ORIGINAL ARTICLE

Moringa oleifera Leaf Ethanol Extract Immunomodulatory Activity Discriminates between Chronic Myeloid Leukaemia Cell Line and Normal Lymphocytes

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ABSTRACT

Introduction: *Moringa oleifera*, a member of the family Moringaceae, is a small-medium sized tree, widely cultivated in Southeast Asia, Polynesia, and the West Indies, where the leaves, fruits and flowers form part of their routine diet. The plant has been reported to possess numerous pharmacological properties; however, its immunomodulatory potentials were least explored, especially on lymphocytes. Therefore, this study aimed to investigate the in vitro immunomodulatory effect of *Moringa oleifera* leaves' ethanol extract (MOETE) on transformed and normal lymphocytes, the leukaemic cell line BV173 and healthy peripheral blood mononuclear cells (PBMCs), respectively. **Methods:** The freshly collected and dried *Moringa oleifera* leaves were extracted using 70% ethanol, and the cytotoxicity activity on transformed and normal lymphocytes was determined using an MTT assay. The immunomodulatory effect was further evaluated through cell proliferation assays, cell cycle analysis and apoptosis assays. **Results:** The ethanolic extract of *Moringa oleifera* leaves showed concentration-dependent cytotoxic effects on the BV173 cell line with an IC_{50} of 125±6 µg/mL while exerting a stimulatory effect on PBMCs (EC_{50} = 28±3 µg/mL). The extract also exerted antiproliferative effects, cell cycle arrest and apoptosis in the BV173 tumour cell but enhanced the viability and proliferation of PBMCs by committing the cells into the cell cycle and reducing apoptosis despite stimulation by phytohemagglutinin (PHA). **Conclusion:** The MOETE has immunostimulatory properties on normal lymphocytes and anti-tumour activity on the leukaemic cell lines.
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Keywords: *Moringa oleifera*, Ethanol extraction, Cell proliferation, Apoptosis, Immunomodulation

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INTRODUCTION

Extrinsic factors such as infectious diseases, environmental stress, age extremities (neonates or old aged), metabolic disorders, malnutrition, and splenectomy, amongst others, are the major causes of secondary immune deficiencies (SIDs), which targets the comprehensive defence apparatus that protects the host organism against harmful microorganisms or substances as well as against malignancies (1, 2). This immunocompromised condition predisposes the body not only to nosocomial infections but also to opportunistic infections and, in addition, increases the susceptibility to developing benign and malignant tumours due to decreased cancer immunosurveillance (3).

Despite the development of many chemotherapeutic interventions against leukaemia, the past few years have, like in other types of cancers, witnessed several cases of multiple drug resistance (4). As a result, efforts to discover new compounds with high sensitivity anticancer properties are expanding. Researchers turn to plants for resources to identify and develop new drugs from phytochemicals. Plant products play pivotal roles in human health and the evolution of medicine as plant materials have been extensively used to treat or alleviate different disease conditions, including cancers (5).The ability of molecules found in medicinal plants to bind to therapeutic targets and modulate proliferative or apoptotic tendencies in cancer cells is indicative of the promising potential of the plant in the treatment of cancers (6).

Similarly, the modulation of immune response in the form of boosting the ability of the body to produce immune cells for the amelioration of disease and

cancer susceptibility as well as a positive response to conventional cancer radio- and chemotherapy can be achieved by the dietary intake and supplementation of plants materials with established immunomodulatory properties (7). Moreover, many plant products have been exploited for the modulation of the immune system in many Ayurvedic formulations, either alone or in groups (9).

Moringa oleifera (*M. oleifera*) is a tree plant utilised for nearly 2000 years in Ayurvedic medicine due to its vast therapeutic potential. Although the tree plant is indigenous to India, it can be found in different geographical locations such as Southeast Asia, Polynesia, South America and Africa (8).As a result of its wide distribution, the *M. oleifera* tree has been cultivated, and its different parts, like leaves, seeds, bark, and flowers, have been used as part of regular diet and medicine. Hence it is often referred to as the 'Tree of life' (9). The biological/pharmacological properties of *M. oleifera* have been extensively explored by many *in vitro* and *in vivo* studies involving diabetes mellitus, liver function, inflammation, obesity and cancer (10, 11). The polyphenolic constituents of *M. oleifera* leaves, stem, roots, and flowers have been investigated using comprehensive analytical techniques ranging from Liquid Chromatography Mass Spectrometry (LC-MS) (12) to High-Performance Liquid Chromatography (HPLC) (13). These investigations have revealed several phytochemicals present in *M. oleifera*. The leaves, however, have mainly flavonoids quercetin and kaempferol (mostly in their glycated form, i.e.isoquercitin and astragalin), chlorogenic acid, as well as other phytochemicals like benzylamine (moringine), niazirin and niazirinin (14). Also, serving as a promising tropical food, the leaves of *M. oleifera* have been a potent dietary source of vitamins A, C, B1, B2 and B3, as well as amino acids, fibre, carbohydrates, potassium, calcium, zinc and magnesium (15). The extracts from the leaves have been obtained using different solvents such as ethanol, water, n-hexane, chloroform and methanol. The extracts as well have been reported to exert hypoglycaemic, hypocholesterolemic, antioxidant, antiarthritic, antinociceptive immunomodulatory, hepatoprotective, anti-inflammatory, antimicrobial, radioprotective and anticancer properties (9).

