



OPEN

Cleistocalyx nervosum var. *paniala* mitigates oxidative stress and inflammation induced by PM₁₀ soluble extract in trophoblast cells via miR-146a-5p

Wittaya Chaiwangyen^{1,6}, Orawan Khantamat^{2,6}, Komsak Pintha¹, Napapan Kangwan³, Amnart Onsa-ard¹, Piyawan Nuntaboon¹, Angkana Songkroo¹, Pilaiporn Thippraphan², Dana Chaiyasit⁴ & Francisco Lázaro Pereira de Sousa⁵

Air pollution poses a significant global concern, notably impacting pregnancy outcomes through mechanisms such as DNA damage, oxidative stress, inflammation, and altered miRNA expression, all of which can adversely affect trophoblast functions. *Cleistocalyx nervosum* var. *paniala*, known for its abundance of anthocyanins with diverse biological activities including anti-mutagenic, antioxidant, and anti-inflammatory properties, is the focus of this study examining its effect on Particulate Matter 10 (PM₁₀) soluble extract-induced trophoblast cell dysfunction via miRNA expression. The study involved the extraction of *C. nervosum* fruit using 70% ethanol, followed by fractionation with hexane, dichloromethane, and ethyl acetate. Subsequent testing for total phenolics, flavonoids, anthocyanins, and antioxidant activity revealed the ethyl acetate fraction (CN-EtOAcF) as possessing the highest phenolic and anthocyanin content along with potent antioxidant activity, prompting its selection for further investigation. In vitro studies on HTR-8/SVneo cells demonstrated that 5–10 µg/mL PM₁₀ soluble extract exposure inhibited cell proliferation, migration, invasion, and induced apoptosis. However, pretreatment with 20–80 µg/mL CN-EtOAcF followed by 5 µg/mL PM₁₀ soluble extract exposure exhibited protective effects against PM₁₀ soluble extract-induced damage, including inflammation inhibition and intracellular ROS suppression. Notably, CN-EtOAcF down-regulated PM₁₀-induced miR-146a-5p expression, with SOX5 identified as a potential target. Overall, CN-EtOAcF demonstrated the potential to protect against PM₁₀-induced harm in trophoblast cells, suggesting its possible application in future therapeutic approaches.

Keywords PM₁₀, *Cleistocalyx nervosum* var. *paniala*, MiR-146a-5p, Trophoblast, Pregnancy, Oxidative stress, SOX5

During the implantation process, the effective penetration of the maternal decidua and myometrium by extravillous trophoblast (EVT) is vital for a successful pregnancy as it facilitates the modification of maternal spiral arteries. This modification results in the widening of these arteries, enhancing the provision of nutrients and facilitating gas exchange for fetal development. There have been reports of a connection between deficient trophoblast invasion and pregnancy complications such as preterm birth, preeclampsia, fetal growth restriction, and spontaneous abortion. The defected trophoblast functions and pregnancy disorders are also associated with environmental pollutants, especially particulate matter (PM) air pollution^{1,2}.

Environmental air pollution, specifically fine PM with diameter <2.5 µm (PM_{2.5}) and coarse PM with diameter <10 µm (PM₁₀), is recognized as having significant adverse effects on maternal and neonatal health³.

¹Division of Biochemistry, School of Medical Sciences, University of Phayao, Phayao 56000, Thailand. ²Department of Biochemistry, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand. ³Division of Physiology, School of Medical Sciences, University of Phayao, Phayao 56000, Thailand. ⁴Clinical Chemistry Laboratory, Chiang Rai Prachanukroh Hospital, Chiang Rai 57000, Thailand. ⁵Department of Gynecology and Obstetrics, UNILUS (Centro Universitário Lusíada), Santos 11050-071, Brazil. ⁶Wittaya Chaiwangyen and Orawan Khantamat have contributed equally to this work. ✉email: wittaya.ch@up.ac.th

Several studies have revealed that PM_{2.5} and PM₁₀ have the capability to breach the lung barrier and access the blood circulation⁴, triggering oxidative stress and inflammation, which in turn elicit systemic responses and contribute to various diseases^{5,6}. PM_{2.5} particles can penetrate deeply into the respiratory system and enter the bloodstream, affecting various organs including the placenta, indicating systemic exposure^{7–11}. Conversely, the systemic translocation of PM₁₀, typically remain in the upper respiratory tract¹², is less well documented. Studies have shown that both PM_{2.5} and PM₁₀ soluble extracts contain similar chemical constituents, including elements and polycyclic aromatic hydrocarbons (PAHs)^{13–15}. This chemical similarity justifies the use of PM₁₀ soluble extracts in the study, which aims to explore trophoblast cell functions through miR-146a-5p expression associated with PM₁₀.

In animal models, exposure to PM_{2.5} has been shown to decrease fetal weight and crown-rump length in mice, along with impairments in placental trophoblast syncytialization¹⁶. Airborne PM_{2.5} exposure has also been linked to disruptions in trophoblast cellular processes, resulting in growth inhibition, inflammation, endoplasmic reticulum stress, and impaired migration and invasion abilities^{2,17}, potentially leading to pregnancy complications. Previous research conducted by our team revealed that exposure to PM₁₀ suppressed the proliferation, migration, and invasion of trophoblast HTR-8/SVneo cells. Furthermore, PM₁₀ exposure induced inflammation in trophoblast cells by upregulating the expression levels of proinflammatory cytokines such as IL-1 β , IL-6, and TNF- α ¹⁸.

Lately, an increasing number of studies have been investigating the relationship between exposure to PM and the expression of miRNAs. This interest stems from the fact that miRNAs have a substantial impact on a range of cellular processes such as cell differentiation, proliferation, migration, invasion, death, and development¹⁹. miRNA, a small non-coding RNA consisting of 21–23 nucleotides, specifically binds to sequences within the 3' UTR of its target mRNAs, resulting in the suppression of gene expression either by inhibiting translation or promoting mRNA degradation²⁰. Throughout pregnancy, miRNAs oversee the regulation of trophoblast cell functions, and any disruption in miRNA expression can result in trophoblast dysfunction, potentially leading to pregnancy complications^{21–23}. Numerous circulating miRNAs were differentially expressed upon exposure to PM including miR-146a-5p^{24–26}.

miR-146a-5p is recognized as a tumor suppressor in various types of cancer, with the ability to inhibit cancer cell proliferation, migration, invasion, and epithelial-mesenchymal transition by targeting SOX5²⁷. Furthermore, miR-146a-5p plays a crucial role in modulating the inflammatory response²⁸. An increase in miR-146a-5p expression in preeclampsia placenta has been documented, with its role in inhibiting trophoblast cell invasion, proliferation, and migration through the regulation of Wnt2 expression²⁹. It has been reported that exosomal miR-146a-5p originating from human umbilical cord mesenchymal stem cells can mitigate trophoblast injury and placental dysfunction. This occurs through the regulation of the TRAF6/NF- κ B axis, leading to the suppression of IL-1 β and IL-18, thereby reducing inflammation³⁰. Despite the insights provided by these studies, the relationship between miR-146a-5p expression in trophoblast cells remains unclear.

Cleistocalyx nervosum var. *paniala* (Ma Kiang), a member of the Myrtaceae family, is a native berry of Thailand. Its dark purple ripe fruit has been shown to contain a significant amount of anthocyanins and to possess various biological activities, including anti-mutagenic, anti-aging, anti-carcinogenic, antioxidant, and anti-inflammatory properties^{31–33}. The *C. nervosum* fruit extract (CNE) has been found to notably inhibit IL-1 β -induced inflammation through the inhibition of the production of inflammatory molecules, such as IL-6, MCP-1, and IL-8, in human retinal pigment epithelial cells³³. Given the biological properties of *C. nervosum* fruit and the association between stress-inducing agent, PM₁₀ soluble extract exposure and miR-146a-5p expression in trophoblast cells as discussed above, our objective is to explore the protective properties of CNE against PM₁₀ soluble extract-induced oxidative stress, inflammation, and trophoblast cell dysfunction by assessing miR-146a-5p expression.

Materials and methods

Plant material collection

Cleistocalyx nervosum fruits were collected in August 2022 in Phayao province, Thailand (a latitude of 19° 04' 21.68' N and a longitude of 99° 53' 3.58' E). A voucher specimen (Code: QBG No. 14630-32) of the plant was acquired and deposited at the Herbarium of the Queen Sirikit Botanic Garden in Chiang Mai, Thailand. The ripe fruit pulp of *C. nervosum* was separated from the seeds and subjected to a 96 h drying process at 40 °C. The resulting dried fruit pulp was finely ground into a powder and stored at –20 °C for future experiments.

Extraction of plant material³⁴

The preparation of the *C. nervosum* fruit extract (CNE) was prepared as follows: the dried powder was initially extracted using 70% ethanol, and the resulting dried crude extract was then fractionated through hexane, dichloromethane, and ethyl acetate. The solvent was eliminated via a rotary evaporator. The water fraction that remained was obtained through the process of freeze-drying. The extracts obtained were weighed for yield assessment and subsequently stored at –20 °C for future analysis. The percentage yield was calculated using the following formula:

$$\% \text{ yield} = [\text{Weight of extract (g)} / \text{Weight of dried material (g)}] \times 100$$

Phytochemical determination

Total phenolic content (TPC)

The TPC in the extracts was assessed using the Folin–Ciocalteu reagent. Concisely, either diluted samples or a standard (Gallic acid) were combined with a solution of Folin–Ciocalteu and sodium carbonate. After 30 min

incubation, the absorbance of the reaction was measured at 765 nm. TPC was quantified in milligrams of gallic acid equivalents per gram of plant dry weight (mg GAE/g extract).

Total flavonoid content (TFC)

The TFC in the extracts was assessed through a colorimetric method using aluminum chloride. Briefly, either diluted samples or a standard (Catechin) were combined with a sodium nitrite solution and allowed to incubate for 5 min at room temperature. Following incubation, an aluminum chloride solution and sodium hydroxide were introduced into the mixture. The absorbance of the resulting reaction was detected after a 10 min incubation period at 510 nm. TFC was measured in milligrams of catechin equivalents per gram of plant dry weight (mg CE/g extract).

Determination of total anthocyanins by pH differential spectroscopic method³⁵

The method for determining total anthocyanin content, as established by Connor et al. in 2002^[61]. In this method, each extract was diluted in a solution of 1% HCl in methanol to achieve an absorbance falling within the range of 0.500 to 1.000 at 530 nm. Briefly, 0.25 mL of the extracts were mixed with one mL of two distinct solutions: one with a pH of 1.0 containing 0.025 M potassium chloride, and the other with a pH of 4.5 containing 0.4 M sodium acetate. Following a 30 min incubation at room temperature, absorbance was measured at 520 and 700 nm. The results were presented as milligrams of cyanidin-3-glucoside (c3g) equivalents per 100 g of fresh weight, using a molar extinction coefficient of 27,900. All measurements were performed in triplicate. The results were computed as follows:

$$A = (A_{520} - A_{700})_{\text{pH } 10.0} - (A_{520} - A_{700})_{\text{pH } 4.5}$$

The total anthocyanin content was calculated as follows:

$$\text{Total anthocyanin content} = (A \times \text{MW} \times \text{DF} \times 1000) / (\epsilon \times \lambda \times m),$$

In this context, MW denotes molecular weight of cyaniding-3-glucoside, ϵ represents molar extinction coefficient of 27,900, DF represents the dilution factor, λ represents the optical path length of the cuvette (1 cm) and m represents the weight of the sample (in g). The total anthocyanin content was indicated as mg of anthocyanin per g of extract.

