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Chemopreventive and immunoadjuvant properties of standardised edible bird's nest extract on human breast cancer cell line

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Abstract

The present work investigated the chemopreventive and immunoadjuvant properties of edible bird's nest (EBN) extract on breast cancer cell line (MCF-7). Specifically, the cytotoxicity level of EBN extracts (HMG, EHMG, pHMG) against MCF-7, human immune cells of cytotoxic T cells, and monocytes ($CD8^+$ and $CD14^+$) were evaluated by measuring the production of pro-apoptotic and anti-apoptotic molecules released in single and co-culture of MCF-7, $CD8^+$, and $CD14^+$ cells, before and after EBN treatment. The highest cytotoxic effect towards MCF-7 using IC_{50} of 15 µg/mL was demonstrated by HMG but no effects on $CD8^+$ and $CD14^+$, with cell viability of more than 90%. At the mRNA level, activated $CD8^+$ and $CD14^+$ depicted increased pro-apoptotic gene expression after HMG treatment in co-culture. Additionally, HMG treatment increased apoptosis by down-regulating the regulation of anti-apoptotic genes and up-regulating the pro-apoptotic genes in MCF-7. ELISA and multiplex assay reflected increased pro-apoptotic factors, and decreased anti-apoptotic soluble factors, by non-activated and activated $CD8^+$ and $CD14^+$, in a single or co-culture with MCF-7 after HMG treatment. In conclusion, HMG extract possesses immunoadjuvant properties that can be a potential anticancer agent without causing any deleterious effects on the human immune cells.

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Introduction

Breast cancer is among the leading causes of mortality in women regardless of their ethnicity. Women between the age of 25 and 59 years are considered to be in the high-risk category. In 2018, approximately 43,837 new cancer cases were reported in Malaysia (GCO, 2018), of which 26,395 deaths (60%) were recorded by the World Health Organisation (WHO).

The National Cancer Institute (NIH, 2015) of the United States revealed that the accredited treatments that are commonly known as allopathic medicine include surgery, chemotherapy, radiation, hormonal, and targeted therapies. Nevertheless, these therapies involve the utilisation of synthetic drugs and medications that can induce adverse effects on cancer patients. Researchers are thus exploring unconventional natural remedies to treat cancer and developing safer alternatives to prevent the related side effects.

Edible bird's nest (EBN) is a secretion from swiftlets (*Aerodramus fuciphagus*), and one of the most expensive animal by-products that have been used in traditional Chinese medicine since the Tang Dynasty (618 - 907 A.D.). Although the content and bioactivity of EBN have been characterised by modern analysis technologies (Hun *et al*., 2015; Lee

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et al., 2019), the scientific evidence of its capability to treat any human diseases remains scarce. Glycoprotein, which is the main content of EBN, has been reported to play a crucial role in its nutritious and therapeutic functions (Kathan and Weeks, 1969). Besides, sialic acid which makes up 9% of the carbohydrates in EBN may also contribute to the healthy intellectual and neurological development of infants (Colombo *et al*., 2003). Carbohydrates and lectins in EBN are also essential in activating the defence mechanism in the immune system by participating in several activities such as the opsonisation of microorganisms, phagocytosis, cell adhesion and migration, cell activation, cell differentiation, and apoptosis (Ni and Tizard, 1996). Elvira and Valentin (2007) reported that lectins can bind with cancer cell membranes and receptors, thus inhibiting tumour growth. Lectins can also trigger the agglutination or aggregation of cancer cells (Elvira and Valentin, 2007).

Regarding the scientific evidence for the therapeutic effects of EBN, a prior study discovered that aqueous extract from EBN demonstrated similar activity to epidermal growth factor (EGF) *in vitro* in a dose-dependent manner, which resulted in the synthesis of DNA in a 3-day transfer inoculum 3×10^5 cells (3T3) fibroblasts (Kong *et al*., 1987). EGF plays an essential role in cellular proliferation, differentiation, and developmental processes. Comparatively, EBN exhibits EGF-like activity and mitogenic effect that improves immunity, delays ageing, and prolongs life expectancy (Yano *et al*., 2003).

In the present work, breast cancer was selected as a model sample given that it is the most common invasive cancer, and the leading cause of death among women of all ethnic backgrounds in Malaysia. The MCF-7 cell line was employed as the *in vitro* breast cancer cell line in the present work since the cell line retains several ideal characteristics similar to that of the mammary epithelium, such as having the oestrogen receptor (ESR) (Soule *et al*., 1973). Current conventional treatments for breast cancer include chemotherapy, which is a systemic treatment that can induce severe side effects by damaging healthy cells besides the cancer cells (Munden *et al*., 2005). Hence, it is vital to identify a safe adjuvant medicine such as EBN, which could reduce the immune impairment and other side effects of chemotherapy. Such treatment, if proven, could enhance cancer patients' quality of life.

Materials and methods

Samples and extractions

The EBN is a house variety (*Collocalia fuciphaga*) collected from a bird's house in Batu Pahat, Johor (Code: J05, ID: 140052; How Tong Peng, Ledang, Johor). The methods adapted from Oda *et al*. (1998) and Goh *et al*. (2000) were used with slight modifications to extract the EBN, which were then denoted as HMG, EHMG, and pHMG. A similar extraction method was employed for HMG and pHMG, except for the state of the extract which was liquid for HMG, and powder for pHMG. The supernatant was transformed to a powdered form using the freeze-drying method, and stored at 4°C until further use. As for the EHMG extract, the aliquot from the HMG extract was double-boiled for 1 h, followed by centrifugation at 4,000 rpm. The supernatant was then stored at 4°C until further use. All extracts were obtained from one batch of extracts to ensure consistent results.

Cell line culture

The MCF-7 cells were purchased from the American Type Culture Collection (ATCC). The method described by Freshney (2010) was applied in performing the cell line culture. Briefly, the cells were cultured using F12:DMEM supplemented with 10% of FBS, 1% of AA, glutaMAX, and vitamin C in the CO_2 incubator, and were subcultured every three days after the cells became confluent. The cells were washed with PBS supplemented with 1% of desired amino acids during subculture, followed by using the TE buffer to detach the cells from the flask surface. The detached cells in the solution were then washed with the TE buffer, and centrifuged for 10 min at 5,000 rpm. The cell pellets were re-suspended with the medium, and cells were counted using a haemocytometer.

Peripheral blood mononuclear, lymphocyte, and monocyte cell isolation

Blood samples were collected from healthy female donors (ethics number: UKM 1.5.3.5/244/FF-2014-105). The peripheral blood mononuclear cells (PBMC) was isolated as described in previous studies (Panda *et al*., 2012; Panda and Ravindran, 2013). The lymphocytes and monocytes were isolated using $CD8^+$ (T lymphocyte) and $CD14^+$ (monocyte) human microbeads together with a MiniMACS starting kit following the manufacturer's instructions (MACS;

Miltenyi Biotec). The isolated cells were assessed for their purity using the FACSVerse Flow cytometer (BD Biosciences, USA).