Since *M. oleifera* leaves have been shown to have anticancer activity, it is essential to closely examine the interactions between the extracts of *M. oleifera* leaves and healthy immune cells, specifically lymphocytes, compared with leukaemic cell lines, within controlled in vitro cultures.

In line with this, the present study evaluated the effect of *M. oleifera* ethanolic extract (MOETE) on the BV173 cell line, which are pre-B cells derived from a lymphoid blast crisis of CML (16) with normal peripheral blood mononuclear cells (PBMCs) as control. The cytotoxicity of the MOETE in these cells was tested along with the cell proliferation, cell cycle progression, and apoptosis assays. The findings of this study will provide valuable insights into the phyto-immunotherapeutic approach toward developing effective therapeutic interventions against secondary immunodeficiencies and leukaemia.

MATERIALS AND METHODS

Source of *M. oleifera* leaves

Approximately 50 kg of *M. oleifera* leaves were harvested from trees aged 4-5 years at a Moringa plantation in Kampar, Perak, Malaysia. The leaves were processed immediately at Ethno Resources Sdn. Bhd., Sungai Buloh, Selangor. The harvested plant segments were identified and authenticated at the Institute for Medical Research (IMR) and Universiti Putra Malaysia (UPM); a specimen with voucher number UPM/FPSK/U1/H0-18 was deposited at the departmental herbarium for future reference. The leaves were washed twice in distilled water and oven dried at 40°C for 72 hours, after which the dried leaves were grounded to powder form (1 mm sieve size), weighed and stored at 4 oC in a chiller until use.

Cells and cell culture conditions

The leukaemic cell line, BV173, was purchased from America Type Culture Collection (ATCC), while frozen PBMCs were obtained from the Immunology Laboratory, Department of Pathology, Universiti Putra Malaysia and used as the source of primary lymphocytes. The PBMCs were carefully thawed, and the suspension cells were grown in Roswell Park Memorial Institute (RPMI 1640) medium (Gibco BRl, Invitrogen) supplemented with 10% fetal bovine serum (Gibco BRL, Invitrogen), 1% of penicillin/ and streptomycin (Gibco, BRL Invitrogen). The cells were incubated at 37°C in a 5% CO2 humidified cell culture incubator, and the media was changed every 3 days.

Stimulation of PBMCs

The PBMCs were stimulated using methods previously described by Ceuppens et al. with slight modifications (17). The PBMCs were stimulated using 5 µg/mL leucocyte-reactive phytohemagglutinin (PHA-L) (Roche Life Science, Catalogue No. 11249738001). Lyophilised powder (5 mg) of the PHA-L was dissolved in 5 mL PBS, yielding a final concentration of 1 mg/mL, and the concentration was adjusted to 5 µg/mL in complete RPMI. The thawed PBMCs were then washed twice in PBS, counted and re-suspended in a T-75 cell culture flask containing the 5µg/mL PHA-supplemented complete RPMI at the density of 2x106 cells/mL. The cells were then incubated vertically for 72 hours prior to subsequent experiments (17).

M. oleifera extraction and storage

The ethanol extraction of *M. oleifera* leaves was conducted at the Phytochemistry Unit, Herbal Medicine

Research Centre (HMRC), and Institute of Medical Research. The dry powdered *M. oleifera* leaves were extracted with 70% ethanol using sonication extraction, each at a 50 g 500 mL ratio for 1 hour at room temperature. The extract was filtered using filter paper, and the filtrate was concentrated under reduced pressure using a vacuum rotary evaporator (Buchi Rotavapor® R210/215, Switzerland) at 40 oC to obtain a crude extract. The concentrated extracts were freeze-dried (Telstar Cryodosto, Spain) to yield dry test material, stored at -20°C until use.

Preparation of *M. oleifera* stock solution

The stock solution of the *M. oleifera* ethanol extract (MOETE) was prepared by dissolving 100 mg of extract in 1 mL of dimethyl sulfoxide (DMSO). The working concentrations of the extracts (0, 0.1, 1.0, 5.0, 10.0, 50.0, 100.0, 200.0, 300.0, 500.0 and 1000.0 µg/mL) were prepared by serial dilutions of the stock solution in complete RPMI media. Fresh stock solutions were made prior to every use.

Cytotoxicity analysis (MTT assay)

Briefly, the cells were washed twice with PBS and suspended into the different working concentrations at a cell density of 5 x 10^4 cells/mL, from which 100 µL (containing 5 x 10^3 cells) were seeded in triplicates into each well of 96-microwell plates. The plates were then incubated for 48 hours. After incubation, 20 µL of MTT solution (Trevigen TACS MTT Cell Proliferation Assay Kit, Catalog No. 4890-25-01) was added per well and incubated for 5 hours in the dark. The formation of purple needle-shaped formazan crystals was confirmed by viewing the plates under an inverted microscope, and then 80 µL DMSO was added per well and shaken for 15 minutes. The absorbance at 570 nm was measured with a microplate reader (MRXII DYNEX Technologies, USA). To account for the potential cytotoxic effect of the vehicle used for ethanol extracts, serial dilutions of DMSO in complete media corresponding to each working concentration were prepared, as indicated in Table I.