High-performance liquid chromatography (HPLC)

HPLC analysis was carried out using a Shimadzu HPLC system integrated with a UV-VIS diode array detector (LC-20 A, Shimadzu, Japan) for quantification at 280 nm. The column utilizing was a C-18 HPLC column (Allure-C18, Restek, Bellefonte, PA, USA). Maintain column temperature at 30 °C during analysis. Prepared samples were injected at a volume of 10 μ L. Solvent A presented of 0.1%TFA in water, where solvent B comprised 0.1% Trifluoroacetic acid (TFA) in methanol. The solvent gradient applied to all samples and reference standards (Cyanidin-3,5-diglucoside, Cyanidin-3-O-glucoside chloride, Cyanidin-3-O-rutinoside chloride, Pelargonidin-3-O-glucoside chloride, and Peonidin-3-O-glucoside chloride) followed this pattern: 0 to 40 min with a flow rate at 1.0 mL/min. The anthocyanin content was assessed using the external standard method, based on a comparison of their retention times with pure standards³⁶.

Antioxidant determinations

DPPH radical scavenging activity

The ability of the CNE to act as hydrogen atoms or electron donors was assessed by observing the lowering of the steady 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical. Various concentration of the CNE or standard Trolox were introduced into a DPPH solution. Following a 30 min incubation period in darkness at room temperature, the absorbance was measured at 515 nm. The percentage of DPPH radical scavenging activity was assessed as the follows:

$$\text{DPPH radical scavenging activity (\%)} = [(A_0 - A_s)/A_0] \times 100$$

where A_0 represents the absorbance of the control and A_s represents the absorbance of the samples. The IC_{50} values were determined by plotting the percentage of inhibition against the concentration of the extracts.

ABTS radical scavenging activity

The assessment of free-radical-scavenging activity was conducted using 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) ABTS radical cation ($\text{ABTS}^{+\cdot}$) decolorization assay (Nile and Park, 2014c)^[62]. $\text{ABTS}^{+\cdot}$ was generated by mixing 7.5 mM ABTS stock solution with 2.45 mM potassium persulfate, allowing it to remain in the dark at room temperature for 12–16 h before utilization. Prior to the investigation, the $\text{ABTS}^{+\cdot}$ mixture was diluted with deionized water to achieve an absorbance value of 0.700 ± 0.02 at 734 nm. The absorbance of the extracts or standard Trolox in the $\text{ABTS}^{+\cdot}$ solution was measured at 734 nm after 6 min of addition. The following formula was used to calculate the percentage of ABTS radical scavenging activity:

$$\text{ABTS radical scavenging activity (\%)} = [1 - A_s/A_0] \times 100$$

where A_0 and A_s correspond to the absorbance of control and the samples, respectively. The IC_{50} values were calculated by graphing the inhibition percentage against the extract concentration.

Ferric reducing antioxidant power (FRAP) assay

The test was developed to assess the ability of an antioxidant compound to turn ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}). This conversion leads to the formation of a blue complex (Fe^{2+} /TPTZ). Briefly, the sample and the FRAP reagent were combined, and the absorbance was obtained at 593 nm following a 30 min incubation period. The data was represented in μM equivalents of ferrous ion per gram of the extract³⁷.

PM₁₀ sample collection

PM₁₀ samples were gathered at the University of Phayao in Phayao, Thailand, during the period of January to March in 2020, which was characterized by a significant occurrence of forest fires. These samples were obtained using 20.3 × 25.4 cm quartz-fiber filters (Toyo Roshi Kaisha, Ltd., Tokyo, Japan) with an Ecotech Model 3000 PM₁₀ high-volume air sampler (Ecotech Pty. Ltd., Melbourne, Australia). The filters were collected and stored at $-20\text{ }^{\circ}\text{C}$ for further use.

PM₁₀ extraction

The filtered samples were divided into small pieces and separated for 15 min in an ultrasonic bath with a 1:1 combination of hexane and dichloromethane. The resulting substance was then filtered using a 0.45 μm Polytetrafluoroethylene (PTFE) filter for removing insoluble substances. The extracted PM₁₀ was subsequently dried with a rotary evaporator before being stored at $-20\text{ }^{\circ}\text{C}$ for investigation¹⁸.

Particle size and elemental analysis of particulate matter

The size of the particles and chemical content were analyzed at the University of Phayao's central laboratory in Phayao, Thailand, using scanning electron microscopy (SEM) (FEI Quanta FEG 250, FEI Company, WA, USA) and an integrated energy-dispersive X-ray system (EDX) (Oxford INCA X-Act, Oxford Instruments). Following SEM determination, filter samples measuring 1 mm × 1 mm were obtained from the middle portion of the filter. A gold sputter coater with a vacuum coating system (Quorum SC7620, East Sussex, UK) was used for coating a thin film of gold (Au) to the sample surface. The micrographs of particulate matter were analyzed using xT microscope control software. To determine the precise chemical composition of PM₁₀, EDX spectra were acquired, and the weight was determined percentage of every compound contained in the spectrum. Fifteen elements were identified by SEM-EDX, including Oxygen (O), Carbon (C), Silicon (Si), Potassium (K), Aluminum (Al), Zinc (Zn), Sulfur (S), Chloride (Cl), Copper (Cu), Magnesium (Mg), Calcium (Ca), Iron (Fe), Nickel (Ni), Sodium (Na), and Nitrogen (N)¹⁸.

Cell line and treatment

The trophoblast cell line, HTR-8/SVneo (RRID: CVCL_7162), was generously provided by Prof. Charles H. Graham from Kingston, Canada. These cells were grown in RPMI medium (Thermo Fisher Scientific, Dreieich, Germany), supplemented with 1% penicillin/streptomycin (Thermo Fisher Scientific, Dreieich, Germany) and 10% heat-activated FBS (Sigma-Aldrich, Darmstadt, Germany) at $37\text{ }^{\circ}\text{C}$ with 5% CO_2 . Cells were subjected to different treatments and divided into six groups: control, 5 $\mu\text{g/mL}$ PM₁₀ soluble extract, 20 $\mu\text{g/mL}$ CN-EtOAcF, 80 $\mu\text{g/mL}$ CN-EtOAcF, pre-treatment for 24 h with 20 $\mu\text{g/mL}$ CN-EtOAcF followed by co-treatment for 24 h with PM₁₀ soluble extract, and pre-treatment for 24 h with 80 $\mu\text{g/mL}$ CN-EtOAcF followed by co-treatment for 24 h with PM₁₀ soluble extract (Fig. 1). After incubation, cells were harvested and analyzed for cell proliferation at 24, 48, and 72 h, as well as for migration, invasion, apoptosis, miR-146a-5p expression, and its potential targets.

Cytotoxicity assay

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay (Sigma-Aldrich, Darmstadt, Germany). A density of 5×10^3 HTR-8/SVneo cells/well was seeded in a 96-well plate. The cells were exposed to PM₁₀ soluble extract or CNE (ethyl acetate fraction; CN-EtOAcF) for 24–72 h, prior to 4 h of MTT solution (100 μL) at $37\text{ }^{\circ}\text{C}$. The formazan crystals were dissolved using an equal volume of dimethyl sulfoxide (DMSO), and the absorbance at 570 nm was measured with a microplate reader.

Matrigel invasion and transwell migration assay

Cell invasion and migration were evaluated using hanging cell culture inserts with a pore size of 8 μm , placed in a 24-well plate (Sigma-Aldrich, Darmstadt, Germany). HTR-8/SVneo cells (1×10^5) were pretreated with CN-EtOAcF and then challenged with PM₁₀ soluble extract in serum-free medium. These cells were subjected to Matrigel pre-coated membranes (Corning, AZ, USA) for the invasion experiment or, alternatively, Transwell uncoated inserts for the migration assay. To act as a chemoattractant, 20% FBS was added to medium in the insert's lower compartment. The cells that invaded the lower part of the membrane were preserved with cold ethanol, followed by staining with crystal violet, and their absorbance was measured at 570 nm after being discolored with acetic acid.

Proliferation assay

The cell proliferation ELISA, BrdU (Sigma-Aldrich, Darmstadt, Germany) was utilized to quantify cell proliferation. HTR-8/SVneo cells (5×10^3 cells/well) were seeded in a 96-well plate and treated to CN-EtOAcF and PM₁₀ soluble extract at various concentrations for 24–72 h. Following this exposure, BrdU-containing medium was introduced for an additional 2 h to allow for the incorporation of BrdU into the cells. The BrdU-incorporated cells were subsequently fixed and subjected to incubation with a monoclonal anti-BrdU antibody

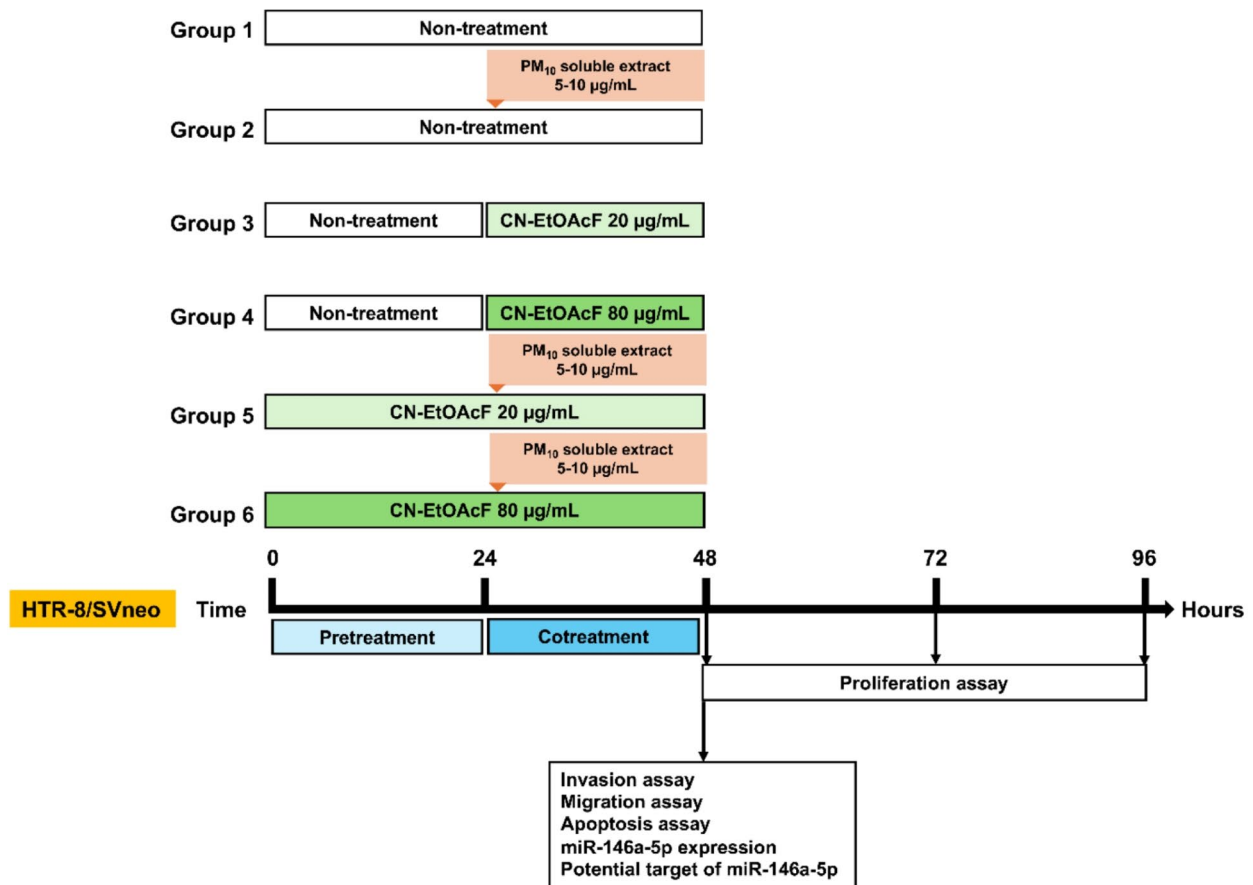


Fig. 1. A schematic diagram of HTR-8/SVneo cell exposure schedule.

conjugated with peroxidase. After washing, the cells underwent incubation with a substrate and the process was completed with 1 M H₂SO₄. Absorbance was evaluated at 450/690 nm using a microplate reader.