$CD8^+$ and $CD14^+$ cells activation

The $CD8^+$ was activated using a T-cell activation kit following the manufacturer's instructions (MACS; Miltenyi Biotec). Meanwhile, the $CD14^+$ cell was activated using lipopolysaccharide (LPS) as described previously (Toossi *et al*., 1992). The cell activation status was then measured by immunostaining technique using flow cytometry following the manufacturer's instructions (BD Biosciences, USA).

Sample culture condition

For the single culture experiment, approximately 1×10^6 cells (either $CD8^+$ or $CD14^+$) in non-activated and activated forms were treated with HMG in 2 mL of serum-free RPMI (Wieckowski *et al*., 2009) for 72 h in six-well plates. As for the co-culture experiment, MCF-7 cells were cultured together with immune cells (either $CD8^+$ or $CD14^+$) in both activated and non-activated forms at the ratio of 2:1. Approximately, 1×10^6 of immune cells (either $CD8^+$ or $CD14^+$) and 5×10^5 of MCF-7 cells were cultured together in the same six-well plates supplemented with the desired concentration of HMG and 2 mL of serum-free RPMI for 72 h. The $CD8^+$ cells were then collected directly from the co-cultured medium as they were suspension cells, whereas MCF-7 cells were collected by trypsinisation. Both collected cells underwent CD8 microbead separation to avoid mixture. The $CD14^+$ cells were collected using a cell scraper (Nest, Bioflow Life Science, China) as they were adherent cells. Each collected cells were then lysed using tri-reagent before proceeding to RT-PCR quantification.

Viability assay

Cytotoxic levels were assessed using the MTT assay as described in a previous study (Mirmalek *et al*., 2015). The MTT assay measures cell proliferation rate by assessing the cell viability, which was calculated using the equation of ([treatment / control] × 100), and the IC_{50} was obtained from the plotted graph.

Morphological analysis

The MCF-7 cells were observed, and their images were captured using an inverted microscope AXIO VER.A1 (Carl Zeiss, Germany) at 20× magnification power pre- and post-HMG treatments. The synergistic effect of HMG in the presence of $CD8^+$ and $CD14^+$ cells in killing the MCF-7 cells was measured by examining the immune cells co-cultured with MCF-7, and their corresponding images were captured at 40× magnification power.

RNA extraction and real-time quantitative reverse transcription-PCR (qRT-PCR)

The total RNA extraction was performed as described by Chua *et al*. (2005). SuperScript™ III First-Strand Synthesis Supermix was used to synthesise cDNA before the qRT-PCR. The manufacturer's intructions (Bioline) were followed to perform the quantitative RT-PCR to quantify the expression level of pro-apoptotic and anti-apoptotic genes in $CD8^+$, $CD14^+$, and MCF-7 cells. The genes selected for $CD8^+$ cells were IFN-γ, GZMB, PRF-1, and TGF-β1, whereas MMP-9, IL-10, IL-12, TNF-α, and TGF-β1 were selected for $CD14^+$ cells. Lastly, CASP-7, BCL-2, BAX, p53, ESR-1, and CYCS were the selected genes for MCF-7 cells (Sagar *et al*., 2014; Mahmoud *et al*., 2016). GADPH was used as the housekeeping gene.

ELISA and multiplex assays

ELISA and multiplex assays utilising the cell culture supernatant as described in the manufacturer's protocol (Wulan Fine Biotech - ELISA; Merck Millipore - multiplex assays) were applied to quantify the amount of pro-apoptotic and anti-apoptotic soluble factors such as IFN-γ, GZMB, PRF-1, and TGF-β1 that were released by the $CD8^+$ cells after the treatment with EBN extract. The same procedure was performed for the MMP-9, IL-10, IL-12, TNF-α, and TGF-β1 released by $CD14^+$ cells after the treatment with EBN extract.

The Human IFN-γ ELISA kit, Human $CD8^+$ T-Cell Magnetic Bead Panel, and TGF-β1 Single Plex Magnetic Bead Kit were used for $CD8^+$ cells, whereas the Human MMP Panel 2 Magnetic Bead Kit, Human Cytokine Magnetic Bead Panel, and TGF-β1 Single Plex Magnetic Bead Kit were used for $CD14^+$ cells.

Statistical analysis

The obtained results were expressed as mean ± standard error of the mean (SEM) from the triplicate values. The Statistical Package for Social Sciences (SPSS) software (International Business Machines Corporation (IBM), SPSS Statistics 24, SPSS Inc.,

USA) was used for the data analysis. A One-way analysis of variance (ANOVA) was employed as the statistical test, and a *p*-value < 0.05 was considered statistically significant (Pallant, 2007).

Results

Cytotoxicity effects of HMG, EHMG, and pHMG on MCF-7 cells

The MTT assay was used to analyse the cytotoxic levels of the EBN extracts. The HMG demonstrated the highest cytotoxic activity towards the MCF-7 cells with the lowest IC_{50} of 0.06% (15 µg/mL), which was lower than the recommended level of 0.08% (20 µg/mL) for crude extract as depicted in Figure 1A. While the EHMG reflected moderate cytotoxic effect towards the MCF-7 cells with an IC_{50} value of 0.08% (21 µg/mL), no cytotoxic effect was exhibited by pHMG towards the MCF-7 cells with an IC_{50} value of higher than 41 µg/mL (1.3% or 325 µg/mL).

Cytotoxic effects of HMG on isolated $CD8^+$ and $CD14^+$ cells from peripheral blood mononuclear cells (PBMCs) of a healthy individual

Further analysis was performed to evaluate the cytotoxic effects of the HMG extract on human immune cells such as cytotoxic T cells and monocytes, given that HMG had demonstrated the highest toxicity activity towards MCF-7 cells. Various concentrations of the HMG ranging from 0 to 0.5% were tested on the isolated $CD8^+$ and $CD14^+$ cells from a healthy individual's PBMCs. The effects of the HMG at 0% concentration were used as a control, in which the values from different concentrations were calculated as the percentage of viable cells relative to the control. Upon HMG treatment, the IC_{50} value revealed the absence of toxicity activity on the isolated $CD8^+$ and $CD14^+$ cells from the healthy individual's PBMCs (Figure 1B). Additionally, no significant changes were observed for the toxicity activity across low and high HMG concentrations, thereby reflecting that HMG would not be toxic to human immune cells.

