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RESEARCH ARTICLE

Physalis angulata leaf extract ameliorates L-N^G-nitroarginine methyl ester (L-NAME)-induced preeclampsia symptoms in rats through improved endothelial progenitor cells and endothelial cells due to reduced angiogenic factor and oxidative stress

[version 1; peer review: 2 approved with reservations]

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Abstract

Background: This study aimed to determine the ameliorative effects of *Physalis angulata* leaf extract on L-N^G-nitroarginine methyl ester (L-NAME)-induced preeclampsia symptoms in rats.

Methods: Phytochemical analysis of the extract was performed with liquid chromatography-high resolution mass spectrometry (LC-HRMS). Pregnant Wistar rats were randomly divided into five groups (n=6). Preeclampsia rats were injected with L-NAME on gestation days 9 to 18 (G9–G18), while sham rats were injected with the same vehicle volume. Three groups of preeclampsia rats were orally supplemented with the extract at doses of 500, 1500, and 2500 mg/kg body weight/day on G12–G18. The tail-cuff method measured blood pressures. Spectrophotometry determined urine protein levels. Serum soluble fms-like tyrosine kinase (sFlt)-1 levels were evaluated using an

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enzyme-linked immunosorbent assay (ELISA). Serum and placental malondialdehyde (MDA) levels, superoxide dismutase (SOD) activities, and nitric oxide (NO) levels were measured by colorimetry. Immunohistochemistry was used to determine tail artery endothelial nitric oxide synthase (eNOS), placental eNOS, and placental hypoxia-inducible factor (HIF)-1 α expressions. Circulating angiogenic cells (CACs) and endothelial colony-forming cells (ECFCs) were counted using flow cytometry.

Results: L-NAME injection increased blood pressures, 24-h total urine protein level, serum sFlt-1 level, serum and placental MDA levels, percentages of CACs and ECFCs, and placental HIF-1 α expression. It also decreased serum and placental SOD activities, serum NO level, tail artery and placental eNOS expressions compared to the sham group. *Physalis angulata* leaf extract administration lowered blood pressures, urine protein level, sFlt-1 level, MDA levels, percentages of CACs and ECFCs, and placental HIF-1 α expression. The extract increased SOD activities, NO level, tail artery and placental eNOS expressions compared to the preeclampsia group.

Conclusions: *Physalis angulata* leaf extract reduces antiangiogenic factor and oxidative stress. It also enhances eNOS/NO signaling. Thus, it improves EPC and endothelial cell function and reverses L-NAME-induced hypertension and proteinuria in preeclampsia rats.

Keywords

Antiangiogenic, Antioxidant, Cutleaf groundcherry, Endothelial cells, Hypertension, Hypoxia, Nitric oxide, Physalis, Preeclampsia, Pregnancy, Stem cells

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article can be found at the end of the article.



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Introduction

Preeclampsia is one of the most severe hypertension conditions during pregnancy. It is distinguished by new-onset hypertension and proteinuria after more than 20 weeks of gestation.¹ Preeclampsia affects 3% of all pregnancies globally.² Preeclampsia is one of the leading causes of maternal mortality in underdeveloped nations, where access to prenatal and intrapartum care is generally limited.³ Preeclampsia also dramatically raises the cost of healthcare services.⁴

Endothelial dysfunction contributes to preeclampsia's pathogenesis, resulting in peripheral vasoconstriction and multi-organ damage.⁵ Endothelial dysfunction persists after birth, increasing the risk of future cardiovascular and cerebrovascular disorders in the mother^{6,7} and the child.^{7,8} This condition underlines the importance of alleviating systemic endothelial dysfunction in preeclampsia.

One essential factor in improving endothelial dysfunction is endothelial progenitor cells (EPCs).⁹ Previous studies showed that EPC function decreases in preeclampsia.^{10,11} Thus, it results in inadequate endothelial cell regeneration (reendothelialization) and impacts various clinical manifestations of preeclampsia.^{9,12}

Although the pathophysiology of preeclampsia is becoming more understood, finding a definitive treatment has been difficult.¹³ Increasing data suggest that treatment strategies to restore the underlying endothelium dysfunction may alleviate the broad systemic manifestations in preeclampsia.¹⁴ A central common metabolite in vascular dysfunction associated with preeclampsia is nitric oxide (NO).¹⁵

One of the natural ingredients to improve nitric oxide bioavailability is *Physalis* leaf extract. Previous study found that *Physalis* leaf extract increases EPCs number and enhances reendothelialization in rats given deoxycorticosterone acetate (DOCA)-salt.¹⁶ The species of *Physalis* found in abundance in Indonesia is *Physalis angulata* L. It is easy to grow and is often only considered a weed.¹⁷ This study aimed to evaluate whether *Physalis angulata* leaf ethanol extract might alleviate L-N^G-nitroarginine methyl ester (L-NAME)-induced preeclampsia symptoms in rats by improving EPC and endothelial cell function. L-NAME is a nonselective NOS inhibitor, thereby decreasing NO production.¹⁸

Methods

Ethical considerations

The institutional research ethics council of Universitas Brawijaya authorized all experimental protocols (No.1191-KEP-UB, December 10, 2019). The reports are compliant with Animal Research Reporting of *In Vivo* Experiments (ARRIVE) guidelines 2.0.¹⁹ All efforts were made to alleviate any suffering of animals. Trained researchers carried out all procedures, including injections and administration of extract through an oral gavage feeding tube.

Plant materials

Physalis angulata leaves were collected from the Research Institute of Spices and Medicinal Plants (BALITTRO) Bogor, Indonesia. The plant was identified as *Physalis angulata* L. (Solanaceae family) by the School of Life Sciences and Technology (SITH) at Institut Teknologi Bandung (ITB), Indonesia (No. 3538/I1.CO2.2/PL/2016, October 13, 2016). The fresh leaves were thoroughly washed to remove soil residues with distilled water and then dried at room temperature (RT) for a week. The dried leaves were then ground using a grinder into a fine powder. The powder was stored in an aluminum foil-wrapped airtight container at -20°C before being used.

Plant extraction

The crude ethanol extract was prepared by maceration. *Physalis angulata* leaf powder (100 g) was stirred mechanically with 900 mL of 96% (v/v) ethanol for 24 h at RT. The residue was extracted thrice. The extract obtained was then filtered through filter papers (Whatman No. 1, Cat# 1001-150). The filtrate was evaporated using vacuum distillation and a rotary evaporator (Janke & Kunkel, IKA-Labortechnik, Germany) at a temperature of $70-80^{\circ}\text{C}$. The crude ethanol extract was then stored in an aluminum foil-wrapped airtight container at -20°C before being used.

Phytochemical analysis with LC-HRMS

Phytochemical analysis of the *Physalis angulata* leaf extract was performed with liquid chromatography-high resolution mass spectrometry (LC-HRMS). The LC-HRMS system was composed of a Dionex™ UltiMate™ 3000 RSLCnano System (Cat# ULTIM3000RSLCNANO) with a micro-flow meter (Cat# 6041.7903A) from Thermo Scientific™. The analytical column used was Thermo Scientific™ Hypersil GOLD™ aQ (1.0 mm $\times$ 50 mm $\times$ 1.9 μm particle size, Cat# 25302-051030). The LC system was linked to a Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap™ MS System (Cat# IQLAAEGAAPFALGMBDK) with heated electrospray ionization (HESI) with positive or negative ionization at 300°C , a nitrogen gas sheath flow of 20 arbitrary units, and an auxiliary nitrogen gas flow of 10 arbitrary units.

The temperature of the analytical column was kept constant at 30°C. (A) MS-grade water (Cat# 900682) with 0.1% (v/v) formic acid and (B) MS-grade acetonitrile with 0.1% (v/v) formic acid (Cat# 900686) were employed as mobile phases (all from Sigma-Aldrich). Separation was accomplished using the following mobile phase gradient: (i) elution with 5% B from the injection time until 2 minutes, (ii) a linear gradient of 5–60% B for the next 13 minutes, (iii) a further linear gradient 60–95% B for 10 minutes, (iv) a further linear gradient 5–95% A for 0.1 minutes, and finally (v) elution with 95% A for 4.9 minutes (total chromatographic run time was 30 minutes). The injection volume was 5 µl, and the flow rate was 40 µl per minute.

