effect in PE. Based on the data, the EVOO, nanoherbal andaliman treatment and a combination of both can affect leukocyte cells in pregnant rats, but the effect was not significant.

Haematocrit

No significant difference was observed, with $p < 0.01$ value ($p = 0.004$), in K^- , K^+ and P2. The highest haematocrit was in the K^- group, and the lowest was in the K^+ and P3 groups (Figure 6). Haematocrit levels in the control increased due to the presence of haemoconcentration and the increased plasma volume by vasospasm, thereby resulting in vasospasm imbalance in the production of substances. EVOO and andaliman caused sustained vasospasm. Excessive dose damaged the blood vessel's endothelial integrity. The plasma in the volume decreased, and haematocrit levels increased. Based on these data, either only EVOO or nanoherbal andaliman treatments can increase the haematocrit rather than a combination of both.

Placental and Foetal number

No significant difference was observed in the number of foetuses, with $p > 0.05$ value, among the groups (Table 2). The control and P2 groups had a greater number of foetuses than K^+ group wherein foetuses were resorbed. The decrease in the number of foetuses in PE may indicate a preimplantation disorder and delay in the blastocyst stage or in cell division, zone of pellucida release, as well as delay in implantation time. Foetal weight of rats showed a significant difference ($p < 0.01$) in K^+ P1 and P3, but the control group had average foetal weight. Furthermore, P1 and P3 had a high foetal weight, whereas K^+ and P2 had a low foetal weight (Table 2). Fat deposit was observed in all treatment groups, except P2, thereby indicating that nanoherbal andaliman had special substances that can reduce foetal weight.

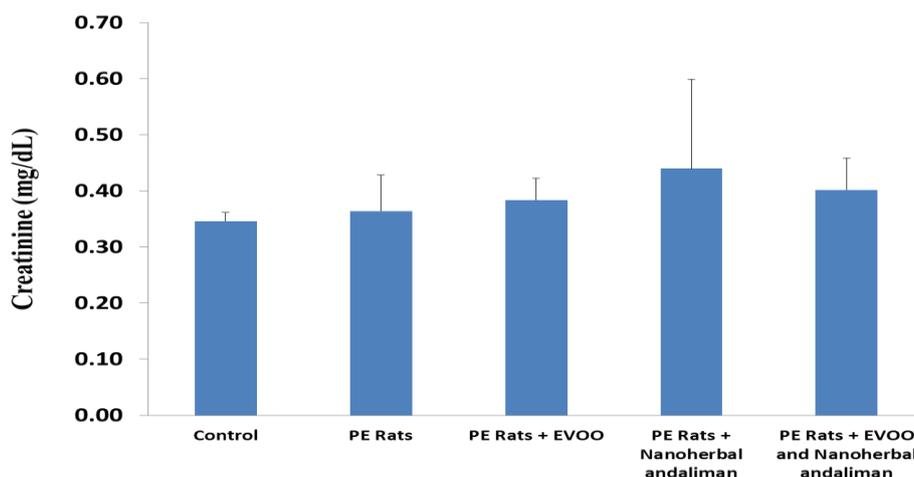


Figure 4. Comparison of creatinine mean values with Kruskal Wallis test. Non-significant difference was found in the creatinine value between the groups ($p = 0.254$).