Intracellular ROS assay

To assess the inhibition of intracellular reactive oxygen species (ROS) linked to *C. nervosum* extract, we employed the fluorometric intracellular ROS kit (MAK143; Sigma, St. Louis, MO, USA) following the manufacturer's instructions. Initially, cells (5×10^3 cells/well) were seeded in a black 96-well plate and maintained overnight. Subsequently, the cells were treated with CN-EtOAcF. Following a 24 h incubation, 100 µM H₂O₂ was added to induce oxidative stress for 1 h. Then, the Master Reaction Mix was introduced into the treated cells, and the intensity of the fluorescence was detected using a fluorescence microplate reader with an excitation/emission wavelength of 490/525 nm.

Apoptosis assay

Apoptosis was evaluated utilizing the Annexin V-FITC Apoptosis Detection Kit (BD, New Jersey, USA) following the manufacturer's instructions. Cells underwent pre-treatment with CN-EtOAcF for 24 h and, followed by co-treatment with PM₁₀ soluble extract for an additional 24 h. Subsequently, cells (1×10^6 cells/mL) were resuspended in 1X annexin-binding buffer. Annexin V and PI were added to cell suspensions and incubated for 15 min in the dark. Analysis of apoptotic cells was conducted using flow cytometry (BD FACSLyric™, Becton Dickinson, Waltham, MA, USA).

RNA isolation

Total RNA was isolated from cells subjected to either pre-treatment with CN-EtOAcF and PM₁₀ soluble extract co-treatment or from untreated cells utilizing Trizol reagent (Invitrogen, Darmstadt, Germany) in accordance with the manufacturer's instructions. The concentrations of total RNA were quantified using a NanoDrop spectrophotometer (PqLab Biotechnologies GmbH, Erlangen, Germany), and samples exhibiting an A260/A280 ratio exceeding 1.8 were preserved at -80 °C for future use.

Quantification of miR146a-5p expression by qRT-PCR

The qRT-PCR experiment was carried out using QIAquant™ 96 (Qiagen, Düsseldorf, Germany) following reverse transcription into cDNA with the miRCURY LNA Reverse Transcription Kit (Düsseldorf, Germany) following the manufacturer's protocol. Subsequently, miRCURY LNA SYBR Green PCR kit was performed

Primers	Forward (5'-3')	Reverse (5'-3')
IL-1β	GCACAGTTCCCCAACTGGTA	AAGACACGGGTTCATGGTG
IL-6	AGACAGCCACTCACCTCTTCAG	TTCTGCCAGTGCCTCTTTGCTG
TNF-α	CCCAGGCAGTCAGATCATCTTC	AGCTGCCCTCAGCTTGA
SOX5	CAGCCAGAGTTAGCACAATAGG	CTGTTGTTCCCGTCGGAGTT
GAPDH	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC

Table 1. List of primers.

Fraction	Weight (g)	% Yield
Ethanol crude extract (ECE)	63.0	31.5
Hexane fraction (CN-HEXF)	0.40	0.6
Dichloromethane fraction (CN-DCMF)	0.17	0.3
Ethyl acetate fraction (CN-EtOAcF)	2.35	3.7
Water fraction (CN-WTF)	54.50	86.5

Table 2. Extraction and partitioning yields of CNE.

for miRNA analysis. Expression of miR-146a-5p (YP0020/NR_002745) (Qiagen, Düsseldorf, Germany) was calculated using the $2^{-\Delta\Delta C_t}$ method, using SNORD48 SNORD48 (YP0020/NR_002745) (Qiagen, Düsseldorf, Germany) as a reference.

Target of miRNA prediction

miRNA targets prediction was conducted using a miRNA target prediction database including TargetScan RRID: SCR_010845 (http://www.targetscan.org/vert_71/), PicTar RRID: SCR_003343 (<https://pictar.mdc-berlin.de>), and miRDB RRID: SCR_010848 (<http://mirdb.org>).

Quantitative RT-PCR (qRT-PCR)

After converting RNA into cDNA using RevertAid RT Reverse Transcription Kit from Thermo Fisher Scientific, Waltham, MA, USA, the expression levels of IL-1β, IL-6, TNF-α, and SOX5 were analyzed with the Maxima SYBR Green qPCR Master Mix from Thermo Fisher Scientific, Waltham, MA, USA, using the QIAquant™ 96 system (Qiagen, Düsseldorf, Germany). Gene expression was quantified the $2^{-\Delta\Delta C_t}$ method and normalized to GAPDH as a reference. The primers used in this study are shown in Table 1.

Statistical analysis

Data from three different experiments were presented as mean ± SD. Statistical analysis was performed using one-way analysis of variance (ANOVA), with significance determined at a *P*-value < 0.05. All statistical assessments were carried out using GraphPad Prism version 8.0, RRID: SCR_002798 (GraphPad Software, CA, USA).

Results

Extraction yield and phytochemical determination

The percentage of the yield for the crude extract was calculated relative to the weight of dried *C. nervosum*, while the yield for the partitioned fraction was determined based on the weight of the initial crude ethanolic extract used³⁸. In this study, we subjected 200 g of *C. nervosum* to a 24 h extraction process using 70% ethanol, resulting in the production of an ethanolic crude extract (ECE) with a yield of 31.5%. The ECE was subsequently subjected to liquid-liquid partitioning using hexane (CN-HEXF), dichloromethane (CN-DCMF), ethyl acetate (CN-EtOAcF), and followed by a water (CN-WTF) extraction, resulting in yields of 0.64%, 0.27%, 3.73%, and 86.51%, respectively (Table 2). Notably, CN-WTF exhibited the highest yield among the partitioned fractions.

Next, we conducted an analysis of the total phenolic content (TPC), total flavonoid content (TFC), and anthocyanin content in the CNE. Our results indicated that CN-EtOAcF had the highest TPC (53.1 ± 0.48 mg GAE/g extract), while the lowest TPC was found in CN-WTF (12.70 ± 0.1 mg GAE/g extract). CN-DCMF exhibited the highest total flavonoid content (3.1 ± 0.84 mg CE/g extract), and CN-EtOAcF and CN-WTF had the lowest TFC, with values of 0.05 ± 0.3 and 0.05 ± 0.2 mg CE/g extract, respectively. Additionally, as shown in Table 3, the highest amount of anthocyanins, 29.5 ± 3.22 mg/g extract, was found in ECE, whereas the lowest amount, 0.03 ± 0.1 mg/g extract, was observed in CN-HEXF. In the present study, five anthocyanin standards analyzed through HPLC at 520 nm revealed retention times (RT) including Cyanidin-3,5-diglucoside (Peak 1, RT = 16.043 min), Cyanidin-3-O-glucoside chloride (Peak 2, RT = 18.466 min), Cyanidin-3-O-rutinoside chloride (Peak 3, RT = 18.933, Pelargonidin-3-O-glucoside chloride (Peak 4, RT = 19.374), and Peonidin-3-O-glucoside chloride (Peak 5, RT = 19.821) (Fig. 2A). The identification and quantification of anthocyanins in the extracts were determined by measuring the retention times and area under the peaks obtained from HPLC. Three anthocyanins were detected in ECE (Fig. 2B), whereas CN-EtOAcF (Fig. 2C) and CN-WTF (Fig. 2D) contained four anthocyanidins. None of anthocyanin was detected in CN-HEXF and CN-DCMF. The main anthocyanin in CN-EtOAcF, ECE, and CN-WTF was Cyanidin-3-O-rutinoside chloride, with concentrations of 79.90, 80.58,

Fraction	TPC (mg GAE/g extract)	TFC (mg CE/g extract)	Anthocyanins (mg/g extract)
Ethanol crude extract (ECE)	18.70 ± 0.4	0.54 ± 0.3	29.52 ± 3.2
Hexane fraction (CN-HEXF)	32.87 ± 0.4	2.87 ± 1.4	0.03 ± 0.1
Dichloromethane fraction (CN-DCMF)	34.66 ± 0.7	3.06 ± 0.8	0.79 ± 0.1
Ethyl acetate fraction (CN-EtOAcF)	53.13 ± 0.5	0.05 ± 0.3	35.04 ± 0.4
Water fraction (CN-WTF)	12.70 ± 0.1	0.05 ± 0.2	24.37 ± 2.0

Table 3. Total phenolics, total flavonoid and anthocyanins content.

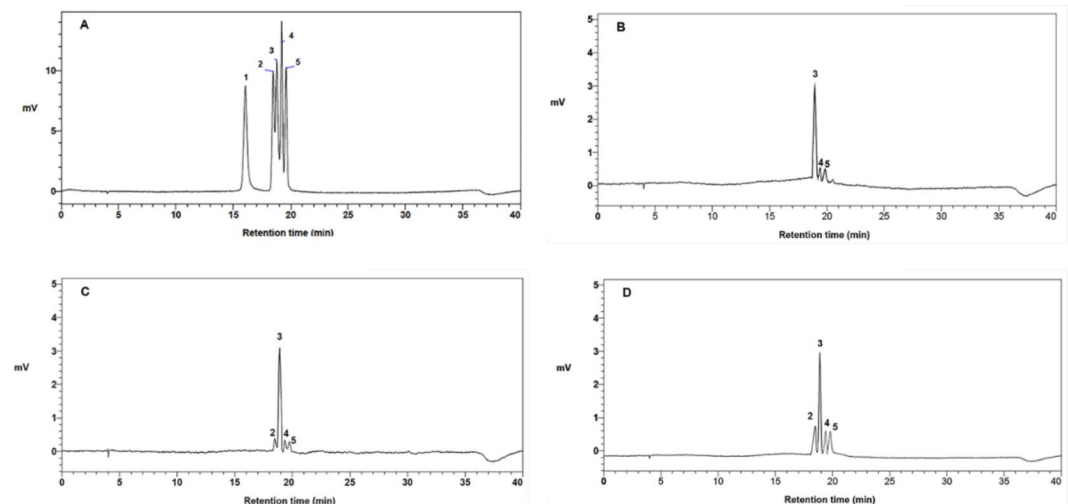


Fig. 2. HPLC chromatograms of standard anthocyanins (A) and analyzed samples of ECE (B), CN-EtOAcF (C) and CN-WTF (D). Peak 1 represents Cyanidin-3,5-diglucoside, Peak 2 represents Cyanidin-3-O-glucoside chloride, Peak 3 represents Cyanidin-3-O-rutinoside chloride, Peak 4 represents Pelargonidin-3-O-glucoside chloride and Peak 5 represents Peonidin-3-O-glucoside chloride.

Anthocyanins	Retention time (min)	Amount of anthocyanins (mg/100 g)				
		ECE	CN-HEXF	CN-DCMF	CN-EtOAcF	CN-WTF
1. Cyanidin-3,5-diglucoside	16.043	nd	nd	nd	nd	nd
2. Cyanidin-3-O-glucoside chloride	18.466	nd	nd	nd	16.44	14.94
3. Cyanidin-3-O-rutinoside chloride	18.933	80.58	nd	nd	79.90	85.34
4. Pelargonidin-3-O-glucoside chloride	19.374	7.42	nd	nd	12.47	7.71
5. Peonidin-3-O-glucoside chloride	19.821	8.77	nd	nd	12.30	9.78
Total anthocyanins		96.77	–	–	121.11	117.77

Table 4. Individual and total anthocyanin content in *C. Nervosum* extracts. *nd not detectable.

and 85.34 mg/100 g extract, respectively (Table 4). Total anthocyanin content was lowest in ECE, with a value of 96.77 mg/100 g extract, while CN-EtOAcF had the highest total anthocyanin content at 121.11 mg/100 g extract. These results indicate that CN-EtOAcF demonstrated the highest total anthocyanin content, which is consistent with the levels of anthocyanins determined using the pH differential spectroscopic method.