Electrospray ionization (ESI)⁺ was carried out with a spray voltage of 3.80 kV, a capillary temperature of 300°C, and a capillary voltage of 3.80 kV. It had a resolution of 70,000 full widths at half maximum peak height (FWHM) at *m/z* 200. A total ion chromatogram (TIC) was obtained using a mass range of 50–750 *m/z*, a target of 3,000,000 charges, and a maximum ionization-time of 250 ms. A complete scan for positive ions (ESI⁺) was performed, and data-dependent MS/MS employed normalized collision energy.

Finally, results were processed for qualitative screening using Thermo Scientific™ Compound Discoverer™ 3.0 (Cat# OPTON-31061) with Thermo Scientific™ High-Resolution Accurate-Mass MS/MS Spectral Library (Cat# IQLAAE-GABSFAPYMBHK). The analyte filter requirements were isotopic pattern matching score >70%, area (max.) >10⁶, and mass deviation for each ion less than 2.5 ppm between observed and theoretical mass. [(Theoretical mass–observed mass)/observed mass] × 10⁶ was defined as mass deviation Δ*m*.

The sample was prepared in the following manner. Ethanol (900 µl) was used to dilute 100 µl of the extract (a dark green paste). The material was vortexed for 1 minute using Thermo Scientific™ Basic Vortex Mixers (Cat# 88882012) before being spun down (6000 rpm, RT, 2 minutes) with Thermo Scientific™ Sorvall™ ST 8 Small Benchtop Centrifuge (Cat# 75007205). The supernatant was collected and placed into a vial after being filtered using a 0.22 µm syringe filter. The sample was placed in the autosampler before it was injected into the LC-HRMS system.

Experimental animals

Healthy virgin female Wistar rats aged 10–12 weeks (160–200 g) and male Wistar rats (200–250 g) were acquired from the Faculty of Pharmacy, Universitas Gadjah Mada, Indonesia. For a week, rats were subjected to laboratory settings. Rats were kept in clean plastic cages in groups, with *ad libitum* access to standard feed and tap water and a 12-hour light/dark cycle at a temperature of 25±2°C.

The sample size was determined *a priori* based on the formula from Steel and Torrie (1995): $(t-1)(r-1) \geq 15$, where *t* is the number of groups and *r* is the number of replications in each group. The minimum number of replications was 4.75 (rounded to 5). In each group, 1 rat was added as a backup in case a rat died or gave birth before the termination time. So, the number of pregnant rats needed for the 5 groups was 30. In this study, no rats died or gave birth before termination time.

The inclusion and exclusion criteria were established *a priori*. The inclusion criteria were healthy pregnant Wistar rats aged 10–12 weeks (160–200 g). Physically healthy rats were characterized by active movement, clear eyes, shiny white fur, no hair loss, and no diarrhea. While the exclusion criteria were rats who died or gave birth before termination time.

Timed pregnant rats were obtained by estrus synchronization with Sardjono *et al.* (2019) methods with modifications. The females were housed together and separated from males for two weeks to become anestrus (the Lee-Boot effect). Females were then exposed to male urine (dirty bedding from the male's cage) for 72 h to induce estrus synchronization (the Whitten effect). Vaginal swab and histological analysis with Giemsa staining were conducted to determine the estrus phase. An estrus female was mated overnight with a male at 1:1. Females with a vaginal plug in the morning after cohabitation were considered pregnant on day 0 (G0; term, G21–23) and were used in this study. The timed pregnancy rate was 70%.²⁰

The randomization was performed on a web-based [Research Randomizer](#). A total of 30 pregnant rats were randomly divided into five groups (n=6). A sham group (sham) received an abdominal subcutaneous injection of phosphate-buffered saline (PBS, bioWORLD Cat# 41620016-2) on G9–G18. A preeclampsia group (PE) received an abdominal subcutaneous injection of L-NAME (Cayman Chemical Cat# 80210) at a dose of 90 mg/kg body weight (BW)/day on G9–G11, then 75 mg/kg BW/day on G12–G18. Treatment groups were preeclampsia rats orally administered with *Physalis angulata* leaf ethanol extract at doses of 500 (D1), 1500 (D2), or 2500 mg/kg BW/day (D3) on G12–G18. The doses of the extract were determined according to our previous study.¹⁶

Blood pressure measurement

Conscious rats were held in a heated plastic chamber, and their systolic and diastolic blood pressures were measured non-invasively using rat tail-cuff blood pressure device. The CODA[®] High Throughput System with 2 activated channels (Kent Scientific Corp. Cat# CODA-HT2) was employed.²¹ Blood pressures were monitored in the morning on G7 (before L-NAME injection), G12, G15, and G19.

Urine protein measurement

Total protein level was measured in 24-h urine samples on G12 and G18. Urine sampling was performed non-invasively by placing the rats in metabolic cages with urine and stool separators. Rats were fasted to avoid contaminating the collected urine. The collected urine was centrifuged (3500 rpm, 4°C, 10 min). Before analysis, the supernatant was decanted, labeled, and stored at −40°C. The total urine protein content was evaluated by measuring absorbance at 280 nm with a Thermo Scientific[™] NanoDrop[™] 1000 spectrophotometer (Cat# ND-1000).²²

Specimen collection

Rats were sacrificed on G19 by terminal anesthetization with ketamine at 40 mg/kg BW intraperitoneally. Maternal blood was collected by cardiac puncture. The blood (2 mL) was put into a tube containing ethylenediaminetetraacetic acid (EDTA). The residual blood volume was centrifuged (4500 rpm, RT, 10 min) to obtain the serum, then labeled and stored at −40°C until analysis.

Laparotomy was performed. The uterus was extracted *in toto* and opened at the anti-mesometrial side. Thus, the placenta remained attached to the uterus. The tail arteries were also collected. The tissues were then fixed in a 10% neutral-buffered formalin solution (pH 7.2, Sigma-Aldrich Cat# HT501128) for 24 h and paraffin-embedded as per the standard procedure.²³ Some placentas were gently perfused with cold PBS before being kept at −40°C until analysis.

Enzyme-linked immunosorbent assay

Serum sFlt-1 levels were determined in duplicate using a rat VEGFR1/Flt-1 ELISA kit (Elabscience Biotechnology Cat# E-EL-R0911). The protocol was carried out following the manufacturer's instructions.

Colorimetry

Serum NO levels (Cat# E-BC-K035-M), serum and placental malondialdehyde (MDA) levels (Cat# E-BC-K025-M), and serum and placental superoxide dismutase (SOD) activities (Cat# E-BC-K020-M) were measured in duplicate using colorimetric assay kits from Elabscience Biotechnology. The protocol was performed according to the manufacturer's instructions.

Immunohistochemistry

The formalin-fixed, paraffin-embedded (FPPE) placenta was sectioned (3 µm thick) parallel to the mesometrial-fetal axis. The FPPE tail artery was also sectioned. After dewaxing, hydrating, and inhibiting endogenous peroxidase activity, sections were treated with the primary antibody overnight at 4°C. Mouse monoclonal IgG_{2a} κ nitric oxide synthase 3 (NOS3) antibody (A-9) (Cat# sc-376751, RRID: AB_2832203) and IgG₁ κ hypoxia-inducible factor (HIF)-1α antibody (28b) (Cat# sc-13515, RRID: AB_627723) were employed as primary antibodies. All from Santa Cruz Biotechnology. According to the manufacturer's instructions, immunoreactivity was detected using N-Histofin[®] Simple Stain MAX PO (MULTI) (Nichirei Biosciences Cat# 414151F).

Stained sections were examined under the microscope (Olympus BX51 Microscope). The sections were then photographed in brightfield imaging mode using Olympus whole-slide scanner (Olympus dotSlide Virtual Microscopy System with Canon Powershot SX10IS Digital Camera) operated with OlyVIA V2.4 (Build 9003) software (Olympus Soft Imaging Solutions GmbH).²⁴

Tail artery eNOS expression was observed throughout the tunica intima, while placental eNOS and HIF-1α expressions were observed in five high-power fields in the basal zone. The analysis was carried out with ImageJ for Windows software (an open-source Java image processing program). The percentage (%) area of the tail artery tunica intima expressing eNOS (brown-stained cells) was calculated compared to the total area of tunica intima. The optical density of eNOS expression in the placental basal zone was measured in arbitrary units. The number of trophoblast giant cells in the placental basal zone expressing HIF-1α was also counted.