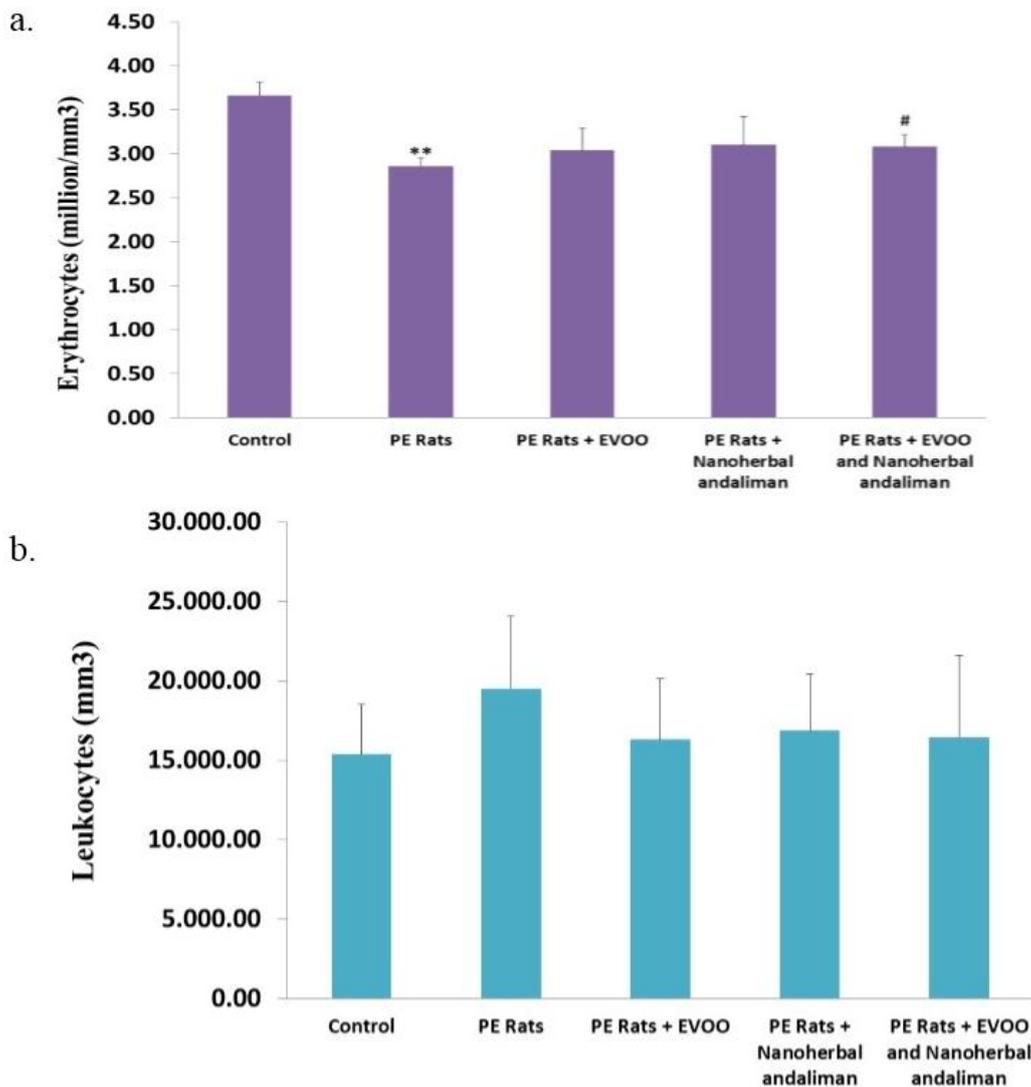


Figure 5. a. Erythrocytes mean values (Mann Whitney test. ** $p < 0.01$ compared to control, # $p < 0.05$ compared to K) b. Leukocytes values (Kruskal Wallis test was applied between all groups. Non-significant difference was found in the leukocytes values between the groups $p = 0.064$)

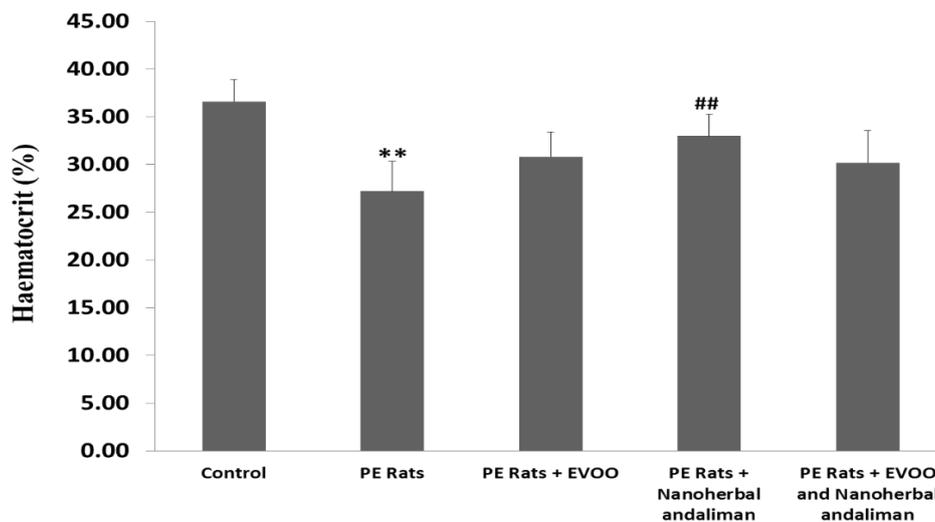


Figure 6. Haematocrit Values (Data are expressed as the mean \pm SEM, Mann Whitney test was applied to compare haematocrit value between all groups, ** $p < 0.01$ compared to control, ## $p < 0.01$ compared to K+).

