

NK Cells Induced by Ethanolic *Nigella sativa* Extract Inhibits Proliferation of Retinoblastoma Y79 Cell Line Through Cyclin D1 Pathway

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ABSTRACT

Nigella sativa can increase the immunomodulatory function of NK cells. This present study aimed to analyze the anti-proliferative effect of NK cells induced by *Nigella sativa* ethanolic extract on Y79 retinoblastoma cell line through the cyclin D1 pathway. Flow cytometry was used on co-cultured Y79 cells with NK cells induced by *Nigella sativa* ethanolic extract at various doses: 0, 25, and 100 µg/ml with incubation for 24 hours and a moi (multiplicity of infection) ratio of 1: 1 and 1: 5. ANOVA results showed a significant correlation ($p < 0.001$) between dose and moi interactions with cyclin D1 expression, and Y79 proliferation. The Tukey Post Hoc results showed the lowest cyclin D1 expression at a dose of 100 µg/ml and moi 1: 5 of 6.75 ± 0.30 . Whereas in the Tukey Post Hoc results, the proliferation showed decreased proliferation at a dose of 100 µg/ml with 1: 1 and 1: 5 moi. Path analysis showed the relationship between the induction of *Nigella sativa* ethanolic extract on NK cells with significant and negative cyclin D1 expression. Meanwhile, the path analysis relationship between cyclin D1 expression and proliferation showed a significantly positive relationship. So it is proven that the NK cell anti-proliferation pathway after induced by the ethanolic *Nigella sativa* extract is indeed through the cyclin D1 pathway. Cyclin D1 is one of the potent proliferation markers, a decrease in cyclin D1 expression will also be followed by a decrease in the proliferation of Y79 retinoblastoma cancer cells.

Keywords: Cyclin D1, retinoblastoma, NK cells, *Nigella sativa*, anti-proliferation

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INTRODUCTION

Retinoblastoma is the most common cancer found in children (Pandey, 2014). The rate of retinoblastoma cases in Indonesia reached 1: 15,000 to 1: 23,000 of total birth and it is accounted for 4% of the total cases of all cancers in children (Dimaras, 2015). Definitive therapy by using chemotherapy may lead to other problems due to the occurrence of side effects that can reduce the quality of life and vision (Del Sole et al., 2014). Therefore, currently, an alternative therapy that has more specific cytotoxic activity without damaging normal cells or giving any side effect is important to be developed.

NK cells use specific molecules on the surface of tumor cells that activate their cytotoxicity, thus enabling protection against normal cells (Barrow, 2019). Cancer patients experience failure to activate NK cells so that they are unable to eliminate tumor cells as they should. The fact that active NK cells have great potential to fight tumor cells can be a special reason to improve the anti-tumor activity by manipulating or inducing NK cells activity. The cytotoxic potential of NK cells against retinoblastoma cancer cells is included their pro-apoptotic and anti-proliferative activities.

Nigella Sativa (black cumin) is known to have immunity-stimulating, anti-inflammatory, hypoglycemic, antihypertensive, anti-plasma, antimicrobial, antiparasitic, antioxidant and anti-cancer effects (Srinivasan, 2018). Many studies have shown that *Nigella sativa* has an immune effect in increasing the response to antibodies and increasing the immune response (Ghasemi, Kasani, & Taherpour, 2014). *Nigella sativa* can increase the potency of cytotoxic of NK cells and functioned as anti-proliferative in tumor cells. Until now, there is still no research focused

on the increase of NK cells activity as immunomodulators using the ethanolic extract of *Nigella sativa* to induce anti-proliferative effects of retinoblastoma cancer cells. This study aims to investigate the anti-proliferative effect of NK cells induced by *Nigella sativa* extract on Y79 retinoblastoma cell line.