Circulating EPC isolation and flow cytometry

According to our previous study, circulating EPCs were isolated with modifications.¹⁶ Briefly, mononuclear cells (MNCs) were isolated by Lymphoprep[™] 1.077 g/mL (Alere Technologies AS Cat# AXS-1114545) density gradient

Table 1. Compounds detected in *Physalis angulata* leaf ethanol extracted by LC-HRMS.

Chemical Family	Analyte	RT [min]	Formula	Theoretical Mass [M + H] ⁺	Observed Mass [M + H] ⁺	Δm (ppm)	Area (Max.)
Amino acids, peptides, and derivatives	Choline	1.053	C ₅ H ₁₃ NO	103.099714	103.09974	-0.3	616,797,316.57
	Betaine	0.948	C ₅ H ₁₁ NO ₂	117.078979	117.07889	0.8	226,806,761.10
	Valine	1.328	C ₅ H ₁₁ NO ₂	117.078979	117.07892	0.5	81,526,589.52
	L-Norleucine	1.345	C ₆ H ₁₃ NO ₂	131.094629	131.09438	1.9	48,881,450.64
	2-Hydroxyphenyl-alanine	1.476	C ₉ H ₁₁ NO ₃	181.073894	181.07367	1.2	25,542,530.11
	L-Phenylalanine	1.483	C ₉ H ₁₁ NO ₂	165.078979	165.07871	1.6	22,476,555.69
	N6,N6-Trimethyl-L-lysine	1.233	C ₉ H ₂₀ N ₂ O ₂	188.152478	188.15220	1.5	22,389,817.55
	L-Pyrogutamic acid	1.452	C ₅ H ₇ NO ₃	129.042594	129.04247	1.0	15,771,178.50
	Proline	1.669	C ₅ H ₉ NO ₂	115.063329	115.06328	0.4	14,605,229.00
	Nicotinic acid	1.430	C ₆ H ₅ NO ₂	123.032029	123.03194	0.7	10,913,391.35
	Pipecolic acid	1.338	C ₆ H ₁₁ NO ₂	129.078979	129.07879	1.5	8,610,852.51
	L-Glutamic acid	0.842	C ₅ H ₉ NO ₄	147.053159	147.05296	1.4	8,428,291.33
	Isoleucine	0.999	C ₆ H ₁₃ NO ₂	131.094629	131.09438	1.9	7,726,199.80
	Leucylproline	1.517	C ₁₁ H ₂₀ N ₂ O ₃	228.147393	228.14699	1.8	5,540,961.06
	N-Acetyl-L-leucine	5.707	C ₈ H ₁₅ NO ₃	173.105193	173.10506	0.8	3,489,258.34
	Kynurenic acid	4.454	C ₁₀ H ₇ NO ₃	189.042594	189.04242	0.9	1,275,019.10
Alkaloids	2,3,4,9-Tetrahydro-1H- β -carboline-3-carboxylic acid	5.272	C ₁₂ H ₁₂ N ₂ O ₂	216.089878	216.08953	1.6	69,503,822.51
	Norharman	6.348	C ₁₁ H ₈ N ₂	168.068748	168.06854	1.2	2,383,323.13
	Tropine	1.476	C ₈ H ₁₅ NO	141.115364	141.11508	2.0	44,001,566.81
	Trigonelline	0.968	C ₇ H ₇ NO ₂	137.047679	137.04743	1.8	22,244,006.33
	DL-Stachydrine	0.962	C ₇ H ₁₃ NO ₂	143.094629	143.09435	1.9	20,384,634.55
	6-Methylquinoline	5.271	C ₁₀ H ₉ N	143.073499	143.07324	1.8	18,254,920.68
	Caffeine	5.005	C ₈ H ₁₀ N ₄ O ₂	194.080376	194.08025	0.6	11,090,480.45
	4-Piperidone	1.692	C ₅ H ₉ NO	99.068414	99.06857	-1.6	8,899,957.00
	N-Acetyltyramine	4.837	C ₁₀ H ₁₃ NO ₂	179.094629	179.09451	0.7	2,498,298.21

Table 1. Continued

Chemical Family	Analyte	RT [min]	Formula	Theoretical Mass [M + H] ⁺	Observed Mass [M + H] ⁺	Δm (ppm)	Area (Max.)
Phenolics	Coumarins						
	7-Hydroxy-coumarine	3.210	C ₉ H ₆ O ₃	162.031695	162.03150	1.2	13,406,442.09
Flavonols	Kaempferol-3-O-β-glucopyranosyl-7-O-α-rhamno-pyranoside	7.561	C ₂₇ H ₃₀ O ₁₅	594.158475	594.15819	0.5	12,234,273.36
	Rutin	7.224	C ₂₇ H ₃₀ O ₁₆	610.153390	610.15300	0.6	8,121,030.26
	Kaempferol	7.561	C ₁₅ H ₁₀ O ₆	286.047740	286.04714	2.1	2,370,680.02
	Quercetin	7.222	C ₁₅ H ₁₀ O ₇	302.042654	302.04198	2.2	1,158,392.56
Phenolic acids	Chlorogenic acid	5.101	C ₁₆ H ₁₈ O ₉	354.095085	354.09471	1.1	14,892,726.50
	Caffeic acid	5.463	C ₉ H ₈ O ₄	180.042260	180.04206	1.1	6,034,960.35
	Ferulic acid	7.321	C ₁₀ H ₁₀ O ₄	194.057910	194.05781	0.5	2,923,936.08
	4-Coumaric acid	6.797	C ₉ H ₈ O ₃	164.047345	164.04713	1.3	2,897,255.73
Nucleobases and derivatives	Adenine	1.365	C ₅ H ₅ N ₅	135.054495	135.05422	2.0	12,630,152.91
	Adenosine	1.465	C ₁₀ H ₁₃ N ₅ O ₄	267.096755	267.09624	1.9	11,394,283.55
	Uracil	1.435	C ₄ H ₄ N ₂ O ₂	112.027278	112.02729	-0.1	4,989,613.51
	Guanine	1.467	C ₅ H ₅ N ₅ O	151.049410	151.04931	0.7	4,548,778.84
	Cytidine	1.376	C ₉ H ₁₃ N ₃ O ₅	243.085522	243.08505	1.9	1,809,831.46
Fatty acids and derivatives	9S,13R-12-Oxophytodienoic acid	10.893	C ₁₈ H ₂₈ O ₃	292.203845	292.20339	1.6	4,175,846.27
	α-Eleostearic acid	17.378	C ₁₈ H ₃₀ O ₂	278.22458	278.22417	1.5	3,660,741.08
	Oleamide	22.325	C ₁₈ H ₃₅ NO	281.271864	281.27123	2.3	3,350,576.89
Organic acids	Indole-3-acrylic acid	3.901	C ₁₁ H ₉ NO ₂	187.063329	187.06308	1.3	26,130,838.47
	(3R,5R)-1,3,5-Trihydroxy-4-[(2E)-3-(4-hydroxy-3-methoxyphenyl)-2-propenyl]oxy} cyclohexane-carboxylic acid	6.311	C ₁₇ H ₂₀ O ₉	368.110735	368.11031	1.2	1,420,786.28
Steroids	Withanolide A	12.441	C ₂₈ H ₃₈ O ₆	470.266840	470.26628	1.2	1,709,017.92
Miscellaneous	(+/-)-C75	5.017	C ₁₄ H ₂₂ O ₄	254.151810	254.15148	1.3	3,548,537.83
	Pyridoxine	1.454	C ₈ H ₁₁ NO ₃	169.073894	169.07359	1.8	3,176,125.50
	4-Indole-carbaldehyde	7.935	C ₉ H ₇ NO	145.052764	145.05263	0.9	2,835,961.24
	Heptanophenone	14.411	C ₁₃ H ₁₈ O	190.135765	190.13556	1.1	2,124,974.85
	Shogaol	13.617	C ₁₇ H ₂₄ O ₃	276.172545	276.17208	1.7	1,868,740.87
	3-Hydroxy-3,5,5-trimethyl-4-(3-oxo-1-buten-1-ylidene) cyclohexyl β-D-glucopyranoside	5.595	C ₁₉ H ₃₀ O ₈	386.194070	386.19359	1.2	1,434,043.83
	Sedanolid	10.952	C ₁₂ H ₁₈ O ₂	194.130679	194.13040	1.4	1,264,413.83

LC-HRMS, liquid chromatography-high resolution mass spectrometry; RT, room temperature.

centrifugation (1600 rpm, RT, 30 min), then washed three times with PBS. Isolated MNCs were then stained with mouse monoclonal IgG κ CD34 antibody (ICO115) conjugated to phycoerythrin (Cat# sc-7324, RRID: AB_2291280), IgG λ Flk-1/KDR/VEGFR2 antibody (D-8) conjugated to fluorescein isothiocyanate (Cat# sc-393163, RRID: AB_2920761), and IgG κ CD45 antibody (35-Z6) conjugated to phycoerythrin-cyanine5 (Cat# sc-1178, RRID: AB_627074) (all from Santa Cruz Biotechnology). EPCs numbers were analyzed using BD FACSCalibur™ Flow Cytometer from BD Biosciences (Cat# 342973).