The cells were treated with DMSO dilutions, and an MTT assay was performed as described earlier. The percentage of cell viability of cells treated with the different MOETE was calculated using the formula below.

$$\% \text{ Cell viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of DMSO treated cells}} \times 100$$

A concentration-response curve was obtained by plotting a graph of % cell viability against the treatment concentrations, and the inhibitory concentration (IC_{50}) or effective concentration (EC50) was estimated using GraphPad Prism version 7.0.

Cell proliferation assay

The BV173 cells and PHA-stimulated PBMCs were seeded in 24 well plates at a density of 5 x 104 cells/well in complete RPMI media containing the MOETE at concentrations of IC_{50} (62.5 µg/mL), IC_{50} (125 µg/mL) and $2IC_{50}$ (250 µg/mL), for BV173 and EC_{50} (14 µg/mL), EC_{50} (28 µg/mL) and $2EC_{50}$ (56 µg/mL), for PBMCs, while cells treated with 0.5% DMSO in complete RPMI were used as vehicle control. The plates were incubated for 48 hours, after which Genecopoeia Cell-Quant™ No Wash Cell Proliferation Assay Kit (Catalogue No. A014) was conducted per the manufacturer's protocol.

After the incubation, the cells were pelleted by centrifugation at 1800 rpm for 10 minutes and re-suspend in 300 µL 1X HBSS buffer, out of which 50 µL aliquots of the cell suspension were dispensed into each well of 96-microwell plate. The cells were then stained with 50 µL of 2X dye binding solution added to each well and incubated at 35°C in the dark for 1 hour. The fluorescence intensity of each sample was measured using a fluorescence microplate reader (Fluoroskan Ascent™ FL, ThermoFisher, USA) with excitation at ~485 nm and emission detection at ~530 nm. Cell numbers were calculated using a standard curve generated on each plate.

Cell cycle analysis

The BV173 cells and PHA-stimulated PBMCs were seeded in 6 well plates at a density of 1 x 106 cells/well in complete RPMI media containing the MOETE at concentrations of IC_{50} (62.5 µg/mL), IC_{50} (125 µg/mL) and $2IC_{50}$ (250 µg/mL), for BV173 and EC_{50} (14 µg/mL), EC_{50} (28 µg/mL) and $2EC_{50}$ (56 µg/mL), for PBMCs, while cells treated with 0.5% DMSO in complete RPMI were used as vehicle control. The plates were incubated for 48 hours, and cell cycle analysis was conducted using the protocol described by Wang et al. with slight modification. After incubation, the cells were harvested, washed with cold PBS and fixed in 70% ethanol overnight at -20oC (18). The fixed cells were then washed in PBS, re-suspended in 0.5 mL of PI/RNase staining buffer and incubated for 30 minutes. The DNA content of the cells was measured by the acquisition of 104PI-labelled cells in BD FACSCantoTM flow cytometer (Becton Dickinson and Company, BD Biosciences, San Jose, USA) and the data obtained were analysed using the FSC Express 6 software (De Novo Software).

Apoptosis assay

The BV173 cells and PHA-stimulated PBMCs were seeded in 6 well plates at a density of 1 x 106 cells/

Table I: Percentage of vehicle (DMSO) in treatment concentrations of the extract

Treatment Conc. (µg/mL)	0.1	1.0	5.0	10.0	50.0	100.0	200.0	300.0	500.0	1000.0
DMSO (% V/V)	0.0001	0.001	0.005	0.01	0.05	0.1	0.2	0.3	0.5	1.0

well in complete RPMI media containing the MOETE at concentrations of IC_{50} (62.5 µg/mL), IC_{50} (125 µg/mL) and $2IC_{50}$ (250 µg/mL), for BV173 and EC_{50} (14 µg/mL), EC_{50} (28 µg/mL) and $2EC_{50}$ (56 µg/mL), for PBMCs, while cells treated with 0.5% DMSO in complete RPMI were used as vehicle control. The plates were incubated for 48 hours, and an apoptosis assay was performed using Annexin V/PI staining kit (Invitrogen, USA) according to the manufacturer's protocol with slight modification. After the respective incubation periods, the cells were harvested, washed with 1x Annexin Binding Buffer (ABB), and stained with 2 µL Annexin V and 2 µL PI. The stained cells were then incubated for 15 minutes in the dark, followed by re-suspension in 500 µL ABB. Cells were finally acquired using BD FACSCantoTM flow cytometer (Becton Dickinson and Company, BD Biosciences, San Jose, USA), and data analysis were performed using FACS Diva software.

Statistical analysis

All values were reported as mean±SD. The results were obtained from treated cells at different *M. oleifera* extract concentrations with those of untreated vehicles by Student's T-test using Graphpad Prism (Version 7), and significant levels were set at p value < 0.05.

