RESEARCH ARTICLE OPEN ACCESS

The Role of Nigella sativa and Phyllanthus urinaria L Extracts Enhance Inflammation Cytokine and Growth factor in Mesenchymal Stem Cells Conditioned Medium

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Submission November 29, 2024
Accepted December 10, 2024
Available online on December 13, 2024

ABSTRACT

Background: Mesenchymal Stem Cells (MSCs) are cells that have the multipotent ability to undergo self-renewal, differentiate and secrete various bioactive substances, such as chemokines, proteins, microRNAs (miRNAs), growth factors, and cytokines. Conditioned medium of MSC is a medium resulting from cell culture enriched with the secretome of the cultured cells. MSC-CM treated with certain factors can increase the production of growth factors such as VEGF and PDGF, which play a role in angiogenesis and tissue repair. Modification of MSC-CM with bioactive compounds can be a promising strategy to increase the effectiveness of MSCs in medical therapy. Therefore, this study aims to examine whether these herbal extracts can modulate the production of pro-inflammatory cytokines (TNF-α, IFN-γ, IL-1β, IL-6) and growth factors (SDF-1, PDGF, VEGF) in MSC conditioned medium. **Methods:** This study used a pre-post research design with four treatment groups. Medium culture of MSCs treated with *Nigella sativa* (doses of 10 µg/mL) and *Phyllanthus urinaria L* (doses of 25 µg/mL), which were cultured for 24 and 48 hours. Measurement of cytokine and growth factor levels was carried out using the Enzyme-Linked Immunosorbent Assay (ELISA) method for quantitative analysis. **Conclusion:** This study showed that the *Nigella sativa* and *Phyllanthus urinaria L* extracts in modifying conditioned medium of Mesenchymal Stem Cells is significant release of pro-inflammatory cytokines and growth factors.

Keywords : Mesenchymal Stem Cells, Conditioned Medium, Secretome, Nigella sativa, Phyllanthus urinaria L, Pro-inflammatory Cytokines, Growth Factors.

INTRODUCTION

Mesenchymal Stem Cells (MSCs) are cells that have the multipotent ability to undergo self-renewal and differentiate into various mesenchymal cell lineages. Mesenchymal stem cells have attracted significant attention due to their interesting cellular biology, broad therapeutic potential, and role as a fundamental component in the emerging field of regenerative therapies. These cells have the potential to homing to damaged tissues through chemo-attraction, making them highly beneficial for a wide range of therapeutics. Recent studies have shown that MSCs can secrete various bioactive substances, such as chemokines, proteins, microRNAs (miRNAs), growth factors, and cytokines, indicating their potential applications[1].

MSC conditioned media is a cell culture fluid enriched with the secretome of the cultured cells. Its composition depends on various factors present in the microenvironment of the cell culture, including inhibition of cell contact, cell aggregation, differentiation ability and chemical parameters. The CM for cartilage regeneration therapy can be obtained from MSC populations derived from embryonic, hematopoietic and induced Pluripotent Stem Cell (iPSC). Isolated homogeneous cell populations are propagated in in vitro culture and when cell confluence is achieved, CM can be collected, pre-cleaned and stored for future use.[2] Current study showed that MSC CM treated with certain factors can increase the production of growth factors such as VEGF and PDGF, which play a role in angiogenesis and tissue repair. In addition, changes in cytokine production, such as decreased TNF-α and IL-6, can improve inflammatory conditions in cell-based therapy. Therefore, modification of MSC CM with bioactive compounds can be a promising strategy to increase the effectiveness of MSCs in medical therapy.[2]

Nigella sativa (NS) has long been used in Iranian traditional medicine for treating various disorders and anti-inflammation. Most of the therapeutic effects of this plant are due to thymoquinone (TQ) which is the main active ingredient in the oil of this plant.[3] Current study showed *Nigella sativa* supplementation significantly increased anti-inflammatory cytokine (IL-10).[3] *Phyllanthus urinaria* Linn. (PU) is a herb belonging to the Euphorbiaceae family, thrives in tropical regions including South-East Asian and Central America. Previous studies have extensively documented the pharmacological potential of *Nigella sativa* and *Phyllanthus urinaria* showcasing a broad spectrum of activities, including anticancer, antivirus, antimicrobial, antidiabetic, hepatoprotective and cardioprotective effects.[4] Both plants known to have anti-inflammatory and immunomodulatory properties. Both of these herbal extracts have the potential to be used to modulate the MSC microenvironment, which can improve or enhance the potential of MSCs in cell-based medicine.