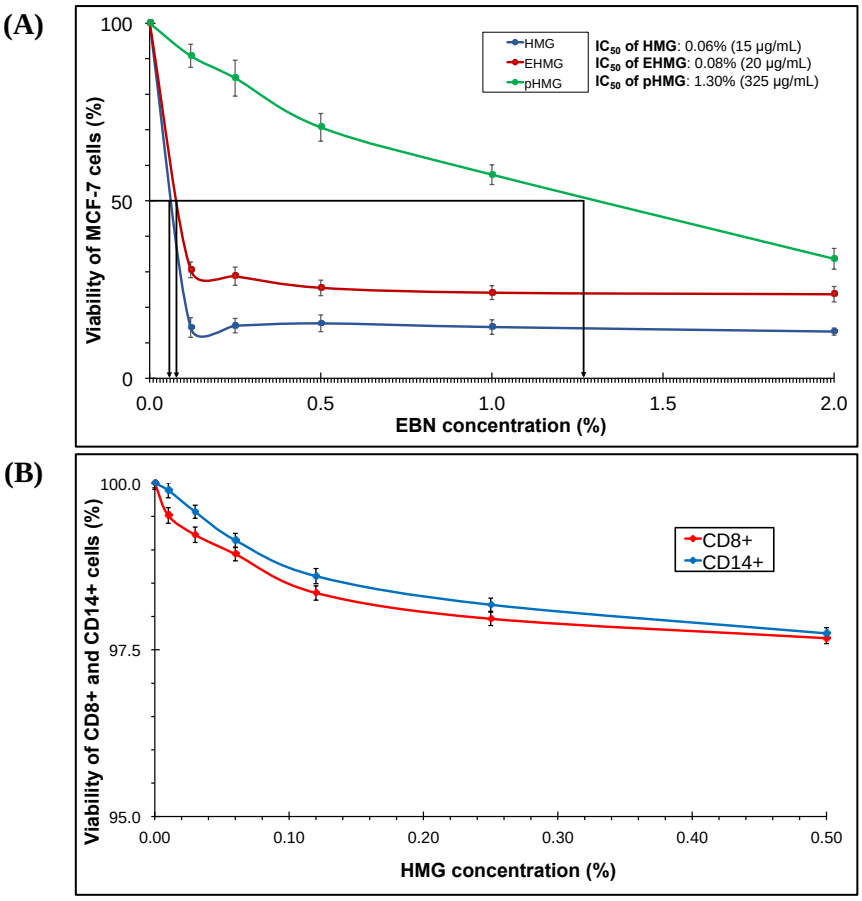


Figure 1. Effect of EBN extracts on MCF-7, $CD8^+$, and $CD14^+$ cells. **(A)** Cytotoxicity activity of HMG, EHMG, and pHMG extracts with various concentrations on MCF-7 cells after 72 hours of treatment to determine the IC_{50}. **(B)** Cytotoxicity effects of the chosen HMG extract with various concentrations on $CD8^+$ and $CD14^+$ cells after 72 hours of treatment to determine the cell viability. Data are mean ± SEM of triplicate (n = 3).

Morphological assessment of MCF-7, CD8$^+$, and CD14$^+$ cells in the presence and absence of HMG

Morphological analysis was conducted to assess the effects of MCF-7 in the presence of HMG. The synergistic effect of HMG after 72 h of treatment was evaluated by preparing single cultures of either non-activated or activated CD8$^+$ or CD14$^+$ cells with the HMG. Microscopic observations depicted that the MCF-7 cell maintained cell adhesions during proliferation (Figure 2A). The cells appeared as large adherence cells with an epithelium that resembled long arms and inconclusive cell boundaries under normal conditions without the HMG. The cells were also viable with an intact nucleus. Meanwhile, the MCF-7 cells demonstrated apoptotic morphological changes after 72 h of treatment with the HMG such as the loss of normal shape, chromatin condensation, nuclear compaction, cell shrinkage, blebbing of the plasma membrane, and collapsing of the cell into small fragments (Figure 2A).

As for co-culture, both activated and non-activated CD8$^+$ or CD14$^+$ cells were introduced to MCF-7 cells, with and without HMG treatment for 72 h. As illustrated in Figures 2B and 2C, the non-activated CD8$^+$ and CD14$^+$ cells reflected normal spherical morphology in both the media with and without the HMG. In contrast, the MCF-7 cells revealed less proliferation with atypical morphology, weak cell adhesion, and some of the cells grew as solid spherical aggregates after 72 h of HMG treatment [Figures 2B (i), (ii); Figures 2C (i), (ii)]. For the activated cells [Figures 2B (a), (b)], the CD8$^+$ cells enlarged in size, and were observed to have grown in clonal suspension with apparent homotypic aggregates around the MCF-7 cells after 72 h of HMG treatment. Some activated CD14$^+$ cells [Figures 2C (a), (b)] were also enlarged and displayed a dendritic phenotype with the development of small cytoplasmic extensions or veils around the MCF-7 cells. In contrast, the proliferation of the MCF-7 cells was reduced with some cells exhibiting apoptotic morphological changes [Figures 2C (i), (ii), (iii), (iv)].

Effects of HMG on pro-apoptotic and anti-apoptotic gene expression levels in CD8$^+$ and CD14$^+$ cells with and without MCF-7 cells

Four different conditions were tested on each isolated cell, namely non-activated, activated, HMG treated non-activated, and HMG treated activated. Each test was performed with and without MCF-7 cells. No up-regulation in the expression of the pro-apoptotic genes [interferon gamma (IFN-γ), granzyme B (GZMB), perforin-1 (PRF-1), and transforming growth factor beta 1 (TGF-β1)] in CD8$^+$ cells were observed under the HMG treatment (Figure 3A) for both single-cultured non-activated CD8$^+$ cells and the non-activated CD8$^+$ cells co-cultured with MCF-7. Similar results were observed in the single culture of activated CD8$^+$ cells under the HMG treatment (Figure 3A - upper). Nevertheless, the activated CD8$^+$ cells recorded significantly higher pro-apoptotic gene expression levels in comparison to the non-activated cells. This result persisted irrespective of whether the activated and non-activated CD8$^+$ cells were single culture or co-cultured with MCF-7. A similar result was also observed in the activated CD8$^+$ cells co-cultured with MCF-7 cells relative to the single-cultured activated CD8$^+$ cells under the HMG treatment (Figure 3A - bottom).

No significant differences in the expression of the pro-apoptotic genes under the HMG treatment were detected for both single-cultured non-activated CD14$^+$ cells and the non-activated CD14$^+$ cells co-cultured with MCF-7 (Figure 3B). Meanwhile, the expression of pro-apoptotic genes [interleukin 10 (IL-10), interleukin 12 (IL-12), tumour necrosis factor (TNF-α), and transforming growth factor beta 1 (TGF-β1)] in CD14$^+$ cells was elevated in both single-cultured and co-cultured activated CD14$^+$ cells in comparison to that in the non-activated cells (Figure 3B). Nevertheless, the expression of matrix metallopeptidase (MMP-9) gene (anti-apoptotic gene) in the activated CD14$^+$ cells decreased relative to the non-activated cells (Figure 3B). Similar to CD8$^+$ cells, the HMG reflected no significant effects on the pro-apoptotic gene expression levels of the single-cultured activated CD14$^+$ cells (Figure 3B - left). On the other hand, the pro-apoptotic gene expression levels of the activated CD14$^+$ cells co-cultured with MCF-7 cells were higher relative to the single-cultured activated CD14$^+$ cells treated with the HMG (Figure 3B - right).