RESULTS

The MOETE dynamically affects BV173 and PBMCs viability

The concentration-response curve obtained from the cytotoxicity analysis of MOETE on the cells showed a concentration-dependent decrease in cell viability in BV173 and improved cell viability in PBMCs (Fig. 1A). The extract exerted inhibitory effects on the BV173 cell line with IC_{50} of 125±6 µg/mL and trophic effect on PBMCs with EC_{50} of 28±3 µg/mL. The impact of serial dilutions of DMSO in complete media corresponding to each treatment concentration on the BV173 and PBMCs after 48 hours indicates that DMSO alone does not exert significant cytotoxic effect on the cells as presented in Fig. 1B.

The MOETE exerted antiproliferative effects onBV173and enhanced the proliferation of PBMCs

The results obtained from the cell proliferation assays indicated that the MOETE selectively exerted antiproliferative effects on the BV173 while improving the induced proliferation of PBMCs. The antiproliferative effect of MOETE on BV173 cells was concentration-dependent as the number of cells significantly decreased by more than 50% at IC_{50}, i.e. from 8959±340 cells in the vehicle group to 3799±152 cells at IC_{50} ($p = 0.00002$). The cell number was further significantly reduced to 2271±233 cells at IC_{50} ($p < 0.00001$) and 1177±161 cells at $2IC_{50}$ ($p < 0.00001$) (Fig. 2A). For the PHA-stimulated PBMCs, MOETE significantly enhanced the proliferation of the stimulated cells at all treatment concentrations (Fig. 2B).

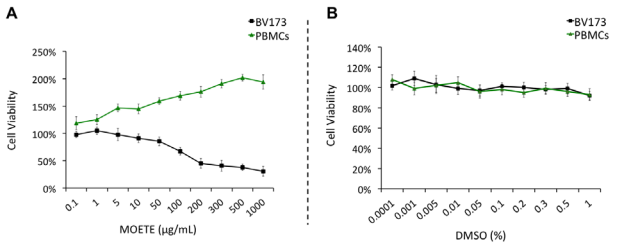

Figure 1: Concentration-response curves of M. oleifera extract cytotoxicity on BV173 and PBMCs. (A) The concentration-response curve obtained from the cytotoxicity analysis of MOETE on BV173 cells and PBMCs. The cells were treated with different working concentrations of MOETE and seeded in triplicates into each well of 96-microwell plates at a density of 5 x 103 cells. The plates were then incubated for 48 hours and MTT assay was performed. The results shown are mean values of 3 independent experiments. (B) The impact of serial dilutions of DMSO in complete media corresponding to each treatment concentration on the BV173 and PBMCs after 48 hours. The percentage of vehicle (DMSO) in each treatment concentration (0.0001% [0.1 µg/mL], 0.001% [1.0 µg/mL], 0.005% [5.0 µg/mL], 0.01% [10.0 µg/mL], 0.05% [50.0 µg/mL], 0.1% [100.0 µg/mL], 0.2% [200.0 µg/mL], 0.3% [300.0 µg/mL], 0.5% [500.0 µg/mL] and 1% [1000.0 µg/mL]). The results shown are mean values of 3 independent experiments.

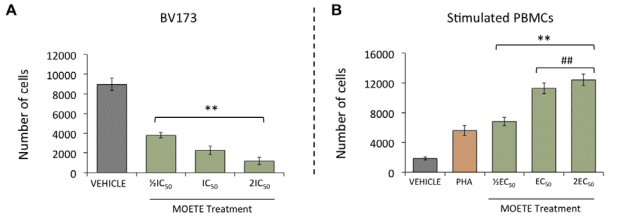

Figure 2: Effect of MOETE on the proliferation of BV173 and PBMCs. (A) The effect of MOETE on proliferation of BV173 cells. The cells were seeded in 24 well plates at a density of 5 x 104 cells/well in complete RPMI media containing the MOETE at concentrations of IC50 (62.5 µg/mL), IC50 (125 µg/mL) and 2IC50 (250 µg/mL). The plates were then incubated for 48 hours, after which proliferation assay was performed. The results shown are mean values of 3 independent experiments. ** shows a statistically significant difference compared to the vehicle control ($p < 0.05$). (B) The effect of MOETE on proliferation of PHA-stimulated PBMCs. The cells were seeded in 24 well plates at a density of 5 x 104 cells/well in complete RPMI media containing the MOETE at concentrations of EC50 (14 µg/mL), EC50 (28 µg/mL) and 2EC50 (56 µg/mL). The plates were then incubated for 48 hours, after which after which proliferation assay was performed. The results shown are mean values of 3 independent experiments. ** shows a statistical significant difference compared to the vehicle control (PBMCs + Vehicle) ($p < 0.05$). ## shows statistical significant difference compared to the positive control (PBMCs + PHA) ($p < 0.05$).