Table 2. Comparison of number of fetus and placental weight mean values.

Treatments	Number of Fetus	Fetus weight (g)	Placental weight (g)
Control	9.4 ± 1.816	1.30 ± 0.225	0.43 ± 0.039
Pregnant Rats (PE)	7.2 ± 1.923	1.04 ± 0.349**	0.34 ± 0.069
PE + EVOO	8.2 ± 2.38	1.23 ± 0.229###	0.37 ± 0.045
PE+ Nanoherbal andaliman	9.4 ± 1.516	1.18 ± 0.094 #	0.36 ± 0.057
PE+EVOO and nanoherbal andaliman	8.8 ± 2.949	1.23 ± 0.139###	0.35 ± 0.055

Number of fetus and placental weight mean values with *Anova test* was applied between all groups. Non-significant difference was found in the mean of number of fetus between the groups ($p = 0.423$) and placental weight ($p = 0.304$). The fetus weight mean values with *Post-hock duncan test* was applied to compare mean of fetus weight value between all groups. ** $p < 0.01$ compared to control, # $p < 0.05$ compared to K^+ , ### $p < 0.01$.

No significant difference in placental weight value was observed ($p > 0.05$), but based on the average, the control group had the highest weight, followed by K^+ (Table 2). Thus, EVOO and nanoherbal andaliman did not significantly affect the placental weight and the number of foetus but can affect the foetal weight.

MDA

No significant difference was observed in MDA value, with $p > 0.05$ ($p = 0.310$) (Figure 7). The highest average MDA value was found in K^+ whereas the lowest was found in P3 due to the increase in ROS, thereby increasing lipid peroxidation in the trophoblastic cells and the core component of placental villi in PE. Oxidative stress is an imbalance in the amount of oxidants and antioxidants in the body. It decreases antioxidant levels and/or increases oxidant levels. Oxidative stress indicated by the presence of lipid peroxidation then plays a role in PE pathogenesis. A combination of EVOO and nanoherbal andaliman reduced the MDA levels in pregnant rats. Andaliman and EVOO have strong antioxidants. Thus, a combination of both can reduce MDA in PE.

HSP-70

No significant difference was observed, with $p > 0.05$ ($p = 0.147$), in HSP-70 levels in the different treatments. The highest HSP-70 levels were found in P1, and such levels were even higher than the control group, whereas the lowest HSP-70 levels were found in K^+ (Figure 8). Oxidative stress can induce the increased production of

HSP-70 in cells. HSP-70 has antiapoptotic effect and repairs damaged cells. HSP-70 levels in normal placenta can still maintain cell function homeostasis and a normal blood pressure, although no significant difference was found among HSP-70 levels in the placenta. Hence, the response to maintain placental homeostasis in PE was still high. Based on the average value, EVOO could increase HSP-70 levels compared with nanoherbal andaliman alone or their combination.

Trophoblastic cells

Trophoblastic cells in placenta was counted with five fields of view in every treatment. A significant difference was observed, with $p < 0.01$ ($p = 0.002$), in K^- with K^+ , P1 and P3. The number of normal cells decreased in K^+ and P2 but increased in P1 with P3 (Figure 9). Nanoherbal andaliman can reduce normal cells of the trophoblasts in the placenta. Trophoblast is important, because it supplies maternal and foetal nutrition and has a role in the PE pathogenesis. Nanoherbal andaliman decreased the number of normal trophoblasts in placenta but increased such number when combined with EVOO.

Histology of Placenta

Based on histological observations (Figure 10), the number of normal trophoblastic cells in K^- was higher than K^+ , and a narrower branching of the villous arteries in blood vessels was observed compared with that in the coronary villous arteries of normal pregnant rats.