Statistical analysis

GraphPad Prism 9.3.1 for Windows (GraphPad Software Inc., San Diego, CA, USA) was used to perform all statistical analyses. Free alternatives such as SOFA or JASP Statistics could be used. The data is displayed as mean $\pm$ standard deviation (SD). The differences in blood pressures and total urine protein levels between groups were examined using a two-way analysis of variance (ANOVA) and Tukey's multiple comparisons test. One-way ANOVA followed by Tukey's multiple comparisons test was used to compare group differences in the other variables. A p-value <0.05 was considered statistically significant. Before we looked at the data, we had determined the analytical stages.

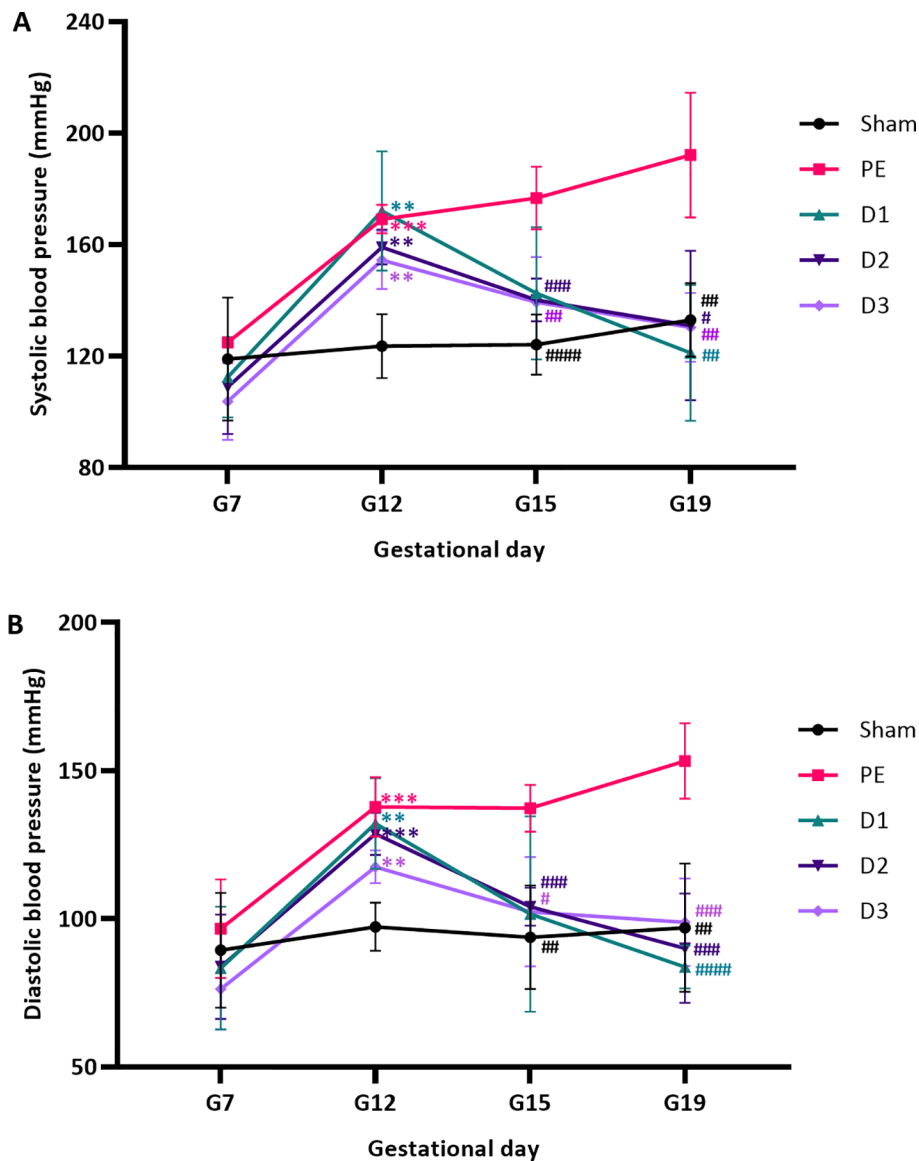


Figure 1. Blood pressure. The panels show the mean $\pm$ SD of (A) systolic and (B) diastolic blood pressures on G7, G12, G15, and G19 for each group (n=6). **p<0.01 vs. Sham; ***p<0.001 vs. Sham; #p<0.05 vs. PE; ##p<0.01 vs. PE; ###p<0.001 vs. PE; ####p<0.0001 vs. PE; D1–D3, preeclampsia rats treated with *Physalis angulata* leaf extract at 500, 1500, and 2500 mg/kg BW, respectively; G, gestational day; PE, preeclampsia.

Results

Phytochemical analysis

As shown in Table 1, compounds observed in *Physalis angulata* leaf ethanol extract were various amino acids, peptides and derivatives, alkaloids, phenolics, nucleobases and derivatives, fatty acids and derivatives, organic acids, steroids, and

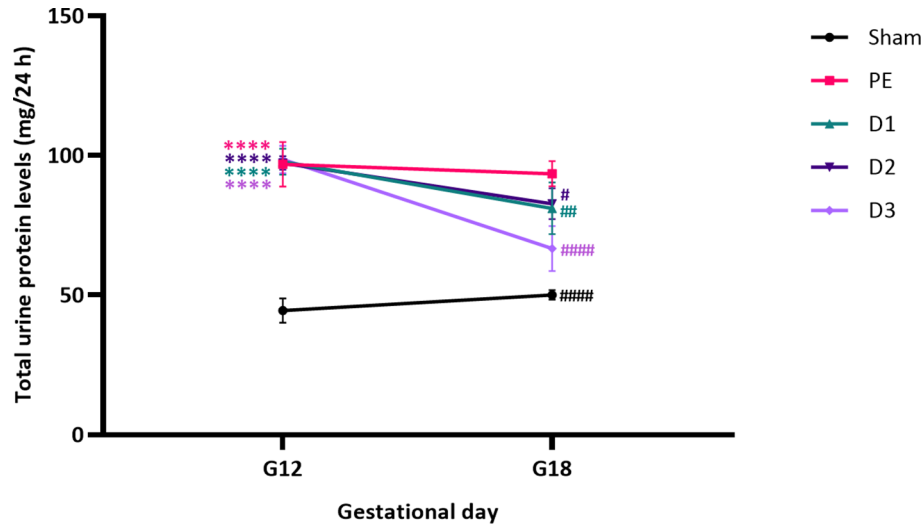


Figure 2. Total urine protein levels. The 24-h total urine protein levels on G12 and G18 for each group (n=6) are presented as mean±SD. ****p<0.0001 vs. Sham; #p<0.05 vs. PE; ##p<0.01 vs. PE; ####p<0.0001 vs. PE; D1–D3, preeclampsia rats treated with *Physalis angulata* leaf extract at 500, 1500, and 2500 mg/kg BW, respectively; G, gestational day; PE, preeclampsia.