The MOETE induced S-phase cell cycle arrest in BV173 and enhanced cell cycle progression in the PBMCs

In the BV173 cells, the percentage of cells in the sub G0 population increased markedly with an increase in MOETE treatment concentration (Fig. 3A).Statistically significant increase in the sub G0 was observed at IC_{50} (Vehicle vs. IC_{50}: 2.70±0.40% vs. 22.10±2.63% ($p = 0.00023$)), IC_{50} (Vehicle vs. IC_{50}: 2.70±0.40% vs. 48.80±2.20% ($p < 0.00001$)) and $2IC_{50}$ (Vehicle vs. $2IC_{50}$: 2.70±0.40% vs. 51.90±3.45% ($p < 0.00001$)) at $p < 0.05$. This was coupled with a significant drop in the G0/G1-phase across all treatment concentrations (i.e. Vehicle vs. IC_{50}: 56.24±4.68% vs. 36.50±3.91%

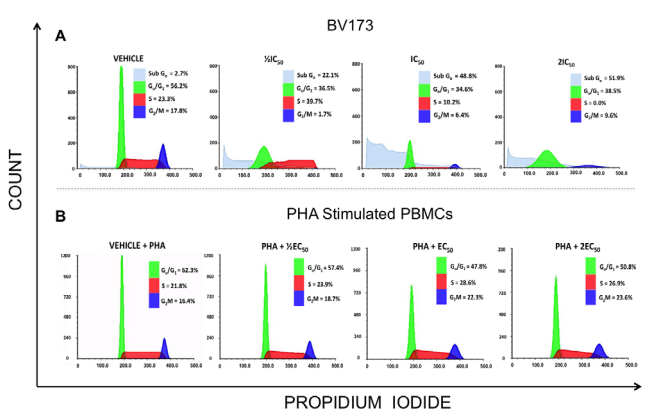

Figure 3: Effect of MOETE on the cell cycle of BV173 and PBMCs. (A) The effect of MOETE on the cell cycle of BV173 cells. The cells were seeded in 6 well plates at a density of 1 x 106 cells/well in complete RPMI media containing the MOETE at concentrations of IC50 (62.5 µg/mL), IC50 (125 µg/mL) and 2IC50 (250 µg/mL). The plates were then incubated for 48 hours, and cell cycle analysis was conducted. The result shown is a representative of 3 independent experiments. (B) The effect of MOETE on the cell cycle of PHA-stimulated PBMCs. The cells were seeded in 6 well plates at a density of 1 x 106 cells/well in complete RPMI media containing the MOETE at concentrations of EC50 (14 µg/mL), EC50 (28 µg/mL) and 2EC50 (56 µg/mL). The plates were then incubated for 48 hours, and cell cycle analysis was conducted. The result shown is a representative of 3 independent experiments.

(p = 0.00497); Vehicle vs. IC_{50}: 56.24±4.68% vs. 34.61±4.67% (p = 0.00478); Vehicle vs. $2IC_{50}$: 56.24±4.68% vs. 38.53±1.79% (p = 0.00361)). Also, S-phase cell cycle arrest was observed at IC_{50}, which was evident from the accumulation of cells in the S-phase (i.e. Vehicle vs. IC_{50}: 23.30±4.94% vs. 39.70±2.46% (p = 0.00676)) at $p < 0.05$. However, the percentage of cells in the S-phase significantly decreased to 10.20±1.17% at IC_{50} compared to the Vehicle (p = 0.01101) as the DNA synthesis phase of the cell cycle completely ceased at $2IC_{50}$ (Table II).

Table II: Mean DNA distribution of cell cycle analysis on MOETE treated BV173 and PBMCs.

	BV173			
	Vehicle	**IC_{50}**	**IC_{50}**	**$2IC_{50}$**
Sub G_0 (%)	2.70±0.40	22.10±2.63**	48.80±2.20**	51.90±3.45**
G_0/G_1 Phase (%)	56.24±4.68	36.50±3.91**	34.61±4.67**	38.53±1.79**
S phase (%)	23.30±4.94	39.70±2.46**	10.20±1.17	0
G_2/M Phase (%)	17.83±1.79	1.70±0.20**	6.42±0.83**	9.60±1.26**
	PHA Stimulated PBMCs			
	Vehicle	**EC_{50}**	**EC_{50}**	**$2EC_{50}$**
G_0/G_1 Phase (%)	66.30±2.63	57.40±3.23	44.50±2.80**	50.82±2.16
S phase (%)	18.80±1.14	23.95±2.29	31.60±2.36**	28.90±2.04**
G_2/M Phase (%)	14.40±1.79	18.53±2.45	24.30±1.26**	20.64±2.02**

BV173 DNA distribution shows significant increase in the Sub-G_0 phase, decrease in G_0/G_1 phase coupled with decrease in the G_2/M phase for all treatment concentrations.
PBMCs DNA distribution shows significant decrease in G_1/G_0 phase at EC_{50} with significant increase in S- and G_2/M phases recorded at EC_{50} and $2EC_{50}$ treatment concentrations.
** Statistical significant difference compared to the vehicle control ($p < 0.05$).