MATERIAL AND METHODS

Preparation of *Nigella sativa* extract

The identification of plant specimens was performed at Aretha Medika Utama, Biomolecular and Biomedical Research Center, Bandung, West Java, Indonesia. *Nigella sativa* seeds (0110320-B019) weighed for 200 gr was soaked in 1500 mL of 70% ethanol then filtered with Whatmann no 1 filtration paper. Further, the seeds were ready for extraction by maceration technique with 70% ethanol solution for 3 days. The filtrate was collected every 24 hours until it has no color. After that, the ethanol filtrate was evaporated until the extract was ready in the paste form (Patent No. IDP000043949, 2014).

Cell line culture and extract induction

The human Natural Killer (NK) cell line and the human retinoblastoma cancer cell line (Y79) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The NK cell line (ATCC® CRL-2408) or the Y79 cell line (ATCC® HTB-18) were inserted in a T25 flask containing 4 ml of complete growth medium (MEM- α (Biowest, L0475-500), 10% Fetal Bovine Serum (Biowest, S1810-5000), Myo-Inositol (SLB 51295), Folic Acid (SLB 57038V), 2-Mercaptoethanol (Gibco, 1628448), and 1% Antibiotic-Antimycotic (ABAM) (Gibco, 1772653)). After that, the cells were incubated in a 5% of CO₂ incubator at 37 °C. The cultured NK cells medium was replaced with the

new medium and added with *Nigella sativa* ethanol extract for 4 hours to induce the activation of NK cells. The cells were collected into a 15 mL tube and centrifuged at 1600 rpm for 5 minutes. The supernatant was removed and the pellet was resuspended with 1 mL of growth medium. The cells in the suspension were counted using a haemocytometer to determine the number of cells in 1 mL of cell suspension. Cells were then incubated in a 5% of CO₂ incubator at 37 °C. The cells were induced by *Nigella sativa* extract concentration 0 µg/ml, 25µg/ml, and 100 µg/ml. The cells were further incubated again in the incubator with 5% of CO₂ and 95% of humidity at 37°C for 4 hours.

Co-culture of Y79 cells and NK cell induced by *Nigella sativa* extract

Co-culture of Y79 cells and NK cells which were already induced by *Nigella sativa* extract was prepared. The co-culture method used was the Transwell method to see the interaction between Y79 retinoblastoma cells with NK cells. NK cells that had been induced by *Nigella sativa* extract were harvested and immediately put into a 15 mL tube, then centrifuged at a speed of 1600 rpm for 5 minutes. The pellet of NK cells was then washed using Phosphate Buffer Saline (PBS). After washing, the cells were again centrifuged to remove the PBS, then resuspended with 1 mL of NK cell growth medium. The NK cells that have been resuspended were inserted into the upper chamber 6-well of transwell plates containing Y79 cells in the lower chamber, with moi 1:1 (2 x 10⁵) and 1:5 (1 x 10⁶). These NK and Y79 cells (co-culture) were incubated for 24 hours in an incubator with a temperature of 37°C containing 5% of CO₂, and 95% of humidity.

Flow cytometry assay for cyclin D1 expression

The cells were washed two times by resuspension using 500 µL FACS Buffer (PBS + 2% FBS Fetal Bovine Serum) then centrifuged at 1600 rpm for 5 minutes. The supernatant was discarded, and the cell pellets were resuspended with 500 µL FACS Buffer. Samples were then stained using 5 µL reagent primary antibody which had

been conjugated with secondary antibody FITC conjugated (FITC: Ab Cyclin D1, 4: 1, mixed and incubated for 15-30 minutes in dark conditions at room temperature) and then incubated for 30-60 minutes at room temperature (dark room). After incubation time, the cells were washed with PBS (Phosphate Buffer Serum) for one time then added with 500 µL of FACS Buffer. The samples were analyzed using flow cytometry.

Flow cytometry assay for proliferation

The cells were washed 2x by resuspension using 500 µL FACS Buffer (PBS + 2% FBS) and then centrifuged at 1600 rpm for 5 minutes. The supernatant was discarded, and the cell pellets were resuspended with 500 µL FACS Buffer. The samples were then stained using 20 µL Ki67 FITC Conjugated and incubated for 30-60 minutes at room temperature (dark room). After incubation time, the cells were washed with PBS for one time then added with 500 µL of FACS Buffer. The samples were analyzed using flow cytometry.

