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Methanolic Leave Extract of *Abrus precatorius* Induces Natural Killer Cell Cytotoxicity Towards Breast Cancer Cells

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Abstract

Abrus precatorius (*A. precatorius*) or locally known as rosary pea, is a type of bean that is traditionally used to treat respiratory disorders. Although the seeds are mainly used, there is lack of focus on the other plant parts, which may be important. In this study, the ability of the *A. precatorius* methanolic leave extract (APME) to induce anticancer immune response by activating NK cells was analysed *via* a co-culture experiment of the NK cells using breast cancer MDA-MB-231 cell lines. Subsequently, analysis of target cell deaths by a flow cytometric analysis was conducted followed by evaluation of cytokines, interleukin-2 (IL-2) and interferon-gamma (IFN-γ) levels. Degranulation of the cytotoxic granules was determined by quantifying perforin (PRF-1) and granzyme B (GzmB) using ELISA. APME activates NK cells obtained from healthy donors since the stimulated NK cells can induce apoptosis in target cells *in-vitro* with increased IFN-γ and PRF-1 levels seen. The findings indicate the ability of *A. precatorius* leaves extract to stimulate NK cells obtained from healthy donors and trigger its cytotoxicity in MDA-MB-231 cells.

INTRODUCTION

Elimination of cancer cells by the immune system is one of the many desirable strategies to combat cancer naturally. The process utilises the highly specific coordinated immune system to eliminate cancerous cells from the host without interfering or damaging the surrounding cells. Therefore, anti-cancer agents that can augment the cytotoxicity of the immune cells in particular the Natural Killer (NK) cells, are in high demand, with many researchers seeking to dwelve deeper into understanding the mechanism [1]. NK cells are large granular lymphocytes that can eliminate various stressed or abnormal cells and even kill target cells without any preliminary sensitization step [2,3]. NK cells, which are characterised by the presence of surface marker protein-expressing CD56 and CD16, are specifically identified by $CD56^{+}/CD3^{-}$, where CD3 is specific for T-lymphocytes and is not expressed by the NK cells [4]. NK cells are the preferred focus in immunotherapy due to its independence on antigen specific T-cells and its rapid pro-inflammatory cytokines secretion that can initiate the adaptive immune response [5]. NK cells release the cytolytic granules upon bridging immune synapses with the target cells including perforin and granzymes. These proteins can induce target cell lysis, leading to degranulation which is an indirect measurement of NK cells cytotoxicity [6].

The discoveries of plant containing immunomodulatory properties remain cutting-edge and is in demand especially to enhance current immunotherapy practices. To date, several studies have been conducted to uncover the potential of plant extracts or isolated phytoconsitutents in immunomodulation of NK cells. Studies have suggested that administration of a high concentration of revasterol promotes target cell apoptosis *via the* caspase signalling pathway [7]. In another study, a fraction from *Caesalpinia spinosa,* is confirmed to be rich in gallotannin which

acts as an anti-tumour agent in breast carcinoma and melanoma [8]. Additionally, Lee and Cho [9] demonstrated that curcumin augments the cytotoxic effect of NK-92 on MDA-MB-231. *Abrus precatorius* (*A. precatorius*) L. (Fabaceae) or also known as rosary pea, is a type of bean that is a thin, perennial ornamental climber with long, pinnate-leafleted leaves. The roots and leaves of *A. precatorius* are utilised in the treatment of respiratory symptoms such as asthma and cough [10] as well as in the treatment of tuberculosis, whooping cough and bronchitis in South Africa [11]. Nevertheless, to date most reported studies on *A. precatorius* are conducted on the seeds, particularly on lectin and abrin. For example, abrin was confirmed to enhance NK cells activity in tumour-bearing mice significantly [12]. Additionally, antibiotic growth promoters (AGPs) and peptides derived from *A. precatorius* seeds have been reported to exert immunostimulatory effects in Dalton's lymphoma (DL)-bearing mice [13]. Another study on the seeds conducted on another *Abrus* species known as *A. agglutinin* also successfully demonstrated their ability to stimulate spleen-derived NK *in vitro* for cytotoxic effect against Dalton's lymphoma ascites cells (DLAC) [14].

In our previous study, we confirmed the ability of *A. precatorius* methanolic leaves extract (APME) in inducing apoptosis in MDA-MB-231 cells [15]. Therefore, in this study, the ability of APME to induce the cytotoxic activity of freshly isolated NK cells from healthy individuals and breast cancer patients against the MDA-MB-231 cells *in vitro* was investigated.

MATERIALS AND METHODS

Plant Collection & Extraction

A. precatorius leaves were collected from Kampung Sabak, Pengkalan Chepa, Kelantan, Malaysia. It was authenticated by Dr. Rahmad Zakaria from the Herbarium Unit, School of Biological Sciences, Universiti Sains Malaysia. The voucher specimen (USM 11730) was also submitted and deposited in the same place for future references. The collected leaves of *A. precatorius* were cleaned and oven-dried at 50ºC, and subsequently grinded to a fine powder using a mechanical grinder.