Pro-inflammatory cytokines, such as TNF-α, IFN-γ, IL-1β, and IL-6 play a key role in upregulating inflammation and the immune response.[5] On the other hand, growth factors such as SDF-1, PDGF, and VEGF are increased tissue repair and angiogenesis.[6] Therefore, through herbal extracts induced in MSCs, it is expected that the modulation of cytokine and growth factor production may be more optimally in regenerative therapy, both in wound healing and degenerative disease therapy.[7] This study aims to assess the effect of *Nigella sativa* and *Phyllanthus urinaria L* extracts on the production of pro-inflammatory cytokines and growth factors in MSC conditioned medium. The results of this study are expected to provide a deeper understanding of the potential use of herbal extracts to enhance the therapeutic capabilities of MSCs in regenerative therapy and cell-based therapy.

MATERIAL AND METHODS

Materials

The herbs of *Nigella sativa* and *Phyllanthus urinaria* were sourced from a supplier at B2P2TOOT (Balai Besar Penelitian dan Pengembangan Tanaman Obat dan Obat Tradisional) in Tawangmangu, Central Java, Indonesia. After collection, the plant materials were dried using a cabinet dryer at a temperature range of 40–50°C. In vitro mesenchymal stem cell culture used Dulbecco Modified Eagle's Medium (DMEM) low glucose (Gibco), fetal bovine serum (Gibco), streptomycin penicillin (Gibco), glutamax (Gibco), Phosphate Buffer Saline (Gibco), T75 flask culture (Nunc) and 12 well plate (Corning). Cytokine testing analysis using specific ELISA kits for SDF1, IL-1B, IL-6, TNF-a, and IFN-g (Elabscience).

Herbs Extraction Procedure

The dried *Nigella sativa* and *Phyllanthus urinaria* herbs were first tested for water content to ensure they met the standards specified in the Farmakope Herbal Indonesia 2nd Edition. Once compliance was confirmed, the materials were ground into a fine powder using a 60-mesh grinder. The powdered *Nigella sativa* and *Phyllanthus urinaria* were then separately subjected to maceration using ethanol as the solvent in a 1:10 ratio (plant material to solvent) for a period of 3 days, followed by a re-maceration process. The resulting filtrate was concentrated by evaporation using a vacuum evaporator at a temperature range of 40–50°C to obtain the final concentrated extract.

hUC-MSCs Isolation and Cultivation

Human umbilical cord-MSCs (hUC-MSCs) were isolated from umbilical cords obtained from donors with written informed consent. The isolation and expansion of hUC-MSCs was done by chopped the umbilical cord into small pieces and then cultured in Dulbecco`s Modified Eagle Media (DMEM) (Sigma-Aldrich, Louis St, MO) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco™ Invitrogen, NY, USA) and 1% antibiotic/antimycotic (Gibco™ Invitrogen, NY, USA) at 37°C and 5% CO2. The medium was renewed every 3 days. The cells were passaged with trypsin- EDTA after 80% confluence. The fourth passage cells were used for experiments.

Characterization of hUC-MSCs

HUC-MSCs-like were fixed with Cytofix™ fixation buffer (554655, BD Biosciences, Franklin Lakes, NJ, USA), and washed twice with stain buffer (554657, BD Biosciences). For the phenotype markers analysis, the cells were stained with phycoerythrin (PE) mouse anti-human CD44 (Clone G44-26, 555479; BD Biosciences), allophycocyanin (APC) mouse anti-human CD73 (Clone AD2, 560847; BD Biosciences), fluorescein isothiocyanate (FITC) mouse anti-human CD90 (Clone 5E10, 561969 BD Biosciences) and PerCP-Cy5.5.1 mouse anti-human CD105 (Clone 266, 560819, BD Biosciences) antibodies. Cells were stained with specific antibody for 30 minutes at room temperature, washed twice with stain buffer (554657, BD Biosciences) and examined with a BD FACSAria™ II flow cytometer (BD Biosciences) and BD FACSDiva™ software (BD Biosciences).