Statistical Analysis

Statistical analysis was conducted using SPSS (version 16.0) software for Windows. Data were presented as Mean ± Standard Deviation. Significant differences among treatments were determined using the one-way Analysis of variance (ANOVA) and p < 0.05 were considered as statistically significant, along with Tukey significant difference, post hoc test and 95% confidence interval, and path analysis.

RESULT

Anti-proliferative effect of NK cells induced by ethanolic *Nigella sativa* extract via the cyclin D1 pathway.

Figure 1 shows flow cytometry with cyclin D1 depiction decreasing by the increase of *Nigella sativa* ethanolic extract doses given and moi NK ratio in co-culture. The results of the ANOVA analysis of the interactions between the extract dose and the moi ratio showed a significant difference to cyclin D1 in co-culture of Y79-NK cells induced by *Nigella sativa* extract with p < 0.001, p < α (0.05).

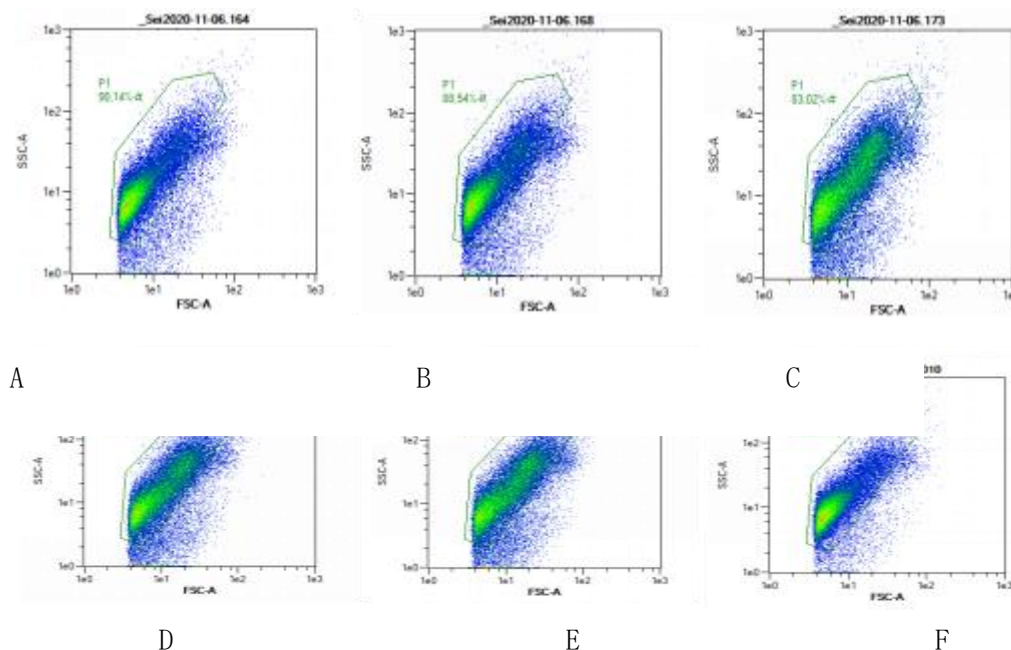


Figure 1. Results of cyclin D1 flow cytometry after Y79 co-culture with NK cells induced by *Nigella sativa* ethanolic extract.

A. cyclin D1 with Y79-NK moi 1: 1 cell co-culture and 0 µg / mL extract dose, B. cyclin D1 with Y79-NK moi 1: 1 cell co-culture and an extract dose of 25 µg / mL, C. cyclin D1 with Y79-NK moi 1: 1 cell co-culture and 100 µg / mL extract dose, D. cyclin D1 with Y79-NK moi 1: 5 cell co-

culture and 0 µg / mL extract dose, E. cyclin D1 with cell co-culture Y79-NK moi 1: 5 and an extract dose of 25 µg / mL, F. cyclin D1 with Y79-NK moi 1: 5 cell co-culture and an extract dose of 100 µg / mL.