About 22 g of ground *A. precatorius* leaves were extracted successively using hexane, ethyl acetate, and methanol in a Soxhlet apparatus (Buchi). Each extraction used 250 mL solvent, maintaining a solvent-to-solid ratio of approximately 11:1 (v/w). Each solvent cycle was conducted for 6 hours at a bath temperature of 65 ± 2°C for hexane and ethyl acetate, and 70 ± 2°C for methanol, corresponding to roughly 8–10 siphon cycles per solvent. Extracts were concentrated using a rotary evaporator under reduced pressure and dried at 40 °C. The extract yields were 4.6% (hexane), 3.2% (ethyl acetate), and 6.9% (methanol) (w/w). The methanolic extract (APME) was previously identified to induce apoptosis in MDA-MB-231 cells [15]. In this study, the ability of APME to induce NK cytotoxicity towards MDA-MB-231 cells was further explored.

MDA-MB-231 cells

Human triple negative breast cancer cell line, MDA-MB-231 was purchased from ATCC (US). The cells were cultured and maintained in a complete Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Foetal Bovine Serum (FBS) (Gibco) and 1% penicillin streptomycin (Gibco). Confluent MDA-MB-231 cells were harvested by trypsinization.

Donors for Isolation of NK Cells

The study protocol was reviewed and approved by the Human Research Ethics Committee of Universiti Sains Malaysia (JEPeM-USM) (JEPeM USM Code: USM/JEPeM/17100566) which complies with the Declaration of Helsinki. Three donors for both healthy and cancer individuals were chosen. All healthy donors were female with no chronic disease, were not consuming any immunosuppressive drugs, were non-smokers and were not pregnant. For cancer patients, the donors were female diagnosed with breast cancer, not receiving any treatment yet including chemotherapy and radiotherapy or consuming any herbal and medicinal plants, were non-smokers and not pregnant.

The cancer patients were at different stages of the disease where two were at stage 4 while another one was at stage 3. Donors were of Malay ethicity, aged between 18 and 45 years, and none reported hormonal therapy or irregular menstrual cycles at the time of sampling, as these variables can affect NK cells function [16]. Each donor provided 20 to 25 ml of peripheral blood, from which 5 ml aliquots were used per independent assay.

Isolation of NK Cell

NK cells were isolated from peripheral blood using the Ficoll density-gradient method as originally described by Böyum [17], followed by negative magnetic selection using the Human NK Cell Isolation Kit (Miltenyi Biotec). Whole blood samples (5 ml) from donors were collected in BD Vacutainer Blood Collection tubes containing EDTA. The collected blood was diluted in PBS with ratio of 1:3 (v/v). The diluted blood was carefully layered on Lymphocyte Separation Media, LSM (Capricorn Scientific) and were centrifuged (400 g for 30 min) at room temperature. The buffy coat was carefully aspirated and transferred into a new 15 ml tube. Approximately 30 ml of PBS was added to the tube before centrifugation (300 g for 10 min). The process was repeated twice.

The pellet collected contained lymphocytes, and the pellet was resuspended in an RPMI 1640 medium before being transferred into a 75 cm^3 flask. The step was followed by an overnight incubation in a humidified 5% carbon dioxide (CO_2) incubator at 37°C. The process was necessary to remove any monocytes which adhere to the flask plastic surface [18]. After a 24 h incubation, the medium was centrifuged to allow collection of lymphocytes. NK cells were isolated using the Human NK Cell Isolation Kit by negative selection, following the manufacturer's instructions. The NK cells count was determined using the Trypan Blue exclusion assay.

NK Cell Staining

The number of NK cells was determined by staining the cells with PE-conjugated anti-CD56 (Santa Cruz) antibodies via a flow cytometry. The collected cells (100 µl) were washed twice with a 1 X PBS. Bovine Serum Albumin (BSA) (1 ml) was added to the cells followed by a 10 min incubation at room temperature. Subsequently, 5 µl of the PE-conjugated anti-CD56 and FITC-conjugated anti-CD3 antibodies were added and were well-mixed. The suspension was incubated for another 30 min on ice. Then, the cells were washed with PBS and were centrifuged (300 g) twice to remove any excess of antibodies. The pellet was resuspended with 500 µl PBS before the determination of NK cells by a flow cytometry FACSCANTO II (BD Bioscience). The data was analysed with an FCS Express Cytometry 7 software (De Novo, Sieera Amdre, USA).