The MCF-7 co-cultured with CD8$^+$ or CD14$^+$ cells were assessed under the same condition for the expression levels of pro-apoptotic [caspase-7 (CASP-7, bcl-2-associated X protein (BAX), tumour protein

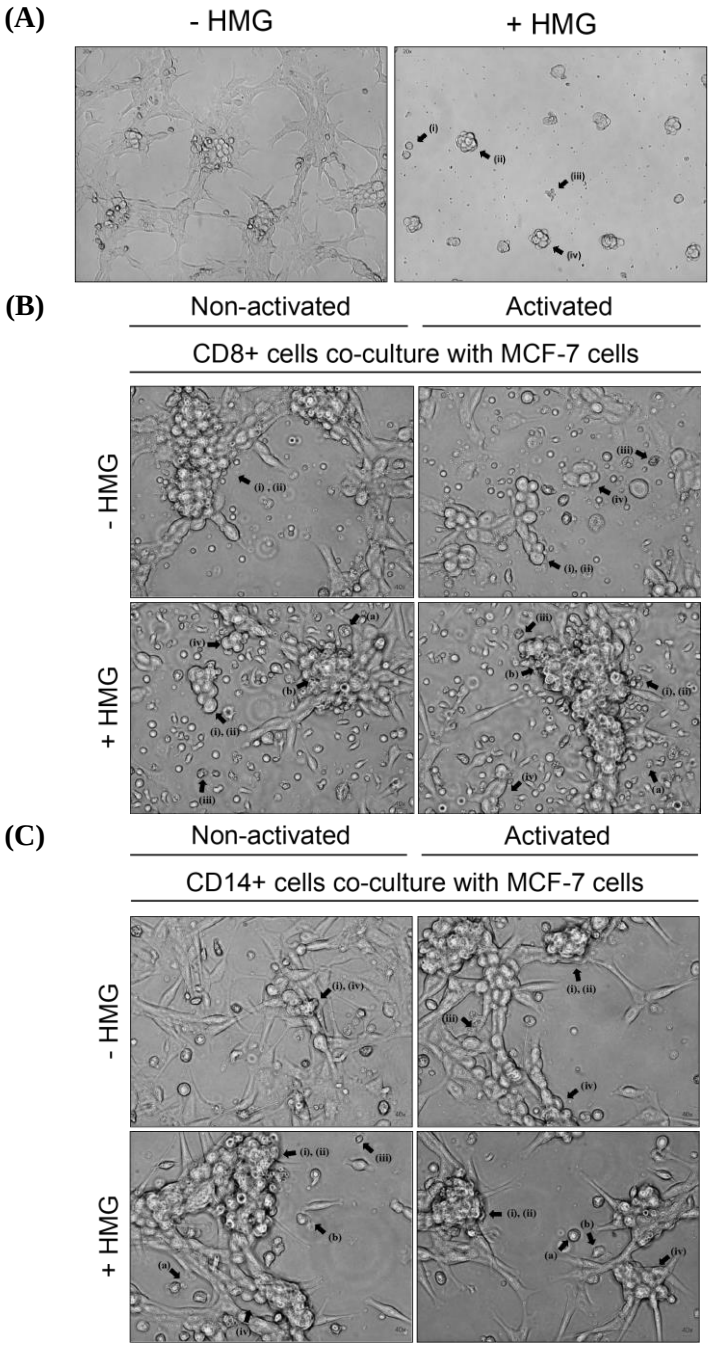
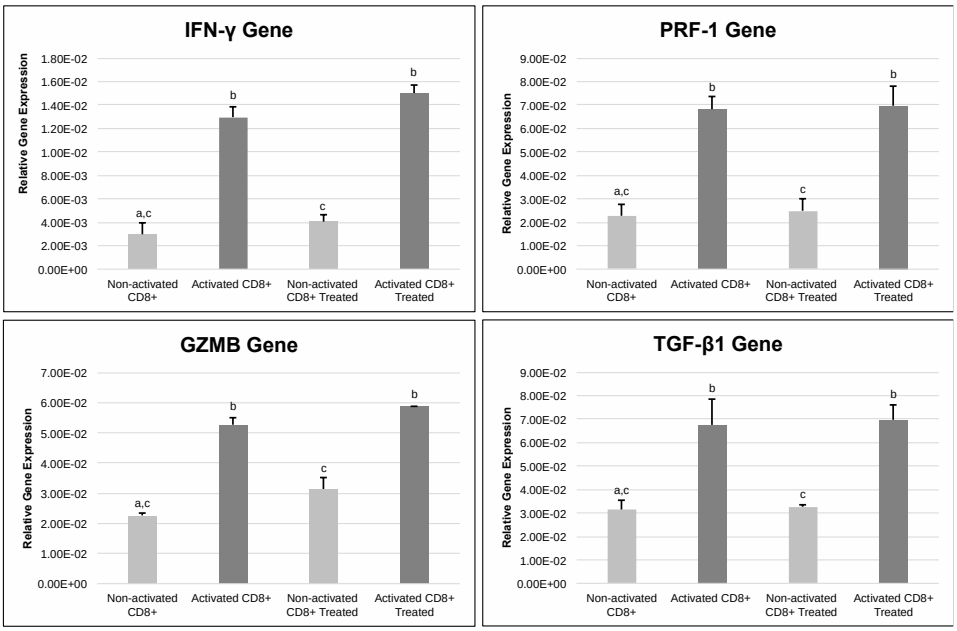