The MOETE enhanced the progression of the PHA-stimulated PBMCs into the cell cycle, which was evident by the observed reduction in G0/G1-phase coupled with an increase in the S-phase as well as the G2/M-phase as shown in (Fig. 3B). However, the statistical significance was observed at EC_{50} S-phase (Vehicle vs. EC_{50}: 18.80±1.14% vs. 31.60±2.36% (p = 0.00107)) and G2/M-phase (Vehicle vs. EC_{50}: 14.40±1.79% vs. 24.30±1.26% (p = 0.00143)) as well as at $2EC_{50}$ S-phase (Vehicle vs. $2EC_{50}$: 18.80±1.14% vs. 28.90±2.04% (p = 0.00170)) and G2/M-phase (Vehicle vs. $2EC_{50}$: 14.40±1.79% vs. 20.64±2.02% (p = 0.01607)), while EC_{50} showed a significant decrease in G0/G1-phase (Vehicle vs. EC_{50}: 66.30±2.63% vs. 44.50±2.80% (p = 0.00060)) at $p < 0.05$ (Table II).

The MOETE induced apoptosis in the BV173 and improved the viability of PBMCs

Apoptotic cell death was induced in the BV173 cells following treatment with MOETE. The percentage of cells at the early and late apoptotic stages (i.e. 2nd and 3rd quadrants) increased as the treatment concentration increases. Conversely, the percentage of viable cells (1st quadrant) decreased as the treatment concentration increases (Fig. 4A). Statistical significant decrease in the percentage of viable cells was observed at IC_{50} (Vehicle vs. IC_{50}: 93.40±4.90% vs. 56.90±2.42% (p = 0.00032)) and $2IC_{50}$ (Vehicle vs. $2IC_{50}$: 93.40±4.90% vs. 43.50±6.54% (p = 0.00045)). Similarly, cells in the late stage of apoptosis significantly increased at IC_{50} (Vehicle vs. IC_{50}: 1.80±0.63% vs. 26.80±3.08% (p = 0.00016)) and $2IC_{50}$ (Vehicle vs. $2IC_{50}$: 1.80±0.63% vs. 44.60±5.13% (p = 0.00014)) at $p < 0.05$ (Table III).

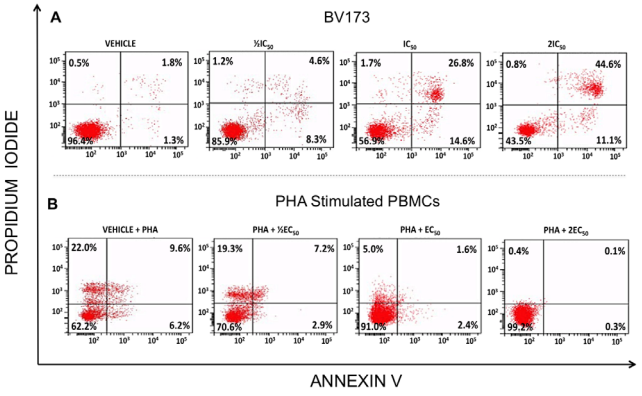

Figure 4: Effect of MOETE on the apoptosis of BV173 and PBMCs. (A) The effect of MOETE on the apoptosis of BV173 cells. The cells were seeded in 6 well plates at a density of 1 x 106 cells/well in complete RPMI media containing the MOETE at concentrations of IC50 (62.5 µg/mL), IC50 (125 µg/mL) and 2IC50 (250 µg/mL). The plates were then incubated for 48 hours, and apoptosis assay was conducted. The result shown is a representative of 3 independent experiments. (B) The effect of MOETE on the apoptosis of PHA-stimulated PBMCs. The cells were seeded in 6 well plates at a density of 1 x 106 cells/well in complete RPMI media containing the MOETE at concentrations of EC50 (14 µg/mL), EC50 (28 µg/mL) and 2EC50 (56 µg/mL). The plates were then incubated for 48 hours, and apoptosis assay was conducted. The result shown is a representative of 3 independent experiments.

Table III: Mean percentage of cells in apoptotic stages of MOETE treated BV173 and PBMCs.

	BV173			
	Vehicle	**IC_{50}**	**IC_{50}**	**$2IC_{50}$**
Viable cells (%)	93.40±4.90	89.90±3.23	56.90±2.42**	43.50±6.54**
Early apoptosis (%)	3.83±0.32	6.30±1.53	14.60±2.19	11.15±2.43
Late apoptosis (%)	1.80±0.63	2.67±0.14	26.80±3.08**	44.60±5.13**
Necrosis (%)	0.95±0.12	1.20±0.14	1.74±0.22	0.80±0.09
	PHA Stimulated PBMCs			
	Vehicle	**EC_{50}**	**EC_{50}**	**$2EC_{50}$**
Viable cells (%)	62.20±5.62	70.66±6.46	91.00±4.55**	97.20±3.91**
Early apoptosis (%)	7.32±1.18	2.94±0.31	2.46±0.30	2.30±0.02
Late apoptosis (%)	9.60±1.63	7.20±0.45	1.60±0.26**	0.10±0.02**
Necrosis (%)	22.10±3.56	19.33±2.41	5.20±0.65	0.40±0.01

BV173 apoptosis analysis shows significant decrease in cell viability and increase in the percentage of cells in late apoptotic stage for IC_{50} and $2IC_{50}$.
Apoptosis analysis for PBMCs shows significant increase in cell viability at concentrations of EC_{50} and $2EC_{50}$, while the percentage of cells in late apoptotic stage and necrosis significantly decreased.
** Statistical significant difference compared to the vehicle control ($p < 0.05$).