NK Cells Proliferation Assay

The optimum period for NK cells proliferation from healthy individuals incubated with APME was determine at three different incubation times (24, 48 and 72 h). Isolated NK cells were first seeded into the 96 wells plate. APME was added into each well starting via serial dilution (200 - 1.56 µg/ml) which included the minimum 50% inhibitory concentration (IC_{50}) (26.4 µg/ml) as previously reported [15]. Dimethyl sulfoxide (DMSO) (<1%) was added into the wells as negative control. After each incubation point, 20 µl of MTT (5 mg/ml) was added into the well, followed by a 4-hr incubation. Then, 100 µl of DMSO was added into the wells to dissolve the formazan and the plate was read at 570 nm which directly represents the relative cell number as compared to the control group [19]. The experiment was done in triplicate each time, using different individuals. Finally, the percentage of cell viability was further determined by dividing the absorbance of the treated cells with the absorbance of the control and multiplied by a hundred.

NK cells co-culture with MDA-MB-231 cells

The experiment was performed to evaluate the potential of APME to induce anticancer immune response by activating the NK cells. Briefly, freshly isolated NK cells (effector) from each donor were pre-incubated with APME at concentration of 26.4 µg/ml for 4 hr before being added into the pre-seeded (overnight) MDA-MB-231 (target) cell culture. The ratio of effector to target was set at 20:1 based on the methods detailed in Ismail et al. [20] and Nishimura et al. [21]. The step was done bearing in mind the fact that NK cells co-culture with adherent cells [22], specifically with MDA-MB-231 cells [23]. Following the incubation step, the media was collected into a fresh tube and the adhered MDA-MB-231 cells were harvested by trypsinization, combined with the collected media followed by centrifugation (300 g) to separate the cell pellet from the supernatant. The media (supernatant) was transferred into fresh 1.5 ml tubes and was kept at -80°C. The collected pellet containing both NK cells and MDA-MB-231 cells were resuspended with RPMI 1640 medium and were divided into two fresh tubes for further analysis.

Apoptosis Assay

Determination of the percentage of apoptotic MDA-MB-231 cells in the culture was done by apoptotic staining of the target cells using the AnnexinV-FITC/PI. The apoptosis assay was performed according to the manufacturer protocol, Annexin V-FITC Detection Kit I (BD Bioscience) and sample readings were done using FACSCANTO II (BD Bioscience). The yielded data was analysed using an FCS Express Cytometry 7 software (De Novo, Sieera Amdre, USA). The percentage of NK cells cytotoxic activity for each donor category was determined by the following formula: ([percentage of cell deaths by effector – percentage of cell deaths without effector] / [100 – percentage of cell deaths without effector]) x 100 [21].

1

Enzyme-linked Immunosorbent Assays

Enzyme-linked Immunosorbent Assays **(**ELISA) were performed to quantify expression levels of cytokines, interleukin-2 (IL-2) and interferon gamma (IFN-γ); and cytotoxic granules protein, perforin (PRF-1) and granzyme B (GzmB). The assay was performed according to the manufacturer's protocol (Elabscience). Then, the optical density (OD value) of each well was determined with a micro-plate reader at absorbance of 450 nm.

Statistical Analysis

The data obtained were expressed as mean ± SD of three repeated experiments. The level of statistical significance among the group was tested using a repeated measure one-way ANOVA, followed by Tukey's multiple comparison test. The level of significance between the two main groups were tested using either paired or unpaired t-tests. The difference was considered significant when $p<0.05$. The normality of each data set was determined by Shapiro-Wilk normality test. Analyses were all done using GraphPad Prism7 (Graphpad Software, La Jolla, CA, USA).

RESULTS

Higher Isolated NK Cell in Healthy Donor

NK cells were isolated from three healthy donors and three breast cancer patients. The average count of NK cells isolated from healthy donors was significantly higher (7.72 x 10^5 cells/ml) than that isolated from cancer donors (3.8 x 10^5 cells/ml). It should be noted that all data were obtained from three independent donors per group as approved by JEPeM-USM, which represents a limited sample size. Therefore, while consistent trends were observed, the following results should be interpreted with caution due to limited statistical power.

Ability of APME to induce proliferation of NK cells

In this experiment, the ability of APME to induce NK cells proliferation, was analysed in various concentrations at three different time points. Generally, the proliferation of NK cells increased with increased in APME concentration **(Fig. 1).** At 24 h, NK cells proliferation increased until 25.0 - 26.4 µg/ml and started to decline at 50 µg/ml, only to increase again at 100 µg/ml. The proliferation pattern was fluctuating at 48 and 72 h. Significant ($p<0.05$) higher NK cells proliferation was observed at APME IC_{50} value at 24 h compared to other time points. Therefore, this APME concentration (26.4 µg/ml) was chosen for the NK co-culture with MDA-MB-231 experiment.