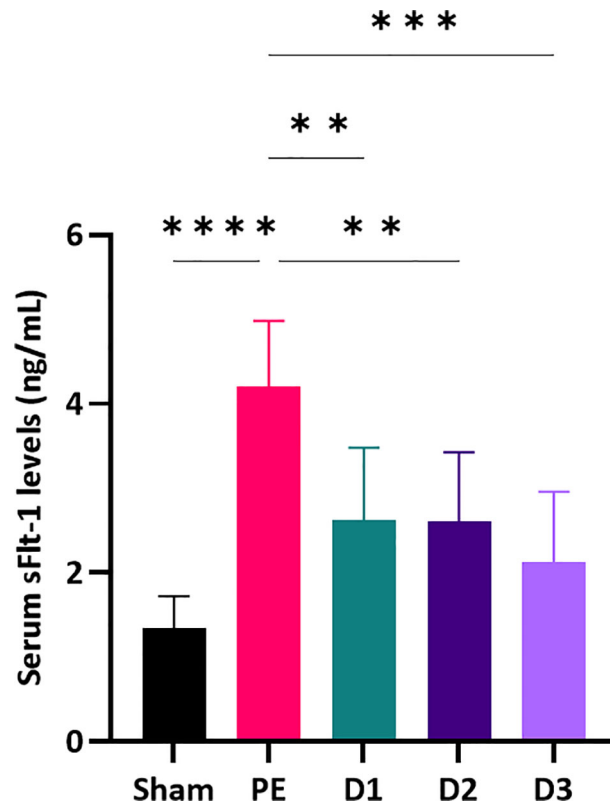


Figure 3. Serum sFlt-1 levels. The serum sFlt-1 levels on G19 for each group (n=6) are presented as mean±SD. **p<0.01; ***p<0.001; ****p<0.0001; D1–D3, preeclampsia rats treated with *Physalis angulata* leaf extract at 500, 1500, and 2500 mg/kg BW, respectively; PE, preeclampsia; sFlt-1, soluble fms-like tyrosine kinase.

miscellaneous. Among the identified alkaloids were trigonelline and DL-stachydrine. While among the identified phenolics were kaempferol, rutin, quercetin, and chlorogenic acid. The steroid detected was withanolide A.¹⁹

Blood pressure

As presented in Figure 1, compared to the sham group, the injection of L-NAME starting on G9 significantly ($p<0.05$) increased systolic and diastolic blood pressures on G12–G19 in the preeclampsia group. Compared to the preeclampsia

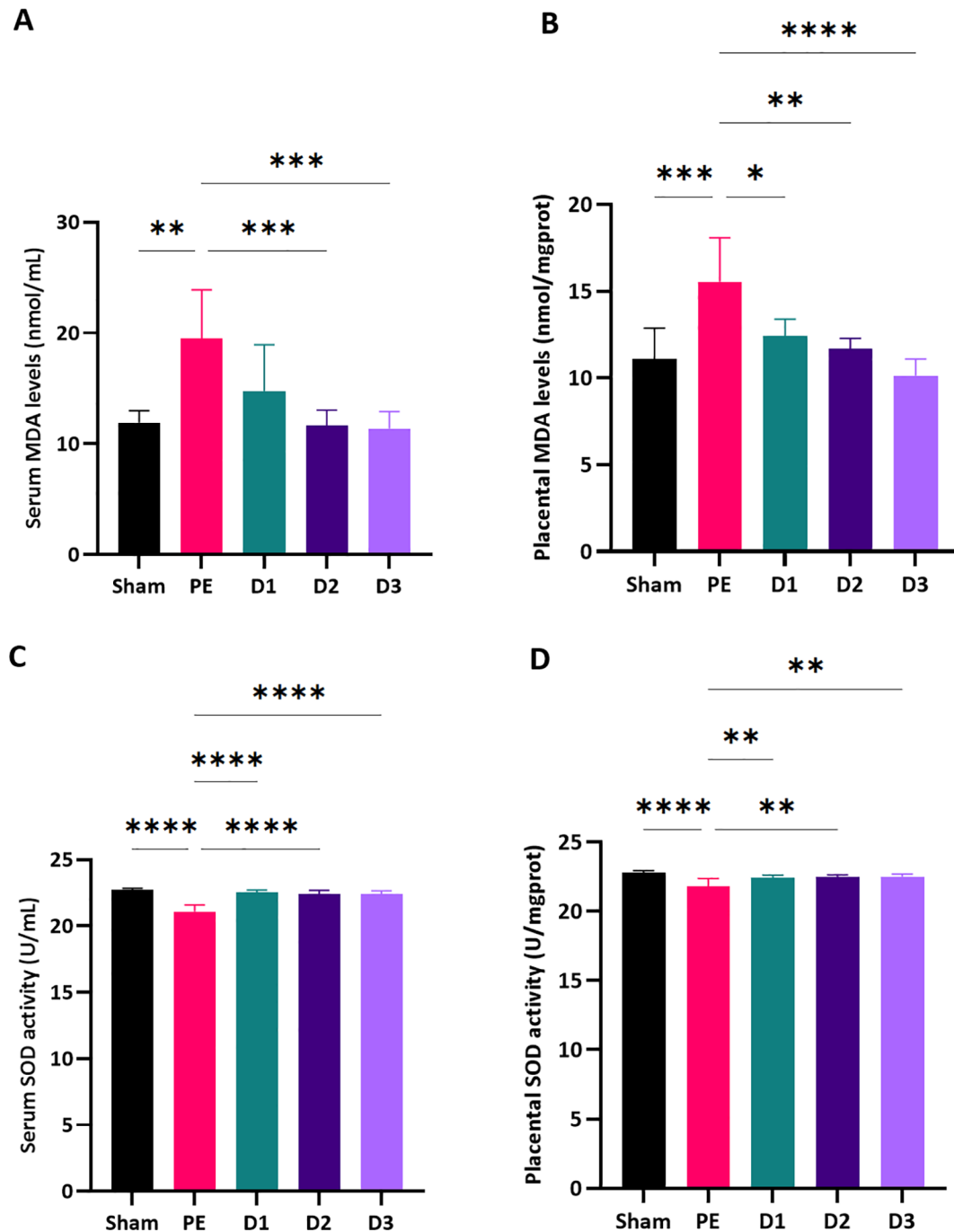


Figure 4. Oxidative stress markers. The serum and placental MDA levels and SOD activities on G19 for each group (n=6) are presented as mean±SD. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$; D1–D3, preeclampsia rats treated with *Physalis angulata* leaf extract at 500, 1500, and 2500 mg/kg BW, respectively; G, gestational day; PE, preeclampsia; MDA, malondialdehyde; SOD, superoxide dismutase.

group, *Physalis angulata* leaf extract treatment at 1500 and 2500 mg/kg BW significantly ($p<0.05$) reduced systolic and diastolic blood pressures on G15. Furthermore, extract treatment at 500, 1500, and 2500 mg/kg BW significantly ($p<0.05$) lowered systolic and diastolic blood pressures on G19, approaching the sham level ($p>0.8$).

Total urine protein levels

The injection of L-NAME starting on G9 significantly ($p<0.0001$) elevated total urine protein levels on G12–G18 in the preeclampsia group compared to the sham group. *Physalis angulata* leaf extract administration at 500, 1500, and 2500 mg/kg BW significantly ($p<0.05$) reduced total urine protein levels on G18 compared to the preeclampsia group (Figure 2).

Serum sFlt-1 levels

As presented in Figure 3, the injection of L-NAME significantly ($p<0.0001$) elevated serum sFlt-1 levels compared to the sham group. *Physalis angulata* leaf extract administration at 500, 1500, and 2500 mg/kg BW significantly ($p<0.01$) reduced serum sFlt-1 levels compared to the preeclampsia group.