In PHA-stimulated PBMCs however, MOETE exhibited the ability to improve cell viability (Fig. 4B). The percentage of cells at the early and late apoptotic stages (i.e. 2nd and 3rd quadrants) decreased as the treatment concentration increases. Conversely, the percentage of viable cells (1st quadrant) increased as the treatment concentration increases (Fig. 4A). Statistical significant increase in the percentage of viable cells was observed at EC_{50} (Vehicle vs. EC_{50}: 62.20±5.62% vs. 91.00±4.55% ($p = 0.00232$)) and $2EC_{50}$ (Vehicle vs. EC_{50}: 62.20±5.62% vs. 97.20±3.91% ($p = 0.00089$)). Similarly, cells in the late stage of apoptosis significantly decreased at EC_{50} (Vehicle vs. EC_{50}: 9.60±1.63% vs. 1.60±0.26% ($p = 0.00110$)) and $2EC_{50}$ (Vehicle vs. EC_{50}: 9.60±1.63% vs. 0.10±0.02% ($p = 0.00054$)). The percentage of cells in the 4th quadrant (representing necrotic cell death) was significantly reduced from 22.10±3.56% (Vehicle) to 5.20±0.65% (EC_{50}) ($p = 0.00127$ and 0.40±0.01% ($2EC_{50}$) ($p = 0.00046$) at $p < 0.05$ (Table III).

DISCUSSION

The tumoricidal activity of *M. oleifera* has attracted more attention from many researchers. The reported antitumour activity is closely related to its immunomodulatory functions, where *M. oleifera* leaves (19), seeds (20), bark (21), and roots (22) have demonstrated a wide range of anti-cancer activities in different experimental animal models. The present study investigated the immunomodulatory and tumouricidal effects of *M. oleifera* leaves in vitro using a standard leukaemic lymphocyte cell line and compared it with normal lymphocytes.

Although ethanol is a non-polar solvent ideal for extracting non-polar compounds like phenolics, alkaloids and terpenes, *M. oleifera* has been reported to be composed of ions, hydrophilic compounds, water-soluble enzymes, glycoproteins, peptides, amino acids, nucleotides, vitamins, sugars and polysaccharides (15). The mixture of ethanol and water at a ratio of 70:30 (70% ethanol) was utilised to extract *M. oleifera* leaves. The cytotoxicity analysis conducted on ethanol and aqueous extracts of *M. oleifera* leaves revealed the ability of the extracts to discriminate between the leukemic cell line and normal lymphocytes by decreasing the viability of the BV173 cells and exerting trophic effects on PBMCs. The observed could be a result of the extract constituting the ideal balance of polyphenols as well as polar nutrients capable of enriching the culture medium and improving the viability and growth of the cells. Although very few studies have investigated the cytotoxic effect of *M. oleifera* on normal non-cancerous cells in vitro, Fernandes et al. correlated the nutritional and polyphenolic constituents with regenerative potentials of extracts of *M. oleifera* leaves and flowers on rat-derived primary fibroblast and mesenchymal stem cells (MSCs) (23). Similar cytotoxic effects of *M. oleifera* leaves extract have been reported in tumour cells like hepatocarcinoma (HepG2), colorectal adenocarcinoma (Caco-2) and breast adenocarcinoma (MCF-7) (11). The cytotoxicity analysis inferred that the 70% ethanol *M. oleifera* leaves extract (MOETE) could efficiently discriminate between the tumour cell and normal PBMCs; this was investigated further.

The immunomodulatory properties of any substance can be evaluated effectively in vitro by analysing its effect on the proliferation of immune cells. Inhibition of proliferation implies immunosuppressive activity while triggering or enhancing cell proliferation indicates immunostimulatory effects. In the MOETE-treated tumour cell, cytotoxicity analysis provided insufficient information on the anti-tumour potentials as it could imply a reduction in cellular metabolic activity, death resulting from loss of membrane integrity leading to cell lysis, i.e. necrosis, inhibition of growth/ proliferation or genotoxic induction of programmed cell death. Further investigations through cell proliferation assays indicated