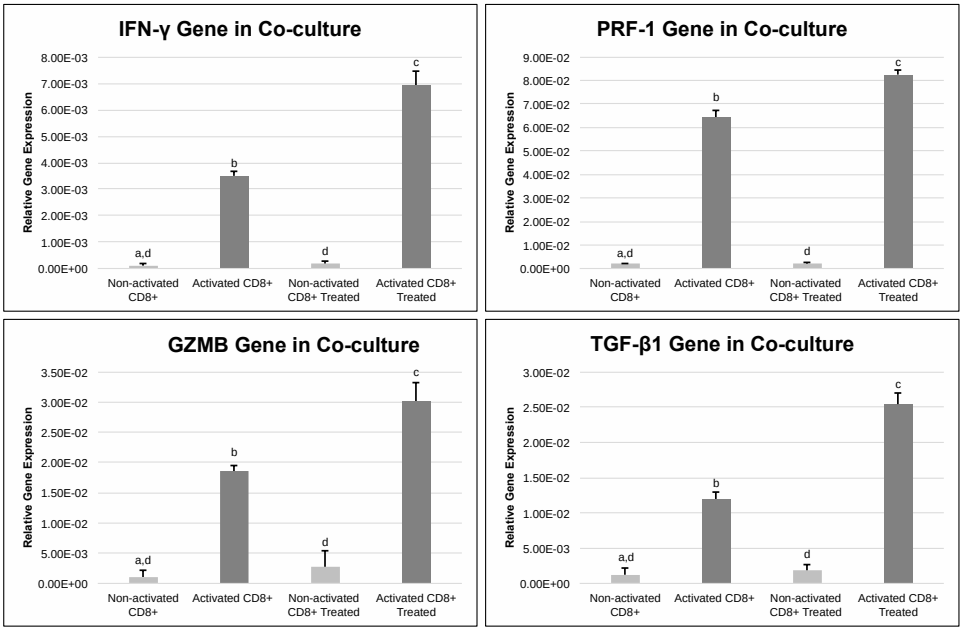
Figure 2. Morphological assessment of MCF-7, $CD8^+$, and $CD14^+$ cells before and after HMG treatment. **(A)** MCF-7 cells exhibited healthy morphology, and later showed apoptotic features after being treated with HMG extract for 72 hours (20× magnification). Apoptotic features such as (i) loss of normal shape and cell shrinkage, (ii) solid spherical aggregates of cells, (iii) cell necrosis, and (iv) plasma membrane blebbing were observed. As for **(B)** $CD8^+$ and **(C)** $CD14^+$, both non-activated cells possessed normal healthy spherical morphology before and after HMG treatment (40× magnification). Some activated **(B)** $CD8^+$ and **(C)** $CD14^+$ cells demonstrated (a) enlarged size and (b) the cells were grown in clonal suspension with apparent homotypic aggregates before and after HMG treatment. MCF-7 cells co-cultured with non-activated and activated $CD8^+$ or $CD14^+$ showed reduced proliferation and apoptotic features such as (i) loss of normal shape and cell shrinkage, (ii) solid spherical aggregates of cells, (iii) cell necrosis, and (iv) plasma membrane blebbing before and after HMG treatment.

(A)

CD8+ cells single culture

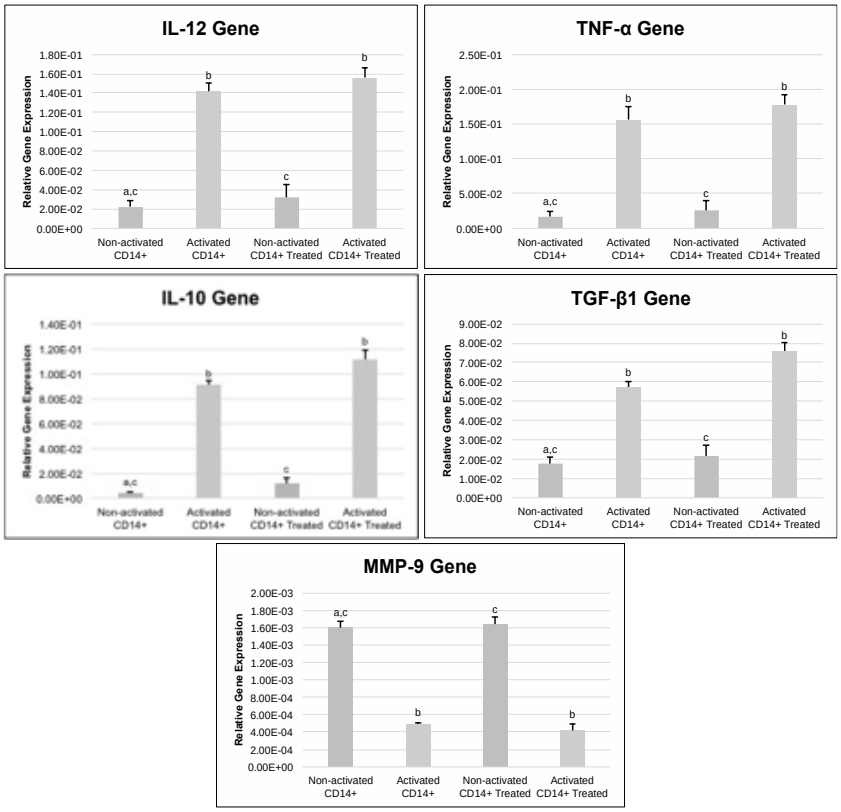


CD8+ cells co-cultured with MCF-7 cells

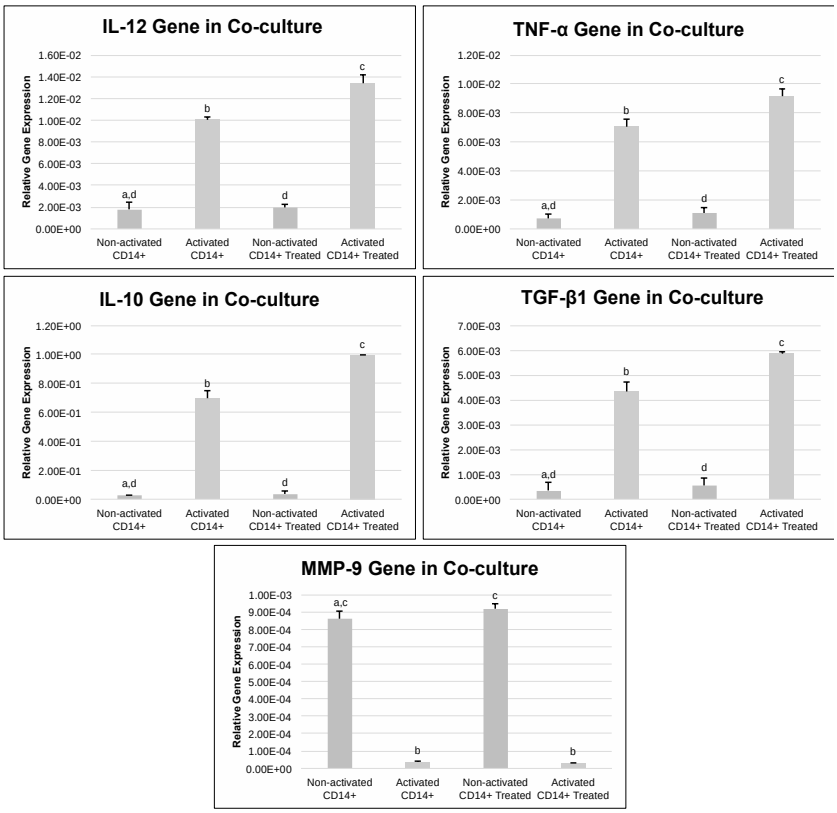


(B)