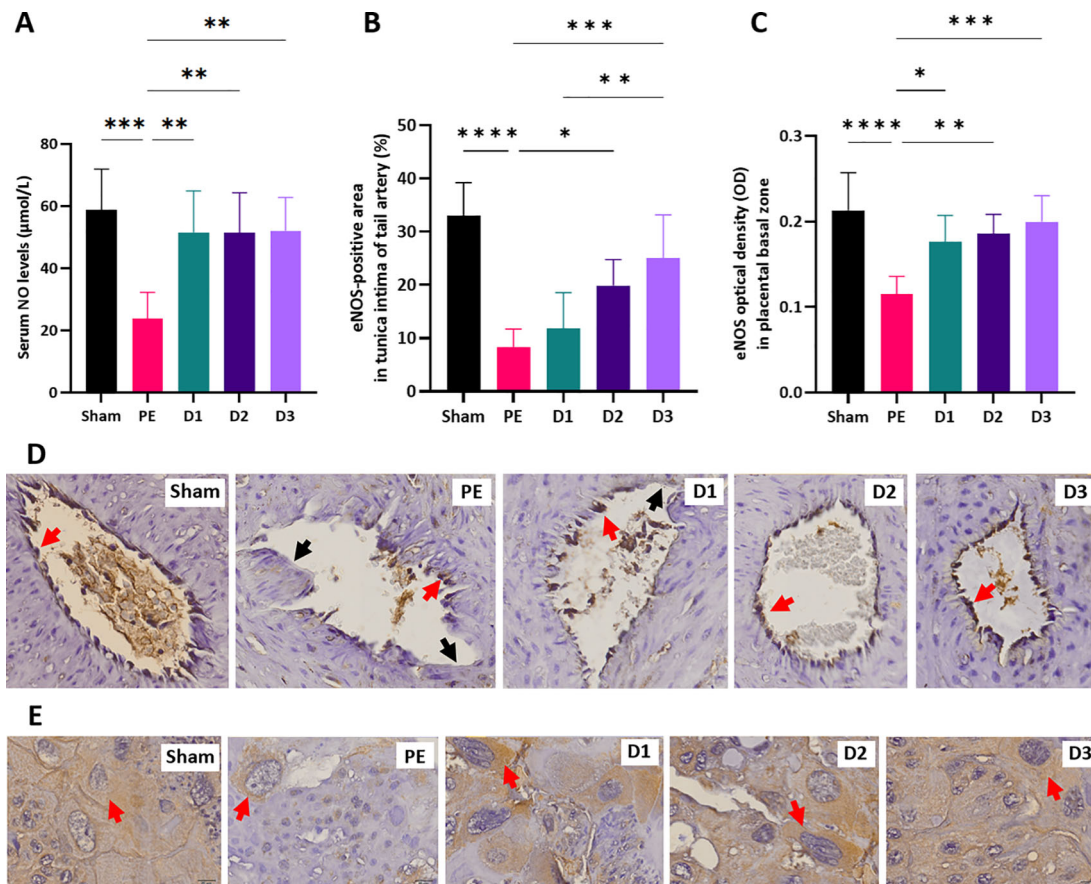


Figure 5. NO levels and eNOS expression levels. (A) The serum NO levels, (B) tail artery and (C) placental eNOS expression levels on G19 for each group ($n=6$) are presented as mean $\pm$ SD. (D) The representative micrographs of the tail artery and (E) placental eNOS expression from five independent experiments in which the same results were obtained are shown. (A) The serum NO level was determined by colorimetry. (B) Tail artery eNOS expression was evaluated in tunica intima by measuring the percentage of eNOS-positive area relative to total area. (C) Placental eNOS expression was evaluated in the basal zone by measuring the optical density (OD, arbitrary unit). (D) The nucleus of an eNOS-positive endothelial cell is brown, marked with a red arrow. Endothelial cell detachment is marked with a black arrow. Magnification: 200 $\times$. (E) The cytoplasm of an eNOS-positive cell is brown, marked with a red arrow. Magnification: 200 $\times$. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$; D1–D3, preeclampsia rats treated with *Physalis angulata* leaf extract at 500, 1500, and 2500 mg/kg BW, respectively; G, gestational day; PE, preeclampsia; NO, nitric oxide; eNOS, endothelial nitric oxide synthase.

Serum and placental MDA levels

The injection of L-NAME significantly ($p<0.01$) raised serum and placental MDA levels compared to the sham group, as seen in [Figure 4A](#) and [B](#). *Physalis angulata* leaf extract treatment at 1500 and 2500 mg/kg BW dose-dependently reduced ($p<0.01$) serum and placental MDA levels compared to the preeclampsia group.

Serum and placental SOD activities

L-NAME injection significantly ($p<0.0001$) lowered serum and placental SOD activities compared to the sham group. Compared to the preeclampsia group, treatment of *Physalis angulata* leaf extract at 500, 1500, and 2500 mg/kg BW significantly ($p<0.01$) increased serum and placental SOD activities ([Figure 4C](#) and [D](#)).

Serum NO levels

As presented in [Figure 5A](#), the injection of L-NAME significantly ($p<0.001$) decreased serum NO level compared to the sham group. *Physalis angulata* leaf extract administration at 500, 1500, and 2500 mg/kg BW significantly ($p<0.01$) increased serum NO levels compared to the preeclampsia group.

Tail artery eNOS expression

Tail artery endothelial nitric oxide synthase (eNOS) expression was evaluated in tunica intima by measuring the percentage of eNOS-positive area relative to total area. [Figure 5B](#) and [D](#) show that the injection of L-NAME significantly ($p<0.0001$) reduced the tail artery eNOS expression compared to the sham group. *Physalis angulata* leaf extract administration at 1500 and 2500 mg/kg BW dose-dependently elevated ($p<0.05$) the tail artery eNOS expression compared to the preeclampsia group. There was endothelial cell detachment in the tail artery of the preeclampsia group, and it was repaired by the administration of the extract ([Figure 5D](#)).

Placental eNOS expression

Placental eNOS expression was evaluated in the basal zone by measuring the optical density (arbitrary unit). [Figure 5C](#) and [E](#) show that the injection of L-NAME significantly ($p<0.0001$) decreased placental eNOS expression compared to the sham group. *Physalis angulata* leaf extract administration at 500, 1500, and 2500 mg/kg BW dose-dependently increased ($p<0.5$) placental eNOS expression compared to the preeclampsia group.

Placental HIF-1 α expression

Placental HIF-1 α expression was evaluated in the basal zone by counting the brown-stained nuclei of trophoblast giant cells. As shown in [Figure 6](#), the injection of L-NAME significantly ($p<0.001$) raised placental HIF-1 α expression compared to the sham group. *Physalis angulata* leaf extract administration at 500, 1500, and 2500 mg/kg BW significantly ($p<0.001$) lowered placental HIF-1 α expression compared to the preeclampsia group.

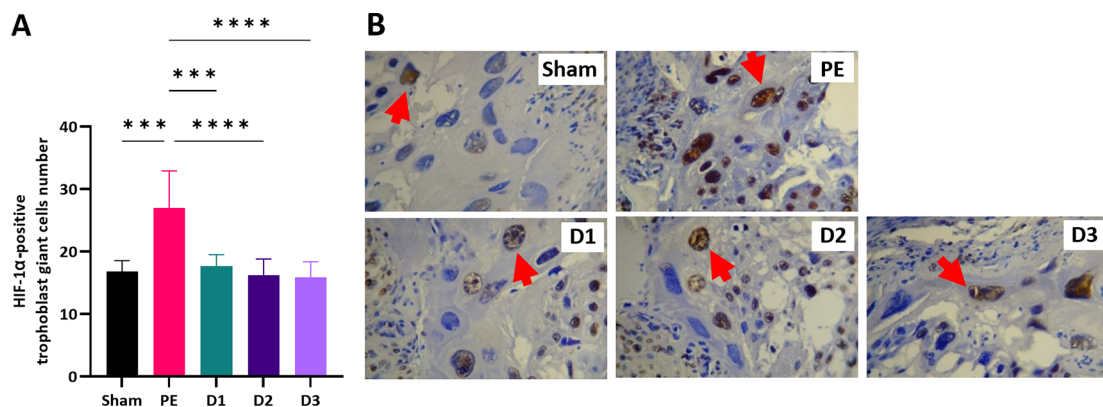


Figure 6. Placental HIF-1 α expression levels. (A) Placental HIF-1 α expression was evaluated in the basal zone by counting the brown-stained nuclei of trophoblast giant cells. The HIF-1 α -positive trophoblast giant cells number on G19 for each group ($n=6$) are presented as mean $\pm$ SD. (B) The representative micrographs of placental HIF-1 α expression (brown-stained nuclei, red arrows) from five independent experiments in which the same results were obtained are shown. Magnification: 200 $\times$. *** $p<0.001$; **** $p<0.0001$; D1–D3, preeclampsia rats treated with *Physalis angulata* leaf extract at 500, 1500, and 2500 mg/kg BW, respectively; G, gestational day; PE, preeclampsia; HIF-1 α , hypoxia-inducible factor-1 α .