In vitro Study design

hUC-MSCs were cultivated under standard culture condition at fourth passage. After reached 80% confluence, the cells were seeded into 12-well plates according to control, treatment 1 (*Nigella sativa* 10 ug/mL) 24 hours, treatment 2 (*Nigella sativa* 10 ug/mL) 48 hours, treatment 3 (*Phyllanthus urinaria L* 25 ug/mL) 24 hours and treatment 4 groups (*Phyllanthus urinaria L* 25 ug/mL) 48 hours. Each treatment was replicated 3 sample.

ELISA Assay

A specific ELISA assay measured the levels of SDF1, IL-1B, IL-6, TNF-a and IFN-g released in the conditioned mediums from the treatment and control groups. The levels of cytokine and growth factor were measured according to the manufacturer's instructions (Elabscience). Colorimetric absorbance was recorded at a wavelength of 450 nm.

Data Analysis

Data were presented as the means ± SD. All calculations were carried out using IBM SPSS 22.0 (IBM Corp., Armonk, NY, USA) was used for statistical analysis. The statistical significance of the

differences between the groups was assessed using one way-ANOVA and continued with Duncan post-hoc analysis. P values: **, $P < 0.001$.

RESULTS

MSC Charachteristic

The morphological analysis of hUC-MSCs under 4X and 10X magnification revealed a spindle-shaped, fibroblast-like morphology, which is characteristic of MSCs (Figure 1). This observation further supports the identity of the cells and their suitability for use in this study. The consistent morphology and marker expression across the cell population indicate that the MSCs were healthy and maintained their stem cell properties throughout the culture period, providing a reliable foundation for the subsequent experiments[8].

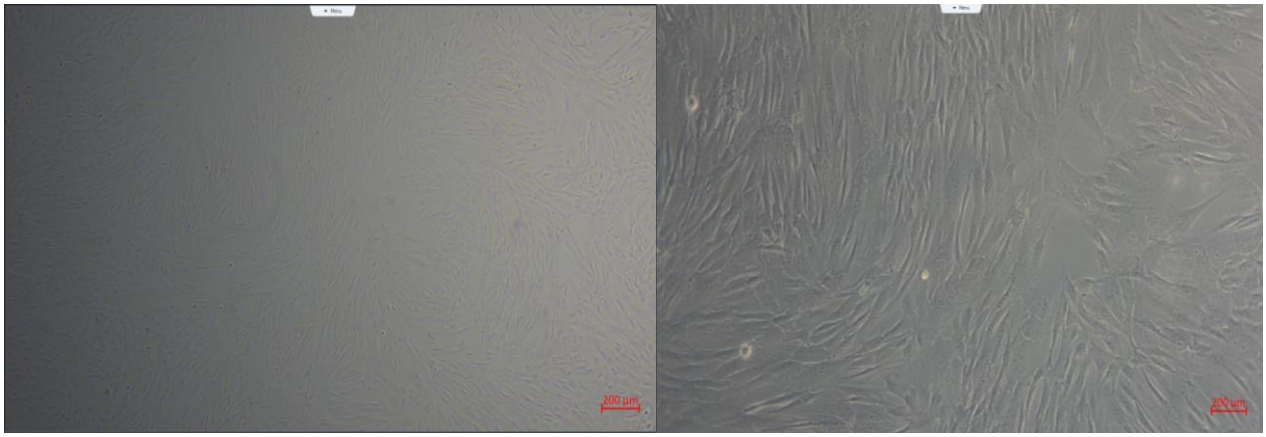

Figure 1. Morphology of MSC derived from human Umbilical Cord in 4X and 10X magnification.