CD14⁺ cells single culture



CD14⁺ co-cultured with MCF-7 cells



(C)

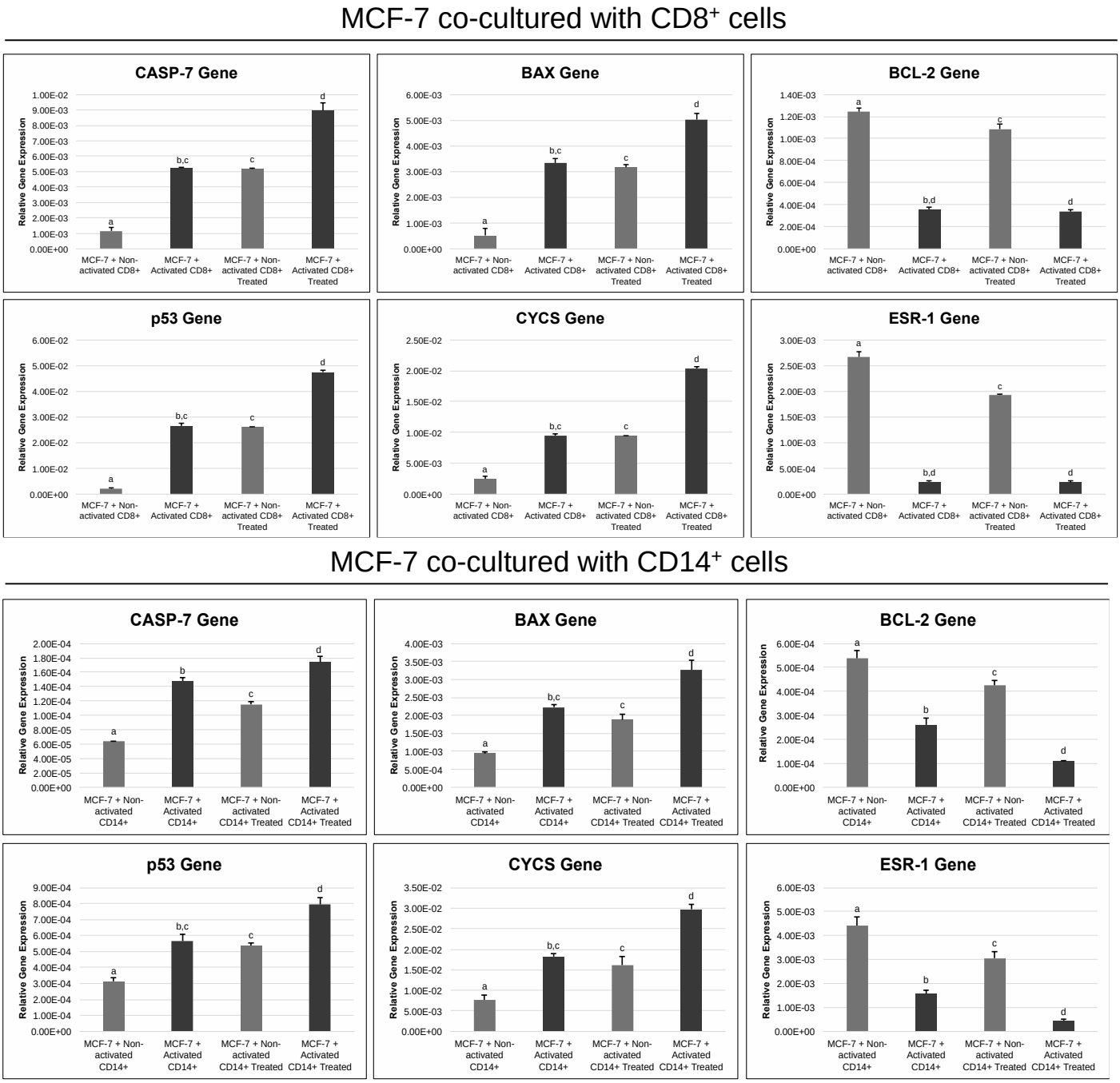


Figure 3. HMG up-regulated the expression level of pro-apoptotic genes, and down-regulated the expression level of anti-apoptotic genes in $CD8^+$, $CD14^+$, and MCF-7 cells co-cultured with human immune cells ($CD8^+$ or $CD14^+$ cells). **(A)** Relative gene expression of IFN-γ, GZMB, PRF-1, and TGFβ-1 genes in non-activated and activated $CD8^+$ cells from single-cultured and co-cultured with MCF-7 cells before and after HMG treatment. **(B)** Relative gene expression of MMP-9, IL-10, IL-12, TNF-α, and TGFβ-1 genes in non-activated and activated $CD14^+$ cells from co-cultured with MCF-7 cells before and after HMG treatment. **(C)** Relative gene expression of CASP-7, BCL-2, BAX, p53, ESR-1, and CYCS genes in MCF-7 cells co-cultured with non-activated and activated $CD8^+$ and $CD14^+$ cells before and after HMG treatment. Data are mean ± SEM of triplicate (n = 3). Bars with different lowercase letters are significantly different ($p < 0.05$).

p53 (p53), and cytochrome c (CYCS)] and anti-apoptotic [B-cell lymphoma 2 (BCL-2) and oestrogen receptor 1 (ESR-1)] genes. A significant increase was observed in the gene expression of the MCF-7 co-cultured with either activated or non-activated $CD8^+$ cells after HMG treatment (Figure 3C - upper). Except for the anti-apoptotic genes, similar results were detected in the expression of pro-apoptotic genes in the MCF-7 co-cultured with either activated or non-activated $CD14^+$ cells (Figure 3C - bottom). The expression of BCL-2 and ESR-1 genes in the MCF-7 co-cultured with activated $CD14^+$ cells treated with HMG decreased significantly in comparison to that of the untreated group.

Effects of HMG on pro-apoptotic and anti-apoptotic soluble factors secreted by $CD8^+$ and $CD14^+$ cells in the presence and absence of MCF-7 cells

Both $CD8^+$ and $CD14^+$ cells treated with the HMG exhibited an elevation in the gene expression or mRNA levels of pro-apoptotic genes. Hence, further analysis was conducted to assess the levels of pro-apoptotic and anti-apoptotic soluble factors in the supernatant of $CD8^+$ and $CD14^+$ cells in single culture and that of co-cultured with MCF-7. The levels of pro-apoptotic soluble factors (IFN-γ, GZMB, PRF-1, and TGF-β1) in the activated $CD8^+$ cell supernatant were significantly higher than that of the non-activated cells (Figure 4A) in both conditions. The activated $CD14^+$ cells also demonstrated significant up-regulation of pro-apoptotic soluble factors (IL-10, IL-12, TNF-α, and TGF-β1) in comparison to that of the non-activated cells (Figure 4B). The result was consistent in both single culture and that of co-cultured with MCF-7 cells. In contrast, the level of the anti-apoptotic soluble factor of MMP-9 in the HMG-treated non-activated $CD14^+$ cells depicted a significant decrease as compared to the non-activated cells.