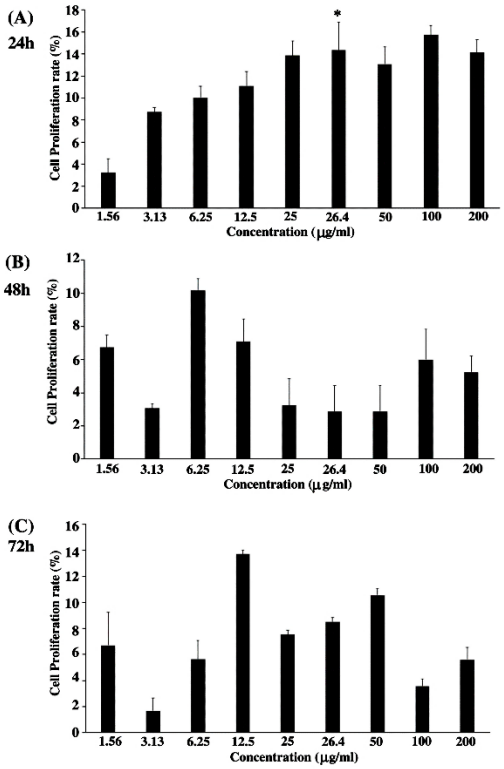

Fig. 1. NK cells proliferation treated with *A. precatorius* methanol extract for (A) 24h, (B) 48h, and (C) 72h. The results were expressed as mean ± SD of three independent experiments. *$p<0.05$, compared between APME IC_{50} (26.4 µg/ml) at 24h to the other time points.