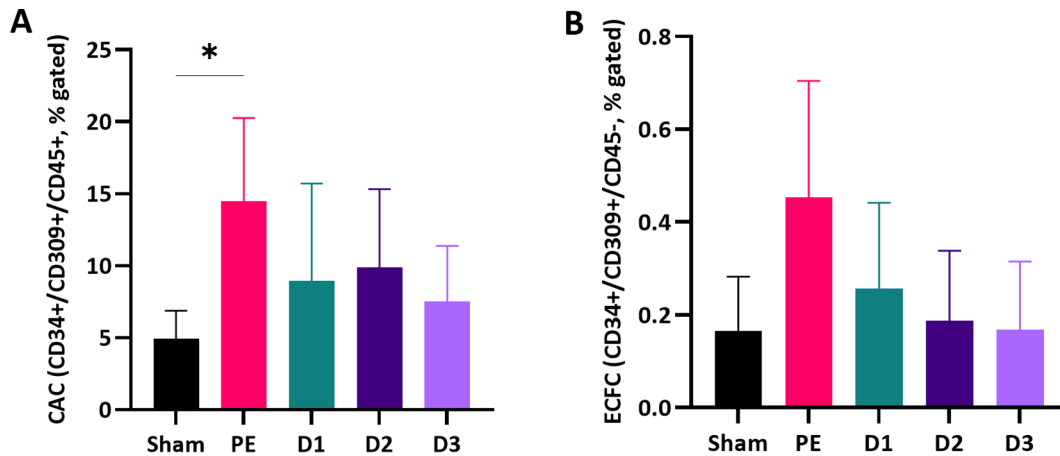


Figure 7. Circulating endothelial progenitor cells. (A) Percentages of CAC and (B) ECFC on G19 for each group (n=6) are presented as mean±SD. *p<0.05; D1–D3, preeclampsia rats treated with *Physalis angulata* leaf extract at 500, 1500, and 2500 mg/kg BW, respectively; G, gestational day; PE, preeclampsia; CAC, circulating angiogenic cells; ECFC, endothelial colony-forming cells.

Circulating EPCs

The injection of L-NAME significantly (p<0.05) increased the percentage of CACs (CD34⁺/CD309⁺/CD45⁺) compared to the sham group. Although not statistically significant (p>0.05), the injection of L-NAME also increased the percentage of circulating ECFCs (CD34⁺/CD309⁺/CD45⁻) compared to the sham group. *Physalis angulata* leaf extract administration at 500, 1500, and 2500 mg/kg BW tended to reduce the percentages of CACs and ECFCs compared to the preeclampsia group (Figure 7).

Discussion

Consistent with Shu *et al.* (2018), our results indicated that subcutaneous injection of L-NAME starting at the ninth gestational day of pregnancy significantly elevated blood pressure and urinary protein excretion, the two symptoms typical in preeclampsia.²⁵ L-NAME is a nonselective NOS inhibitor, thereby decreasing NO production.¹⁸ In line with Motta-Mejia *et al.* (2017), rats injected with L-NAME had a reduced placental eNOS expression, thus diminishing NO production.²⁶ Preeclampsia rats' placenta also had a higher MDA level, and a lower SOD activity indicated oxidative stress. Among the mechanisms involved, reduced NO bioavailability and increased oxidative stress play a critical role in placental dysfunction in preeclampsia. Increased reactive oxygen species (ROS) production inhibits the expression and function of eNOS. Scavenging of NO by ROS reduces NO bioavailability and forms the metabolite peroxynitrite (ONOO⁻). Both interfere with critical vascular signaling pathways, impairing vascular function and growth associated with poor placentation.^{14,27,28} This condition leads to placental hypoperfusion and hypoxic microenvironment, marked with increased placental HIF-1α expression.^{29,30}

Ischemia-reperfusion injury to the placenta causes the release of soluble mediators into the maternal circulation. Soluble fms-like tyrosine kinase (sFlt)-1, a soluble form of the vascular endothelial growth factor receptor (VEGFR)-1 generated by alternative splicing of VEGFR-1 mRNA, is one of the mediators.³¹ Overproduction of the antiangiogenic factor sFlt-1 may antagonize the vasodilatory properties of VEGF. The sFlt-1 binds free VEGF and inhibits activation of VEGFR-2 and eNOS so that NO synthesis decreases.^{32,33} Furthermore, the NO bioavailability decreases due to rapid scavenging by ROS.²⁸ In the present study, preeclampsia rats had an increased serum sFlt-1 level, reduced tail artery eNOS expression, and lower serum NO level, indicating a systemic maternal endothelial dysfunction. Preeclampsia rats also had a higher serum MDA level, and lower serum SOD activity showed oxidative stress. These results align with previous studies.^{34–36}

The balance of stimulatory and inhibitory substances in peripheral blood may also be important in controlling the number and function of circulating EPCs, which leads to endothelial homeostasis. Its disruption may play a role in the pathophysiology of preeclampsia via endothelial dysfunction.¹⁰ There are two forms of EPCs: circulating angiogenic cells (CACs, CD34⁺/CD309⁺/CD45⁺) and endothelial colony-forming cells (ECFCs, CD34⁺/CD309⁺/CD45⁻).¹¹ Circulating angiogenic cells release paracrine factors that promote ECFCs' migration, proliferation, and differentiation into mature endothelial cells at vessel formation or injuries.³⁷ There were higher percentages of CACs and ECFCs in preeclampsia rats in the present study. This condition might happen because preeclampsia causes maternal endothelial damage, which drives EPCs to mobilize from the bone marrow. However, the cells could not fulfill their normal function due to oxidative stress. A lower eNOS expression in tunica intima of the tail artery in preeclampsia rats indicated this

Endothelial progenitor cells play a role in reproductive organ neovascularization for implantation and pregnancy maintenance. A decrease in the quantity and function of EPCs may lead to inadequate endothelial cell regeneration in compromised uteroplacental circulation.^{38–40} As in the peripheral blood, the endothelial injuries in uteroplacental circulation may induce EPCs mobilization. However, the cells were dysfunctional due to oxidative stress, thereby not regenerating injured endothelial cells or promoting neovascularization. Thus, the placental eNOS expression was lower, and the placental HIF-1 α expression was higher in preeclampsia rats.

Physalis angulata leaf ethanol extract contains active compounds. They are trigonelline, stachydrine (alkaloid), chlorogenic acid (polyphenol), quercetin, kaempferol (flavonoid), and withanolide (steroidal lactone). Many studies have revealed that these compounds have antioxidant activity.^{41–47} Quercetin improves the angiogenic function of EPCs, i.e., migration, proliferation, differentiation, and survival, by increasing the SIRT1/AMPK/Akt/eNOS,⁴⁸ ERK/eNOS,⁴⁹ and PI3K/Akt/eNOS signaling pathways.^{49,50} Quercetin has also been shown to prevent the reduction of eNOS phosphorylation, intracellular NO, and cGMP production in EPCs.⁴⁸ *In vitro* study by Li *et al.* (2016) has revealed that withaferin A, a withanolide, dose-dependently increases VEGF secretion in endothelial cells, thereby increasing the proliferation and migration of endothelial cells.⁵¹ Many studies showed that stachydrine,⁵² chlorogenic acids,^{46,53,54} quercetin,^{55–57} and kaempferol⁵⁸ improve endothelial cell function by increasing the eNOS/NO signaling pathway. Trigonelline has also increased the Ca²⁺-dependent eNOS/NO signaling pathway.⁵⁹ In this study, the administration of *Physalis angulata* leaf ethanol extract in preeclampsia rats was proved to reduce oxidative stress and maintain NO bioavailability. It restored EPC function in improving the damaged endothelial cells. Thus, vascular signaling pathways and vascular function and growth are restored, placentation is improved, marked with decreased placental HIF-1 α expression and reduced production of the antiangiogenic factor sFlt-1. This condition leads to improved preeclampsia symptoms, i.e., reduced blood pressure and proteinuria.