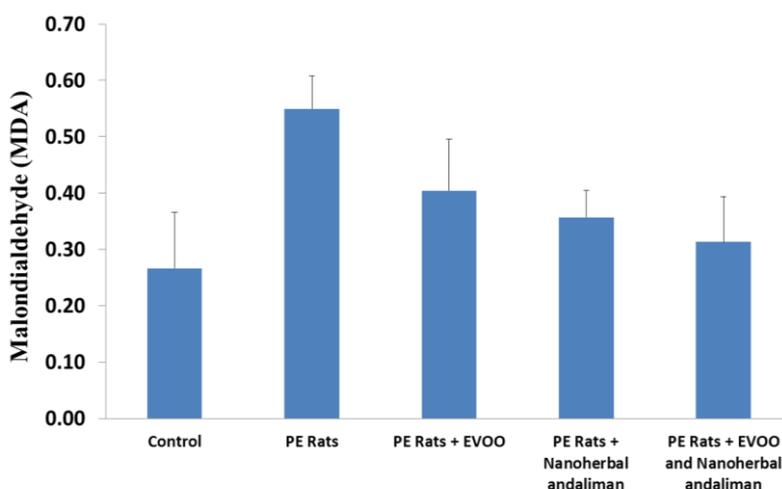


Figure 7. Comparison of MDA values with Kruskal Wallis test was applied. Non-significant difference was found in the MDA value between the groups ($p = 0.310$).

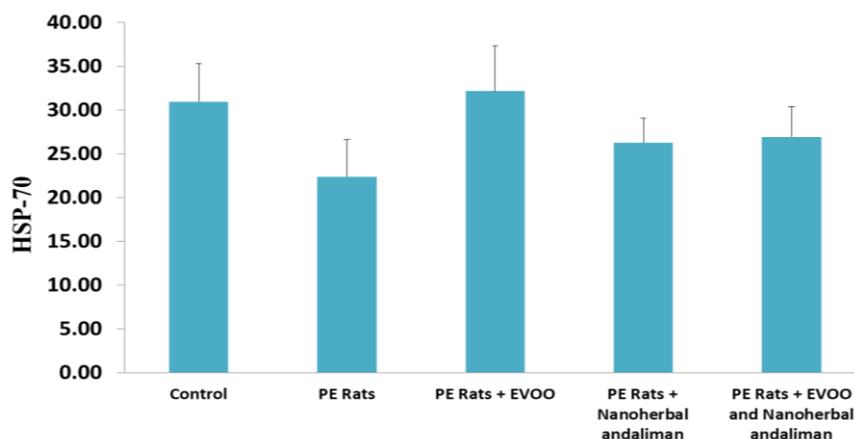


Figure 8. Comparison of HSP-70 values between all groups with *Kruskal Wallis* test was applied to compare mean value of parameters between all groups. Non-significant difference was found in the HSP-70 value between the groups ($p = 0.147$).

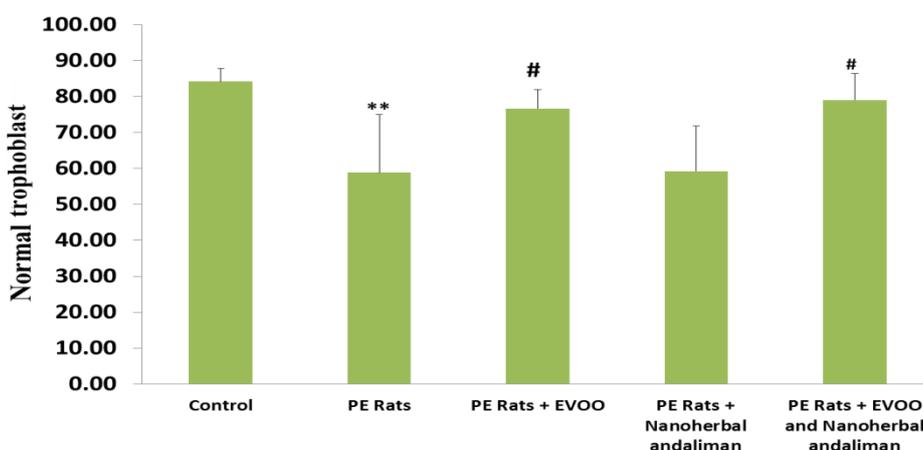


Figure 9. Data are expressed as the mean \pm SEM, *Mann Whitney* test was applied to compare trophoblast cells value between all groups, ** $p < 0.01$ compared to control, # $p < 0.05$ compared to K^+ .