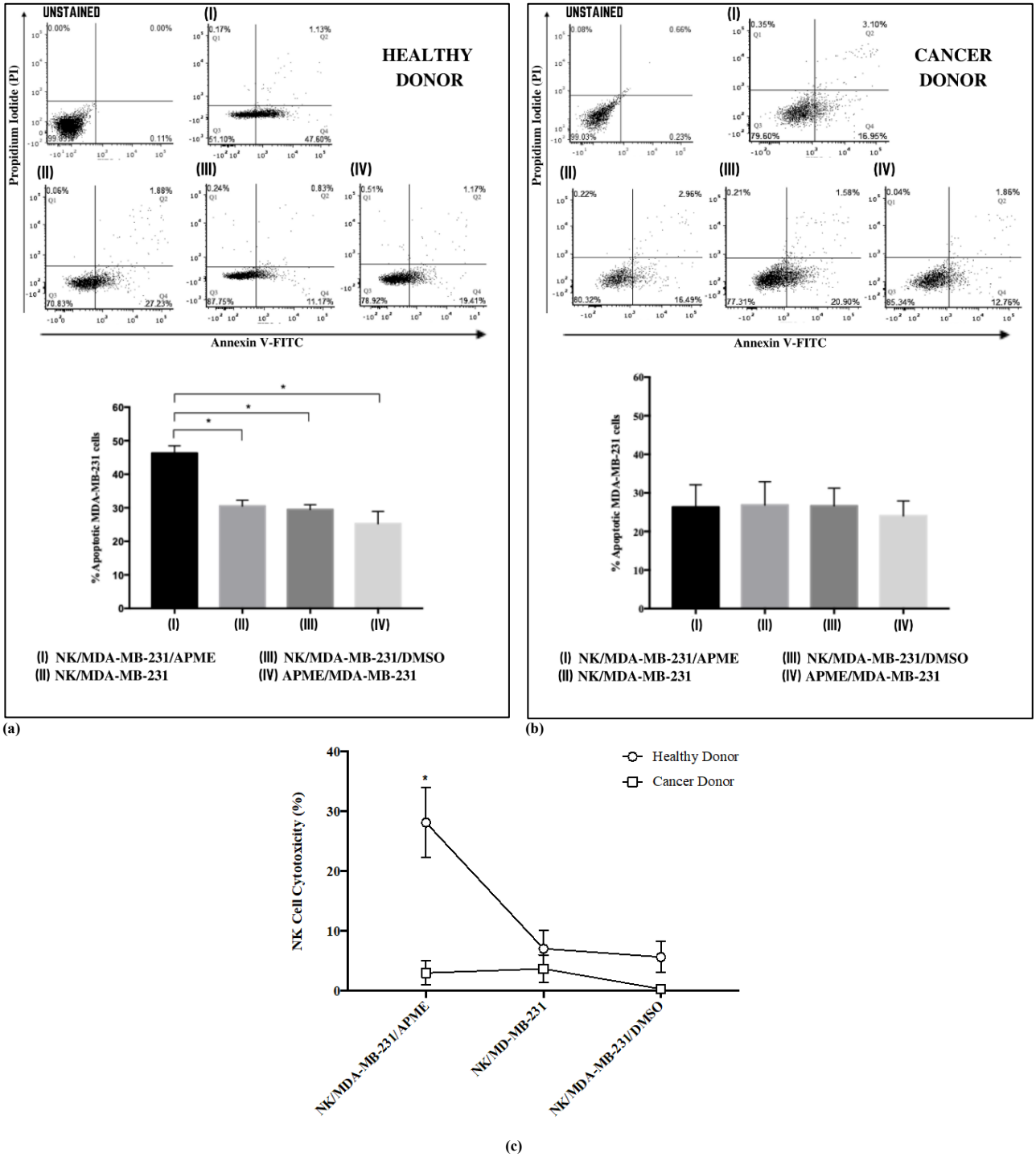

Fig. 2. APME-treated NK cells induced MDA-MB-231 cell death. Percentage of apoptotic MDA-MB-231 cells induced by NK from **(a)** healthy donors and **(b)** cancer donors. **(c)** Calculated NK cells cytotoxic activity. The results were expressed as mean ± SD of three independent donor for each group. *p<0.05, compared between the MDA-MB-231 apoptotic cell death induced by APME-treated NK cells (NK/MDA-MB-231/APME) to the rest of the groups.

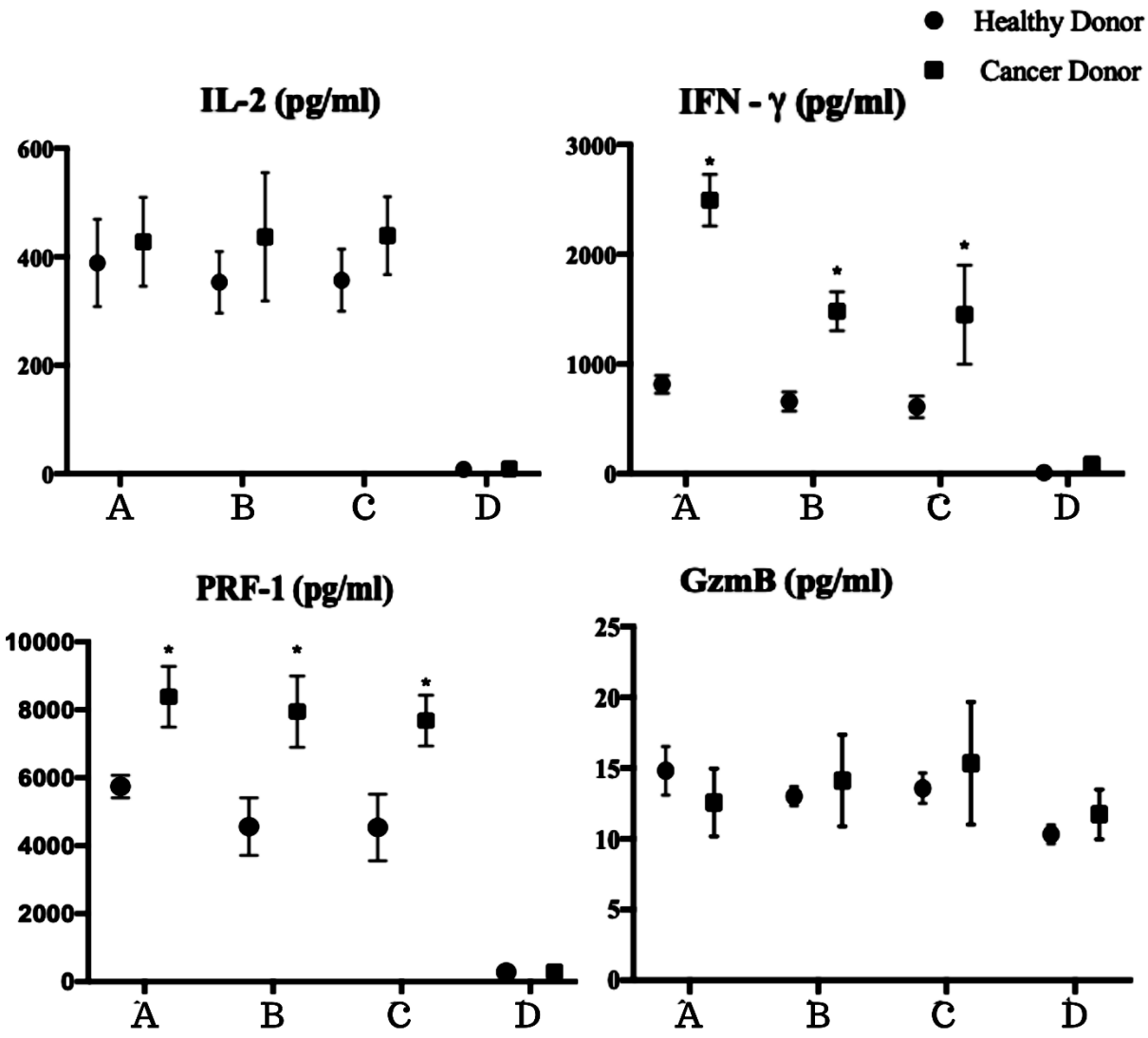

Fig. 3. Cytokines and Cytotoxic Granules Protein quantification following AMPE-induced NK cells co-culture with MDA-MB-231 cells. (a) IL-2 and, (b) IFN-γ secretions from NK cells obtained from healthy and cancer donors. (c) PRF-1 and (d) GzmB proteins expression from both NK cells obtained from healthy and cancer donors. The results were expressed as mean ± S.D of three independent donor for each group. *p<0.05, compared between healthy and cancer donors; #p<0.05, compared between APME-treated NK cells (NK/MDA-MB-231/APME) to the rest of the groups, in both donors respectively. In the graphs, the letters A, B, C and D in the x-axes indicate A- NK/MDA-MB231/APME, B- NK/MDA-MB-231, C- NK/MDA-MB231/DMSO and D- NK only.