Antioxidant capacity of *C. Nervosum* extracts

When evaluating the antioxidant capacity of CNE using a range of methods, including ABTS, DPPH, and FRAP, consistent results from all three methods indicated that CN-EtOAcF exhibited the highest radical scavenging activity, followed by CN-HEXF, CN-DCMF, ECE, and finally, CN-WTF, as indicated in Table 5. The IC_{50} value of ABTS assay ranged from 43.77 ± 0.7 (CN-EtOAcF) to 302.45 ± 11.2 $\mu\text{g/mL}$ (CN-WTF). The DPPH assay showed that IC_{50} was lowest at 79.98 ± 4.4 $\mu\text{g/mL}$ (CN-EtOAcF) and highest at 710.44 ± 15.1 $\mu\text{g/mL}$ (CN-WTF). In FRAP assay, CN-WTF had the lowest value at 54.10 ± 7.0 $\mu\text{M Fe}^{2+}/\text{g}$, while the highest value, 564.18 ± 9.3 $\mu\text{M Fe}^{2+}/\text{g}$, was found in CN-EtOAcF.

Fraction	ABTS IC ₅₀ (µg/mL)	DPPH IC ₅₀ (µg/mL)	FRAP µM Fe ²⁺ /g
Ethanollic crude extract (ECE)	223.26 ± 10.7	457.03 ± 7.8	68.85 ± 4.1
Hexane fraction (CN-HEXF)	83.83 ± 2.6	250.15 ± 35.1	98.07 ± 8.8
Dichloromethane fraction (CN-DCMF)	96.96 ± 6.1	402.66 ± 15.8	87.02 ± 7.9
Ethyl acetate fraction (CN-EtOAcF)	43.77 ± 0.7	79.98 ± 4.4	564.18 ± 9.3
Water fraction (CN-WTF)	302.45 ± 11.2	710.44 ± 15.1	54.10 ± 7.0

Table 5. Antioxidant activities of CNE.

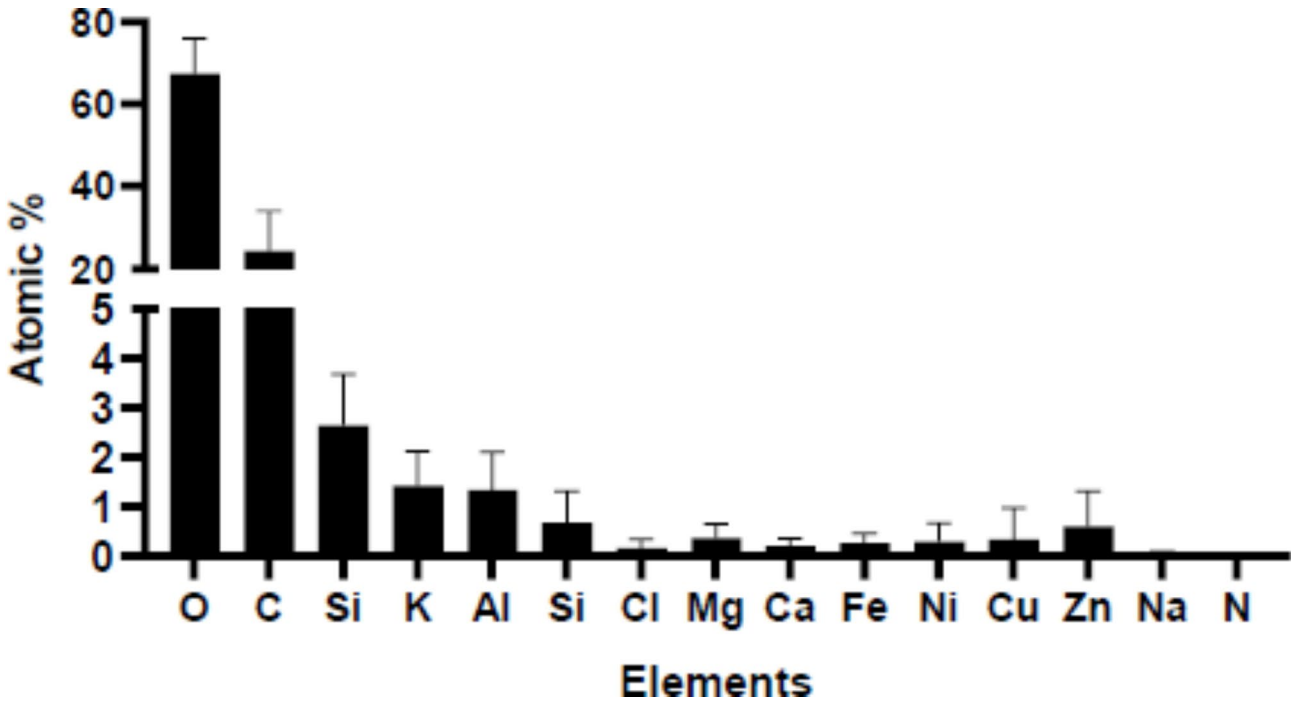


Fig. 3. The elements components in PM₁₀ samples expressed as atomic percentages.

Chemical characteristics and size distribution of PM₁₀

To carry out elemental composition (O, C, Si, K, Al, S, Cl, Mg, Ca, Fe, Ni, Cu, Zn, Na, and N) of PM₁₀, the filter was sectioned and subjected to SEM-EDX. The findings indicated that O was the predominant element (67%), followed by C (24%), Si (2.6%), K (1.4%), and Al (1.3%), as indicated in Fig. 3. The other elements were found less than 1%, whereas N was not detectable. Furthermore, the size of PM₁₀ was determined to be below 10 µm, and it displayed a nearly spherical form, as illustrated in Fig. 4.

Effect of PM₁₀ soluble extract and CN-EtOAcF on trophoblast cell cytotoxicity

First, we assessed the cytotoxicity of PM₁₀ soluble extract and CN-EtOAcF on HTR-8/SVneo cells using the MTT assay, and non-toxic doses were subsequently employed for further experiments. The results revealed that PM₁₀ soluble extract significantly reduced HTR-8/SVneo cell viability at concentrations ranging from 10 to 30 µg/mL for 24–72 h (Fig. 5A). Furthermore, CN-EtOAcF, at concentrations of 100–160 µg/mL, exhibited toxic effects on HTR-8/SVneo cell viability over the same 24–72 h period (Fig. 5B).

Effect of PM₁₀ soluble extract and CN-EtOAcF on trophoblast migration and invasion

We analyzed the impact of CN-EtOAcF on trophoblast migration and invasion after PM₁₀ soluble extract treatment. The outcomes revealed a notable increase in PM₁₀ soluble extract-induced trophoblast migration when CN-EtOAcF was present, in comparison to PM₁₀ soluble extract treatment alone (Fig. 6A). Likewise, at a concentration of 5 µg/mL, PM₁₀ soluble extract significantly inhibited cell invasion. Trophoblast invasion was elevated in the pre-treatment of CN-EtOAcF and followed by PM₁₀ co-treatment (Fig. 6B). These results suggest that CN-EtOAcF holds promise in preventing complications related to PM₁₀ exposure during pregnancy.

Effect of PM₁₀ soluble extract and CN-EtOAcF on trophoblast cell proliferation

To investigate the effect of PM₁₀ soluble extract and CN-EtOAcF on trophoblast cell proliferation, a BrdU incorporation assay was performed over 24–72 h. Treatment of HTR-8/SVneo cells with 5 µg/mL PM₁₀ soluble

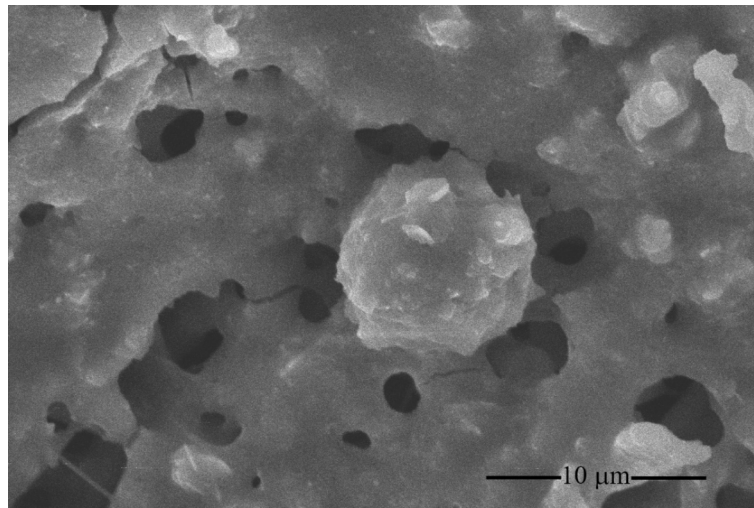


Fig. 4. SEM image depicting PM₁₀ particles at a ×12,000 magnification level.

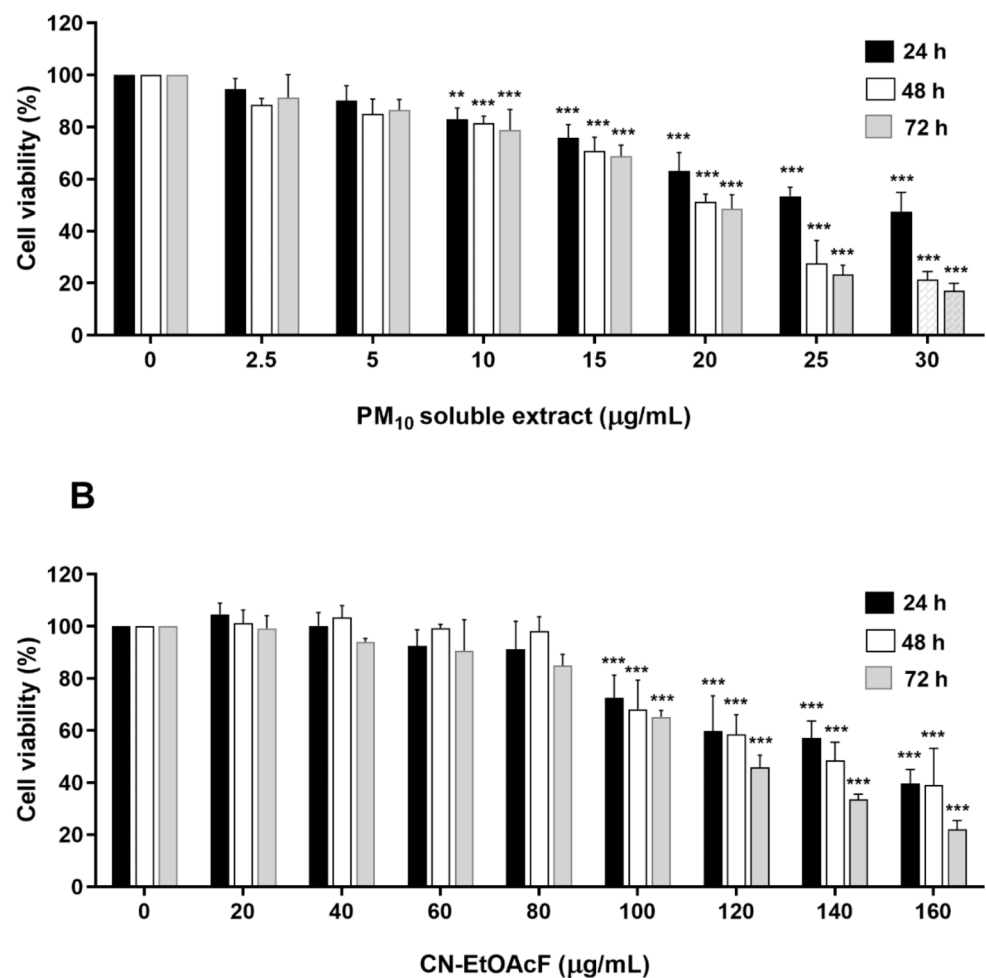


Fig. 5. Cell viability of PM₁₀ soluble extract (A) and CN-EtOAcF (B) on HTR-8/SVneo cells. MTT assay was conducted to evaluate PM₁₀ soluble extract and CN-EtOAcF-treated cells for 24–72 h.