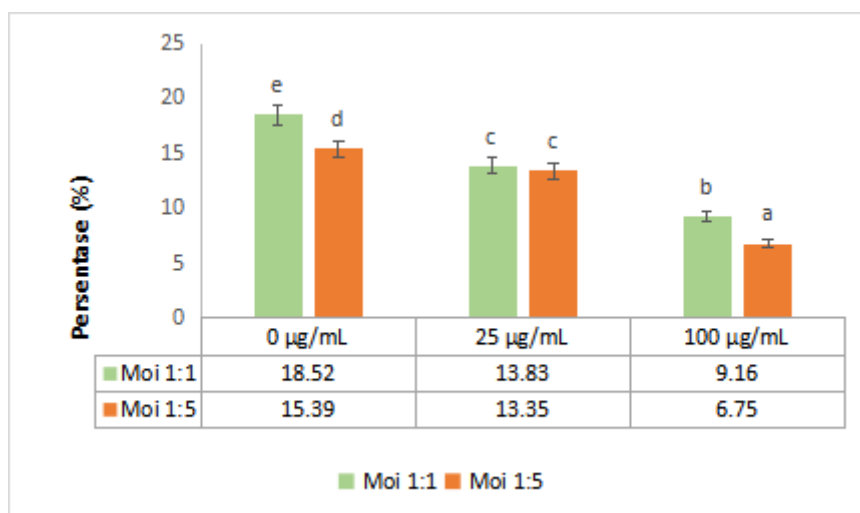


Figure 2. Examination of cyclin D1 expression after co-culture of Y79-NK cells induced by ethanolic *Nigella sativa* extract using flowcytometry.

Two doses of extract treatment were used, namely 25 and 100 µg / mL with one control dose of 0 µg / mL. The green color in the bar chart shows the cyclin D1 expression at the moi 1: 1 ratio and the orange color show the cyclin D1 expression at the 1: 5 moi ratio. ANOVA test showed significant results with $p < 0.001$. Letters a, b, c, d, e, and f show the results of the Tukey Post Hoc analysis test. Letter a indicates the lowest cyclin D1, b has higher cyclin D1 than a, C has higher than a and b, and d has highest cyclin D1. In the control group, the letter e = 18.52 ± 1.15 cyclin D1 is the highest, d = 15.39 ± 0.19 is lower than e, c = 13.83 ± 0.51 and 13.35 ± 0.13 is lower than d and e, b = $9.16 \pm$

0.18 is lower than c, d, e, while the letter a show the lowest cyclin D1 expression, namely 6.75 ± 0.30 .

Furthermore, the Tukey Post Hoc test was carried out (Figure 5.6.2), the induction of NK cells by *Nigella sativa* extract in various dose and the ratio of moi resulting different effects to cyclin D1 expression. It appears that cyclin D1 is significantly decreased at the extract dose of 100 µg / mL with moi 1: 5.

The NK cell proliferative test induced by ethanolic *Nigella sativa* extract used Ki67 test.

Y79 cells that have been co-cultured with NK cells induced by *Nigella sativa* ethanolic extract were then taken for proliferation examination using the Ki67 test.