Discussion

Toxicity assessment of EBN extraction

Several studies have evaluated the therapeutic effects of EBN extracts (*i.e*., EHMG, pHMG, and HMG) using different extraction methods (Abidin *et al*., 2011; Chua *et al*., 2013). The chemical compositions of these EBN extracts, which include the proximate composition, minerals, metabolites, and amino acids have also been characterised separately in previous studies (Lee *et al*., 2019; Tong *et al*., 2020). In the present work, the HMG was chosen for subsequent analysis as it demonstrated the highest cytotoxic activity towards MCF-7 cells in comparison to other extracts. Subsequently, the HMG was employed to test the $CD8^+$ and $CD14^+$ cells isolated from a healthy individual's PBMCs. A similar result was reported by Roh *et al*. (2012), in which EBN extracts at 500 and 8,000 µg/mL extracted using different extraction methods depicted low or no cytotoxic effect on the MCF-7 cells. Likewise, EBN extract was found to promote the proliferation of human adipose-derived stem cells (hADSCs) and normal human fibroblast (NHFs) murine cell lines, but not human cancer cell lines (MCF-7 and Hep2B). This result suggested that the EBN extract would not promote the proliferation of MCF-7 cells, and that the active properties of the EBN extract could be affected by the extraction methods.

In the present work, no cytotoxic effects were observed in the $CD8^+$ T cells treated with the EBN extract. This was consistent with a previous study conducted by Zhao *et al*. (2016), where EBN extract accelerated the proliferation of B-cells at different EBN extract concentrations, but not in the case of T cells. The difference in the effects of EBN extract on B- and T-cells indicated that other soluble factors such as EGF play a pivotal role in interacting with the EBN extract, which subsequently affects the proliferation, differentiation, and survival of human immune cells (Herbst, 2004). Nevertheless, these results were not congruent with previous studies (Ng *et al*. 1986; Kong *et al*., 1989), in which EBN extract exhibited the potential of enhancing the stimulation of proliferative properties and potentiate the mitogenic response of human peripheral blood monocytes $CD14^+$ cells (Ng *et al*., 1986) and human lymphocyte $CD8^+$ cells (Kong *et al*., 1989). Therefore, it can be hypothesised that interaction between EBN properties and other growth factors might initiate cell proliferation and differentiation of human lymphocytes and monocytes. Further studies are required to test the hypothesis, which may add value to the existing findings.

Morphological assessment of MCF-7, $CD8^+$, and $CD14^+$ cells

In the present work, the MCF-7 cells exhibited an intact nucleus and cobblestone morphology with an epithelium-like shape under normal conditions, which corroborated the results of other authors (Faraj

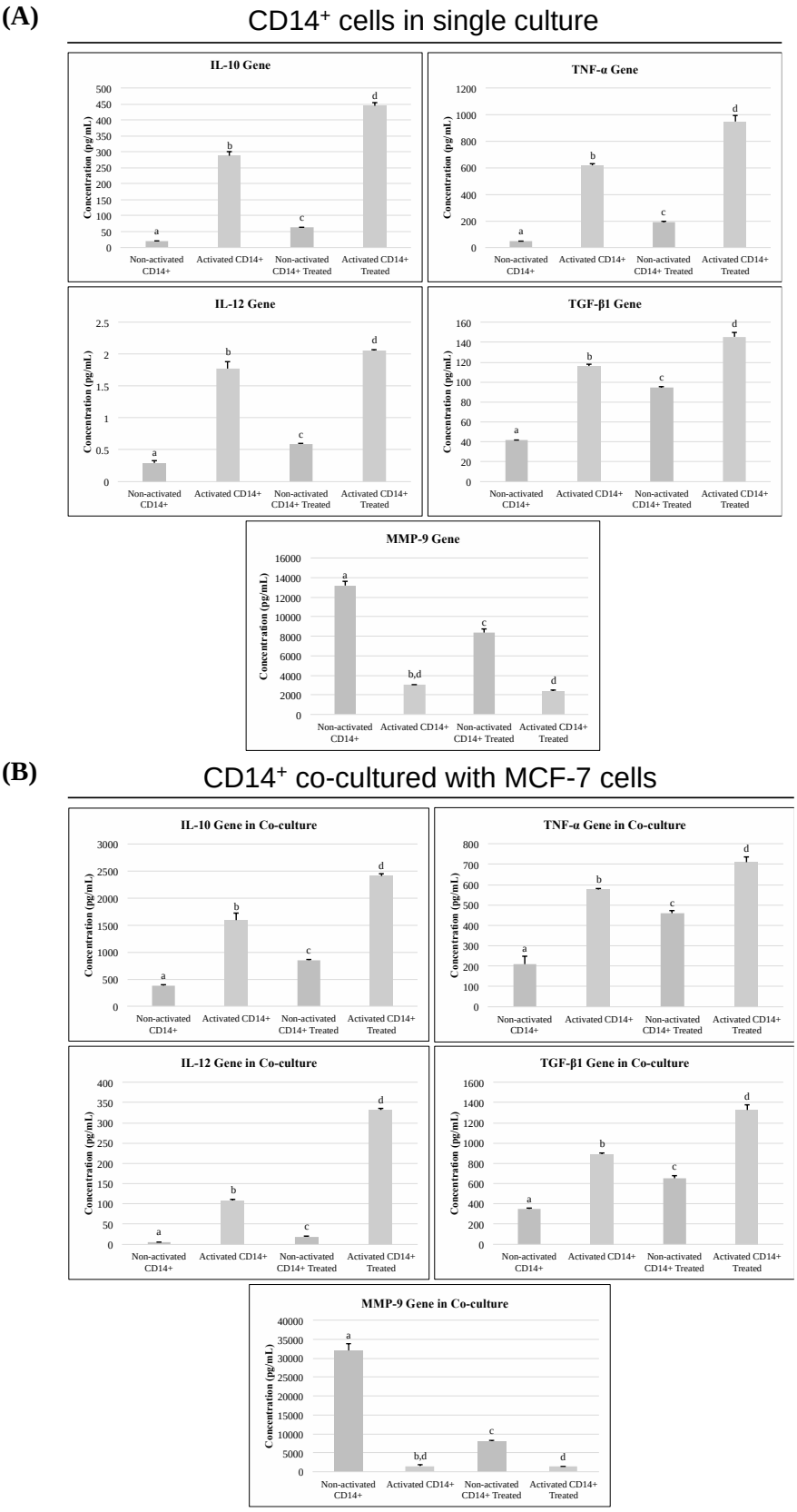


Figure 4. HMG treatment results in the upregulation of pro-apoptotic and downregulation of anti-apoptotic soluble factors in non-activated and activated $CD8^+$ and $CD14^+$ cells for both single and co-cultured with MCF-7 cells. **(A)** Concentration or pro-apoptotic soluble factors of IFN-γ, GZMB, PRF-1, and TGFβ-1 in non-activated and activated CD8+ cells from single-cultured and co-cultured with MCF-7 cells before and after HMG treatment. **(B)** Concentration of pro-apoptotic and anti-apoptotic soluble factors of MMP-9, IL-10, IL-12, TNF-α, and TGFβ-1 in non-activated and activated $CD14^+$ cells from single-cultured and co-cultured with MCF-7 before and after HMG treatment. Data are mean ± SEM of triplicate (n = 3). Bars with different lowercase letters are significantly different ($p < 0.05$).