The arteries in the K^+ or PE group were also narrowed, possibly caused by the lack of supply of oxygen to the blood vessels, especially arterioles, leading to the migration of tunica smooth muscle cells to the intima tunica and eventually, proliferation. The proliferation was characterised by the thickening of the tunica intima, resulting in the narrowing of the arteries. The muscular layer on the walls of the spiral arteries appeared stiff, so that it could not smoothly cause perfusion to the placenta. The number of trophoblast cells on K^- and P3 was almost the same, but the giant cells in group K^- was higher and more neatly arranged. The number of trophoblastic cells also increased in P1 but decreased in P2 (Figure 10). Allegedly, nanoherbal andaliman can reduce trophoblastic cells through apoptosis. In group P2, although normal trophoblast cells were decreased, the arteries were not entirely narrowed.

Discussion

A constraint that often occurs in herbal medicines is that active substances can difficultly penetrate the lipid membrane of body cells because they have a large molecular size and low solubility in water.²⁴ However, this limitation can be overcome by nanoscale due to

considerably numerous important metabolic contents in these plants that can disappear during the maceration or heating process in making methanol, ethanol, n-hexane, extract and more. According to Tensisca et al. (2003),²⁵ andaliman fruit extract with ethanol and hexane has a different antioxidant activity, which is highest in water systems during emulsion and oily systems though having a moderate activity. The content of important compounds is relatively stable during heating, but in heating up to 175 °C, it can reduce up to 17%. Andaliman in the form of extract also has different contents and activities when exposed to heat, fluorescent light and ultraviolet light.²⁶ One of the diagnostic criteria to determine PE is having a blood pressure of more than 120/80 mmHg. When the pregnant rats were injected with 6% NaCl, the systolic blood pressure increased (Figure 2). This finding can be useful for researchers who want to create PE mouse models. However no significant increase was found in diastolic blood pressures due to immunity, activity and endurance of different rat bodies. Therefore, evaluating the dose of NaCl is important. Administering a combination of EVOO and nanoherbal andaliman has shown significance, given that it reduced the systolic blood pressure in PE rats injected with 6% NaCl.

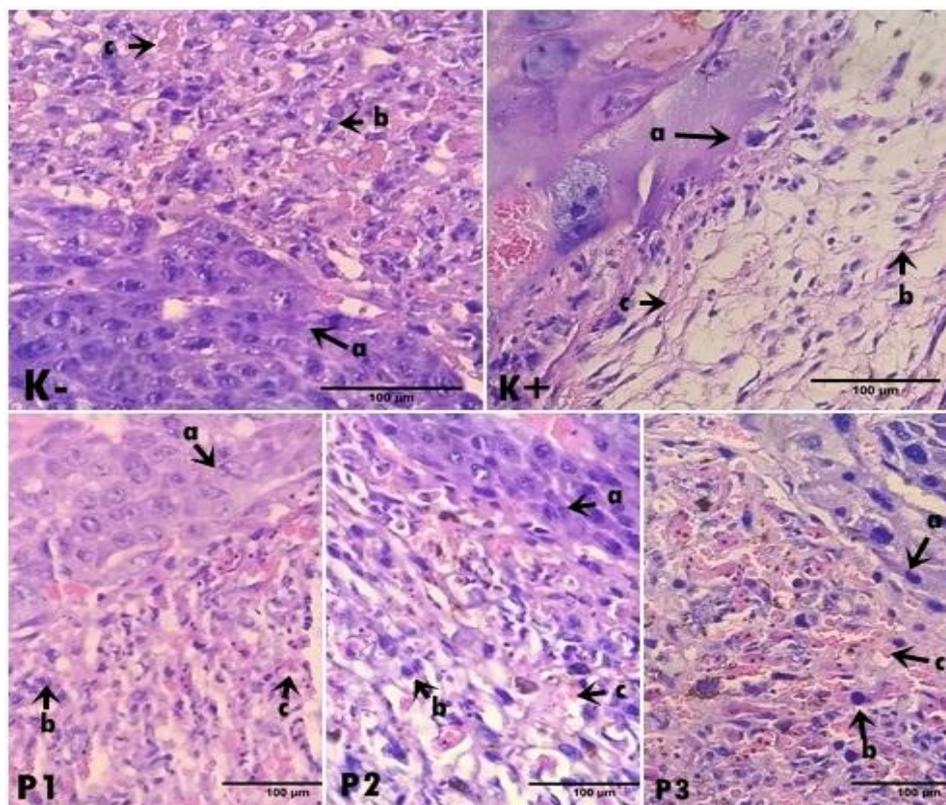


Figure 10. Histology of Placental : K⁻ : Control; K⁺ : PE, P1: EVOO, P2: Nanoherbal andaliman, P3: Combination of EVOO and nanoherbal andaliman. (a) Giant Cell (b). Trophoblast, (c) Blood vessels (40x).