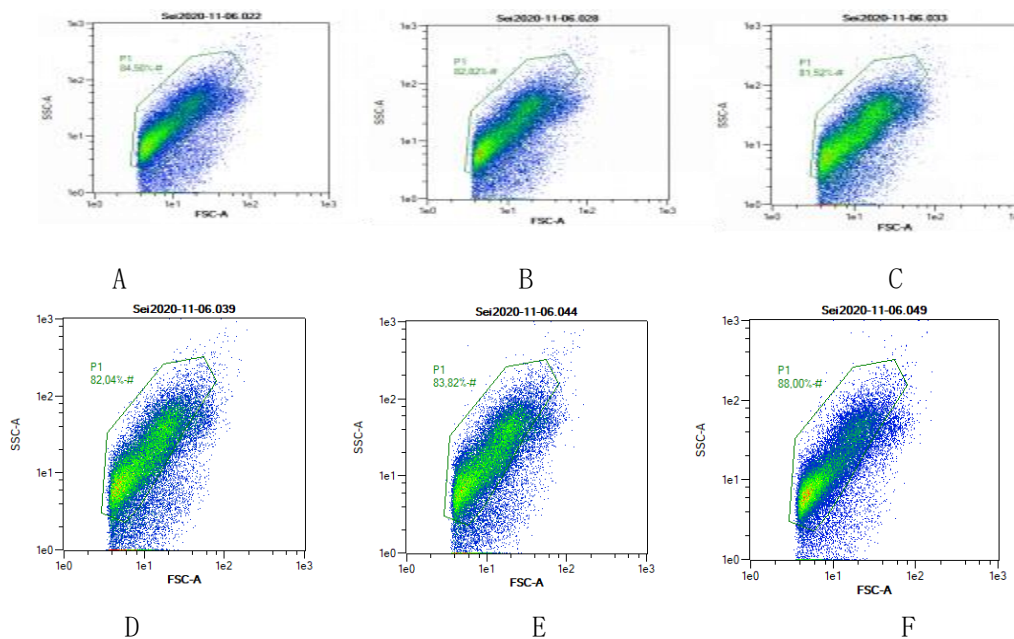


Figure 3. Results of flowcytometry of Y79 cell proliferation with KI67 after co-culture with NK cells induced by *Nigella sativa* ethanolic extract.

A. Y79 cells with 1: 1 moi co culture and 0 µg / mL extract dose, B. Y79 cells with 1: 1 moi co culture and 25 µg / mL co culture extract, C. Y79 cells with 1: 1 co culture moi and extract dose 100 µg / mL, D. Y79 cells with 1: 5 co culture moi and 0 µg / mL extract dose, E. Y79 cells with 1: 5 co culture moi and 25 µg / mL extract doses, F. Y79 cells with Moi co culture 1: 5 and extract dose of 100 µg / mL.

Figure 3 shows flow cytometry with a picture of proliferating cells decreasing by the increase of the *Nigella sativa ethanolic* extract doses and the ratio of moi NK in co-culture. The results of the ANOVA analysis of the interactions between the extract dose and the moi ratio showed a significant difference in Y79 cell proliferation in co-culture with NK cells induced by *Nigella sativa ethanolic* extract with $p < 0.05$, $p < \alpha (0.05)$.

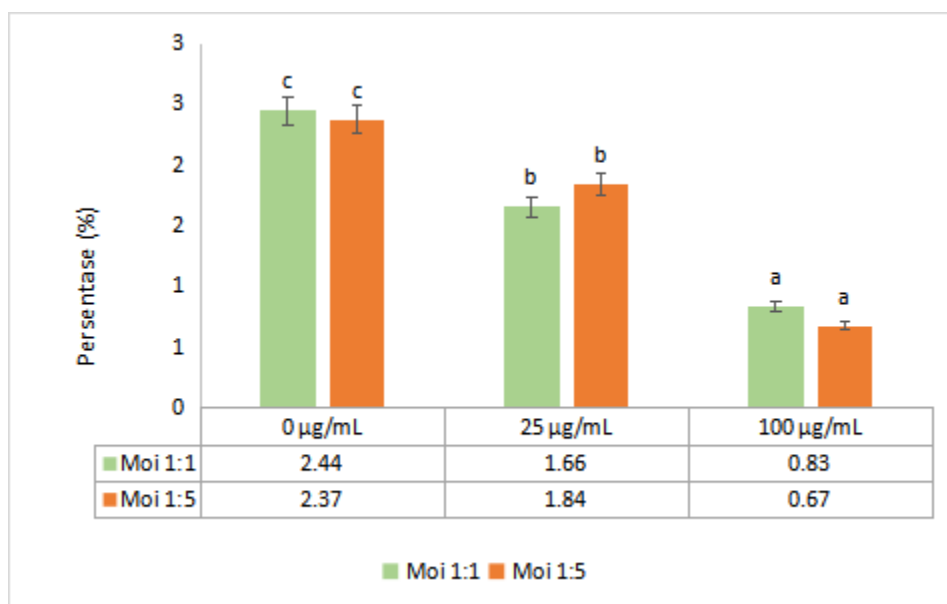


Figure 4. Y79 cell proliferation test results during co-culture with NK cells induced by ethanolic *Nigella sativa* ethanolic extract using flow cytometry.