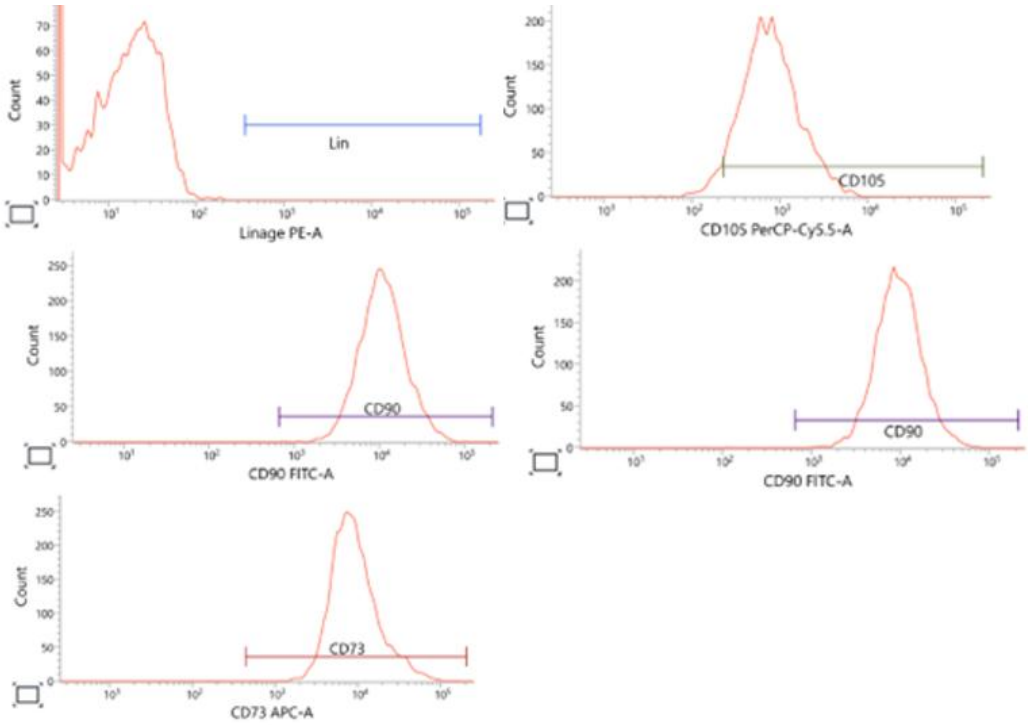

Figure 2. Flow cytometry of human umbilical cord mesenchymal stem cells that express negative surface marker HLA and positive surface marker CD73, CD105, CD44, CD90.

The flow cytometry analysis demonstrated that the hUC-MSCs expressed the typical surface markers CD73, CD105, CD44, and CD90 (Figure 2), which are widely recognized as defining characteristics of MSCs[9]. These markers are associated with the multipotency and immunomodulatory properties of MSCs, confirming that the cells used in this study were indeed mesenchymal stem cells. The expression of these markers is crucial for ensuring that the cells possess the necessary regenerative and immunomodulatory capabilities to respond to the herbal extracts.

Table 1. Level of SDF-1, IL-1B, TNFa and IFNg post treatment *Nigella sativa* (black cumin) and *Phyllanthus urinaria* L. extracts.

Sample	SDF-1	IL-1β	IL-6	TNF-α	IFN-γ
NS-1 24h	154,7	66,6	114,6	7,4	23,9
NS-1 48h	261,3	53,0	31,3	19,8	0
PU-1 24h	0,00	32,6	115,9	16,3	0
PU-1 48h	27,5	48,0	37,9	0,9	0

DISCUSSION

The results of this study provide valuable insights into the effects of Nigella sativa and Phyllanthus urinaria L. extracts on the production of cytokines and growth factors in mesenchymal stem cell (MSC) conditioned medium. The data reveal distinct patterns in the modulation of protein levels, particularly for SDF-1, IL-1β, IL-6, TNF-α, and IFN-γ, which are critical players in inflammation and tissue regeneration. Additionally, the characterization of human umbilical cord-derived MSCs (hUC-MSCs) through flow cytometry and morphological analysis confirmed the identity and quality of the MSCs used in this study, which is essential for ensuring the reliability of the experimental results.

One of the most notable findings is the significant increase in SDF-1 levels in the Nigella sativa 48-hour treatment group (261.3 pg/mL) compared to the 24-hour group (154.7 pg/mL). SDF-1, a chemokine involved in cell migration and homing, plays a crucial role in tissue repair by recruiting stem cells and progenitor cells to sites of injury[10]. The observed increase in SDF-1 levels suggests that Nigella sativa may enhance the regenerative potential of MSCs by promoting cell recruitment and tissue repair over time[11]. This aligns with previous studies that have demonstrated the ability of Nigella sativa and its active compound, thymoquinone, to support tissue regeneration and angiogenesis[11-13]. In contrast, the Phyllanthus urinaria L. 48-hour group showed a modest increase in SDF-1 levels (27.5 pg/mL), indicating that Nigella sativa may have a more pronounced effect on SDF-1 production compared to Phyllanthus urinaria L.