Necrosis can occur due to increased levels of salt in the blood. The TGF- β gene activity was stimulated due to the occurrence of physiological processes of cells caused by the administration of a high salt content (NaCl 6%). A high salt content disrupts cell membranes, leading to an increased activity of death receptors such as Death Receptor (DR) or Tumour Necrosis Factor (TNF α). The activity of receptors stimulates procaspase 8 to caspase 8, resulting in an increase in caspase 3. Receptor stimulant may also increase Bax or Bid expression, causing disruption to mitochondrial membranes; eventually, the cell cytochrome is removed, joining Apaf-1 and Procaspase 9 and forming apoptosome; an increased TGF- β can lead to cell death (apoptosis).²³

This phenomenon is similar to that of the proteinuria value, which also decreased after the administration of the combined EVOO and nanoherbal andaliman (Figure 3), as exhibited in the dipstick test. PE can be determined by identifying hypertension and proteinuria after 20 weeks of pregnancy and changes in angiogenic factors in pregnant women.^{27,28} In proteinuria examination, a dipstick test was used, and an abnormally high concentration of urine sample with creatinine indicates such disorder.²⁹ Furthermore, urine protein and adipsin have increased significantly, and the ratio of creatinine to adipsin correlates with urine protein, for 24 h in 124 patients, combined with an increase in diastolic blood pressure (≥ 90 mm Hg).³⁰ However, the value of proteinuria of >3 g/L in severe PE indicates that the value of proteinuria is not always related to PE.³¹ Other than proteinuria, the

creatinine ratio is more reliable in detecting proteinuria in PE. Concomitant symptoms such as oedema and hypertension also need to be monitored. Rat activity, body weight and urinary system disorders also affect protein values.

Creatinine values plays a role in PE cases, but with the combined treatment, creatinine results were insignificant. Nanoherbal andaliman can increase creatinine when compared with EVOO or a combination of both (Figure 4). However, the combined treatment is still considered safe for the kidneys in patients with PE, but the kidneys will be disrupted and damaged if given at a higher dose. Andaliman ethanol extract at a dose of 15 mg/BW significantly affected the renal ratio and increased the number of leukocyte granulocytes, thereby affecting the cellular immunostimulatory system.³² This plant is often used as a spice ingredient in traditional dishes, such as arsik and naniura in Medan, Indonesia. Its chemical contents such as flavonoids, alkaloids, saponins and terpenoids, given at certain doses, is useful for the health. The albumin/creatinine ratio is also an independent prognostic factor suspected of PE.³³ Combined nanoherbal andaliman and EVOO can reduce blood pressure levels and proteinuria but cannot significantly affect the creatinine levels in pregnant rats.

EVOO or nanoherbal andaliman increased haematocrit rather than a combination of both (Figure 5), but this increment remained insignificant. The imbalance of substances in herbs caused endothelial dysfunction, resulting in vasospasm, and the plasma volume also

decreased, as evidenced by an increase in haematocrit levels. EVOO, nanoherbal andaliman and a combination of both can significantly improve erythrocytes in pregnant women and decrease leukocytes but remained insignificant (Figure 6). Active leukocytes, such as monocytes and granulocytes, produce excess ROS generated by oxidative stress.³⁴ Compared with normal pregnancies, pregnant rats with PE have higher levels of calprotectin, which is a protein involved in various physiological inflammatory processes, indicating a leukocyte activation.³⁵ Increased secretion of TNF by leukocytes is also detected in the blood of patients with PE.³⁶ Therefore, leukocyte cell activity can affect PE.

Nanoherbal andaliman has toxic compounds that can reduce foetal weight significantly (Table 2). Decreased foetal weight was observed in PE and andaliman treatment. However, the results in the weight of the placenta and number of foetuses were insignificant.