Two doses of extract treatment were used, namely 25 and 100 µg / mL with one control dose of 0 µg / mL. The green color in the bar chart shows the proliferation at the 1: 1 moi ratio and the orange color shows the proliferation expression at the 1: 5 moi ratio. ANOVA test showed significant results with $p < 0.05$. Letters a, b, and c show the results of the Tukey Post Hoc analysis test. Letter a indicates the lowest proliferation, letter b has higher proliferation than a, and c has higher proliferation than and b. In the control group, the letter c = 2.44 ± 0.09 and

2.37 ± 0.10 , $b = 1.66 \pm 0.21$ and 1.84 ± 0.17 were lower than c, while letter a showed the lowest proliferation, namely 0.83 ± 0.07 and 0.67 ± 0.07 .

Furthermore, the Tukey Post Hoc test was carried out (Figure 5.8.2), the results of the NK cells induction by *Nigella sativa* ethanolic extract with different dose and the ratio of moi showed different results on Y79 cell proliferation. It appears that Y79 cell proliferation is significantly reduced at the extract dose of 100 µg / mL with moi 1: 5 or 1: 1.

Path analysis of the correlation between NK cells induced by ethanolic *Nigella sativa* extract and Y79 cells proliferation through the cyclin D1 pathway.

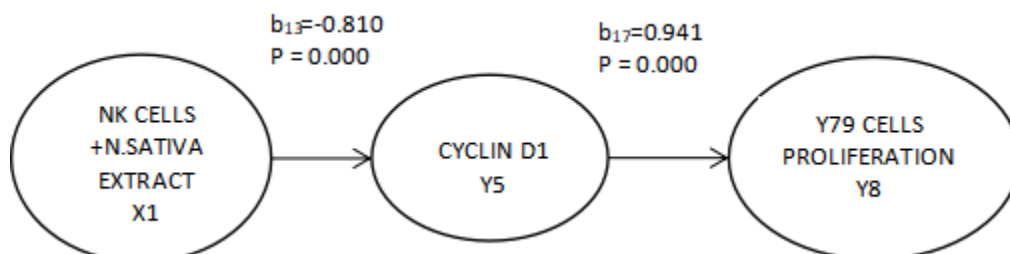


Figure 5. Path analysis of the direct correlation between NK cells induced by ethanolic *Nigella sativa* extract with cyclin D1 expression and direct correlation of cyclin D1 with Y79 cell proliferation.

The direct effect of NK cells that have been induced by *Nigella sativa ethanolic* extract (X1) on cyclin D1 (Y3) shows a significance value of 0.000, and it is known that NK cells induced by *Nigella sativa* have a direct effect on cyclin D1 by 65.6% (R2). From the regression equation, it was found that if there was an increase in NK cells induced by *Nigella sativa* extract by 1%, a 1.8% reduction in cyclin

D1 would be obtained with a strong effect ($R = 0.810$). The path coefficient (b) between the NK cell interactions induced by *Nigella sativa* ethanolic extract (X1) against Cyclin D1 (Y5) is negative / inversely proportional to - 0.810 with a p value of 0.000.

The direct effect of cyclin D1 (Y5) on the proliferation (Y8) of Y79 cells shows a significance value of 0.000, and it is

known that cyclin D1 has a direct effect on the proliferation of 88.5% (R2). From the regression equation, it was found that if there was an increase in cyclin D1 by 1%, there would be an increase in the proliferation of 16.7% with a fairly strong effect ($R = 0.941$). The path coefficient (b) between the variable of Cyclin D1 (Y5) on Y79 cell proliferation (Y8) is positive / proportional to the value of 0.941 with a value of $p = 0.000$.