Furthermore, various compounds in *Physalis angulata* leaf ethanol extract can help decrease blood pressure. Quercetin and kaempferol inhibit cGMP hydrolysis by the Ca²⁺-dependent phosphodiesterase.^{60,61} Kaempferol has been shown to reduce myosin light-chain kinase activity,⁶² inducing myosin light-chain dephosphorylation and triggering vasorelaxation.⁶³ Besides, chlorogenic acid and quercetin inhibit angiotensin-converting enzyme (ACE) in endothelial cells.⁶⁴ Chlorogenic acid⁶⁵ and quercetin^{66,67} have also been proven to improve endothelial-dependent vasodilation and reduce blood pressure in hypertensive subjects. The presumption mechanism of action of active compounds in *Physalis angulata* leaf extract in ameliorating the EPC and endothelial cell function is presented in Figure 8.

The novelty of this study is that the EPCs' involvement in preeclampsia pathogenesis has just been widely studied in the past decade. The effect of antihypertensive drugs on improving the number and function of EPCs in preeclampsia patients was also recently published by Wang *et al.* in 2019.⁶⁸ In addition, studies of the benefits of herbs in preeclampsia are still focused on preventive models. Until now, no studies have proven the roles of herbs in improving EPCs in preeclampsia models. So, this is the first study.

The study has limitations that need to be considered while interpreting the results. Although prolonged NO suppression by L-NAME in pregnant rats leads to many clinical manifestations of preeclampsia, this is not a physiologically accurate representation of the pathogenesis of human preeclampsia. However, this approach emphasizes the significance of NO in pregnancy.⁶⁹ Furthermore, the development of animal models that mimic the pathogenesis of preeclampsia in humans still needs to be continued to discover treatments for preeclampsia.

Conclusions

In conclusion, we suggest that the ameliorative effects of *Physalis angulata* leaf ethanol extract on preeclampsia symptoms may be achieved by improving EPC and endothelial cell function due to reduced antiangiogenic factor and oxidative stress and also restored eNOS/NO signaling. As a result, the extract has the potential to be used as a treatment for preeclampsia. Furthermore, our data highlight the significance of EPCs as the target for biological therapy for preeclampsia.

Data availability

Underlying data

Figshare: Data for Journal F1000: *Physalis angulata* Leaf Extract Ameliorates L-NAME-induced Preeclampsia Symptoms in Rats through Improved Endothelial Progenitor Cells and Endothelial Cells due to Reduced Antiangiogenic Factor and Oxidative Stress. <https://doi.org/10.6084/m9.figshare.20057093>.¹⁹

This project contains the following underlying data:

- Data LC-HRMS 1 (Best Match).xlsx (mzCloud best match compounds identified by LC-HRMS)

- Data LC-HRMS 2 (Best Similarity Match).xlsx (mzCloud best similarity match compounds identified by LC-HRMS)
- Serial Data Systole-Diastole-Protein Urine.xlsx (Serial data of systole and diastole blood pressures on G7, G12, G15, G19, and also 24-h total urine protein levels on G12 and G18)
- All Data Gestational Day 19.xlsx (Raw data on s-Flt1, MDA, SOD, NO, eNOS, HIF-1 α , CAC, and ECFC)
- Raw images_Placental eNOS expression.zip (Raw unedited microscope images of placental eNOS expression-200x magnification)
- Raw images_Placental HIF-1 α expression.zip (Raw unedited microscope images of placental HIF-1 α expression-200x magnification)
- Raw images_Tail artery eNOS expression.zip (Raw unedited microscope images of tail artery eNOS expression-200x magnification)

Data are available under the terms of the [Creative Commons Attribution 4.0 International license](#) (CC-BY 4.0).

Reporting guidelines

Figshare: ARRIVE checklist for 'Physalis angulata leaf extract ameliorates L-NAME-induced preeclampsia symptoms in rats through improved endothelial progenitor cells and endothelial cells due to reduced antiangiogenic factor and oxidative stress'. <https://doi.org/10.6084/m9.figshare.20057093>.¹⁹

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Natasha De Alwis

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This study by Nugrahenny et al. assesses the ability of *Physalis angulata* leaf extract to ameliorate a preeclampsia-like phenotype induced by administration of L-NAME. Overall this paper was well-written; the methods were appropriately detailed and figures descriptive. However some of the conclusions made are not supported completely by the results – please see my comments below.

Major Comments:

1. The aim of the study was to assess whether the leaf extract could alleviate the preeclampsia phenotype by “by improving EPC and endothelial cell function”. However EPC function was not specifically assessed. Similarly mentioned in the discussion: “It restored EPC function in improving the damaged endothelial cells.” While I think the authors can certainly speculate that improvement of EPC/endothelial cell function may have been the mechanism by which the leaf extract worked, they did not test whether EPCs were specifically responsible for this in functional studies. This should be amended throughout the manuscript.
2. In the discussion, the authors state: “vascular function and growth are restored”. However, I don’t think improvement in vascular growth was proven. Also while anti-angiogenic factors were reduced, angiogenic factors like VEGF were not measured. As such, it should be clear that this is speculative.
3. Further, the discussion states: “placentation is improved” – however there is no study of placental structure or size, nor is there any data for fetal outcomes to show whether placental growth and function might have been altered. This is important data in a pregnant animal model and should be included, particularly when assessing a candidate therapeutic, as an important aspect of treatment should be safety for the developing fetus.

Minor comments:

1. Introduction: “Preeclampsia is one of the most severe hypertension conditions during pregnancy” – change hypertension to hypertensive
2. Introduction: “Preeclampsia also dramatically raises the cost of healthcare services.” – This is

too vague – please describe what this means.

3. Methods: Why was L-NAME dose reduced later in gestation? Please detail this in method.
4. Methods: Statistical analysis states “Free alternatives such as SOFA or JASP Statistics could be used” – does this mean that the authors used these programs? If not I would exclude.
5. Methods: In statistical analysis, it is unclear what “Before we looked at the data, we had determined the analytical stages” means. Does this mean that the method of statistical analysis had been determined before deciding the method? It is unclear then whether the authors had considered the assumptions of their ANOVA tests, i.e. whether the data was normally distributed and equal variance. Please include this information.
6. Results: Figure 1 – include an annotation showing where the treatment was started.
7. Results: In results text, please write if D1-D3 was not significantly different to the sham group.
8. Results: SOD data – please split the y-axis so that the differences between the groups can be visualised easily.
9. Results: Fig 5 – “The serum NO level was determined by colorimetry.” – Would change this to “colorimetric assay”.
10. Results: Fig 7 legend – include that it was measured via flow cytometry.
11. Discussion: The leaf extract contained around 50 different compounds but the discussion focused on just 6 active compounds. What about the other compounds? Could they have an effect as well? This should be discussed.
12. Discussion: Please include what the next steps in this research are if being considered a potential treatment for preeclampsia. This study focused on oxidative stress and nitric oxide pathways. Would you next perhaps look at the effect of leaf extract on the systemic inflammation associated with preeclampsia?

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: preeclampsia, pregnancy, rodent models

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 22 September 2022

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Eighty Mardiyani Kurniawati 

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Abstract

- The objectives have just been written, not yet briefly described the reasons why this research needs to be done- The method is too long, and can be summarized.
- In the results section, there are no statistical test results so it needs to be added
- Recommendations need to be added to the conclusion, especially the novelty of this study

Introduction

- What is the prevalence of pre-eclampsia? If discussing preeclampsia in Indonesia, please add data on preeclampsia in Indonesia
- It is necessary to add the causes of preeclampsia in addition to directing the pathophysiology towards endothelial dysfunction
- The role of EPC in preeclampsia is incomplete
- Why is it necessary to go natural, does medical administration of drugs not work?
- Please add to the previous research using Physalis leaf extract in the field of obstetrics.
- Why is it aimed at the plant Physalis leaf extract? Aren't there any other herbs to consider?
- Why was this research conducted on animals, especially rats? Do previous studies recommend doing it on animals?
- What are the benefits of this research going forward?

Discussion

- The arrangement of the discussion needs to be corrected, starting with the results of the

research in the first sentence, followed by a comparison with other studies. Do not directly mention other sources. Add other sources related to how alternative medicine such as other plants can help treat preeclampsia.

- If it can't be identified in humans, what steps can be taken and what are the obstacles why this research can't be done on humans?
- What is the relevance while in your conclusion you state "as a result, the extract has the potential to be used as a treatment for preeclampsia".? Preeclampsia for animals?
- If for humans, it is necessary to add management problems in human preeclampsia.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: obstetrics and gynecology, urogynecology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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