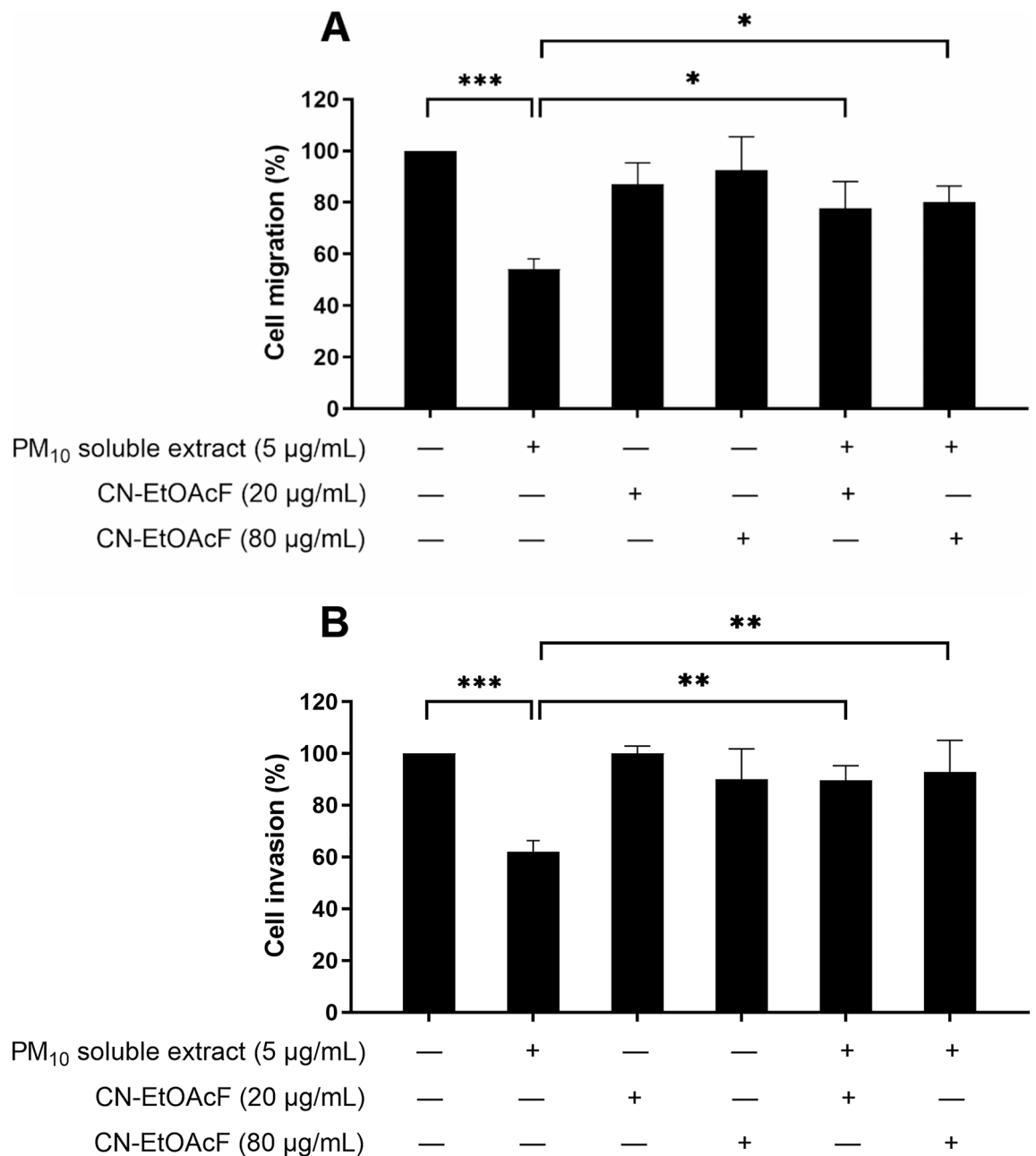


Fig. 6. Cell migration (A) and invasion (B) of PM₁₀ soluble extract and CN-EtOAcF on HTR-8/SVneo cells were assessed. Transwell migration and Matrigel invasion assays were conducted to evaluate the effect of CN-EtOAcF on PM₁₀-induced cell migration and invasion for 24 h.

extract resulted in a suppression of cell proliferation specifically noticeable at 48–72 h (Fig. 7). Interestingly, pre-treatment of CN-EtOAcF at concentrations of 20 and 80 µg/mL followed by co-treatment of PM₁₀ soluble extract demonstrated the ability to counteract the inhibitory effects of PM₁₀ soluble extract on cell proliferation. These findings suggest that CN-EtOAcF could protect trophoblast cells from PM₁₀ soluble extract-induced suppression of proliferation.

Effect of CN-EtOAcF on intracellular ROS

The elevation of ROS is toxic and is associated with pollutants such as PM₁₀, causing DNA damage. As shown in Fig. 8, we observed a significant dose-dependent reduction in intracellular ROS levels in CN-EtOAcF-pre-treated cells, followed by H₂O₂ co-treatment compared to H₂O₂-treated cells. Our findings suggest that CN-EtOAcF alleviates ROS production in trophoblast cells.

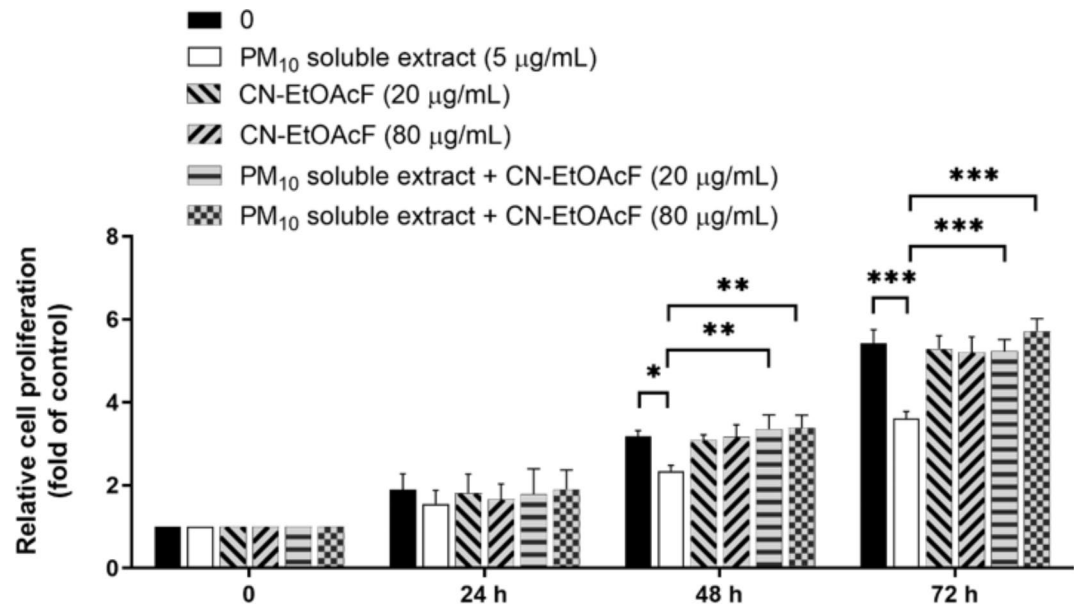


Fig. 7. Cell proliferation of HTR-8/SVneo cells pre-treated with CN-EtOAcF and followed by PM₁₀ soluble extract co-treatment was assessed. The BrdU assay was conducted to evaluate the effect of CN-EtOAcF on PM₁₀ soluble extract-induced cell proliferation for 24–72 h.

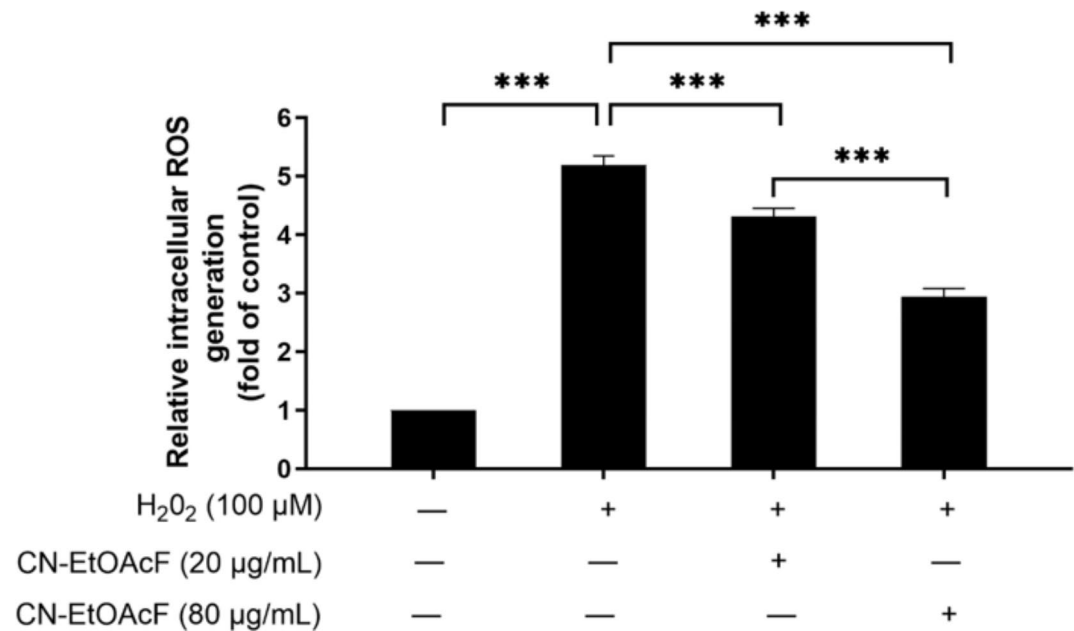


Fig. 8. Intracellular ROS in HTR-8/SVneo cells. The fluorescence intensity was measured to evaluate the effect of CN-EtOAcF on H₂O₂-induced intracellular ROS.

Anti-inflammation by CN-EtOAcF

To evaluate the anti-inflammatory properties of CN-EtOAcF on PM₁₀ soluble extract-induced pro-inflammatory cytokine expression, we examined the levels of IL-1 β , IL-6, and TNF- α in HTR-8/SVneo cells pre-treated with CN-EtOAcF and then co-treated with 5 µg/mL PM₁₀ soluble extract (Fig. 9). Treatment with PM₁₀ soluble extract alone led to a significant increase in the expression of IL-1 β , IL-6, and TNF- α . Conversely, pre-treatment with CN-EtOAcF followed by co-treatment with PM₁₀ soluble extract resulted in the suppression of these cytokines. These findings indicate that CN-EtOAcF exhibits anti-inflammatory activity in the presence of PM₁₀ soluble extract.