DISCUSSION

The cytotoxic potential of NK cells against retinoblastoma cancer cells includes their pro-apoptotic and anti-proliferative cancer cells. NK cells that have been induced by *Nigella sativa* ethanolic extract at 0 µg/ml, 25 µg/ml, and 100 µg/ml and co-culture with Y79 retinoblastoma cancer cells were then tested by flow cytometry on Y79 cells at moi 1:1 and 1:5 to see the proliferation that occurs after treatment. One of the proteins that plays a role in cell survival is cyclin D1. Cyclin D1 functions as a key sensor and cell extracellular signal integrator in the early to mid-G1 phase, mediating the binding of Cyclin Dependent Kinase (CDK) and histone acetylase [p300 / cAMP response element-binding protein-binding protein (CBP) and P / CAF] and histone deacetylase to modulate structure local chromatin from genes involved in the regulation of cell proliferation and differentiation.

The results of ANOVA analysis of variance (table 1) of this study regarding cyclin D1 expression showed that the interaction between doses of the ethanolic extract of *Nigella sativa* given and the ratio of moi Y79 cells and NK cells in co-culture showed a significant difference with $p < 0.001$, $p < \alpha (0.05)$. This means that the expression of cyclin D1 is determined by the extract dose and the number of NK cells in the co-culture.

The results of ANOVA test (table 2) of our study on proliferation (Ki67) showed that the interaction between *Nigella sativa* ethanolic extract doses and the ratio of moi gave significant differences with p value < 0.05 , $p < \alpha (0.05)$. The Tukey Post Hoc follow-up test (Figure 4) showed that three doses of *Nigella sativa* ethanolic extract induced on NK cells and the comparison of moi gives different results on Y79 cell proliferation. The higher the extract dose induced on NK cells, the decrease in Y79 cell proliferation will be obtained. The best dose that results in the lowest Y79 cell proliferation was at a dose of 100 µg / mL both at 1: 1 moi (0.83 ± 0.07) and 1: 5 moi (0.67 ± 0.07) ratios. This means that the proliferation of Y79 cell is determined by the extract dose and the number of NK cells in the co-culture.

The results of the Tukey Post Hoc follow-up test (Figure 2) showed that there were differences in the expression of cyclin D1 in the three induction doses of the ethanolic extract of *Nigella sativa* on NK cells that were co-culture with Y79 cells. It can be seen from the bar chart illustration, the higher the dose of the extract induced in NK cells and the greater the ratio of moi ratios given in the treatment will decrease the cyclin D1 expression. The percentage of cyclin D1 expression appeared to be the lowest at the 100 µg / mL *Nigella sativa* ethanolic extract dose with a moi ratio of 1: 5 co-culture Y79-NK (6.75 ± 0.30). The Tukey Post Hoc follow-up test (Figure 4) showed the three doses of *Nigella sativa* ethanolic extracts induced on NK cells and the ratio of moi gave different results on Y79 cell proliferation. The higher the extract dose induced on NK cells, the decrease in Y79 cell proliferation will be obtained. The best dose that produced the lowest Y79 cell

proliferation was at a dose of 100 µg / mL both at 1: 1 moi ratio (0.83 ± 0.07) and 1: 5 moi (0.67 ± 0.07).

Similar to previous studies that examined *Nigella sativa* ethanolic extract and its active ingredient thymoquinone (TQ), it was found that there was the suppression of tumor regulation accompanied by decreased p-Akt, IAP1, IAP2, Bcl-2, Bcl-xL, XIAP, survivin, COX-2, cyclin. D1, and VEGF as gene products regulated by NF-κB due to their inhibitory effect on TNF-α (Mollazadeh, Afshari, & Hosseinzadeh, 2017). Cyclin D1 is induced by growth factors including epithelial growth factor and IGF-I and IGF-II; amino acid; lysophosphatidic acid; and hormones including androgens, retinoic acid, and peroxisome proliferator-activated receptor (PPAR); cyclin D1 is secreted by adipocytes and gastrointestinal hormones such as gastrin, each of which regulates cyclin D1 expression specifically according to the target cell type (Love, 2018).