et al., 2014; Shaheen *et al*., 2018). The cells underwent apoptotic morphological changes following the HMG treatment, similar to the reports by Kwan *et al*. (2016) as the MCF-7 cells exhibited apoptotic changes after being treated with *Euphorbia hirta* L. (Euphorbiaceae). Some of the treated cells also demonstrated signs of secondary necrosis - an autolytic process of the apoptotic or necrotic cells that failed to be engulfed by the phagocytes.

In the present work, images obtained by the inverted microscope were insufficient to explain the effects of the HMG on the single-cultured $CD8^+$ and $CD14^+$ cells. No significant difference was observed in changes in cell morphology between the control and treatment groups. Moreover, the changes in $CD8^+$ and $CD14^+$ cells might have been indistinguishable due to their small sizes. No proliferation was detected in the non-activated $CD8^+$ and $CD14^+$ cells, but a small amount of apoptosis was observed in the absence of any stimulation. Some cells also lost their viability due to insufficient nutrients and growth factors (River, 2018). Therefore, future studies could propose several analyses using a specific marker to induce the expression of pro-apoptotic factors. For instance, the research gap can be addressed by designing experiments adopting the pro-apoptotic and anti-apoptotic genes by making constructs with different fluorescent probes. Results from such analyses will further elucidate and provide more detailed information to justify the present findings.

Effects of EBN on gene expression/mRNA level of pro-apoptotic and anti-apoptotic genes in $CD8^+$, $CD14^+$, and MCF-7 cells

Apoptosis is a conserved genetic programme that is critical for the development and homeostasis of the immune system. The dynamic balance between anti-apoptotic and pro-apoptotic factors assists in the proper initiation of apoptosis (Strasser *et al*., 2011). Both $CD8^+$ and $CD14^+$ cells play vital roles in the immune system, which is linked to the elevated level of cytotoxic $CD8^+$ T cells in the tumour microenvironment with positive anti-tumour effects in breast cancer (Kim and Ahmed, 2010). While IFN-γ, GZMB, PRF-1, and TGF-β1 are the genes expressed by cytotoxic $CD8^+$ T cells, the genes expressed by $CD14^+$ cells to kill virally-infected cells and tumours include IL10, IL12, TNF-α, and TGF-β1 genes. These genes possess the strongest pro-apoptotic functions to induce apoptosis (Wissinger, 2016). Meanwhile, the MMP-9 gene expressed by the $CD14^+$ cells is an anti-apoptosis gene that suppresses the apoptotic process (Trypuc *et al*., 2016).

The present results revealed that the expression of pro-apoptotic genes increased while the expression of anti-apoptotic genes decreased in the activated $CD8^+$ and $CD14^+$ cells in comparison to the non-activated cells. This finding might be attributed to the progression of $CD8^+$ T cells from an inactive stage to a fully activated stage with further proliferation, which is a crucial step in initiating an immune response (Sagerstrom *et al*., 1993). Similarly, dendritic cells are the most potent antigen-presenting cells based on their capacity to present tumour antigens to naive or effector T lymphocytes while playing a crucial role in the initiation, programming, and regulation of tumour-specific immune responses (Li *et al*., 2016).

Effect of HMG on pro-apoptotic and anti-apoptotic soluble factors secreted by $CD8^+$ and $CD14^+$ cells in conditions of single or co-culture with MCF-7 cells

Immune cells such as $CD8^+$ and $CD14^+$ cells secrete pro-apoptotic and anti-apoptotic soluble factors to eliminate tumour cells through apoptosis. IFN-γ, IL-10, IL-12, TNF-α, and TGF-β1 are all cytokines. Meanwhile, IFN-γ, IL-12, and TNF-α can be further classified as pro-inflammatory cytokines by acting as signalling molecules that regulate anti-inflammatory activity and promote inflammation (Scarpioni *et al*., 2016). Pro-inflammatory cytokines such as TNF-α are vital to activate macrophages, which then phagocytose and kill mycobacteria and other pathogens (Kevin, 2006). In contrast, IL-10 and TGF-β1 are anti-inflammatory cytokines that control the pro-inflammatory cytokine responses. Anti-inflammatory cytokines such as IL-10 are equally important in limiting the host immune response to pathogens, thereby preventing damage to the host and several human malignancies. Examples of these functions include the degradation of collagen IV in the basement membrane and extracellular matrix that can accelerate tumour progression, which includes invasion, metastasis, growth, and angiogenesis (Groblewska *et al*., 2012).

In the present work, immune-enhancing properties of EBN were reflected by the increased production of pro-apoptotic soluble factors and the lower synthesis of anti-apoptotic soluble factors. These events were elicited by the HMG in the single-cultured non-activated and activated immune cells and those that were co-cultured with MCF-7. Overall,

these results suggested the potential of EBN as an immunoadjuvant and chemopreventive or alternative treatment for cancer.

Conclusion

The present work revealed the potential of EBN extract (HMG) as an anticancer agent without exhibiting cytotoxic effects on the human immune cells. The morphological assessment also demonstrated a direct cytotoxic effect of the HMG on MCF-7 cells. Meanwhile, the cancer cells supplemented with the HMG exhibited apoptotic features. The higher expression of pro-apoptotic genes and lower expression of anti-apoptotic genes by the HMG treatment in the activated $CD8^+$ and $CD14^+$ cells co-cultured with MCF-7 depicted that HMG could act as an immunoadjuvant in combating breast cancer. Furthermore, the HMG also demonstrated anticancer potential and immune-enhancing properties in MCF-7 co-cultured with non-activated and activated immune cells by upregulating pro-apoptotic genes and downregulating anti-apoptotic genes. The present work might contribute to the development of anticancer therapeutic modalities from EBN extracts in the future. The present work also contributed toward elucidating and validating the medical potential of this ancient animal-based traditional medicine.

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