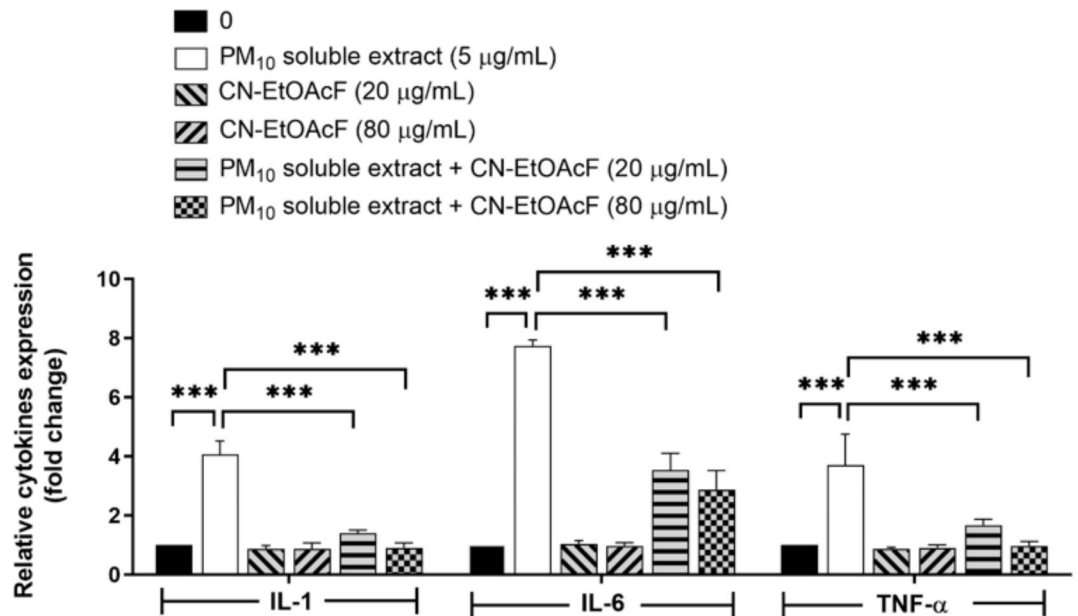


Fig. 9. Cytokine expression in HTR-8/SVneo cells. Cells were pre-treated with CN-EtOAcF followed by co-treatment with PM₁₀ soluble extract. RNA was isolated, and the expression levels of IL-1 β , IL-6, and TNF- α were assessed using qRT-PCR.

Effect of PM₁₀ soluble extract and CN-EtOAcF on apoptosis

Subsequently, we investigated the impact of CN-EtOAcF on apoptosis induced by PM₁₀ soluble extract in trophoblast cells through Annexin V/PI staining using flow cytometry. Our findings reveal that apoptosis was induced in HTR-8/SVneo cells at a concentration of 10 μ g/mL of PM₁₀ soluble extract, with no significant effect observed at 5 μ g/mL (Fig. 10A,B). Pre-treatment with CN-EtOAcF at concentrations of either 20 μ g/mL or 80 μ g/mL, followed by co-treatment with 10 μ g/mL of PM₁₀ soluble extract, effectively reversed apoptosis in HTR-8/SVneo cells. Thus, our results suggest that CN-EtOAcF has the potential to shield trophoblast cells from the detrimental effects of PM₁₀ soluble extract by inhibiting cell apoptosis.

Effect of CN-EtOAcF on PM₁₀ soluble extract-induced miR-146a-5p expression and its potential target

Since CN-EtOAcF can restore growth inhibition, impaired migration and invasion, inflammation, and cell apoptosis caused by PM₁₀ soluble extract in trophoblast cells, we hypothesize that pre-treatment of CN-EtOAcF could protect trophoblast cells from PM₁₀ soluble extract co-treatment by modulating miR-146a-5p expression. Our results showed that following a 24 h treatment with 5 μ g/mL PM₁₀ soluble extract, there was an upregulation of miR-146a-5p in HTR-8/SVneo cells. Nevertheless, the administration of CN-EtOAcF at 20 and 80 μ g/mL, subsequent to PM₁₀ soluble extract co-treatment, led to a substantial downregulation of miR-146a-5p compared to PM₁₀ soluble extract alone (Fig. 11A). We proceeded to predict the potential target of miR-146a-5p utilizing bioinformatics platforms. The finding indicated that 109 potential target genes of miR-146a-5p, including SOX5 were related to cell proliferation, migration and invasion (Supplementary Table 1). The results revealed that miR-146a-5p targeted a 7mer-A1 site located within the 3' untranslated region (UTR) of SOX5, precisely at transcript positions 883–889 (Fig. 11B). Consequently, SOX5 was identified as a candidate target of miR-146a-5p, and its expression was validated through qRT-PCR authentication. The expression level of SOX5 was found to be opposite to that of miR-146a-5p (Fig. 11C). Our results suggest that CN-EtOAcF modulates the expression of miR-146a-5p, targeting SOX5.

Discussion and conclusion

The trophoblast cell plays a pivotal role in ensuring a successful pregnancy, and any dysfunction in trophoblast activities can lead to pregnancy complications. Environmental pollutants such as PM_{2.5/10} trigger oxidative stress and inflammation, leading to the suppression of trophoblast cell growth, migration, invasion, and induction of cell cycle arrest and apoptosis^{2,17,18}. PM₁₀, predominantly composed of carbon and oxygen and extracted within a range of less than 10 μ m, was identified as containing substances toxic to trophoblast cells. This discovery aligns with our prior findings¹⁸, suggesting that PM₁₀ inhibits trophoblast cell proliferation, migration, invasion, and induces apoptosis. These factors contribute to adverse pregnancy outcomes, including hypertensive disorders, preeclampsia, preterm birth, low birth weight, and gestational diabetes mellitus^{39,40}.

C. nervosum fruit extract (CNE) has been reported to possess various beneficial properties, including antidiabetic, neuroprotective, antiaging, antioxidant, and anti-inflammatory effects^{32,41–43}. However, there is no existing report on the effectiveness of CNE in trophoblast cells. This study investigates and reports the protective effects of CNE on stress-inducing agent (PM₁₀ soluble extract)-induced trophoblast cell dysfunction through the

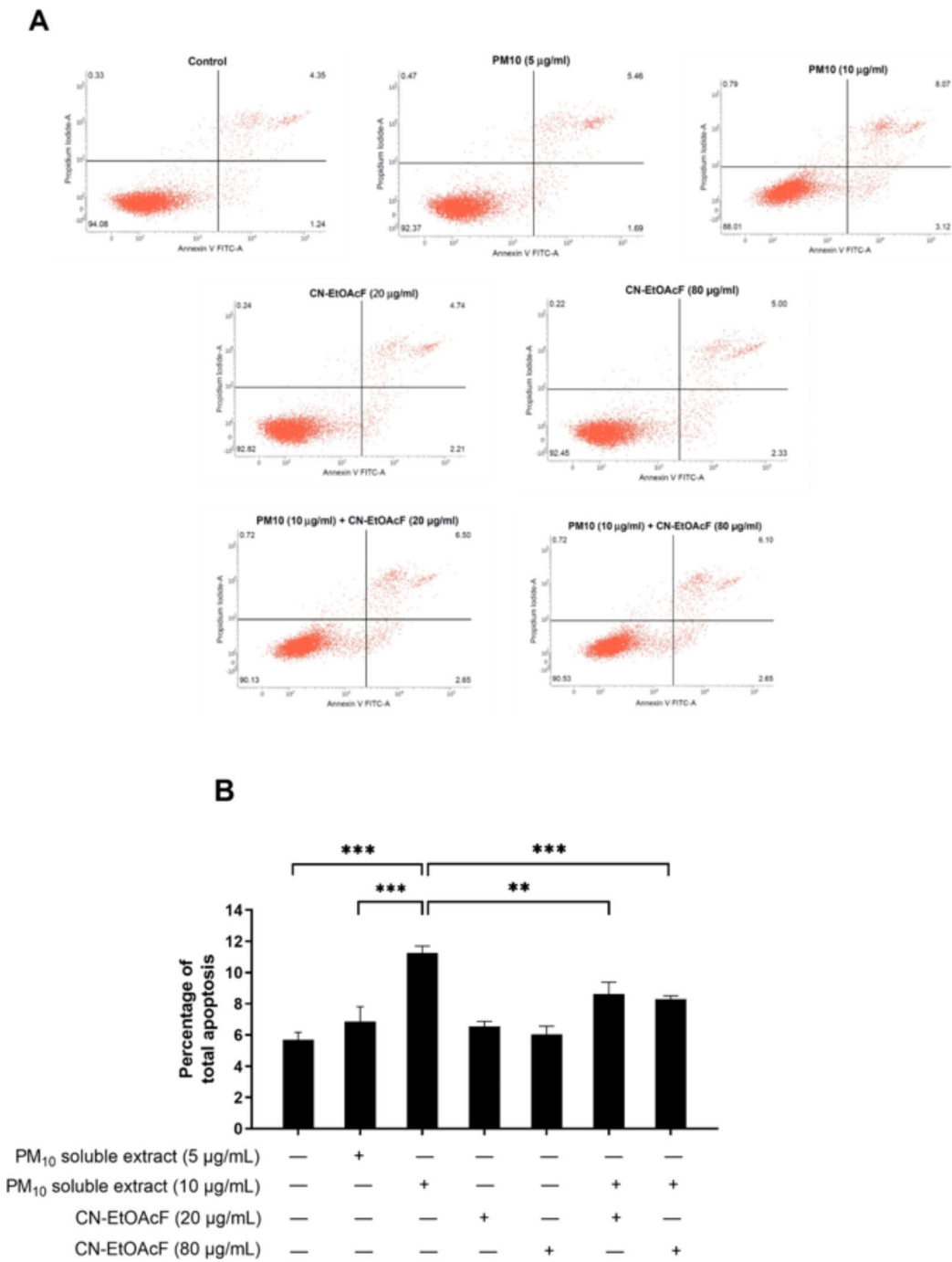


Fig. 10. Apoptosis in HTR-8/SVneo cells. The Annexin V/PI apoptosis assay was performed on HTR-8/SVneo pretreated with CN-EtOAcF followed by PM₁₀ soluble extract co-treatment, using flow cytometry (A). The histogram depicted the relative levels of apoptosis observed in each group (B).

modulation of miR-146a-5p expression. Recently, miRNAs have emerged as crucial regulators of cell processes and homeostasis, operating at the post-transcriptional level through mRNA degradation or translational inhibition. Several miRNAs are associated with trophoblast functions, and altered miRNA expression leading to trophoblast cell dysfunction contributes to pregnancy complications⁴⁴. One such miRNA is miR-146a-5p, known as a tumor suppressor in various cancers, capable of inhibiting cancer cell proliferation, migration, and invasion²⁷. It also acts as an inflammatory miRNA, involved in regulating the NF-κB and NLRP pathways²⁸. miR-146a-5p can be upregulated through stimulation by TNF-α, IL-1β, and LPS⁴⁵, and its high expression level has been shown to decrease trophoblast cell proliferation, migration, and invasion²⁹.