APME-treated NK cells induced MDA-MB-231 cell death

Next, to assess the ability of APME-induced NK cells in inducing apoptosis and cell death in MDA-MB-231 cells, the cells collected following treatment were subjected to an Annexin V/PI apoptosis assay. The treatment groups were 1) NK/MDA-MB-231/APME; 2) NK/MDA-MB-231; 3) NK/MDA-MB-231/DMSO and 4) APME/MDA-MB-231. In this experiment, APME-treated NK cells from healthy donors significantly ($p<0.05$) induced MDA-MB-231 apoptosis (46.28 ± 2.2%) when compared to other treatment groups **(Fig. 2a)**. However, it could not stimulate NK cells from cancer donors **(Fig. 2b)**. The percentage of NK cell cytotoxicity demonstrated that APME-treated NK cells from healthy donors significantly ($p<0.05$) showed a higher percentage of NK cells cytotoxic activity when compared to the other treatment group **(Fig. 2c).**

Cytokines and cytotoxic granules protein quantification following AMPE-induced NK cells co-cultured with MDA-MB-231 cells

ELISA was performed to quantify the levels of IL-2, IFN-γ and cytotoxic granules protein like PRF-1 and GzmB in the media collected following NK cells co-culture with MDA-MB-231 cells. The expression of IL-2, IFN-γ, PRF-1 and GzmB presents the treatment groups 1) NK/MDA-MB-231/APME 2) NK/MDA-MB-231, 3) NK/MDA-MB-231/DMSO and 4) NK only **(Fig. 3)**. In healthy donors, IL-2 secretion in Group 1 was slightly higher (388.7 ± 80.6 pg/ml) as compared to Groups 2 (353 ± 56.5 pg/ml) and 3 (356.9 ± 57.33 pg/ml) although the finding was not significant **(Fig. 3a)**. In cancer donor, the IL-2 expression in Group 1 was 427.5 ± 82 pg/ml as compared to Groups 2 (436.9 ± 118 pg/ml) and 3 (439.1 ± 71.7 pg/ml). IL-2 expressions in cancer donors were higher in all treatment groups when

compared to that for healthy donors although again, the finding is not significant. Quantification of IFN-γ in healthy donor indicated that the expression of IFN-γ was significantly ($p<0.05$) higher in Group 1 in both healthy and cancer donors (**Fig. 3b**). As for the cytotoxic granule protein levels in healthy donors, the expression of PRF-1 was significantly highest in Group 1 (**Fig. 3c).** The expression level of PRF-1 was also the highest in Group 1 cancer donors although the finding was not statistically significant. Overall, the expression of PRF-1 in cancer donors was significantly ($p<0.05$) higher compared to the expression in healthy donors. Another cytotoxic granule protein measured was GzmB. In healthy donor, Group 1 had the highest GzmB protein expression, although it was not statistically significant. **(Fig. 3d)**. In cancer donor, Group 3 has the highest GzmB protein expression, followed by Groups 2, 1 and 4 although again the finding was not statistically significant. Similarly, healthy and cancer donor had similar GzmB expressions.

DISCUSSION

To our knowledge, this is the first study to report on the ability of APME to activate NK cells activity upon co-culture with MDA-MB-231 cells. NK cell activation can be measured by cytototoxic analysis of the target cell death, quantification of soluble target cell death markers, quantification of cytokine relased upon NK cell activation and finally the evaluation of the degranulation indicators [24,25]. The NK cells used in this study were freshly isolated from healthy (n=3) and cancer patients (n=3) who were on the waiting list for chemotherapy at the time of blood collection.

The amount of NK cells isolated from the healthy donors were two-fold higher than that yielded from cancer donors. An experiment on NK cells expansion comparing between healthy and cancer donors indicated that NK cell counts in healthy donors were also higher. A similar trend was also reported [26,27] where a reduced number of NK cells from breast cancer patients was seen as compared to healthy donors. NK cells from cancer patients exhibited inhibitory phenotype as characterised by a decrease in the expression of activating markers including natural killer group 2 member D (NKG2D) and natural cytotoxicity receptors (NCRs). In contrast, the inhibitory markers such as killer-cell immunoglobulin-like receptors (KIRs) and NKG2A were upregulated, overall leading to the inability of NK cells of cancer patients to exhibit cytotoxicity compared to the NK cells from healthy donors [28,29].