that MOETE effectively inhibits the expansion of the BV173 cells. A similar antiproliferative effect of *M. oleifera* has been reported in colon cancer cells (24) and cancerous human alveolar epithelial cells (25).Although the IC50 recorded from the cytotoxicity analysis of MOETE-treated BV173 cells was 125 µg/mL, more than 50% inhibition of proliferation was observed at IC_{50}, i.e. concentration of 63 µg/mL, suggesting that non-proliferating viable cells may not have been captured by the cytotoxicity analysis, thus to clarify this, cell cycle analysis was conducted. Cell cycle analysis of the BV173 cells following treatment with MOETE revealed a concentration-dependent accumulation of cells in the Sub G0 phase. Cells are detected within these peaks due to DNA degradation or fragmentation as the shortened DNA fragments leak out of cells during sample preparation and staining process, yielding cells with less than normal diploid (2n) DNA content (26).Similarly, increased treatment concentration reduced cells in the G0/G1 phase. The cells were arrested in the S-phase at IC_{50}, indicating that inhibition of proliferation preceded the induction of cell death. *M. oleifera* leaves and seeds have been reported to induce G2/M cell cycle arrest in colorectal cancer cells (27), while the accumulation of cells in S phase following treatment with *M. oleifera* has been previously reported in Dalton's lymphoma cells (28). Interestingly, Gismondi et al analysed the effect of different plant extracts on melanoma cells and reported G0/G1 cell cycle arrest with *M. oleifera* leaves as the only plant extract inducing a significant accumulation of sub-G0 cells (29). This is suggestive that *M. oleifera* does not only inhibit proliferation via arrest of the cell cycle, it also induces tumour cell death. Although the sub-G0 population confirms cell death, the population does not distinguish between apoptotic and non-apoptotic cell death. The apoptosis assay conducted helped to identify the mechanism of MOETE-induced cell death in BV173 cells and revealed a concentration-dependent increase in the percentage of cells in the late apoptotic stage (3rd quadrant | Ann+PI+) coupled with a negligible number of cell death from non-apoptotic or necrotic process (4th quadrant |Ann-PI+) indicates that MOETE exerts its anti-tumour effects by inducing apoptosis rather than noxious and inflammatory necrotic cell death capable of affecting normal cells within the physiological milieu. In contrast with the present study's findings, de Andrale Luz et al. reported necrotic cell death in B16-F10 melanoma cells following treatment with lectins from *M. oleifera* seeds (30).However, lectins have been shown to trigger an increase in mitochondrial reactive oxygen species (ROS) generation (31), thereby inducing oxidative stress-mediated necrotic cell death. Apoptotic effects of *M. oleifera* on tumour cells, in agreement with the findings of this study, have been previously reported (32-34). It is suggested that MOETE-mediated induction of apoptosis may result from modification of gene expression of apoptotic proteins and cytokines involved in the apoptotic process; however, this requires investigation in future studies.

Interestingly, however, cell proliferation assays conducted on stimulated PBMCs revealed improvement in cell proliferation after treatment with MOETE. This outcome indicates that MOETE exerts an immunostimulatory effect on normal PBMCs. Activating proliferation in PBMCs requires interaction between cell surface receptors (T-cell receptor, TCR or B- cell receptor, BCR) with a specific antigen or an equivalent mitogenic substance (35).In cell culture, PHA and concanavalin A (Con A) is used as mitogens for T-cell activation, while lipopolysaccharide (LPS) and pokeweed mitogen (PWM) are utilised for B-cell activation. It is important to note, however, that PHA, Con A and PWM are carbohydrate-binding proteins known as lectins (36).These lectins bind to the sugars or fucose residues on the cell surface proteins like the TCR/BCR, thereby triggering proliferation (37). Many studies have reported the identification of mitogenic lectins from *M. oleifera* seeds, and a few have identified the same from the leaves of *M. oleifera* (38, 39). However, whether the stimulatory effect of the extract on PBMCs can be ascribed to these proteins is yet to be deciphered. Similar immunostimulatory effects of *M. oleifera* leaves have been reported in CD4+, CD8+ and B220 cells (40), while Jayanthi et al. reported in vitro stimulation of splenocyte proliferation in mice by *M. oleifera* leaves extracts (41). In addition to the stimulatory effect on PBMC proliferation, MOETE exhibited an ability to commit PBMCs into the cell cycle by enhancing progression into the S-phase and improving the viability of the PBMCs by reducing the number of apoptotic cells. Similarly, the ability of MOETE to ameliorate apoptosis and improve cell viability of PHA-stimulated PBMCs can be associated with the buoyant nutritional composition of the extract, which enriches the culture medium and nourishes the cells. Although many studies have explored the effect of *M. oleifera* on the apoptosis of tumour cells, only a few studies have investigated its potential effects on the apoptosis of healthy cells. Fernandes et al. reported an improvement in cell viability of mesenchymal stem cells (MSC) after treatment with extracts of *M. oleifera* seeds and leaves (23). Likewise, kaempferol from *M. oleifera* has shown anti-apoptotic effects on isoproterenol-induced cardiac toxicity in rats (42).

CONCLUSION

Although the inhibitory effect MOETE on the leukemic cell line reveals its immense anticancer potential, its immunostimulatory impact on PBMCs highlights the ability to differently modulate the viability of cancerous and normal lymphocytes; an indication of more substantial therapeutic potential, especially in cancer immunotherapy and in improving the immune competence of immunocompromised individuals. Future investigations on the extract's ability to modulate cytokine secretion and expression of apoptotic proteins in treated leukemic cells as well as the exploration of the

extract's anticancer effects in suitable in vivo models is recommended. These findings would promote the use of *M. oleifera* leaves for herbal supplementation aimed at strengthening the immune system to support aged and immunocompromised individuals and serve as adjuvant in therapies against blood cancers.

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