The correlation between TPC, anthocyanin levels, and radical scavenging activity is well-established in various fruits and vegetables^{46–48}. High levels of TPC and anthocyanins contribute to robust antioxidant capacity,

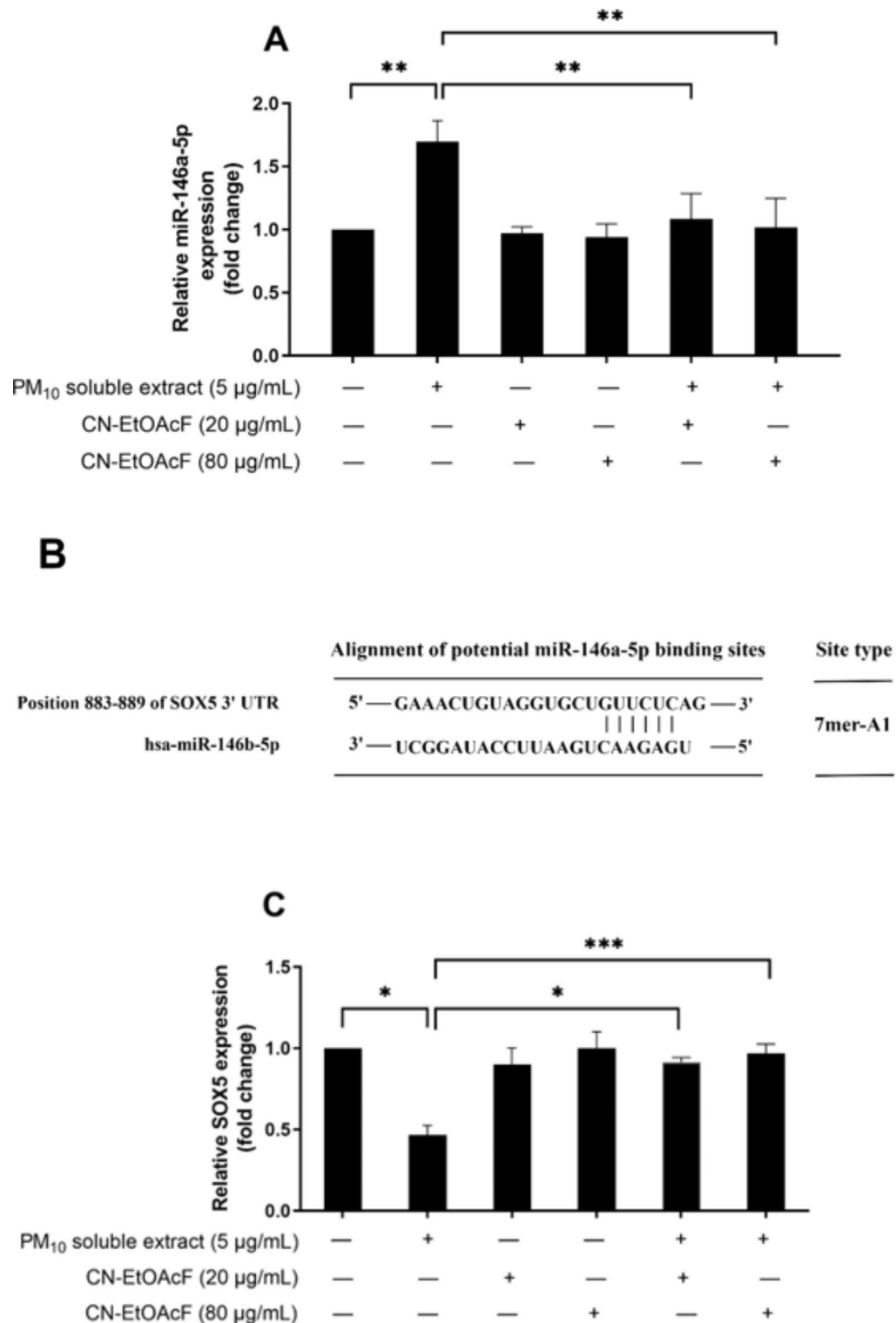


Fig. 11. miR-146a-5p expression (A), the TargetScan database predicted the binding site of miR-146a-5p with SOX5 and SOX5 expression (C) in CN-EtOAcF-treated PM₁₀ soluble extract-induced HTR-8/SVneo cells. Cells were pre-treated with CN-EtOAcF followed by PM₁₀ soluble extract co-treatment for 24 h. The qRT-PCR was employed to quantify the expression of miR-146a-5p and SOX5.

reducing intracellular ROS levels and inflammation⁴⁹. In our study, we observed that TPC, anthocyanidin content, and antioxidant activity in CNE, particularly in *C. nervosum* fruit fractionated by ethyl acetate (CN-EtOAcF), correlate with its protective potential in trophoblast cells exposed to PM₁₀ soluble extract. This protective effect is evident from the reversal of PM₁₀ soluble extract-induced impairments in cell proliferation, migration, invasion,

and apoptosis. CN-EtOAcF's ability to mitigate ROS production and inflammatory cytokine expression further supports its role in protecting trophoblast cells from environmental stressors. It is well known that excessive intracellular ROS can damage proteins, nucleic acids, lipids, membranes, and organelles, potentially leading to the activation of apoptosis⁵⁰. Our investigation revealed that pre-treatment with CN-EtOAcF, which exhibited the highest concentrations of TPC, antioxidant activities, and anthocyanins compared to other fractions, demonstrated a protective effect against PM₁₀ soluble extract-induced cell damage by reducing intracellular ROS levels and reversing apoptosis in HTR-8/SVneo cells.

Additionally, CN-EtOAcF treatment led to decreased levels of inflammatory cytokines IL-1 β , IL-6, and TNF- α in PM₁₀-induced HTR-8/SVneo cells, which was associated with reduced expression of the inflammatory miRNA, miR-146a-5p. Pre-treatment with CN-EtOAcF prior to PM₁₀ soluble extract exposure downregulated miR-146a-5p expression. This miRNA, whose gene is located on chromosome 10 (10q24.32), is known to regulate inflammation by targeting upstream of NF- κ B signaling pathways^{51–53}. Using the TargetScan database, we identified 109 potential target genes of miR-146a-5p involved in key cellular processes such as proliferation, migration, and invasion. Among these, SOX5 emerged as a notable target, linked to cell proliferation, migration, and invasion^{27,54}. The expression pattern of SOX5 was inversely related to miR-146a-5p, with high levels of SOX5 regulating these processes. Our results suggest that miR-146a-5p influences trophoblast cell functions through a network of target genes, providing insights into its regulatory mechanisms in both physiological and pathological conditions. Further research, including gain- and loss-of-function studies, is needed to elucidate specific role of SOX5 and validate other identified target genes to assess the potential of miR-146a-5p as a therapeutic target for pregnancy-related complications.

Oxidative stress, characterized by an overproduction of ROS, which can be activated by PM₁₀, leads to cellular damage, including DNA damage^{55,56}. This damage contributes to cellular dysfunction, such as impaired cell proliferation, migration, invasion, and increased apoptosis^{18,57,58}. The study revealed that PM₁₀ soluble extract increased oxidative stress, suppressed trophoblast cell proliferation, migration, and invasion, and induced apoptosis. Remarkably, pre-treatment with CN-EtOAcF reduced oxidative stress, enhanced cell proliferation, migration, and invasion, and decreased apoptosis in trophoblast cells exposed to PM₁₀ soluble extract. Several studies have demonstrated that CNE provides cellular protection through its antioxidant properties, anti-inflammatory effects, and its ability to prevent apoptosis across various cell types^{32,59,60}. Consequently, our study highlights that CN-EtOAcF's ability to reduce ROS levels, modulate inflammatory responses, and prevent apoptosis underscores its protective role against oxidative stress induced by PM₁₀. Considering the essential role of trophoblast cells in placenta function during implantation and throughout pregnancy, enhancing their functionality including proliferation, migration, invasion while reducing apoptosis through pre-treatment with CN-EtOAcF, followed by co-treatment with the PM₁₀ soluble extract, can mitigate the adverse effects of PM₁₀ soluble extract. This approach could therefore contribute to a successful pregnancy by protecting trophoblast cells from oxidative stress and cellular damage induced by the environmental pollutant, PM₁₀. It is indeed intriguing to elucidate whether the extract prevents PM₁₀ from causing cellular damage through mechanisms such as precipitation, inactivation, and inhibition of cellular penetration and activation. Further investigation will be crucial for validating the proposed protective effects of the extract and understanding its potential therapeutic applications.

In summary, this study highlights the severe detrimental effects of PM₁₀ soluble extract on trophoblast cells, which manifest through oxidative stress, particularly upregulation of miR-146a-5p, and subsequent disruptions in gene expression. These molecular changes impair key cellular processes such as proliferation, migration, and invasion, ultimately increasing apoptosis and compromising trophoblast cell function. However, the study demonstrates that pre-treatment with CN-EtOAcF effectively mitigates these harmful effects. By reducing oxidative stress, lowering ROS levels, and downregulating miR-146a-5p expression, CN-EtOAcF reverses the PM₁₀ soluble extract-induced suppression of trophoblast cell proliferation, migration, invasion, and apoptosis. These findings underscore the protective potential of CN-EtOAcF in safeguarding trophoblast cells from PM₁₀ soluble extract-induced damage, offering a promising therapeutic approach for maintaining normal cellular functions and supporting successful pregnancies in environments burdened by pollutants. Further investigations are crucial to fully elucidate the molecular mechanisms involved and to explore the broader therapeutic applications of CN-EtOAcF in counteracting environmental pollutant-induced cellular damage.

Data availability

The data produced by the present study can be accessed upon request from the corresponding author.

Received: 7 May 2024; Accepted: 12 September 2024

Published online: 16 October 2024

References

1. Bearblock, E., Aiken, C. E. & Burton, G. J. Air pollution and pre-eclampsia; Associations and potential mechanisms. *Placenta* **104**, 188–194 (2021).
2. Familiari, M. *et al.* Exposure of trophoblast cells to fine particulate matter air pollution leads to growth inhibition, inflammation and ER stress. *PLoS ONE* **14**, e0218799 (2019).
3. Bravo, M. A. & Miranda, M. L. A longitudinal study of exposure to fine particulate matter during pregnancy, small-for-gestational age births, and birthweight percentile for gestational age in a statewide birth cohort. *Environ. Health*. **21**, 9 (2022).
4. Chen, H. *et al.* Effects of air pollution on human health: Mechanistic evidence suggested by in vitro and in vivo modelling. *Environ. Res.* **212**, 113378 (2022).
5. Gangwar, R. S., Bevan, G. H., Palanivel, R., Das, L. & Rajagopalan, S. Oxidative stress pathways of air pollution mediated toxicity: Recent insights. *Redox Biol.* **34**, 101545 (2020).