Lymphocytes activation, survival, proliferation and differentiation are regulated by cytokines. Interleukin, in particular IL-2, IL-15, IL-12, IL-18 and IL-21 can promote NK cells proliferation and improve their anti-tumour activities [1,30]. However, in this study, freshly isolated unstimulated NK cells from healthy and cancer donors were used for the co-culture assay to measure the NK cells cytotoxicity with the target cells in the presence of plant extract [20]. Therefore, prior to the co-culture assay with the target cell, the optimal concentration in which APME could induce proliferation of NK cells was determined.

The ability of APME to proliferate NK cells was evaluated by an MTT assay [31,32]. The ability of APME (26.4 µg/ml) to induce NK cell cytotoxicity was assessed in the experiment of NK cells co-culture with MDA-MB-231 cells. Three parameters were measured in this experiment the 1) effect on NK cells counts following the incubation 2) effect on cell deaths measured by apoptosis Annexin V/PI assay and 3) concentration of IL-2, IFN-γ, PRF-1 and GzmB. Following the co-culture incubation, APME-treated NK cells from healthy donor significantly induced MDA-MB-231 cell death when compared to the non-treated NK cells. On the other hand, there was no significant difference in the percentage of cell deaths in all treatment groups from the cancer donor. In particular, APME-treated NK cells from healthy donors exhibited significant cytotoxic activity when compared to APME-treated NK cells from cancer donors which was supported by the percentage of NK cytotoxicity calculated based on a formula as previously described [21,33]. The finding demonstrated that APME can further promote NK cell cytotoxicity in NK cells obtained from the healthy donor but not from cancer donors.

As previously described [27], compared to healthy donors the number of NK cells from cancer patients was not only reduced but there was also less cytotoxic activity. It is plausible that the said phenomenon occurred due to the alteration of cell receptors including decreased inhibitory receptors or/and increased activating receptors [34]. Mamessier et al. [26] reported a decreased expression of the activating NK cell receptors and an increased expression of the inhibitory receptors with cancer progression. Therefore, in immunotherapy treatment involving NK cells, immune activation is facilitated by adding cytokines and antibodies to sensitize the NK cells for modulation towards its anti-tumour response [1].

Recent advances further reinforce the potential of NK cell-based strategies in cancer therapy. Li et al. [35] highlighted innovations in CAR-NK cell engineering for solid tumors, while Li et al. [36] described how metabolic reprogramming shapes NK-cell activity within the tumor microenvironment. Kumar and Tanwar [37] reviewed next-generation NK therapies emphasizing expansion and activation protocols applicable to translational immunotherapy. Complementarily, Yang et al. [38] demonstrated that multiple plant-derived natural products can enhance NK cell-mediated tumor immunity through cytokine modulation. Finally, Barshidi et al. [39] discussed NK-cell exhaustion in cancer, offering a plausible mechanistic explanation for the impaired cytotoxicity observed in cancer donors in our study.

IL-2 was the first cytokine administered to metastatic melanoma patients [40] since it affects many immune system cells, including T, B and NK cells [41]. In our experiment, there was no significant difference in IL-2 levels in all treatment groups. The presence of IL-2 indicates that the isolated NK cells were contaminated with other IL-2 secreting lymphocytes since the purity of NK cells in our study was <90%. Although IL-2 is a 15K-kDa cytokine mainly produced by activated $CD4^+$/ $CD8^+$ T cells, there were reports on the secretion of IL-2 by dendritic and NK cells [42,43] as well. Nevertheless, the relevance of IL-2 production by these cells remains unknown [44].

IFN-γ, is the cytokine involved mainly in modulating host immune response, particularly controlling the spread of pathogenic infection and defence against cancers. IFN-γ is also released by activated TH1, $CD8^+$ T and NK T, besides NK cells [45]. Our results showed that the level of IFN-γ production was significantly increased in APME-treated NK cells. The production of IFN-γ indicates NK cells activation. Although NK cells produce a high level of IFN-γ exhibited low cytotoxicity, on the contrary, cytotoxic NK cells demonstrated a low level of IFN-γ production [46] which corroborates with our findings where even in extremely high IFN-γ expression is seen in cancer donors, the cytotoxicity of NK cells from healthy donor remained higher.

It was also reported that $CD56^{dim}CD16^{+}$ NK cells can produce IFN-γ during their cytolytic activities. Activated NK cells which produce intrinsic cytokines such as IFN-γ and TNF-α promote target cell lysis while neutralization of these cytokines causes the impaired function of the cell lysis [47]. Based on these findings, APME can stimulate NK cells from both healthy and cancer donors by producing higher IFN-γ levels compared to non-treated NK cells.