Foetus was also not macroscopically defective; bleeding, wrinkled skin, stunted growth, defects in the legs and tail were noted even after nanoherbal andaliman, EVOO and a combination of both were administered. Alkaloid compounds in andaliman may cause the number of live and dead foetuses. Alkaloids contain at least one atom, and most nitrogen atoms are part of heterocyclic rings and have certain physiological activeness.³⁷ Alkaloids can cause the number of implants and live foetuses to decrease significantly, causing antifertility.³⁸

Meanwhile, MDA is found in almost all biological fluids of the body.^{39,40} PE rats have higher plasma MDA levels than normal rats (Figure 7). Increased MDA levels are suspected because of oxidative stress. As described, oxidative stress is an imbalance in the amount of oxidants and antioxidants in the body; it can either result in a decrease in antioxidant levels and/or an increase in oxidant levels. Oxidative stress indicated by the presence of lipid peroxidation plays a role in the pathogenesis of PE. Oxidative stress occurs when an imbalance occurs

between pro-oxidants and antioxidants. Levels of deficiency in vitamins E and C indicate a lipid peroxide chain that violates antioxidants inhibiting NADPH oxidase in placental tissue.⁴¹ Combination of EVOO and nanoherbal andaliman can reduce MDA levels in pregnant rats. Andaliman has strong antioxidants, and EVOO has a strong vitamin E, so a combination of both can reduce MDA level in PE. Nutritional balance in pregnant women, such as food antioxidant intake and oxidative stress status in maternal, can cause IUGR.⁴² IUGR often occurs in PE but the plasma levels of pregnant women influenced by vitamin C and E contents significantly increase live births with normal weight and length.⁴³

HSP-70 originates from ischemic and PE placenta that is oxidatively suppressed; it is regulated in pre-eclamptic placental tissue but not in normal placenta.⁴⁴⁻⁴⁷ Oxidative stress can increase the production of HSP-70 in cells. HSP has anti-apoptotic effect and repairs damaged cells. EVOO increases HSP-70 production in cells because it contains antioxidants and tocopherol (Vitamin E).¹⁷ The use of EVOO is beneficial for the supplementation of various other antioxidants, such as Vitamin C.⁴⁸ In addition to tocopherols, EVOO also has components such as phenolics and carotenoids, which have antimicrobial, antioxidant and anti-inflammatory properties.⁴⁹ Combination of nanoherbal andaliman and EVOO can decrease PE but not significantly (Figure 8). HSP-70 levels in normal placenta can maintain cell function homeostasis and normal blood pressure, but the difference among the levels was insignificant in each treatment.

HSP-70 levels in PE have considerably lower levels according to Molvarec.⁵⁰ Intracellular HSP-70 has an important role in maintaining cellular homeostasis, but extracellular HSP-70 originates from stress or damaged cells. Necrotic cells can cause innate proinflammatory immune responses and eventually adapt. Increased HSP-70 levels can reflect the systemic inflammation of oxidative stress and hepatocellular injury in PE.^{50,51}