6. Leni, Z. *et al.* Oxidative stress-induced inflammation in susceptible airways by anthropogenic aerosol. *PLoS ONE* **15**, e0233425 (2020).
7. Li, D. *et al.* Fluorescent reconstitution on deposition of PM(2.5) in lung and extrapulmonary organs. *Proc. Natl. Acad. Sci. USA* **116**, 2488–2493 (2019).
8. Son, T. *et al.* Monitoring in vivo behavior of size-dependent fluorescent particles as a model fine dust. *J. Nanobiotechnol.* **20**, 227 (2022).
9. Gonzalez-Ramos, S. *et al.* Integrating 4-D light-sheet fluorescence microscopy and genetic zebrafish system to investigate ambient pollutants-mediated toxicity. *Sci. Total Environ.* **902**, 165947 (2023).
10. Bongaerts, E. *et al.* Maternal exposure to ambient black carbon particles and their presence in maternal and fetal circulation and organs: An analysis of two independent population-based observational studies. *Lancet Planet. Health* **6**, e804–e811 (2022).
11. Manjunatha, B., Deekshitha, B., Seo, E., Kim, J. & Lee, S. J. Developmental toxicity induced by particulate matter (PM(2.5)) in zebrafish (*Danio rerio*) model. *Aquat. Toxicol.* **238**, 105928 (2021).
12. Fongsodsri, K. *et al.* Particulate matter 2.5 and Hematological disorders from Dust to diseases: A systematic review of available evidence. *Front. Med. (Lausanne)* **8**, 692008 (2021).
13. Jahedi, F. *et al.* Polycyclic aromatic hydrocarbons in PM(1), PM(2.5) and PM(10) atmospheric particles: Identification, sources, temporal and spatial variations. *J. Environ. Health Sci. Eng.* **19**, 851–866 (2021).
14. Dong, D. *et al.* The chemical characterization and source apportionment of PM2.5 and PM10 in a typical city of Northeast China. *Urban Clim.* **47**, 101373 (2023).
15. Nuchdang, S. *et al.* Metal composition and source identification of PM2.5 and PM10 at a suburban site in Pathum Thani, Thailand. *Atmosphere*. **14**, 659 (2023).
16. Wang, Y. *et al.* Gestational exposure to PM(2.5) disrupts fetal development by suppressing placental trophoblast syncytialization via progranulin/mTOR signaling. *Sci. Total Environ.* 171101 (2024).
17. Qin, Z. *et al.* Fine particulate matter exposure induces cell cycle arrest and inhibits migration and invasion of human extravillous trophoblast, as determined by an iTRAQ-based quantitative proteomics strategy. *Reprod. Toxicol.* **74**, 10–22 (2017).
18. Chaiwangyen, W. *et al.* PM10 alters trophoblast cell function and modulates miR-125b-5p expression. *Biomed. Res. Int.* 3697944 (2022).
19. Saliminejad, K., Khorshid, K., Soleymani Fard, H. R. & Ghaffari, S. H. An overview of microRNAs: Biology, functions, therapeutics, and analysis methods. *J. Cell. Physiol.* **234**, 5451–5465 (2019).
20. O'Brien, J., Hayder, H., Zayed, Y. & Peng, C. Overview of microRNA biogenesis, mechanisms of actions, and circulation. *Front. Endocrinol. (Lausanne)* **9**, 402 (2018).
21. Liang, L. *et al.* MicroRNAs: Key regulators of the trophoblast function in pregnancy disorders. *J. Assist. Reprod. Genet.* **40**, 3–17 (2023).
22. Ali, A. *et al.* MicroRNA-mRNA networks in pregnancy complications: A comprehensive downstream analysis of potential biomarkers. *Int. J. Mol. Sci.* **22** (2021).
23. Hayder, H., Shan, Y., Chen, Y., O'Brien, J. A. & Peng, C. Role of microRNAs in trophoblast invasion and spiral artery remodeling: Implications for preeclampsia. *Front. Cell. Dev. Biol.* **10**, 995462 (2022).
24. Hou, T., Chen, Q. & Ma, Y. Elevated expression of miR-146 involved in regulating mice pulmonary dysfunction after exposure to PM2.5. *J. Toxicol. Sci.* **46**, 437–443 (2021).
25. Cheng, M. *et al.* MicroRNAs expression in relation to particulate matter exposure: A systematic review. *Environ. Pollut.* **260**, 113961 (2020).
26. Hubert, A. *et al.* The relationship between residential exposure to atmospheric pollution and circulating miRNA in adults living in an urban area in northern France. *Environ. Int.* **174**, 107913 (2023).
27. Si, C., Yu, Q. & Yao, Y. Effect of miR-146a-5p on proliferation and metastasis of triple-negative breast cancer via regulation of SOX5. *Exp. Ther. Med.* **15**, 4515–4521 (2018).
28. Hou, J. *et al.* MicroRNA-146a-5p alleviates lipopolysaccharide-induced NLRP3 inflammasome injury and pro-inflammatory cytokine production via the regulation of TRAF6 and IRAK1 in human umbilical vein endothelial cells (HUVECs). *Ann. Transl. Med.* **9**, 1433 (2021).
29. Peng, P. *et al.* miR-146a-5p-mediated suppression on trophoblast cell progression and epithelial-mesenchymal transition in preeclampsia. *Biol. Res.* **54**, 30 (2021).
30. Lv, Q. *et al.* Exosomal miR-146a-5p derived from human umbilical cord mesenchymal stem cells can alleviate antiphospholipid antibody-induced trophoblast injury and placental dysfunction by regulating the TRAF6/NF-kappaB axis. *J. Nanobiotechnol.* **21**, 419 (2023).
31. Prasanth, M. L. *et al.* Functional properties and bioactivities of *Cleistanthus nervosum* var. Paniala berry plant: A review. *Food Sci. Technol. (Campinas)* **40**, 369–373 (2020).
32. Nantacharoen, W. *et al.* *Cleistanthus nervosum* var. Paniala berry promotes antioxidant response and suppresses glutamate-induced cell death via SIRT1/Nrf2 survival pathway in hippocampal HT22 neuronal cells. *Molecules* **27** (2022).
33. Srimard, P., Muangnoi, C., Tuntipipat, S., Charoenkiatkul, S. & Sukprasansap, M. *Cleistanthus nervosum* var. Paniala fruit extract attenuates interleukin-1 β -induced inflammation in human retinal pigment epithelial cells. *J. Nutr. Assoc. Thailand* **57**, 18–31 (2022).
34. Sawong, S. *et al.* Calotropis gigantea stem bark extracts inhibit liver cancer induced by diethylnitrosamine. *Sci. Rep.* **12**, 12151 (2022).
35. Nile, S. H., Kim, D. H. & Keum, Y. S. Determination of anthocyanin content and antioxidant capacity of different grape varieties. *Cienc. T c. Vitiv.* **30**, 60–68 (2015).
36. Ryu, S. N., Park, S. Z. & Ho, C. T. High performance liquid chromatographic determination of anthocyanin pigments in some varieties of black rice. *J. Food Drug Anal.* **6**, 729–736 (1998).
37. Fernandes, R. P. *et al.* Evaluation of antioxidant capacity of 13 plant extracts by three different methods: Cluster analyses applied for selection of the natural extracts with higher antioxidant capacity to replace synthetic antioxidant in lamb burgers. *J. Food Sci. Technol.* **53**, 451–460 (2016).
38. Reddy, A. S., Malek, A., Ibrahim, S. N. & Sim, K. S. Cytotoxic effect of *Alpinia scabra* (Blume) Naves extracts on human breast and ovarian cancer cells. *BMC Complement. Altern. Med.* **13**, 314 (2013).
39. Chiarello, D. I. *et al.* Cellular mechanisms linking to outdoor and indoor air pollution damage during pregnancy. *Front. Endocrinol. (Lausanne)* **14**, 1084986 (2023).
40. Zhai, Y. *et al.* Smog and risk of maternal and fetal birth outcomes: A retrospective study in Baoding, China. *Open. Med. (Wars)* **17**, 1007–1018 (2022).
41. Chukitsiri, S., Wongsrangsap, N., Ratanabunyong, S. & Choowongkomon, K. In vitro evaluation of antidiabetic potential of *Cleistanthus nervosum* var. Paniala Fruit Extract. *Plants (Basel)* **12** (2022).
42. Prasanth, M. I. *et al.* Stress resistance, and neuroprotective efficacies of *Cleistanthus nervosum* var. paniala fruit extracts using *Caenorhabditis elegans* model. *Oxid. Med. Cell Longev.* 7024785 (2019).
43. Janpaijit, S. *et al.* *Cleistanthus Nervosum* var. Paniala Berry seed protects against TNF-alpha-stimulated neuroinflammation by inducing HO-1 and suppressing NF-kappaB mechanism in BV-2 microglial cells. *Molecules* **28** (2023).
44. Shang, R., Lee, S., Senavirathne, G. & Lai, E. C. MicroRNAs in action: Biogenesis, function and regulation. *Nat. Rev. Genet.* **24**, 816–833 (2023).

45. Olivieri, F. *et al.* miR-21 and miR-146a: The microRNAs of inflammaging and age-related diseases. *Ageing Res. Rev.* **70**, 101374 (2021).
46. Choi, Y. M. *et al.* Isoflavones, anthocyanins, phenolic content, and antioxidant activities of black soybeans (*Glycine max* (L.) Merrill) as affected by seed weight. *Sci. Rep.* **10**, 19960 (2020).
47. Brito, A., Areche, C., Sepúlveda, B., Kennelly, E. J. & Simirgiotis, M. J. Anthocyanin characterization, total phenolic quantification and antioxidant features of some Chilean edible berry extracts. *Molecules* **19**, 10936–10955 (2014).
48. Pattananandecha, T. *et al.* Anthocyanin profile, antioxidant, anti-inflammatory, and antimicrobial against foodborne pathogens activities of purple rice cultivars in Northern Thailand. *Molecules* **26**, 5234 (2021).
49. Ma, Z., Du, B., Li, J., Yang, Y. & Zhu, F. An insight into anti-inflammatory activities and inflammation related diseases of anthocyanins: A review of both in vivo and in vitro investigations. *Int. J. Mol. Sci.* **22** (2021).
50. Redza-Dutordoir, M. & Averill-Bates, D. A. Activation of apoptosis signalling pathways by reactive oxygen species. *Biochim. Biophys. Acta* **1863**, 2977–2992 (2016).
51. Liao, Z., Zheng, R. & Shao, G. Mechanisms and application strategies of miRNA-146a regulating inflammation and fibrosis at molecular and cellular levels (review). *Int. J. Mol. Med.* **51** (2023).
52. Zhang, H., Chen, D., Ji, Q., Yang, M. & Ding, R. miR-146a-5p promotes the inflammatory response in PBMCs Induced by microcystin-leucine-arginine. *J. Inflamm. Res.* **16**, 1979–1993 (2023).
53. Shang, Y. *et al.* microRNA-146a-5p negatively modulates PM(2.5) caused inflammation in THP-1 cells via autophagy process. *Environ. Pollut.* **268**, 115961 (2021).
54. Shi, Y. *et al.* Transcription factor SOX5 promotes the migration and invasion of fibroblast-like synoviocytes in part by regulating MMP-9 expression in collagen-induced arthritis. *Front. Immunol.* **9**, 749 (2018).
55. Möller, P. *et al.* Oxidative stress and inflammation generated DNA damage by exposure to air pollution particles. *Mutat. Res. Rev. Mutat. Res.* **762**, 133–166 (2014).
56. Lionetto, M. G. *et al.* Oxidative potential, cytotoxicity, and intracellular oxidative stress generating capacity of PM10: A case study in South of Italy. *Atmosphere* **12**, 464 (2021).
57. Kello, M. *et al.* Oxidative stress-induced DNA damage and apoptosis in clove buds-treated MCF-7 cells. *Biomolecules* **10** (2020).
58. Martins, S. G., Zilhão, R., Thorsteinsdóttir, S. & Carlos, A. R. Linking oxidative stress and DNA damage to changes in the expression of extracellular matrix components. *Front. Genet.* **12** (2021).
59. Sukprasansap, M., Chanvorachote, P. & Tencomnao, T. *Cleistocalyx nervosum* var. Paniala berry fruit protects neurotoxicity against endoplasmic reticulum stress-induced apoptosis. *Food Chem. Toxicol.* **103**, 279–288 (2017).
60. Panritdum, P., Muangnoi, C., Tuntipipat, S., Charoenkiatkul, S. & Sukprasansap, M. *Cleistocalyx nervosum* var. Paniala berry extract and cyanidin-3-glucoside inhibit hepatotoxicity and apoptosis. *Food Sci. Nutr.* **12**, 2947–2962 (2024).
61. Connor, A. M., Luby, J. J., Tong, C. B., Finn, C. E., & Hancock, J. F. Genotypic and Environmental Variation in Antioxidant Activity, Total Phenolic Content, and Anthocyanin Content among Blueberry Cultivars. *Am. Soc. Hortic. Sci.* **127**, 89–97 (2002).
62. Nile, S. H., Park, S. W. HPTLC analysis, antioxidant, anti-inflammatory and antiproliferative activities of *Arisaema tortuosum* tuber extract. *Pharm. Biol.* **52**, 221–7 (2014).

Author contributions

Conceptualization and designation: W.C., O.K., K.P., N.K., and A.O.; Methodology: W.C. and K.P.; Formal analysis and investigation: W.C., O.K., N.K. A.O., P.N., A.S., P.T., D.C., and F.P.; Validation: W.C., O.K., and N.P.; Writing - original draft preparation: W.C.; Writing - review and editing: W.C., O.K., and N.P.; Funding acquisition: W.C.; Resources: W.C.; Supervision: W.C. All authors read and approved the final manuscript.

Funding

This research project was supported by the Thailand science research and innovation fund (Fundamental Fund 2023 Grant No. FF66-RIM067), School of Medical Sciences Research Grant (MS 231001), the University of Phayao, and National Research Council of Thailand (NRCT) and the University of Phayao (N42A66043).

Declarations

Competing interests

The authors declare no competing interests.

Ethical approval

This research has not involved human participants and/or animals.

Additional information

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1038/s41598-024-73000-y>.

Correspondence and requests for materials should be addressed to W.C.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

© The Author(s) 2024