Cyclin D1 is a holoenzyme regulatory subunit that phosphorylates and, together with sequential phosphorylation of cyclin E / CDK2, inactivates the cell cycle inhibiting the function of the retinoblastoma protein (pRb). pRb serves as the gatekeeper of the G1 phase and passes the boundary point to DNA synthesis. Overexpression of cyclin D1 is known to correlate with early cancer and the risk of tumor development and metastasis. However, a number of studies have shown a lack of correlation between increased cyclin D1 expression and increased DNA synthesis in tumors (Reena Rachel John, N Malathi, C Ravindran, 2017). pRb is thought to suppress certain genes that are active in the S phase of the cell cycle through active repression of E2F transcription activity, and this activity is stopped by cyclin D1. Genetic aberrations in the regulatory circuits that regulate cell transit through the G1 phase of the cell cycle are common in human cancers, and overexpression of cyclin D1 is one of the most frequently observed changes. One study showed that cyclin D1 overexpression could serve as a determinant oncogene in cell cycle regulatory function. Increased cyclin D1 occurs early in tumorigenesis. Cyclin D1 not only controls cell proliferation but also contributes to hormonal secretion abnormalities (Reena Rachel John, N Malathi, C Ravindran, 2017).

In most types of cancer, including lung, breast, sarcoma, and colon cancer, cyclin D1 results from overexpression of oncogenic signal induction, not through clonal somatic mutation mechanisms or rearrangements in the cyclin D1 gene. The common single nucleotide A / G polymorphism (A870G) in intron 4 of the cyclin D1 gene results in two mRNA transcript differences (isoforms a and b). Spliced transcript RNA (isoform b) alternately encodes a protein in which the last 55 amino acids from the C end of cyclin D1 are replaced by a shorter sequence encoded by intron 4. This truncated form of cyclin D1 is associated with the incidence of tumors, including lung cancer, colon cancer, and other types of cancer. Mice deficient in Cyclin D1 show retinal apoptosis, failure of terminal alveolar growth in response to pregnancy, changes in fat metabolism and hepatic steatosis, as well as defects in macrophage cell migration (Ortiz et al., 2017).

The path analysis test (Figure 5) shows that there is a significant, negative, and inversely proportional direct effect between NK cells that have been induced by *Nigella sativa* ethanolic extract on cyclin D1 expression of 65.6%, where every 1% increase of the *Nigella sativa* extract induced on NK cells will decrease cyclin D1 expression by 1.8%. While the results of the path analysis also showed

that there was a direct effect of cyclin D1 on the proliferation which was significant, positive, and directly proportional to 88.5%, where every 1% increase in cyclin D1 would also increase the Y79 cell proliferation by 16.7%. It means that anti proliferative effect of NK cell induced by ethanolic extract primarily due to cyclin D1 expression.

This study is in line with the results of other studies using aqueous and ethanolic extracts of *Nigella sativa* seeds, either alone or in combination have been shown to cause anti-proliferative effects of MCF-7 human breast cancer cells with or without H₂O₂ (IO, Rowshan A, & Begum', 2003). The anti-proliferative activity of *Nigella sativa* works synergistically with the function of NK cells as an anti-cancer agent through anti-proliferative mechanisms, as in previous studies, which stated that NK cells inhibit tumor growth and cytotoxic activity in killing ovarian cancer cells through apoptosis induction, inhibition of proliferation and suppression, invasion and migration of tumor cells (Sun et al., 2018). Researchers assume the active ingredient that role in the ethanolic extract of *Nigella sativa* is thymoquinone, which will be confirmed again with the results of the next study.

CONCLUSION

This study succeeded in proving the effect of increasing the potency of NK cells using the induction of the ethanolic *Nigella sativa* extract as an anti-proliferation against Y79 cancer cells through the cyclin D1 pathway. This study gives hope that there is an alternative therapy for retinoblastoma sufferers that is safer and work more specific so that it reduces the possibility of damage to surrounding normal cells and does not interfere with the patient's quality of life.

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