PRF-1 and GzmB are two granule proteins released by NK cells upon activation by stimuli from the target cells. The synergistic effects of PRF-1 and GzmB lead to lysis of the target cell. PRF-1 is a potent pore-forming protein that allows the entrance of cytotoxic proteases such as GzmB into the target cell cytoplasm. The pore-forming process by PRF-1 is crucial in order to facilitate the entry of granzyme proteases into the cytoplasm of target cells. Defects in the said pathway, particularly lacking in perforin molecule, will result in human disorders termed as perforinopathies, which include immunoproliferative disease Familial Haemophagocytic Lymphohistocytosis (FHL) type 2, some haemotological malignancies as well as protracted viral infections [48,49].

In our current study, PRF-1 expression was significantly higher in the APME-treated NK cells coming from healthy donors. A similar trend was seen with its counterpart from the cancer donor group although the the difference was only slight and was therefore not statistically significant. Nevertheless, overall, the expression of PRF-1 in NK cells from cancer donors was significantly increased when compared to that for healthy donors. Although there was a decreased NK cells cytotoxicity in cancer donors, the PRF-1 expression was extremely high, which was parallel to the higher production of IFN-γ in NK cells as compared to the cancer donor. The phenomenon was due to the fact that NK cells isolated from cancer donors have “memory” and are activated upon stimulation by the target cells. Fehniger and Cooper [50] suggested that enhanced NK cell function was observed in antigen-specific stimulation; termed as “memory” or “memory-like” depending on how NK cells were activated. NK cells memory is beneficial to be used for anti-tumour immunotherapy, especially in *in vitro* expansion capability [51].

GzmB induces cell death which occurs by both caspase-dependant and independent manners [52]. GzmB is initially expressed as an inactive precursor protein that bears an N-terminal signal peptide. The terminal direct packaging of GzmB into secretory granules is followed by the removal of dipeptide glycine and glutamate by cysteine protease cathepsin C. Upon removal of those peptides, GzmB becomes activated and is kept together in lytic granules together with other granzymes and perforin. GzmB enters the target cell following perforin activation and instantly induces apoptosis either by caspase-dependant or independent manners [53].

In the current study, GzmB were lowly expressed in both healthy and cancer donors. Nevertheless, coupled with the very low GzmB expression levels detected in all groups, there were also no significant differences in GzmB expressions between healthy and cancer donors. AMPE-treated NK cells cannot exhibit any significant differences in GzmB expression from the co-culture experiment. We postulated that NK-induced cytotoxicity on cancer cells occur *via* granzyme-independent pathway. Another way to measure GzmB expression is by observing the activation of caspase 3/7, 8 and 10 since GzmB activates proapoptotic through caspase-dependant pathways [54].

The *in vitro* experiment conducted may have contributed to the very low amount of GzmB expressions detected. Madakkannu and Ravichandran [55] have successfully demonstrated that *Indigofera tinctoria* and *Scoparia dulcis* aqueous extracts exhibited immunopreventive role in noise-stress rats by elevation of GzmB *in vivo*. Another study [56] involving a plant known as sea buckthorn or *Hippophae rhamnoides* L. exhibited an elevated level of GzmB and PRF-1 expression in chronic-stress rat model all of which may be incorporated in future studies.

Our data demonstrated that APME increases the proliferative capacity of NK cells, induce NK cell cytotoxicity thus promoting cell death *via* apoptosis, and increased the levels of IFN-γ and PRF-1 protein expressions by NK cells isolated from healthy donors. As for the cancer donor, although higher expressions of IFN-γ and PRF-1 were observed in comparison to the healthy donor, the NK cells cytotoxicity was impaired with no significant changes seen following APME administration. Nevertheless, based on our findings on healthy cells, it will be an interesting idea if *A. precatorius* is introduced in the diet of healthy individuals to determine if the herb can help prevent cancer.

Although *A. precatorius* leaves extract in this study exhibited immunostimulatory potential, its seeds contain the highly toxic protein abrin, and accidental ingestion has resulted in fatalities [57]. Therefore, any dietary or prophylactic applications should be approached with extreme caution, emphasizing that only standardized and purified leaf extracts should be considered for future nutraceutical or therapeutic exploration.

LIST OF ABBREVIATIONS

Abrus precatorius methanolic leave extract (APME)
Natural killer (NK)
Interleukin-2 (IL-2)
Interferon-gamma (IFN-γ)
Perforin (PRF-1)
Granzyme B (GzmB)
Antibiotic growth promoters (AGP)
Dalton’s Lymphoma (DL)
Dalton’s lymphoma ascites cells (DLAC)
Lymphocyte Separation Media (LSM)
Natural killer group 2 member D (NKG2D)
Natural cytotoxicity receptors (NCRs)
Killer-cell immunoglobulin-like receptors (KIRs)
Familial Haemophagocytic Lymphohistocytosis (FHL)

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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