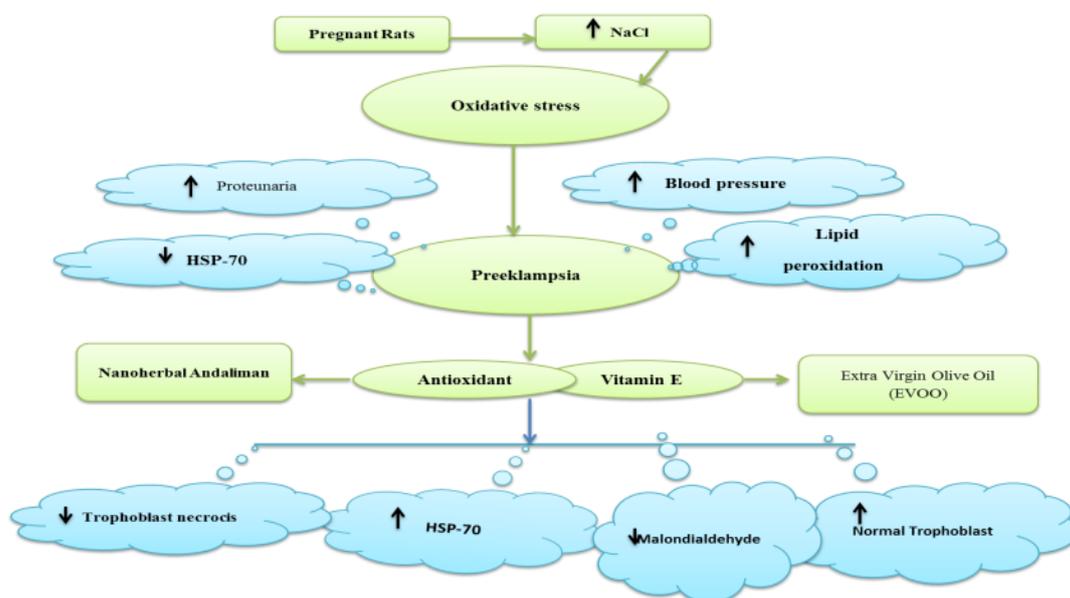


Figure 11. The possible protective effects of EVOO and nanoherbal andaliman against preeclampsia.

Proinflammatory immune responses and eventually adapt. Increased HSP-70 levels can reflect the systemic inflammation of oxidative stress and hepatocellular injury in PE.^{50,51} HSP-70 can also have anti-inflammatory properties. However, TNF- α and IL-1 β have extremely short half-lives in the maternal circulation, thereby possibly explaining the lack of correlation between serum levels of HSP-70 and cytokines.^{52,53} The response in maintaining placental homeostasis in PE remains high. HSP-70 has anti-apoptotic effect. It reduces radical compounds and regulates homeostasis in the placental trophoblasts. Combined nanoherbal andaliman and EVOO can reduce MDA, proteinuria, blood pressure levels and decrease HSP-70 levels in pregnant rats (Figure 11).

The invasion of trophoblastic cells is disrupted.⁵⁴ Nanoherbal andaliman can reduce the number of normal cells of the trophoblasts in the placenta. Trophoblast is extremely important, because it is a supplier of maternal and foetal nutrition. Placenta also plays an important role in the pathogenesis of PE and reduction of uteroplacental perfusion, which develops as a result of abnormal invasion of the cytotrophoblast, and spiral arterioles trigger a cascade, leading to maternal disorders. Placental hypoxia causes irregular parts of the villi terminals and trophoblast to build up, thereby causing the formation of syncytial nodes.⁵⁵ Placental ischemia also causes the release of placental factors which are classified as antiangiogenic or pro-inflammatory.

Based on a histological observation, a significant difference was found in trophoblast structure in each treatment (Figure 10). Trophoblast exposure to hypoxia in vitro results in bonding to the apoptotic process, which is associated with increased expression of p53 and Bax proteins and decreased expression of anti-apoptotic Bcl-2.^{56,57} According to Allaire et al. (2000),⁵⁸ placental apoptosis index increased in PE compared with normal pregnancy. However, their research with Ishihara et al. (2002)⁵⁹ indicates insignificant difference in the control group with PE in apoptotic parameters with Bcl-2. Placental insufficiency causes hypoxia/ischemia with increasing sFlt-1 and ROS in PE.⁶⁰

The arteries in the K⁺ were narrowed (Figure 10) because of the lack of oxygen supply to the blood vessels, especially the arterioles. Subsequently, tunica smooth muscle cells migrate to the intima tunica and undergo proliferation, as characterised by the thickening of the tunica intima, thereby resulting in the narrowing of the arteries. Muscular layers on the walls of the spiral arteries appear stiff due to the rough perfusion in the placenta. In group P2 (Figure 10), although normal trophoblastic cells were reduced, the arteries were not entirely narrowed due to high terpenoid content and the presence of andaliman antioxidants.

Conclusion

Nanoherbal andaliman is a novel herbal medicine compound that can be used as medicine. The administration of nanoherbal andaliman and EVOO can

reduce preeclampsia, as shown by a significant difference ($p < 0.05$) in blood pressure, proteinuria, foetal weight, number of haematocrit and erythrocytes and trophoblastic cells in pregnant preeclampsia rats. However, insignificant differences were found for placental weight, number of foetus, leukocytes cells, MDA and HSP-70 levels ($p > 0.05$). Further examination with immunohistochemistry is necessary.

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Conflict of interests

The authors claim that there is no conflict of interest.

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