The data also revealed that the levels of IL-1β were consistently higher in the Nigella sativa group compared to the Phyllanthus urinaria L. group. IL-1β is a pro-inflammatory cytokine that plays a role in initiating and amplifying inflammatory responses[14-16]. While elevated IL-1β levels may indicate a stronger inflammatory response, they could also reflect the activation of immune pathways that are necessary for tissue repair. This finding suggests that Nigella sativa may modulate the inflammatory microenvironment in a way that supports both inflammation and subsequent tissue regeneration. In contrast, the lower IL-1β levels in the Phyllanthus urinaria L. group may indicate a

more suppressive effect on inflammation, consistent with its known anti-inflammatory properties[17-19].

Interestingly, the expression of IL-6, another pro-inflammatory cytokine, was higher after 24 hours of treatment in both the Nigella sativa and Phyllanthus urinaria L. groups. IL-6 is involved in both inflammatory and regenerative processes, and its transient increase at 24 hours may reflect an initial inflammatory response that is later downregulated[15]. This pattern is consistent with the role of IL-6 in the early stages of tissue repair, where it helps to recruit immune cells and initiate the healing process[18-20]. However, the subsequent decrease in IL-6 levels at 48 hours, particularly in the Phyllanthus urinaria L. group, suggests that this extract may help to control excessive inflammation over time, creating a more balanced microenvironment for tissue regeneration[21,22].

The levels of TNF-α and IFN-γ were also higher in the Nigella sativa group compared to the Phyllanthus urinaria L. group. TNF-α is a key mediator of inflammation, while IFN-γ is involved in immune regulation and defense against pathogens[18]. The elevated levels of these cytokines in the Nigella sativa group may indicate a stronger immune activation, which could be beneficial in contexts where a robust immune response is needed, such as in combating infections or promoting tissue repair. However, the lower levels of TNF-α and IFN-γ in the Phyllanthus urinaria L. group suggest that this extract may have a more suppressive effect on inflammatory pathways, which could be advantageous in conditions where excessive inflammation is detrimental.

Overall, the results suggest that Nigella sativa and Phyllanthus urinaria L. extracts have distinct but complementary effects on the MSC microenvironment. Nigella sativa appears to enhance the production of growth factors like SDF-1 and pro-inflammatory cytokines such as IL-1β, TNF-α, and IFN-γ, which may support tissue repair by promoting cell migration and immune activation. In contrast, Phyllanthus urinaria L. seems to exert a more suppressive effect on inflammation, particularly by reducing IL-6 and TNF-α levels over time, which could help to create a more balanced and conducive environment for tissue regeneration.

These findings are consistent with previous studies that have highlighted the anti-inflammatory and immunomodulatory properties of Nigella sativa and Phyllanthus urinaria L.[23-24]. However, further research is needed to fully understand the mechanisms by which these extracts modulate cytokine and growth factor production in MSCs. In vivo studies will be particularly important to validate the therapeutic potential of these extracts in animal models of tissue injury or disease.

CONCLUSIONS

In conclusion, this study demonstrates that *Nigella sativa* and *Phyllanthus urinaria L*. extracts can differentially modulate the MSC secretome, with Nigella sativa promoting growth factor production and immune activation, while Phyllanthus urinaria L. exerts a more suppressive effect on inflammation. These findings suggest that these herbal extracts could be used as adjuvants in MSC-based therapies to enhance tissue repair and regeneration, particularly in conditions where inflammation and tissue damage are key challenges.

Acknowledgement

The authors would like to acknowledge the support of the Institute Karya Mulia Bangsa for providing the facilities and resources necessary to conduct this research. Special thanks to the donors who provided umbilical cord samples for the isolation of MSCs.

Authors' contributions

Dian Respati Ayu designed the study, conducted the experiments, and analyzed the data. Risky Chandra Satria Irawan contributed to the experimental design, data interpretation, and manuscript preparation. Salindri Prawitasari contributed in statistical analysis. Meirista Shindy assisted in the laboratory work and data collection. All authors reviewed and approved the final manuscript.

Competing Interests

The authors declare that there is no conflict of interest.

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